Genetic and Functional Analyses of \textit{SHANK2} Mutations Suggest a Multiple Hit Model of Autism Spectrum Disorders

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Abstract

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders with a complex inheritance pattern. While many rare variants in synaptic proteins have been identified in patients with ASD, little is known about their effects at the synapse and their interactions with other genetic variations. Here, following the discovery of two \textit{de novo} \textit{SHANK2} deletions by the Autism Genome Project, we identified a novel 421 kb \textit{de novo} \textit{SHANK2} deletion in a patient with autism. We then sequenced \textit{SHANK2} in 455 patients with ASD and 431 controls and integrated these results with those reported by Berkel et al. 2010 (n = 396 patients and n = 659 controls). We observed a significant enrichment of variants affecting conserved amino acids in 29 of 851 (3.4%) patients and in 16 of 1,090 (1.5%) controls (P = 0.004, OR = 2.37, 95% CI = 1.23–4.70). In neuronal cell cultures, the variants identified in patients were associated with a reduced synaptic density at dendrites compared to the variants only detected in controls (P = 0.0013). Interestingly, the three patients with \textit{de novo} \textit{SHANK2} deletions also carried inherited CNVs at 15q11–q13 previously associated with neuropsychiatric disorders. In two cases, the nicotinic receptor \textit{CHRNA7} was deleted and in one case the synaptic translation repressor \textit{CYFIP1} was deleted. These results strengthen the role of synaptic gene dysfunction in ASD but also highlight the presence of putative modifier genes, which is in keeping with the “multiple hit model” for ASD. A better knowledge of these genetic interactions will be necessary to understand the complex inheritance pattern of ASD.
Introduction

Autism spectrum disorders (ASD) are characterized by impairments in reciprocal social communication and stereotyped behaviors [1]. The prevalence of ASD is about 1/100, but closer to 1/300 for typical autism [2]. ASD are more common in males than females, with a 4:1 ratio. Previously, twin and family studies have conclusively described ASD as the most “genetic” of neuropsychiatric disorders, with concordance rates of 82–92% in monozygotic twins versus 1–10% in dizygotic twins [3], but a recent study finds evidence for a more substantial environmental component [4]. In the absence of Mendelian inheritance patterns, ASD were first considered to be polygenic, i.e., a disorder caused by multiple genetic risk factors, each of weak effect. More recently, an alternative model was proposed that considered ASD as a group of disorders caused by heterogeneous genetic risk factors influencing common neuronal pathways [5,6]. It was supported by the identification of apparently monogenic forms of ASD, each affecting a limited number of patients (1–2% for the most replicated genes) [7–14]. In this model, eventually a single highly penetrant mutation would be sufficient to produce ASD. However, the occurrence of two or more deleterious copy number variants (CNV) or mutations in a subset of patients also suggested that independent loci could act in concert to induce the development of ASD [9,13–16]. In line with these findings, the recent observation that patients with a deletion at 16p11.2 were more likely to carry an additional large CNV agrees with a “two-hit model” for developmental disorders [17].

The genetic causes of ASD are diverse [18], but the main category of genes associated with the disorder is related to the development and function of neuronal circuits [6,19]. Mutations of genes coding for synaptic cell adhesion molecules and scaffolding proteins, such as neuroligins (NLGN), neurexins (NRXN) and SHANK, have been recurrently reported in patients with ASD [7–10,13,14,20]. These proteins play a crucial role in the formation and stabilization of synapses [21], as well as in synaptic homeostasis [22]. SHANK2 and SHANK3 code for scaffolding proteins located in the postsynaptic density (PSD) of glutamatergic synapses. Deletions of ProSAP2/SHANK3 at chromosome 22q13 are one of the major genetic abnormalities in neurodevelopmental disorders [20], and mutations of ProSAP2/SHANK3 have been identified in patients with ASD, intellectual disability (ID) and schizophrenia [7,23–25]. Mutations of ProSAP1/SHANK2 have also recently been reported in both, ASD and ID [9,26]. The difference in clinical outcome of mutation carriers has been attributed to the presence of still uncharacterized additional genetic, epigenetic and/or environmental factors [27].

In order to better understand the role of the NRXN-NLGN-SHANK pathway in ASD, we first aimed to describe SHANK2 isoform expression in different tissues of healthy individuals. To investigate the role of this pathway in ASD, we screened for SHANK2 CNVs and coding mutations in a large sample of patients with ASD and controls. We provide genetic and functional evidence that SHANK2 is associated with ASD, and that its mutations affect the number of synapses. Additionally, we report the co-occurrence of SHANK2 de novo deletions and inherited CNVs altering neuronal genes, suggesting that epistasis between specific loci in the genome could modulate the risk for ASD.

Results

SHANK2 isoforms are differentially expressed in human tissues

In order to characterize all isoforms of SHANK2, we scanned genomic databases for specific Expressed Sequence Tags (ESTs) and spliced isoforms. The human SHANK2 gene (NM_012309.3) spans 6211.8 kb and contains 25 exons (Figure 1). The longest SHANK2 isoform (SHANK2E, AB208025) contains ankyrin (ANK) repeats at the N-terminus, followed by a Src homology 3 (SH3) domain, a PSD95/DLG/ZO1 (PDZ) domain, a proline-rich region and a sterile alpha motif (SAM) domain at its C-terminus region. All these domains are involved in protein-protein interactions that bridge glutamate receptors, scaffolding proteins and intracellular effectors to the actin cytoskeleton [28,29]. Two additional isoforms, ProSAP1 (AB208026) and ProSAP1 (AB208027), originating from distinct promoters, were previously detected in the rat [30,31]. Finally, the shortest isoform (AF114901), also originally described in the rat, results in premature termination of the transcription before the SAM domain due to an alternative 3’ end in exon 22 [32] (Figure 1A). To validate these SHANK2 isoforms in humans, we used specific RT-PCRs and sequencing (Figure 1B). Almost all tissues tested (brain, liver, placenta, kidney, lung, pancreas and lymphoblastoid cell lines) expressed SHANK2 mRNA, except heart and skeletal muscle, for which no expression was detected. We observed inter-individual differences in the relative amount of SHANK2 mRNA that were confirmed by using independent RT-PCRs and primers (not shown). Such differences have been previously reported for other synaptic genes such as NLGN1–4Y, PCDH11X/Y, and SHANK3 [7,8,33] and might be the consequence of polymorphisms located in specific regulatory sequences and/or activity dependent expression of this family of post-synaptic proteins [34]. Notably, exons 19, 20 and 23 were found to be expressed only in brain in all individuals tested (Figure 1C). Such brain specific splicing has been already observed for exon 18 in SHANK3 [7], which is similar to exon 19 and 20 in SHANK2. These ‘brain-
Author Summary

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders with a complex inheritance pattern. While mutations in several genes have been identified in patients with ASD, little is known about their effects on neuronal function and their interaction with other genetic variations. Using a combination of genetic and functional approaches, we identified novel SHANK2 mutations including a de novo loss of one copy of the SHANK2 gene in a patient with autism and several mutations observed in patients that reduced neuronal cell contacts in vitro. Further genomic analysis of three patients carrying de novo SHANK2 deletions identified additional rare genomic imbalances previously associated with neuropsychiatric disorders. Taken together, these results strengthen the role of synaptic gene dysfunction in ASD but also highlight the presence of putative modifier genes, which is in keeping with the “multiple hit model” for ASD. A better knowledge of these genetic interactions will be necessary to understand the complex inheritance pattern of ASD.

A de novo deletion of SHANK2 in a patient with ASD

Berkel et al. 2010 recently identified two independent de novo SHANK2 deletions in two patients, one with ID and another one with ASD [26]. In addition, whole genome analysis performed by the Autism Genome Project (AGP) using Illumina 1M single nucleotide polymorphism (SNP) arrays detected one additional de novo SHANK2 deletion in a patient (6319_3) with ASD [9] (the second patient described by the AGP, 5237_3, is patient SK0217-003 reported in Berkel et al. 2010 [26]). Recently, a 3.4 Mb de novo deletion including SHANK2 was observed in a female patient with speech and developmental delay [35]. To follow up on these results, we genotyped an independent sample of 260 patients with ASD and 16 of 1090 (1.5%) controls (Fisher’s exact test 1-sided, P = 0.004, OR = 2.37, 95% CI = 1.23–4.70). A total of 8 variants were identified in patients and controls. Among these 8 variants, 2 affected conserved amino acids (R818H and S557N). The variant S557N was observed in 9 of 851 (1.06%) independent families with ASD and in 3 of 1090 (0.28%) controls (Fisher’s Exact Test one sided, P = 0.029, OR = 3.87; 95% CI = 0.96–22.29). It affects a conserved serine with a high probability of being phosphorylated and located in the SH3 domain of all SHANK proteins. This domain binds to GRIP and b-PIX, two proteins linking SHANK to glutamate AMPA receptors and actin skeleton, respectively [36]. In our initial mutation screen, R818H was observed in 5 of 230 patients with ASD and 0 of 230 controls. In order to determine if R818H was more frequent in the patients with ASD, we screened an additional sample of 3020 individuals with ASD, 1785 controls from European descent, and the Human Genome Diversity Panel (HGDP) control dataset (Table S3 and S4). R818H was virtually absent outside Europe and had the highest allelic frequency (2.57%) in Finland, but overall its frequency was not higher in patients with ASD compared with controls (ASD 0.2% vs. 0.1% of controls; OR 1.37, 95% CI = 1.05–1.80; Fisher’s exact test 1-sided, P = 0.007) (Table S3).

Finally, and unexpectedly, during this additional mutation screening, we detected a variation (IVS22+1G>T) altering the consensus donor splice site of exon 22 in a Swedish control, SWE_Q56_508 (Figure 3A). This variant was predicted to disrupt all SHANK2 isoforms by deleting the proline rich and the SAM domain, except for the shortest isoform AF141901 where the mutation is located in the open reading frame (ORF) and should lead to a G263V change. This variant was not observed in 1786 patients or 1407 controls, and is not listed in dbSNP.

SHANK2 coding variants affecting conserved amino acids are enriched in patients with ASD

To probe for additional mutations, we first sequenced all exons of the longest SHANK2 isoform in 230 patients with ASD and 230 controls. We then sequenced an additional sample of 225 patients and 201 controls (Table S1) for the ProsAP1A isoform that corresponds to the major SHANK2 isoform in the brain. Since we screened all SHANK2 isoforms, we used a nomenclature including the SHANK2 isoform that differed from Berkel et al. 2010 [26]. Within the 9 coding exons specific to SHANK2, we identified R174C (rs7926203) listed in dbSNP in 2 independent patients with ASD and R185Q in one patient with ASD. For this isoform, no variant was identified in the control sample. Within the ProsAP1A isoform, we identified 24 non-synonymous variations. When these results are combined with those obtained by Berkel et al. 2010, a total of 40 variants of ProsAP1A including 3 already reported in dbSNP were identified (Figure 2B, Table 1, Figure S3). Only two variants (Y967C and R369H) with MAF>1% are detected and there is no enrichment of rare variants of SHANK2 (MAF<1%) in patients with ASD compared with controls. Because variants affecting conserved amino acids in the SHANK proteins are most likely to have a functional effect, we tested whether there was an enrichment of these variants in patients compared to controls. The alignment of the SHANK protein sequences and the conservation of the variants are indicated in the Table S5. In both mutation screening studies, the first performed by Berkel et al. 2010 and the second presented here, we observed an enrichment of variants affecting conserved amino acids in patients compared with controls (Figure 2C, Table S5 and Table S7). Overall, 12 of 15 (80%) of the variants identified only in the patient sample affected conserved amino acids compared with only 6 of 17 (35.3%) in controls (Fisher’s exact test 1-sided, P = 0.013, OR = 6.83, 95% CI = 1.19–53.40). Because several independent patients carried these variants (Table 1), the enrichment is even more significant when the number of carriers was considered. The variants affecting conserved amino acids were observed in 29 of 851 (3.4%) patients and in 0 of 1090 (0.15%) controls (Fisher’s exact test 1-sided, P = 0.004, OR = 2.37, 95% CI = 1.23–4.70). A total of 12 of 15 (80%) of the variants identified only in the patient sample affected conserved amino acids compared with only 6 of 17 (35.3%) in controls (Fisher’s exact test 1-sided, P = 0.013, OR = 6.83, 95% CI = 1.19–53.40).
Figure 1. Genomic structure, isoforms, and expression of human SHANK2. A. Genomic structure of the human SHANK2 gene. Transcription of SHANK2 produces four main mRNA from three distinct promoters: SHANK2E (AB208025), ProSAP1A (AB208026), ProSAP1 (AB208027) and AF141901. There are three translation starts: in exon 2 for SHANK2E, in exon 1b for ProSAP1A, and in exon 1c for ProSAP1 and AF141901; and two independent stop codons: in exon 22b for AF141901 and in exon 25 for SHANK2E, ProSAP1A and ProSAP1. Conserved domains of protein interaction or protein binding site are represented in color: ANK (red), SH3 (orange), PDZ (blue) and SAM (green), H (pink), D (dark blue) and C (purple). Black stars identify the alternative spliced exons (‘brain-specific exons’ in turquoise: 19, 20 and 23). B. RT-PCRs of SHANK2 isoforms on RNA from different human control tissues (Clontech), and different brain regions of four controls (2 males and 2 females). The amplified regions specific to each isoform of SHANK2 are
lar risk factors such as blood pressure and levels of all major hormones. Additionally, it was ascertained for axis I psychiatric disorders and personality traits using the Temperament and Character Inventory (TCI) [38] and the Karolinska Scales of Personality (KSP) [39]. Notably, despite the predicted deleterious effect of the mutation, this subject had no major somatic or psychiatric health problems. Regarding personality traits, none of her scores for TCI items were different from those found in the general population. KSP assessment showed that her scores for neuroticism (51.3), nonconformity/aggressiveness (56.7), and psychotism (50.5) were not different from the general population (mean ± SD = 50 ± 10). However, she displayed a high score (61.4) for the extraversion factor, and for one of its subscales, monotony avoidance [40].

Several SHANK2 variants identified in patients alter synaptic density in cultured neurons

In order to establish the functional impact of SHANK2 variations, we performed expression studies in primary neuronal cell cultures after over-expression of wild-type vs. mutant ProSAP1A/Shank2A cDNA (Figure 4). All the variants (n = 16) identified by our first screen of 230 patients and 230 controls were tested: 5 were identified only in patients (V717F, A729T, G1170R, D1535N and L1722P), 6 were detected in patients and controls (S557N, R569H, K780Q, R318H, Y967C and P1586L) and 5 were only found in controls (L629P, A822T, V823M, R1290W and Q1308R). All variants were predicted as damaging by Polyphen2 DIV except Q1308R identified only in controls and predicted as benign [41]. In the patient sample, 5/5 variants affected conserved amino acids in the SHANK proteins compared with only 2/6 in the group of variants identified in patients and controls, and 2/5 in the control group. All mutation sites were introduced into the rat ProSAP1A cDNA and confirmed by sequencing. The effect of the Shank2 variants was further investigated in cultured hippocampal neurons. Upon transfection, Western blot analysis revealed that the different GFP-Shank2 fusion proteins were expressed with the expected size (Figure S4). Results from quantification showed that none of the variants affected the cluster formation of Shank2 protein along the dendrites, the number or the general branching pattern of dendrites (Figure S4). In contrast, 5/5 variants affected conserved amino acids in the SHANK proteins with a higher reduction of synapse density when compared with those shared by patients and controls (Student’s t-test 2-sided P = 0.022) or those only detected in the controls (Student’s t-test 2-sided P = 0.001). As expected, variants affecting conserved amino acids were associated with a significant decrease of synaptic density in vitro compared with those shared by patients and controls (Student’s t-test 2-sided P = 0.022) or those only detected in the controls (Student’s t-test 2-sided P = 0.001). Variant identified in patients were associated with a significant decrease of synaptic density in vitro compared with those shared by patients and controls (Student’s t-test 2-sided P = 0.022) or those only detected in the controls (Student’s t-test 2-sided P = 0.001).
This deletion was observed in 4 of 1257 patients with ASD (0.32%) and in 4 of 1577 controls (0.25%) (Fisher’s exact test, 2-sided \( P = 0.74 \)). The BP1-BP2 deletion is associated with phenotypic variability and has been reported in individuals with neurodevelopmental disorders \[20\], schizophrenia \[53,60\], ASD \[44–46,49\], and epilepsy \[61\].

In a recent screen for large CNVs (>400 kb) performed on 15,767 children with ID and various congenital defects, and 8,329 unaffected adult controls \[20\], deletions affecting \textit{CIFIP1}, \textit{NIPA1}, \textit{NIPA2} and \textit{TUBGCP5} were associated with neurodevelopmental disorder \( P = 4.73 \times 10^{-6} \), epilepsy \( P = 1.48 \times 10^{-7} \) and autism \( P = 1.99 \times 10^{-5} \).
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<td><strong>Total variants</strong></td>
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<td>Fisher's exact test 1-sided, P = 0.34, OR = 1.08, 95% CI = 0.79–1.47</td>
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**Total variants with MAF<1%**  

43 (5.1%) 38 (3.5%) Fisher's exact test 1-sided, P = 0.06, OR = 1.47, 95% CI = 0.92–2.37

**Total conserved variants**  

29 (3.4%) 16 (1.5%) Fisher's exact test 1-sided, P = 0.004, OR = 2.37, 95% CI = 1.23–4.70
Several additional CNVs also altered compelling candidate genes for susceptibility to ASD. In patient AU038_3 we detected a previously unreported paternally inherited intronic duplication of CAMSAP1L on chromosome 1q32.1, coding for a calmodulin regulated spectrin-associated protein highly expressed in the brain. Patient 5237_3 carried a de novo deletion altering the coding sequence of the tyrosine phosphatase DUSP22 on chromosome 6p25.3 and a maternally inherited intronic duplication of NLGN1 on chromosome 3q26.3 [9]. These CNVs were observed at similar frequencies in patients with ASD compared with controls. DUSP22 deletions were observed in 8 of 1257 patients with ASD (0.64%) and in 14 of 1577 controls (0.89%), while NLGN1 intronic duplications were observed in 60 of 1257 patients with ASD (4.77%) and in 62 of 1577 controls (3.93%). Finally, patient 6319_3 carried an unreported maternally inherited intronic deletion of contactin CNTN4, a gene on chromosome 3p26.3 associated with ASD [62], as well as a paternally inherited deletion within the protocadherin PCDHA1-10 gene cluster on chromosome 5q31.3. Interestingly, this deletion removes the first exon of both PCDH8 and PCDH9 and was significantly less frequent in patients with ASD compared with controls (ASD: 62 of 1257; controls: 132 of 1577; Fisher’s exact test, 2-sided P = 0.0003; OR = 0.57; 95% CI = 0.41–0.78).

We also analyzed the genome of the Swedish control SWE_Q56_508 carrying the SHANK2 splice mutation using the Human Omni2.5 BeadChip array from Illumina (Figure 3B). Two close duplications on 2p25.3 were detected, altering four genes, LOC339822, SNTG2, PXDN and MYT1L. The inheritance of these two duplications could not be investigated, because DNA samples from the parents were not available. However, 2 of 1577 controls also carried the same close duplications, suggesting that these CNVs are located on the same chromosome. Among the affected genes, syntrophin-c (SNTG2) and myelin transcription factor 1-like (MYT1L) are expressed in the brain. Alterations of SNTG2 and MYT1L have been previously reported in patients with ASD [20,63,64] and schizophrenia [65], respectively. SNTG2 is a scaffolding protein interacting with the NLGN3/4X proteins [66].

**Figure 3. Genetic alterations identified in the control subject SWE_Q56_508.** A. SHANK2 splice mutation (IVS22+1G>T) detected in a Swedish female control, SWE_Q56_508. The mutation altered the donor splicing site of exon 22 and led to a premature stop in all SHANK2 isoforms except for the AF141901 isoform, where it altered the protein sequence (G263V). B. CNVs in the same individual altering LOC339822, SNTG2, PXDN and MYT1L. The two close duplications span 264 kb and 245 kb on chromosome 2 and altered LOC339822 and SNTG2, PXDN and MYT1L, respectively. Dots show the B allele frequency (BAF; in green), Log R ratio (LRR; in red), and QuantiSNP score (in blue). Lower panel: all CNVs listed in the Database of Genomic Variants (DGV) are represented: loss (in red), gain (in blue), gain or loss (in brown). H, homer binding site; D, dynamin binding site; C, cortactin binding site.

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and a component of the dystrophin glycoprotein complex [67]. MYT1L is a myelin transcription factor required to convert mouse embryonic and postnatal fibroblasts into functional neurons [68].

Discussion

Deleterious SHANK2 variations are enriched in patients with ASD, but also observed in controls

The identification of mutations in synaptic proteins such as NRXN1, NLGN3/4X and SHANK2/3 has demonstrated that a synaptic defect might be at the origin of ASD [5,6]. Here we confirm the presence of SHANK2 de novo deletions in individuals with ASD, with a prevalence of 0.38% (1/260) in our cohort of ASD patients analyzed with the Illumina 1M SNP array. This frequency is similar to the one reported previously by the AGP in a larger sample of 996 patients with ASD (0.2%) [9]. SHANK2 deletions altering exons were not detected in controls, in agreement with previous findings [9,26]. As reported for SHANK3 [7], no other coding variations were detected in the remaining SHANK2 allele of the deletion carriers, suggesting that, in some

![Figure 4. Characterization of the functional impact of SHANK2 mutations in cultured neuronal cells. A. The colocalization of ProSAP1A/SHANK2-EGFP (postsynaptic marker) and Bassoon (presynaptic marker) indicated that the mutations did not disturb the formation of SHANK2 clusters at excitatory synapses along the dendrites. B. The quantification of synapse density was performed on 20 transfected hippocampal neurons per construct from at least three independent experiments. The majority of the ProSAP1A variants affecting a conserved amino acid among SHANK proteins reduced significantly the synaptic density compared with the variants that affect amino acid non conserved among SHANK proteins (Mann-Whitney U-test: nWT = 20, nmut = 20; U557N = 82.5, P557N = 0.001; U629R = 124, P629R = 0.04; U629W = 149, P629W = 0.17; U717F = 114, P717F = 0.02; U717W = 73, P717W = 0.000; U717Q = 154, P717Q = 0.221; U818H = 108, P818H = 0.012; U967C = 154.5, P967C = 0.224; U967N = 129, P967N = 0.056; U967G = 137, P967G = 0.910; U1271P = 79, P1271P = 0.001, *p<0.05, **p<0.01, ***p<0.001). C. Effect of the variants on synaptic density. The y-axis represents \(-\log P\) compared to WT (P obtained with Mann-Whitney test). After Bonferroni correction for 16 tests, only P values<0.003 were considered as significant. Variants represented in red were specific to ASD, in orange were shared by ASD and controls, and in green were specific to the controls. Open circles and filled circles represent non conserved and conserved amino acids, respectively. Prim, primary; second, secondary. doi:10.1371/journal.pgen.1002521.g004)
individuals, a *de novo* deletion of a single allele of *SHANK2* might be sufficient to increase the risk for ASD. In one case, a patient carried two rare *SHANK2* variants predicted as deleterious and inherited from different parents, indicating that they were separate alleles.

For the remaining *SHANK2* variants, patients were heterozygous for non-synonymous rare variations inherited from one of their parents, indicating that they were separate alleles.

For the remaining *SHANK2* variants, patients were heterozygous for non-synonymous rare variations inherited from one of their parents, indicating that they were separate alleles. However, we observed a significant enrichment of *SHANK2* variants affecting conserved amino acids in patients with ASD compared with controls. This was also the case in the previous mutation screening by Berkel et al. 2010 [26]. The majority of the variants affecting conserved residues and identified in the patients were shown to alter the ability of *SHANK2* to increase the number of synapses in *vivo*. Importantly, the assays performed in this study show that the variants can potentially impact on the function of the protein, but they do not confirm that they have deleterious effects on neuronal function in *vivo* in people that carry them. However, these results are consistent with...
previous findings showing that inherited variants of SHANK2 and SHANK3 cause synaptic defects in vitro [7,69,70]. Recently, Berkel et al. 2011 showed that two inherited (L1008_P1009dup, T1127M) and one de novo (R462X) SHANK2 mutations identified in patients with ASD affect spine volume and reduced Shank2 cluster sizes [70]. This deleterious effect was also observed in vivo since mice expressing rAAV-transduced Shank2-R462X present a specific long-lasting reduction in miniature postsynaptic AMPA receptor currents [70].

In patients, the only feature associated with carriers of SHANK2 mutations compared with other patients was a trend for low IQ (P = 0.025, OR = 3.75, 95% CI = 1.1–20.0) (Table S8). But, as observed for SHANK3 mutations, this correlation could differ from one individual to another (i.e. the patient with a SHANK2 de novo stop mutation reported by Berkel et al. 2010 presented with high-functioning autism [26]).

Our result also showed that potentially deleterious SHANK2 variants were detected in a heterozygous state in parents and in the general population without causing severe phenotypic consequences. Indeed, we showed that almost 5% of the Finnish population is heterozygous for the SHANK2 R818H variation, which modifies a conserved amino acid and is associated with
lower synaptic density in vitro. Furthermore, we identified a SHANK2 splice site mutation in a control female without any apparent psychiatric disorders. Similarly, two frame-shift mutations and one splice site mutation of SHANK2 are listed in dbSNP and in the 1000 genomes project [71]. These nonsense variants should be interpreted with caution since none of them has been validated by Sanger sequence technology. Taken together, variants affecting conserved amino acids of SHANK2 might act as susceptibility variants for ASD, but, in some cases, additional genetic, epigenetic or environmental factors seem to be necessary for the emergence of the disorder.

Additional CNVs in subjects with SHANK2 mutations may modulate the risk for ASD

In order to detect risk and protective genetic factors, we analyzed the CNV burden of the individuals carrying deleterious variations of SHANK2. Notably, the three ASD patients with de novo SHANK2 deletions also carried CNVs on chromosome 15q11–q13, a region associated with ASD [43,47,48,50–52,72]. In contrast, the patient reported by Berkel et al. 2010, who did not meet all the diagnostic criteria for ASD, seemed to have no CNV at chromosome 15q [26]. Although the probability to observe the co-occurrence of a de novo SHANK2 deletion and a duplication of CHRNA7 at 15q is very low, two of the three patients carrying a de novo SHANK2 deletion also carried the CHRNA7 duplication. While the numbers are small, this finding could suggest epistasis between these two loci. The role of CHRNA7 in ASD was recently supported by the observation of low levels of CHRNA7 mRNA in the post-mortem brain from patients with ASD [73]. Interestingly, it was also found that, in contrast to the gene copy number, the transcript levels CHRNA7 were reduced in neuronal cells [74] or brain samples with maternal 15q duplication [75]. Finally, functional studies have shown that NLGN and NRXXN, which belong to the same synaptic pathway, are key organizers of the clustering of nicotinic receptors at the synapse [76–78]. Therefore the co-occurrence of a deletion of SHANK2 and a duplication of the nicotinic receptor CHRNA7 could act together within the same pathway to increase the risk of ASD in patients AL038_3 and 5237_3. In patient 6319_3 carrying the BP1–BP2 deletion, several genes might also play a role in the susceptibility to ASD. Among them, NIPA1 and TUBGCP5 encoding a magnesium transporter and a tubulin gamma associated protein, respectively, are highly expressed in the brain. However, the most compelling candidate in the detected region is CYFIP1 [45,53], which codes for a binding partner of FMRP, the protein responsible for fragile X syndrome. Both CYFIP1 and FMRP are involved in the repression of synaptic translation [79], one of the major biological mechanisms associated with ASD [80]. Therefore, the co-occurrence of a loss of one copy of SHANK2 and CYFIP1 might increase the risk of abnormal synaptic function in patient 6319_3.

If some individuals have a higher risk to develop ASD when a deleterious SHANK2 variant is present, others individuals may experience a protective effect by additional genetic factors. For example, control SWE_Q56_508 carried a SHANK2 splice mutation, but clinical examination revealed no major disorders. In addition, this control individual also carried a partial duplication of SNTG2 and MYT1L. Based on a single control subject, it is not possible to formally prove that these additional hits at SNTG2 and/or MYT1L acted as suppressor mutations, counteracting the phenotypic effects of the SHANK2 splice mutation. However, the encoded proteins may interact with the NRXXN-NLGN-SHANK pathway. Both SNTG2 and SHANK2 are scaffolding proteins localized in actin rich structures [81–83] and bind directly to neuroligins [66]. Furthermore, mutations of NLGN3/4X identified in patients with ASD decrease their protein binding to SNTG2 [66]. In addition, MYT1L is a myelin transcription factor that is sufficient, with only two other transcription factors, ASCL1 and BRN2, to convert mouse embryonic and postnatal fibroblasts into functional neurons in vitro [67]. Therefore, alterations of SNTG2 and/or MYT1L might modulate synapse physiology and counteract the effect of the SHANK2 splice site mutation. We recently highlighted the key role of synaptic gene dosage in ASD and the possibility that a protein imbalance at the synapse could alter synaptic homeostasis [6]. In the future, animal models should be developed to test whether the effect of a primary mutation in a synaptic protein complex (e.g. Shank2), can be reduced or suppressed by a second mutation (e.g. Sntg2 or Mytl1). A similar suppressor effect has been demonstrated by the decrease of abnormal behavior of the Fmr1 mutant mice carrying a heterozygous mutation of the metabotropic glutamate receptor mGlur5 [84].

Conclusions and perspectives

In summary, we confirmed that de novo SHANK2 deletions are present in patients with ASD and showed that several SHANK2 variants reduce the number of synapses in vitro. The genomic profile of the patients carrying deleterious de novo SHANK2 deletions also points to a possible genetic epistasis between the NRXXN-NLGN-SHANK pathway and 15q11–q13 CNVs. CHRNA7 and CYFIP1 were already proposed as susceptibility genes for neuropsychiatric disorders [43,45,49,51], and our study provides additional support for this association. Therefore, as previously observed for ID [85], our results suggest that the co-occurrence of de novo mutations, together with inherited variations might play a role in the genetic susceptibility to ASD. Finally, our analyses suggest the interesting possibility that deleterious mutations of neuronal genes (e.g. SNTG2 and MYT1L) could potentially counteract the effect of synaptic deleterious mutations (e.g. SHANK2). The identification of risk and protective alleles within the same subject is one of the main challenges for understanding the inheritance of ASD. Initial results from the 1000 genomes project has estimated that, on average, each person carries approximately 250 to 300 loss-of-function variants in annotated genes and 50 to 100 variants previously implicated in inherited disorders [71]. To date, it is not clear how many loci can regulate synaptic homeostasis and how these variants interact with each other to modulate the risk for ASD [6]. A better knowledge of these genetic interactions will be necessary to understand the complex inheritance pattern of ASD.

Materials and Methods

Ethics statement

This study was approved by the local Institutional Review Board (IRB) and written inform consents were obtained from all participants of the study. The local IRB are the “Comité de Protection des Personnes” (Ile-de-France Hôpital Pitié-Salpêtrière Paris) for France; the Salighrenska Academy Ethics committee, University of Gothenburg for Sweden; the local IRB of the medical faculty of JW Goethe University Frankfurt/Main for Germany; the Committee #3 of the Helsinki University Hospital, Finland; the “Comitato Etico IRCCS Fondazione Stella Matris” at Stella Maris Institute, Calambrone (Pisa), Italy; the “Comitato Etico Azienda Ospedaliera-Universitaria Policlinico-Vittorio Emanuele”, Catania, Italy.

Patients

Patients with ASD and analyzed for CNV analysis and/or mutation screening are presented in Table S1. Patients were
recruited by the PARIS (Paris Autism Research International Silhouette) study at specialized clinical centers disposed in France, Sweden, Germany, Finland, UK. The Autism Diagnostic Interview-Revised (ADI-R) and Autism Diagnostic Observation Schedule (ADOS) were used for clinical evaluation and diagnosis. In Sweden, in some cases, the Diagnostic Interview for Social and Communication Disorders (DISCO-10) was applied instead of the ADI-R. Patients were included after a clinical and medical check-up with psychiatric and neuropsychological examination, standard karyotyping, fragile-X testing and brain imaging and EEG whenever possible. All patients were from Caucasian ancestry.

The patient AU038_3 with a de novo SHANK2 deletion is an 11.05 year-old boy diagnosed with autism and moderate ID (Table S2). He was the only child of non-consanguineous parents from European descent. His parents had no relevant personal and familial history of psychiatric or medical illness. He was born at 40 weeks of gestation, after normal pregnancy and delivery. Birth weight, length and occipitofrontal head circumference were 2500 g (5th percentile), 48 cm (22nd percentile) and 31 cm (2nd percentile), respectively. Apgar scores were 7 and 10 at 1 and 5 minutes, respectively. In the first year of life, the pediatrician reports did not mention signs of hypotonia. At 2 months, he was operated for an inguinal hernia. Motor acquisition was apparently normal (sitting at 6 months), but with a late acquisition of walking, at 18 months. Speech was severely delayed, without any apparent regressive phase. Only a few words and sentences appeared when he was 4 y and 6.5 y, respectively. His expressive language remained limited to restrictive sentences, mainly dyssyntactically. A formal diagnostic assessment for autism was performed when he was 11 years old. The scores of the Autism Diagnostic Interview-Revised (ADI-R) domains were: social 24, communication 23, and behavior 6 (cut-offs for autism diagnosis are 10, 8 (verbal autism) and 3, respectively); the age at first symptoms was before 36 months. Cognitive evaluation with the Kaufman Assessment Battery for Children (K-ABC) showed moderate intellectual deficit (composite score 40). He required assistance with basic activities such as eating and dressing. At examination, he had a normal facial appearance, with a prominent chin. General and neurological examinations were normal, except for hypermetropia and astigmatism. High-resolution karyotype, fragile-X testing and brain imaging and EEG were all normal. No significant epileptic event was reported on the electroencephalogram.

The two male patients with de novo SHANK2 deletions reported by Pinto et al. 2010 [9] (5237_3 and 6319_3) shared several clinical features with patient AU038_3. Patient 5237_3 is a Canadian subject diagnosed with autism (based on ADI-R and ADOS) associated with below average non verbal IQ (IQ <1st percentile) and language (<1st percentile). He had minor dysmorphic features including 5th finger clinodactyly and several curled toes, and no history of epilepsy. Patient 6319_3 was recruited in the same geographic area as patient AU038_3 (Grenoble, France) and was clinically diagnosed with PDD-NOS. The ADI-R scores were: social 14, communication 8, behaviors 2 (cut-off for autism: 3); with an age at first symptoms <36 months. He had mild ID as evaluated with the WISC-III (full scale IQ 60, performance IQ 60, verbal IQ 67). His language was delayed (first words 24 m, first sentences 48 m), but functional. He had no history of regression or epilepsy. The physical exam was normal, except for large and prominent ears and flat feet; the neurological exam was also normal. Similarly to patient AU038_3, he had hypermetropia.

The control female carrying the splice site mutation (IVS22+1G>T) was part of a cohort of 172 females recruited for a study on obesity, anthropometrics, and cardiovascular risk factors [37]. In addition, these women were assessed for axis I psychiatric disorders and for personality traits using the Temperament and Character Inventory (TCI) [30] and the Karolinska Scales of Personality (KSP) [39]. This subject had no psychiatric disorders and her TCI and KSP scores were similar to those found in the general population.

Genomic structure and transcripts analysis of SHANK2

To define the genomic structure of the human SHANK2 gene, we used the two reference sequence genes from UCSC (NM_012309 and NM_133266), one human mRNA from GenBank (DQ132234) and three Rattus reference sequence genes from UCSC (NM_201389, NM_133441 and NM_133440). SHANK2 is transcribed in four isoforms described in GenBank (AB208025, AB208026, AB208027 and AF141901) and is composed of 25 exons. Transcript analysis of SHANK2 was performed in human brain regions from four independent controls (two females and two males) and in human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas and B lymphoblastoid cell lines) using the Clontech Multiple Tissue cDNA panel (Clontech). Total RNA was isolated from control human brain tissues by the acid guanidinium thiocyanate phenol chloroform method and reverse transcribed by oligoDT priming using SuperScript II Reverse Transcriptase (Invitrogen). The PCR was performed with HotStar Taq polymerase (Qiagen) and the protocol used was 95°C for 15 min, followed by 40 cycles at 95°C for 30 sec, 55 to 58°C for 30 sec, 72°C for 30 sec to 1 min, with a final cycle at 72°C for 10 min. PCR primers were designed to detect the ANK domain, the SH3 domain, the PDZ domain, and the SAM domain in order to distinguish the four SHANK2 isoforms and are indicated in Table S11. All RT-PCR products were directly sequenced. The expression of SHANK2E isoform was also studied by SYBR-Green real-time PCR approach. The fluorescence was read with the Applied Biosystems 7500 Real-Time PCR System. Each assay was conducted in three replicates. GAPDH was used for the ΔCt calculation and total brain was used as the reference for relative quantification calculation (RQ). The relative RQ of transcripts was calculated as 2^{-ΔΔCt} with the magnitude of upper error as 2^{-ΔΔCt+SEM, 2^{-ΔΔCt}−SEM} and the magnitude of lower error as 2^{-ΔΔCt−SEM, 2^{-ΔΔCt}+SEM}. The primers specific to SHANK2E isoform are indicated in Table S11. In situ hybridization was performed essentially as described previously [28]. Transcripts encoding the different ProSAP1/Shank1 cDNAs (ProSAP1/Shank1 starting with the PDZ domain, ProSAP1, starting with the SH3 domain and ProSAP1E/Shank2E, starting with the ankyrin repeats) were detected with isoform specific S35 labeled cDNA antisense oligonucleotides purchased from MWG-Biotech (Ebersberg, Germany) directed against the ATG regions of the different mRNAs. All variants were evaluated for potential pathogenicity using the HumDIV method for rare alleles of PolyPhen2 [41].

CNV detection and validation

DNA was extracted from blood leukocytes or B lymphoblastoid cell lines. The SHANK2 CNV was detected with the Illumina Human 1M-Duo BeadChip, which interrogates 1 million SNPs distributed over the human genome. For the Swedish control SWE_Q56_508 carrying the SHANK2 splice mutation we used the Illumina Human Omni2.5 BeadChip array. The genotyping was performed at the Centre National de Génotypage (CNG) and the Institut Pasteur. Only samples that met stringent quality control (QC) criteria were included: call rate ≥99%; high confidence score
log Bayes factor ≥15; standard deviation of the log R ratio (LRR) ≤0.35 and of the B allele frequency (BAF) ≤ 0.13; number of consecutive probes for CNV detection ≥5; CNV size ≥1 kb. When the QC criteria were met, we used two CNV calling algorithms, QuantiSNP [86] and PennCNV [87], and the CNV viewer, SnapPeep (http://snappeep.sourceforge.net/). To obtain high-confidence calls, the CNVs identified by QuantiSNP were validated by visual inspection of the LRR and BAF values. PennCNV was used to confirm inheritance status of the resulting CNV calls. CNVs were validated by quantitative PCR analysis using the Universal Probe Library (UPL) system from Roche. UPL probes were labeled with FAM and the fluorescence was read with the Applied Biosystems 7500 Real-Time PCR System. Each assay was conducted in four replicates for target region probe-set and control region probe-set. Relative levels of region dosage were determined using the comparative CT method assuming that there were two copies of DNA in the control region. The relative copy number for each target region was calculated as $2^{-\Delta CT}$ with the magnitude of upper error as $2^{-\Delta CT-\text{SEM}}$ and the magnitude of lower error as $2^{-\Delta CT+\text{SEM}}$. UPL probes and primers are indicated in Table S12. For comparisons between patients and controls, statistical significance for each CNV was assessed using a 2-sided Fisher’s exact test.

### Mutation screening

The 24 coding exons of SHANK2 were amplified and sequenced for mutation screening. The PCR was performed on 20–40 ng of genomic DNA template with HotStar Taq polymerase from Qiagen for all exons the protocol used was 95°C for 15 min, followed by 35–40 cycles at 95–97°C for 30 sec, 55–62°C for 30 sec, 72°C for 30 sec to 90 sec, with a final cycle at 72°C for 10 min. Sequence analysis was performed by direct sequencing of the PCR products using a 373A automated DNA sequencer (Applied Biosystems). Genotyping of R185Q, V717F, A729T, R818H, G1170R, D1535N and L1722P was performed by direct sequencing or Taqman SNP Genotyping Assays system from Applied Biosystems designed with Custom TaqMan Assay Design Tool. All primers are indicated in Table S9. Enrichment of SHANK2 variations in the ASD sample compared with controls was assessed using a 1-sided Fisher’s exact test (hypothesizing that cases will show an excess of SHANK2 variants compared to controls).

### In vitro mutagenesis and transfection studies in hippocampal neurons

Rat GFP-ProSAP1A/Shank2A cDNA was mutated according to the human mutations using the site directed mutagenesis kit (Stratagene). The mutagenesis primers were listed in Table S10. We have tested all the variants (n = 16) identified in our first screen of 230 patients with ASD and 230 controls: 5 were detected only in patients (V717F, A729T, G1170R, D1535N and L1722P), 6 were detected in patients and controls (S557N, R569H, K780Q, R818H, Y967C and P1586L) and 5 were only found in controls (L629P, A822T, V823M, R1290W and Q1308R). All mutated amino acids were conserved among human, rat and mouse ProSAP1/Shank2. All cDNAs were sequenced and subsequently tested for expression by Western blot analysis. After expression of the constructs in Cos7 cells, the cell homogenate was separated on a gel, transferred to a nitrocellulose membrane and subsequently protein bands were detected using a rabbit anti-GFP antibody. Thereafter, the cDNAs were transfected into primary hippocampal neurons. Cell culture experiments of rat hippocampal primary neurons (embryonic day 18–21: E18-21) were performed as described previously [88]. In brief, after preparation, hippocampal neurons were seeded on poly-L-lysine (0.1 mg/ml; Sigma-Aldrich, Steinheim, Germany) coated coverslips at a density of 4 × 10^4 cells/well (transfection experiments) or 2 × 10^5 cells/well (immunohistochemical staining). Cells were grown in Neurobasal medium (Invitrogen, Karlsruhe, Germany), complemented with B27 supplement (Invitrogen), 0.5 mM L-glutamine (Invitrogen), and 100 U/ml penicillin/streptomycin (Invitrogen) and maintained at 37°C in 5% CO₂. Hippocampal cells were transfected using Lipofectamine 2000, according to the manufacturer’s recommendation (Invitrogen). Fluorescence images were obtained using a camera attached to a fluorescence microscope. For immunohistochemical staining, the primary cultures were fixed with ice cold 4% paraformaldehyde/1.5% sucrose/PBS for 20 min at 4°C and processed for immunohistochemistry. After washing three times with 1× PBS for 5 min at room temperature the cells were permeabilized for 3 min on ice in a buffer containing 0.1% Triton X-100/0.1% Na-Citrate/PBS and washed again three times with 1× PBS. Blocking was performed with 10% fetal calf serum/PBS for 1 h at room temperature followed by incubation with the primary antibody (mouse anti-Bassoon) overnight at room temperature. After a further washing-step the cells were incubated with the secondary antibody coupled to Alexa555 (red) (Molecular Probes, Invitrogen) for 90 min at room temperature, washed first with 1× PBS and then with aqua bident for 5 min and mounted in Moviol (with or without DAPI for staining of the nucleus).

### Image acquisition and quantification

In morphological studies, dendrites were considered primary when processes extended directly from the cell body, and secondary when processes branched off primary dendrites. Twenty transfected neurons were chosen randomly for quantification from at least three independent experiments for each construct. Morphometric measurements were performed using Axiovision Zeiss microscope and Axiowire software with a 40× magnification. For the quantification of excitatory synapse number, cells were counterstained with anti-Bassoon antibodies. From randomly chosen transfected neurons, Bassoon-positive spots from primary dendrites were counted and the length of dendrites was measured. The total number of spines was expressed as density per 10 μm length of dendrite. Measured data were exported to Excel software (Microsoft), and the data of each variant were compared by using the Mann-Whitney U test. The comparisons of synaptic density for each phenotypic or conservation categories were performed using the Student’s t test.

### Supporting Information

#### Figure S1

**SHANK2E expression in human multiple tissue panel and in rat embryos.** A. Quantitative RT-PCR in human tissues. Primers and probe were designed to detect SHANK2E isofrom. GAPDH was used for the ΔCt calculation and total brain was used as the reference for relative quantification calculation (RQ ± SEM). B. In situ hybridization of rat fetus sagittal sections with ProSAP1/Shank2 isoform specific oligonucleotides. The ProSAP1/Shank2 isoform starting with the PDZ domain is solely expressed in brain, brain stem and medulla. The same holds true for the ProSAP1E/Shank1E isoform that starts with the SH3 domain. In some sections bone tissue also gave some moderately positive signals. The expression of ProSAP1E/Shank1E (with the ankyrin
repeats) is especially seen in the liver and some glandular tissue. In the brain, the ProSAP1A/SHANK2 mRNA is only detectable within the cerebellum (arrow).

**Figure S2** Characterization and validation of the SHANK2 CNV in family AU038. A. Pedigree of the AU038 family showing that the deletion is de novo on the maternal chromosome. SNPs were genotyped using the Illumina 1M duo array. B. SHANK2 CNV validation by quantitative PCR of exon E4–E6, E15–E17 of SHANK2 using genomic DNA from the father, mother and the proband of family AU038. Results from QPCR analysis confirmed that the deletion is de novo and removes exon E5 to Exon E16. Bars represent mean of RQ ± SEM.

**(TIF)**

**Figure S3** Pedigree of families with ASD carrier of non-synonymous SHANK2 mutations. Variations specific to ASD or shared by ASD and controls are indicated in red and orange, respectively. Clinical phenotypes are specified in the figure.

**(TIF)**

**Figure S4** Functional analysis of shank2 mutations in cultured hippocampal neurons. A. Rat hippocampal neurons transfected with wild-type or mutated ProSAP1A/SHANK2-EGFP cDNA constructs were detected by EGFP expression. B. The quantification of dendrite number was performed on 20 transfected hippocampal neurons per construct from at least 3 independent experiments. No mutation affected the number of dendrites per neuron (Bars represent mean ± SD, Mann-Whitney U test and Kolmogorov-Smirnov Z test; no significant differences; nWT = 20, nmut = 20). Western blot analysis of ProSAP1A/SHANK2-EGFP cDNA constructs revealed similar sizes of WT fusion ProSAP1A/SHANK2-EGFP protein and the constructs carrying the mutations. C. The variants identified in patients with ASD were associated with reduced synaptic density compared with those identified in controls. D. The variants affecting conserved amino acids in other SHANK proteins were associated with reduced synaptic density compared with those identified in patients with ASD and controls. We used the Wilcoxon test for the continuous traits and the Fisher’s exact test (2-sided) for the discontinuous traits. OR is given with 95% confidence interval. OR, odds ratio; P, p-value; ADI-R, Autism Diagnosis Interview revised.

**(DOC)**

**Table S7** Distribution of SHANK2 variants affecting conserved or non conserved amino acids. All variants came from this study and from Berkel et al. 2010 (24). *Several variants shared by patients with ASD and controls were identified in both studies.

**(DOC)**

**Table S8** Clinical comparison of patients with ASD carrying SHANK2 variants with the rest of the cohort of patients. We used the Wilcoxon test for the continuous traits and the Fisher’s exact test (2-sided) for the discontinuous traits. OR is given with 95% confidence interval. OR, odds ratio; P, p-value; ADI-R, Autism Diagnosis Interview revised.

**(DOC)**

**Table S9** Primers used for mutation screening.

**(DOC)**

**Table S10** Primers used for in vitro mutagenesis.

**(DOC)**

**Table S11** Primers used for mRNA analysis of SHANK2 isoforms. * Primers were used for relative quantification study of SHANK2 isoform. The other primers were used for RT-PCR analysis of each SHANK2 isoform.

**(DOC)**

**Table S12** Primers used for CNV validation.

**(DOC)**

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**Author Contributions**

References


