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SYN1 loss-of-function mutations in autism and partial epilepsy cause impaired synaptic function

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Several genes predisposing to autism spectrum disorders (ASDs) with or without epilepsy have been identified, many of which are implicated in synaptic function. Here we report a Q555X mutation in synapsin 1 (*SYN1*), an X-linked gene encoding for a neuron-specific phosphoprotein implicated in the regulation of neurotransmitter release and synaptogenesis. This nonsense mutation was found in all affected individuals from a large French-Canadian family segregating epilepsy and ASDs. Additional mutations in *SYN1* (A51G, A550T and T567A) were found in 1.0 and 3.5% of French-Canadian individuals with autism and epilepsy, respectively. The majority of these *SYN1* mutations were clustered in the proline-rich D-domain which is substrate of multiple protein kinases. When expressed in synapsin I (SynI) knockout (KO) neurons, all the D-domain mutants failed in rescuing the impairment in the size and trafficking of synaptic vesicle pools, whereas the wild-type human SynI fully reverted the KO phenotype. Moreover, the nonsense Q555X mutation had a dramatic impact on phosphorylation by MAPK/Erk and neurite outgrowth, whereas the missense A550T and T567A mutants displayed impaired targeting to nerve terminals. These results demonstrate that *SYN1* is a novel predisposing gene to ASDs, in addition to epilepsy, and strengthen the hypothesis that a disturbance of synaptic homeostasis underlies the pathogenesis of both diseases.

INTRODUCTION

Autism spectrum disorders (ASDs) are a heterogeneous group of disorders affecting approximately 1 in 1000 children. These diseases are characterized by impaired social relationships, rigid and repetitive behavior, restricted interests and abnormal language development (1). An important genetic contribution has been consistently observed for ASDs, as demonstrated by the 25-fold greater risk of recurrence in siblings than in the general population (2), and the high concordance rate (70–90%) of monozygotic twins compared with dizygotic twins (0–10%) (3,4). Overall, genetic epidemiology studies suggest an interaction between several epistatic genes (polygenic inheritance), although the mechanism of inheritance of ASDs remains largely unknown (5). The male:female ratio is estimated at 4:1, which may be partly explained by X-linked inheritance in some families (5–7).

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Linkage studies allowed the identification of numerous susceptibility loci for ASDs, but only a small fraction of these positive findings have been replicated in independent studies (5). In turn, the search for rare genetic variants having a more dramatic contribution to the ASD phenotype was found to be more successful, including the identification of an increasing number of *de novo* mutations (5,8). Among these methodological approaches, the search for chromosomal deletions and duplications, combined with resequencing of candidates genes mapping to these regions, allowed the identification of several predisposing genes for the disease, including genes coding for the postsynaptic proteins neuroligins 3 and 4 (*NLGN3*, *NLGN4*), their cytoplasmic interactors, *SHANK2* and *SHANK3*, as well as their presynaptic partner neurexin-1 (*NRXN1*) (6,9–16). Rare and common genetic variants in contactin-associated protein-like 2 (*CNTNAP2*), another member of the neurexin superfamily, are also associated with either ASDs or epilepsy (17–20). More recently, mutations in *IL1RAPL1* and *RIMS3/NIM3*, two additional synaptic genes, have been found to predispose to ASDs (7,21). Interestingly, most of the ASD-predisposing genes thus far identified encode synaptic proteins, including adhesion molecules of the synaptic cleft or cytoplasmic molecules that associate with synaptic receptors, leading to the hypothesis that ASDs are due to abnormal synaptic function and/or neural connectivity in the time window in which neuronal circuits are remodeled by experience (8,22).

Epileptic seizures are observed in up to one-third of ASD individuals (1,23). Conversely, autistic features are commonly observed in severe forms of epilepsy associated with mental retardation (24). The frequent association between epileptic and autistic phenotypes suggests that these diseases may share common predisposing genes. Most of the known epilepsy-predisposing genes implicate voltage-gated or ligand-gated ion channels (25). Defects in synaptic proteins implicated in neurotransmitter release and synaptic vesicle (SV) trafficking are also interesting in this respect, as suggested by the epileptic phenotype of mouse models with mutations in *SYN1*, *SYN2* and *SV2A* (25–29) encoding the SV proteins synapsins I/II (SynI/II) and SV2A. However, the only mutation potentially causing a defect in SV trafficking and neurotransmitter release in humans was described by Garcia *et al*. (30), who reported a W356X mutation in *SYN1* in a family segregating a mixture of neurodevelopmental disorders associated with epilepsy. Synapsins are a family of neuron-specific SV phosphoproteins implicated in synaptic transmission and plasticity. Synapsins regulate SV trafficking between the reserve pool (RP) and the readily releasable pool (RRP) and participate in the regulation of SV availability for release and in short-term plasticity. Synapsins have also been implicated in neuronal development, synaptogenesis and maintenance of mature synapses (31).

We have identified one nonsense and three missense mutations in *SYN1* associated with ASDs, epilepsy, or both. Three out of four mutations were clustered in the SynI D-domain and were associated with functional defects in nerve terminal function. These results demonstrate that *SYN1* is a novel predisposing gene to ASDs, in addition to epilepsy, and provide further support to the 'synaptic autism pathway'.

RESULTS

Identification of SYN1 mutations in individuals with epilepsy and autism

We identified a large French-Canadian family segregating complex partial epilepsy over four generations with a pattern compatible with a recessive X-linked transmission (Fig. 1A). We scanned the X chromosome by using 10 evenly distributed microsatellite markers and found evidence for linkage on chromosome Xp11–q21, with a maximum LOD score of 2.53 at $\theta = 0$ for marker *DXS7132* (Supplementary Material, Table S1). Key recombinants allowed us to refine a candidate region of 27 cM between markers *DXS8042* and *DXS6799*, with a maximum LOD score of 3.23 for marker *DXS6949*. By sequencing candidate genes mapping to the minimum candidate interval, we identified a stop codon (Q555X) in *SYN1* in all affected individuals from the family (Fig. 1B). We sequenced *SYN1* coding regions in 85 additional individuals of French-Canadian origin with partial epilepsy and found two missense mutations affecting a total of three unrelated individuals, including the same missense mutation (A550T) in two unrelated epileptic individuals (Table 1). In addition to epilepsy, males carrying the Q555X mutation in *SYN1* also presented learning difficulties and low average IQ, including two individuals meeting criteria for ASDs (IV-01 and IV-02) (Supplementary Material, Table S2).

To validate the hypothesis that additional mutations in *SYN1* could be associated with ASDs with reduced penetrance, we first sequenced the complete open reading frame of this gene in 191 individuals (189 males, 2 females) from our large cohort of autistic individuals. We identified missense variants in three ASD individuals (Supplementary Material, Tables S1 and S2): A51G and A550T, previously identified in the epileptic cohort, and T567A. Interestingly, an affected individual carried two missense variants (A51G and T567A), which may result in an epistatic effect on synapsin dysfunction. Two missense variants (A550T, T567A), as well as the nonsense mutation (Q555X), were located in exon 12 of the *SYN1* gene. We therefore sequenced this exon in 150 additional ASD cases and identified the A550T variant in another affected individual. In total, the A550T variant was found in four affected individuals: two with epilepsy, one with ASD and another one with ASD and epilepsy (Table 1). Because all the individuals ($n = 4$) with epilepsy or ASDs (or both) carrying the A550T variant are of French-Canadian origin, we suspected a founder mutation. Consistent with this hypothesis, we confirmed significant haplotype sharing by genotyping 10 polymorphic microsatellite markers spanning 9 Mb in the vicinity of the *SYN1* gene (Supplementary Material, Table S3). Finally, the A51G and T567A variants were also found in two individuals with either ASD or epilepsy, but from various ethnic origins. No significant haplotype sharing was found between these individuals (Supplementary Material, Table S3). The missense and nonsense variants in *SYN1* were not detected in at least 418 control chromosomes. We also sequenced all the *SYN1* coding regions in 190 control individuals (101 females, 89 males), for a total of 291 control chromosomes. We did not find any rare variant in the controls. Statistical analysis has shown that the excess of rare missense variants in our ASD cohort is significant

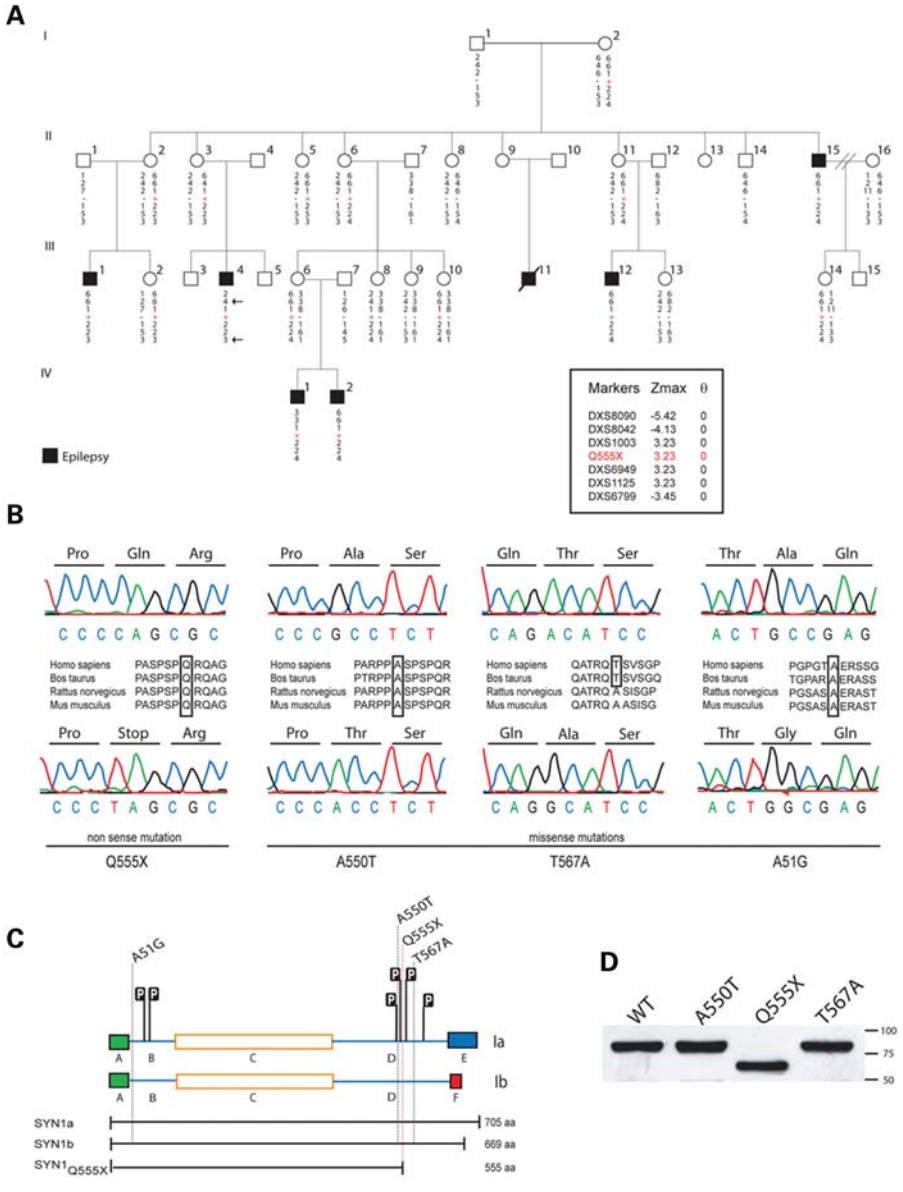

Figure 1. Nonsense and missense mutations identified in *SYN1*. (**A**) A large French-Canadian family with X-linked epilepsy was analyzed. Alleles were numbered according to their respective size corresponding to the CEPH genotyping database. The maximum LOD scores obtained with these markers and for the Q555X mutation (+) are shown in the lower right. Key recombinants are indicated by arrows. (**B**) Sequence analyses showing the Q555X, A51G, A550T and T567A mutations in *SYN1*. (**C**) Domain model of hSynI showing the location of the phosphorylation sites (P) and of the mutations identified in this study. In the figure, highly conserved domains are depicted as thick boxes. Three out of the four mutations associated with autism and epilepsy reside in the D-domain of the protein. (**D**) Expression of D-domain hSynI mutants in COS-7 cells. Either WT-hSynI or its Q555X, A550T, T657A mutants were expressed in COS-7 cells transfected with a pcDNA3.1 vector containing the corresponding SynI cDNAs. Cells were harvested 48 h after transfection, and expression of recombinant SynI was analyzed by SDS–PAGE and immunoblotting with SynI-specific antibodies.

($P = 0.0261$). We are not aware of any affected sibling with ASDs in *SYN1* mutation carriers. In all these ASD individuals, the mutation has been transmitted by the mother.

The Q555X mutation dramatically affects the molecular interactions of the SynI C-terminal domain

Synapsins are composed of a mosaic of conserved (A–C, E) and individual domains (D, F–J) (31). Interestingly, three of the four mutations were clustered in the middle part of the D-domain of SynI (Fig. 1C and D) a multifunctional domain which associates with SVs (32), regulates presynaptic targeting (33), binds SH3 domains (34) and contains phosphorylation sites for CaMKII, MAPK/Erk and cdk5 which regulate SV trafficking (31,35). We first analyzed whether the mutations affected the ability of recombinant WT or mutant SynI DE-domains to associate with SVs and be phosphorylated. The Q555X mutation virtually abolished the DE-domain binding to SVs, whereas the missense mutations were ineffective (Fig. 2A). The Q555X mutation also abolished or dramatically reduced phosphorylation by CaMKII and Mapk/Erk, whereas phosphorylation of the A550T and T567A

Table 1. *SYN1* missense mutations in epileptic and autistic individuals

ID	Sex	Exon	ADNc	Protein	Phenotype
30904	F	1	c.152C>G	p.A51G	Epilepsy
26755	M	12	c.1648G>A	p.A550T	Epilepsy
14026	F	12	c.1648G>A	p.A550T	Epilepsy
23679	F	12	c.1648G>A	p.A550T	ASDs/epilepsy
17546	M	12	c.1648G>A	p.A550T	ASDs
17851	M	1	c.152C>G	p.A51G	ASDs
17851	M	12	c.1699A>C	p.T567A	ASDs
19718	M	12	c.1699A>C	p.T567A	ASDs

Three missense mutations were identified in a total of seven individuals affected with either epilepsy ($n = 3$), ASDs ($n = 3$) or both ($n = 1$). Individual 17851 carries two missense mutations on the same chromosome. All mutations were found in at least two affected individuals, suggesting either hotspots for mutation or a founder effect (see Supplementary Material, Table S3).

DE-domains by these kinases was virtually unaffected (Fig. 2B). No change in phosphorylation of mutant DE-domains by cdk5 was found (Supplementary Material, Fig. S1). We next tested the ability of mutant SynI expressed in COS-7 cells to bind to an array of SH3 domains that were previously shown to interact with the SynI D-domain (34). The D-domain contains a major canonical Class II motif ($Syn^{594-605}$) binding the SH3 domains of c-Src, PI3K, endophilin and intersectin, together with an additional consensus sequence for PLCγ located N-terminal of the major site. Although the missense mutations were not overlapping with any of the SH3-binding regions and did not alter the binding of either SH3 domain, the Q555X mutation abolished the interaction with all SH3 domains except for PLCγ, whose binding site is located N-terminal to the truncation (Fig. 2C).

SYN1 mutations affect neuronal development and nerve terminal targeting

The synapsins are implicated in early stages of neuronal development, and Syn knockout (KO) mice display delayed and/or abnormal neurite outgrowth (31,36). Although expression of WT-hSynI in SynI KO neurons did not enhance axon elongation, expression of Q555X-hSynI impaired axon elongation both at 3 DIV (Fig. 3) and at later stages of development (Supplementary Material, Fig. S2), whereas the missense mutations were ineffective. Neither WT-hSynI nor hSynI mutants affected dendritic arborization (not shown). We also studied the expression levels and nerve terminal targeting of SynI mutants in SynI KO hippocampal neurons. Both WT-hSynI and its mutants displayed a similar expression level (Supplementary Material, Fig. S3A) and presynaptic targeting based on co-localization with either bassoon (Fig. 4A) or synaptotagmin (Supplementary Material, Fig. S3B). Interestingly, although the presynaptic targeting of Q555X-hSynI was comparable with that of WT-hSynI, the presynaptic localization of A550T and T567A was significantly impaired (Fig. 4B).

SYN1 mutations disrupt SV pools

We then performed dynamic imaging of exo-endocytosis to assess the effects of the mutations on SV trafficking at the level of single synaptic boutons of SynI KO neurons. SynI deletion is known to be associated with a decrease in SV density in nerve terminals and an impaired SV availability for release (26,27,31). SynI KO hippocampal neurons were co-transfected with the Cherry variant of either WT or mutant hSynI and with synaptophysin-pHluorin (SypHy), a chimeric SV probe whose fluorescence is low in the acidic intravesicular environment and increases when exposed to the extracellular medium during exocytosis (37–39). To evaluate the effect of mutations on SV trafficking, stimulation protocols were applied to estimate the release from either the RRP (40 action potentials at 20 Hz) or the RP (900 action potentials at 20 Hz in the presence of bafilomycin) (Fig. 5A). Each experiment was followed by exposure to the alkaline agent NH_4Cl to quantify the total SV pool. Expression of WT-hSynI in KO neurons significantly increased the release from both the RRP and the RP, although expression of the A550T, T567A or Q555X hSyn mutant was virtually unable to correct the KO phenotype, indicating a complete loss of function (Fig. 5B–E). The effect was particularly intense for the Q555X mutant which further decreased the release from the RRP with respect to SynI KO neurons (Fig. 5B and D). Neither WT-hSynI nor hSynI mutants affected the resting levels of SypHy, although the total SypHy fluorescence determined by NH_4Cl was significantly lower in KO and Q555X-hSynI-expressing neurons (Fig. 5F and G).

DISCUSSION

We describe here a large French-Canadian family with epilepsy in males carrying a Q555X mutation in *SYN1*. Although the most robust phenotype in our family is epilepsy, which was found in virtually all the male mutation carriers, learning and behavioral disturbances were also common among these mutation carriers, including two males with overt ASD. By further screening affected individuals from our population, we have found that 1.0 and 3.5% of ASD and epilepsy cases, respectively, exhibit missense mutations in *SYN1*, including a founder mutation (A550T) found in the two cohorts. Both nonsense and missense mutations in *SYN1* found in our population lead to a loss of function. Overall, these results indicate that *SYN1* is a predisposing gene for ASDs and epilepsy, with a lower penetrance for the former phenotype. These observations are consistent with the mixture of phenotypes reported in the nine male carriers of the W356X mutation in SYN1 (30), which included epilepsy ($n = 8$), learning disorders ($n = 3$), episodic aggressive outbursts ($n = 3$) and autism ($n = 1$). Collectively, the two nonsense *SYN1* mutations identified so far are associated with ASDs in a total of three males among 15 mutation carriers (20%).

Several 'synaptic' ASD candidate genes have been identified, including NLGN3, NLGN4, SHANK2/3 and IL1RAPL1 encoding postsynaptic proteins, as well as NRXN1 and its homolog CNTNAP2 and RIMS3/NIM3, encoding presynaptic proteins (6,7,9–21,40). Although mutations in these genes account only for a small number of ASD cases, they led to the hypothesis that ASDs are due to abnormal synaptic function and/or neural connectivity in the

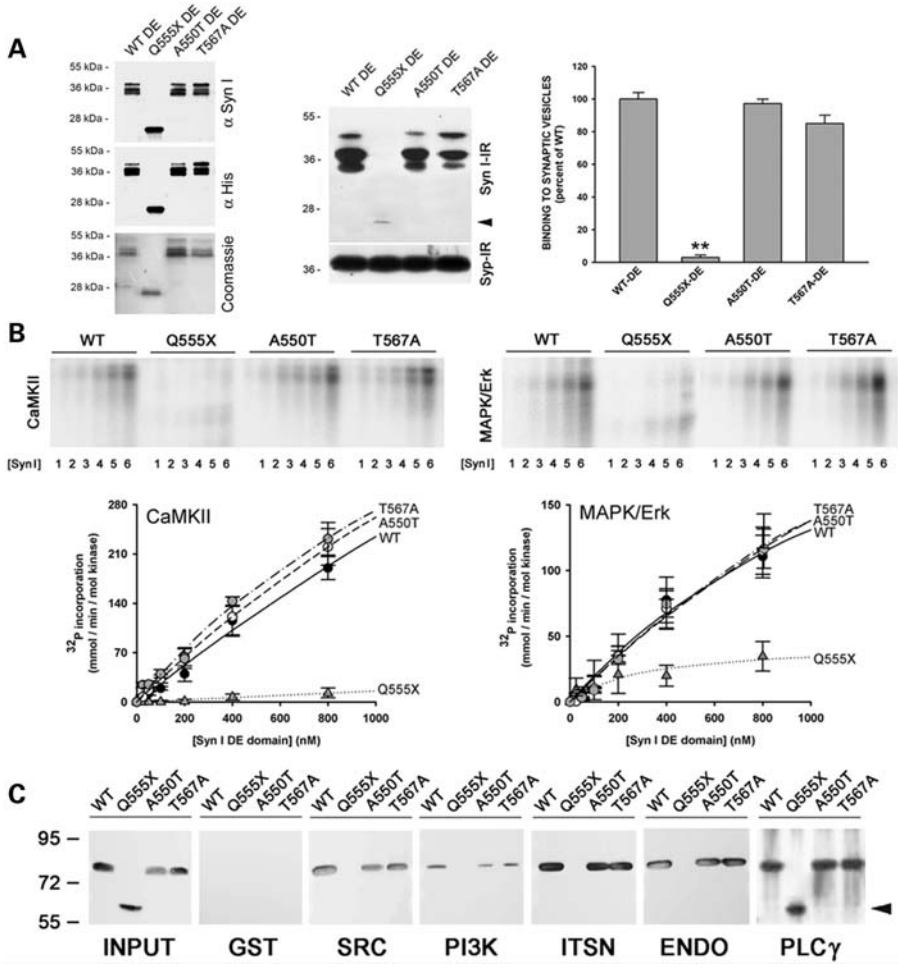

Figure 2. Biochemical characterization of hSynI mutants. (**A**) Binding of WT and mutant hSynI DE-domains to SVs. His-tagged hSynI DE-domains (600 nM) were incubated with SynI-depleted SVs (10 μg/sample), and the bound fraction was separated by high-speed sedimentation. Left panel: purified His-hSynI DE proteins added to the samples as revealed by anti-SynI/anti-His antibodies and Coomassie staining. Middle panel: corresponding anti-His immunoblot of bound hSynI DE proteins. Synaptophysin (syp) immunoreactivity is shown to monitor SV recovery in the pellets. Right panel: the binding of DE-domains is expressed as a percentage of the WT binding (means ± SEM; $n = 5$). $^{**}P < 0.01$; Bonferroni's multiple comparison test. (**B**) Phosphorylation of mutant hSynI DE-domains. Upper panels: representative autoradiograms from *in vitro* phosphorylation assays at increasing substrate concentrations (1, 25 nM; 2, 50 nM; 3,100 nM; 4, 200 nM; 5, 400 nM; 6, 800 nM) in the presence of either CaMKII (1 U/sample; left) or MAPK/Erk (0.1 U/sample; right). Lower panels: ^{32}P-incorporation into either WT (solid circle), Q555X (gray triangle), A550T (open circle) or T567A (gray circle) hSynI DE-domains by CaMKII (left) and MAPK/Erk (right). Data are means ± SEM ($n = 6$). Curves were fitted with one-site-binding isotherms, using Sigmaplot 10.0. (**C**) Binding of mutant hSynI to SH3 domains. Extracts from Cos-7 cells expressing either WT, Q555X, A550T or T567A hSynI, normalized by the amount of expressed SynI (input), were added to glutathione–Sepharose previously coupled with either GST alone or the indicated GST-SH3 domains. Bound proteins were eluted and analyzed by SynI immunoblotting. The arrowhead in (A) and (C) indicates the migration of the truncated mutant of hSynI.

time window in which neuronal circuits are extensively remodeled by experience (22,41,42). *SYN1* fully conforms to the requirements of the synaptic autism pathway, as it belongs to a family of synaptic genes essential for the regulation of synapse formation, excitation/inhibition balance and activity-dependent synaptic rearrangements.

Similar to the neuroligins, synapsins are not essential for exocytosis, but are believed to be important for synapse maturation and remodeling. SynI expression peaks with synaptogenesis *in vitro* and reaches a maximum 3–4 weeks after birth (31), a developmental period characterized by an extensive synaptic rearrangement. Consistent with this developmental pattern, epilepsy linked to SynI deletion in mice, as well as to the human mutations described here, is not present at birth but develops later in life, a temporal pattern that resembles their expression profile. This pattern is also shared by ASD manifestations which begin in the second to third year of life, a time window in which refinement, remodeling and experience-dependent plasticity of synapses and neuronal circuits are thought to take place (22). Interestingly, Syn KO mice exhibit various impairments in cognitive and social behavior that are compatible with an ASD phenotype in human (31,43,44).

All but one of the mutations reported here are clustered in the D-domain of hSynI. Although this domain is poorly conserved during evolution and across Syn isoforms, it is a target of multiple molecular interactions and signal transduction pathways (31). None of the identified mutations

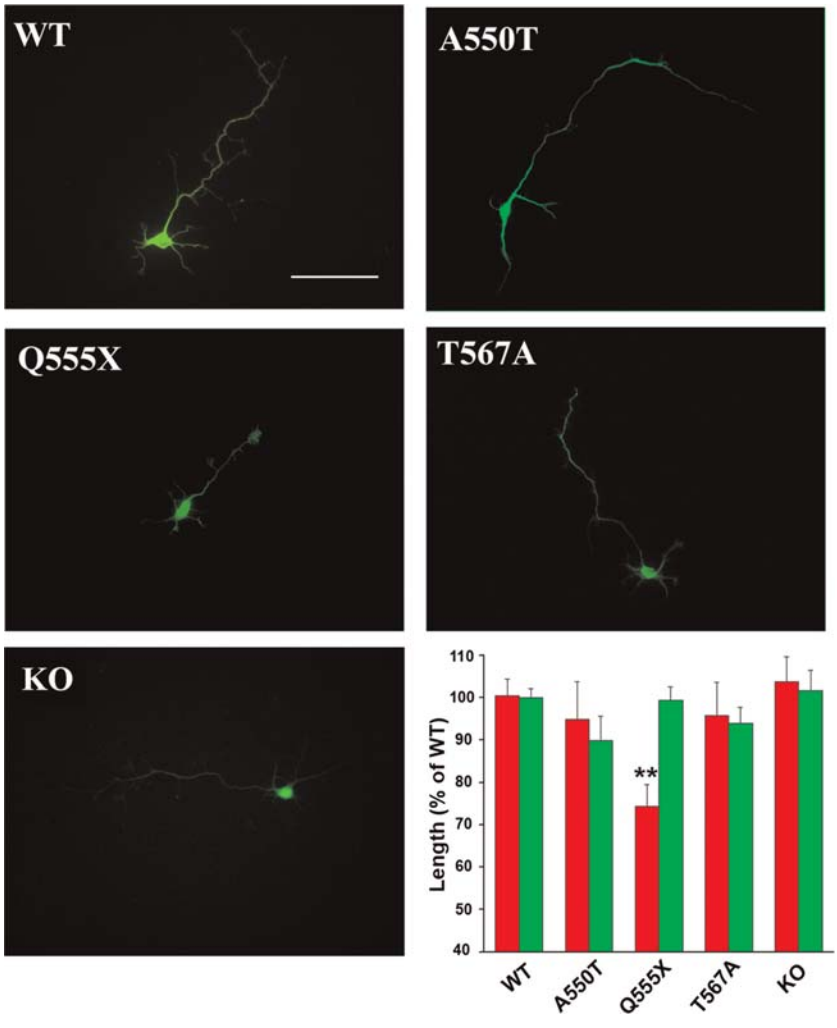

Figure 3. Effects of hSynI mutations on SynI KO hippocampal neurons at early stages of *in vitro* development. Representative images of SynI KO hippocampal neurons transfected at 0 DIV with either GFP-labeled WT, A550T, Q555X or T567A hSynI or with GFP alone (KO). Bar: 100 μm. The length of the axons and dendrites at 3 DIV was measured using the ImageJ software based on either GFP fluorescence (lower right panel) or soluble RFP (not shown), yielding virtually identical results. Axons were retrospectively identified as MAP-2-negative processes of characteristic morphology. Axon length was calculated by tracing the entire process, whereas dendritic arborization was calculated as the sum of the length of all dendrites per neuron. Within each experimental session, the length of processes measured in mutant SynI-transfected neurons was normalized to the average length of processes measured in neurons transfected with WT-SynI. The plot shows the mean length values ($\pm$SEM) of axons (red bars) and dendrites (green bars) calculated from $n = 150$ (WT), 35 (A550T), 62 (Q555X) and 50 (T567A) transfected neurons from $n = 6$ independent experiments. $^{**}P < 0.01$ versus WT; one-way ANOVA followed by Bonferroni's multiple comparisons test.

dramatically impacted with the targeting of mutant hSynI to nerve terminals. However, once it reached the nerve terminal, virtually all mutants displayed a clear neuronal phenotype with alterations in SV trafficking and pool sizes, which were particularly dramatic with the Q555X mutation. The release from both the RRP and the RP was significantly impaired with respect to the levels achieved by expression of WT-hSynI, with effects ranging from a loss of function of the missense mutations on both RRP and RP to a dramatic inhibition of the RRP release by the nonsense mutation, indicating a defect in the activity-dependent presynaptic function of hSynI. The unique phenotype of the Q555X mutation on the RRP suggests that the nonsense mutation has a strong impact on the post-docking steps of exocytosis.

These effects of the Q555X mutation can be explained by the combined losses of several key components of the protein, including: (i) the CaMKII phosphorylation sites, regulating SV availability for exocytosis in response to low stimulation frequencies (35); (ii) the E-domain, playing a key role in SV clustering and in post-docking steps of exocytosis (45–47) and (iii) the major SH3-binding domain for Src, PI3K, ITSN and endophilin which are potentially implicated in the SV reclustering after activity (34). In addition, the truncated D-domain displays an impaired SV binding, consistent with the loss of binding determinants (32), and loses its substrate properties for MAPK/Erk phosphorylation, although the phosphorylation site *per se* is not deleted by the truncation. As SynI phosphorylation by MAPK/Erk has been reported to

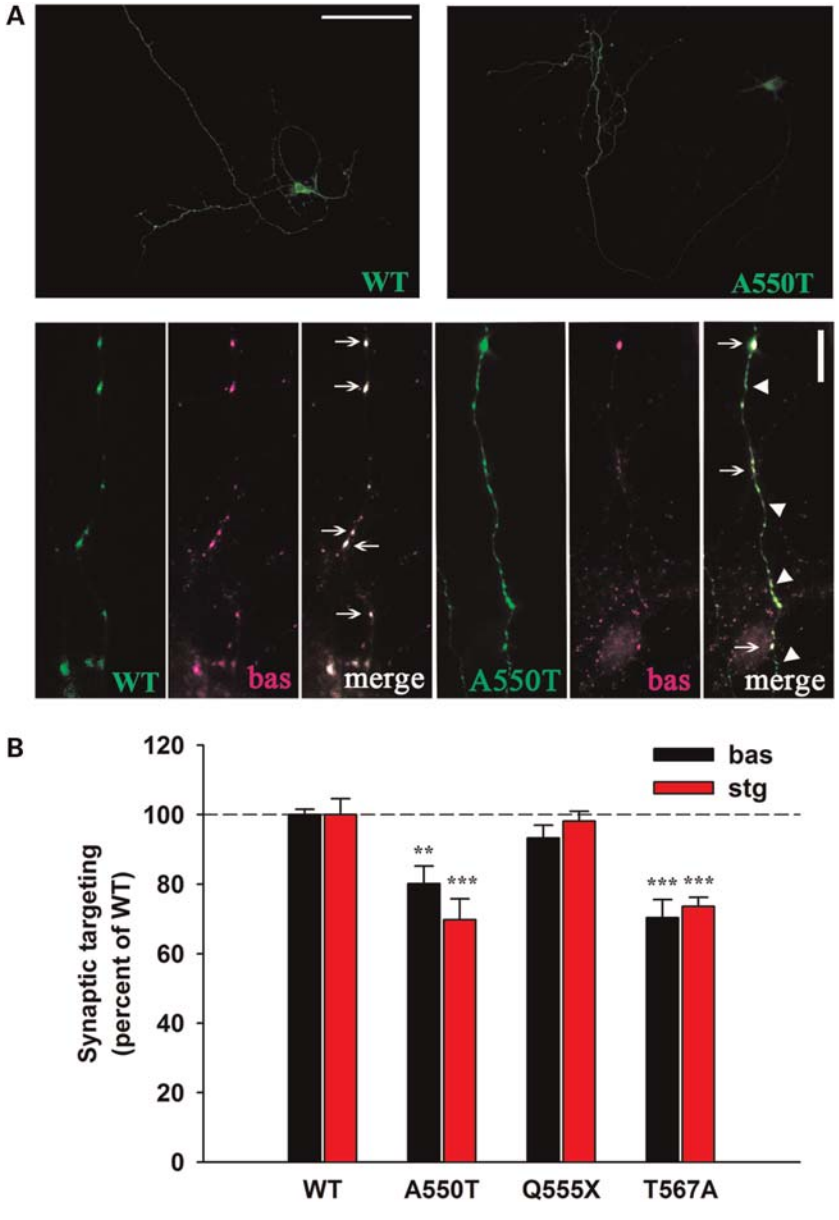

Figure 4. Targeting of hSynI mutants in SynI KO hippocampal neurons. (**A**) Representative images of the expression of either GFP-labeled WT or GFP-labeled A550T hSynI in SynI KO hippocampal neurons transfected at 7 DIV and analyzed at 10 DIV at low (upper panels) and high (lower panels) magnification. In the latter panels, presynaptic boutons were labeled with the scaffold protein bassoon. Arrows: boutons positive for both hSynI and bassoon (bas). Arrowheads: hSynI-positive, basson-negative puncta. Bars: 100 μm (upper panels), 10 μm (lower panels). (**B**) Quantification of the targeting of GFP-labeled WT, A550T, Q555X or T567A SynI to presynaptic terminals in SynI KO hippocampal neurons. Presynaptic targeting was assessed by retrospective labeling with either basson (bas; black bars) or synaptotagmin (stg; red bars). Puncta positive for either presynaptic marker and GFP–hSynI were counted and normalized to the total number of GFP–SynI-positive puncta. Data, obtained from 9–13 fields/transgene and 3 independent preparations, are expressed as a percentage of the average value observed in neurons transfected with WT-hSynI (means ± SEM). $^{**}P < 0.01$; $^{***}P < 0.001$ versus WT; one-way ANOVA followed by Bonferroni's multiple comparison test.

control SV mobilization at both low and high stimulation frequencies and to be involved in the expression of short-term plasticity (35,48), the impaired phosphorylation can directly contribute to the defective sizes and kinetics of SV pools. The developmental impairment associated with the Q555X mutant could also result from defective MAPK/Erk phosphorylation and/or c-Src or PI3K interactions, although a dominant-negative effect towards the endogenous SynII/III isoforms involved in earlier stages of development cannot be ruled out (31,48).

Although *in silico* analysis by SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and POLYPHEN (http://genetics.bwh.harvard.edu/pph/) did not predict a strong impact of the A550T and T567A substitutions on hSynI function, a significant decrease in nerve terminal targeting was observed for these mutants. It is possible that the missense mutations alter the negative targeting determinants of the D-domain (33) and that the loss of function of these mutants observed in the size of RRP and RP is due to a decreased nerve terminal targeting which makes them unable to rescue the SynI KO phenotype.

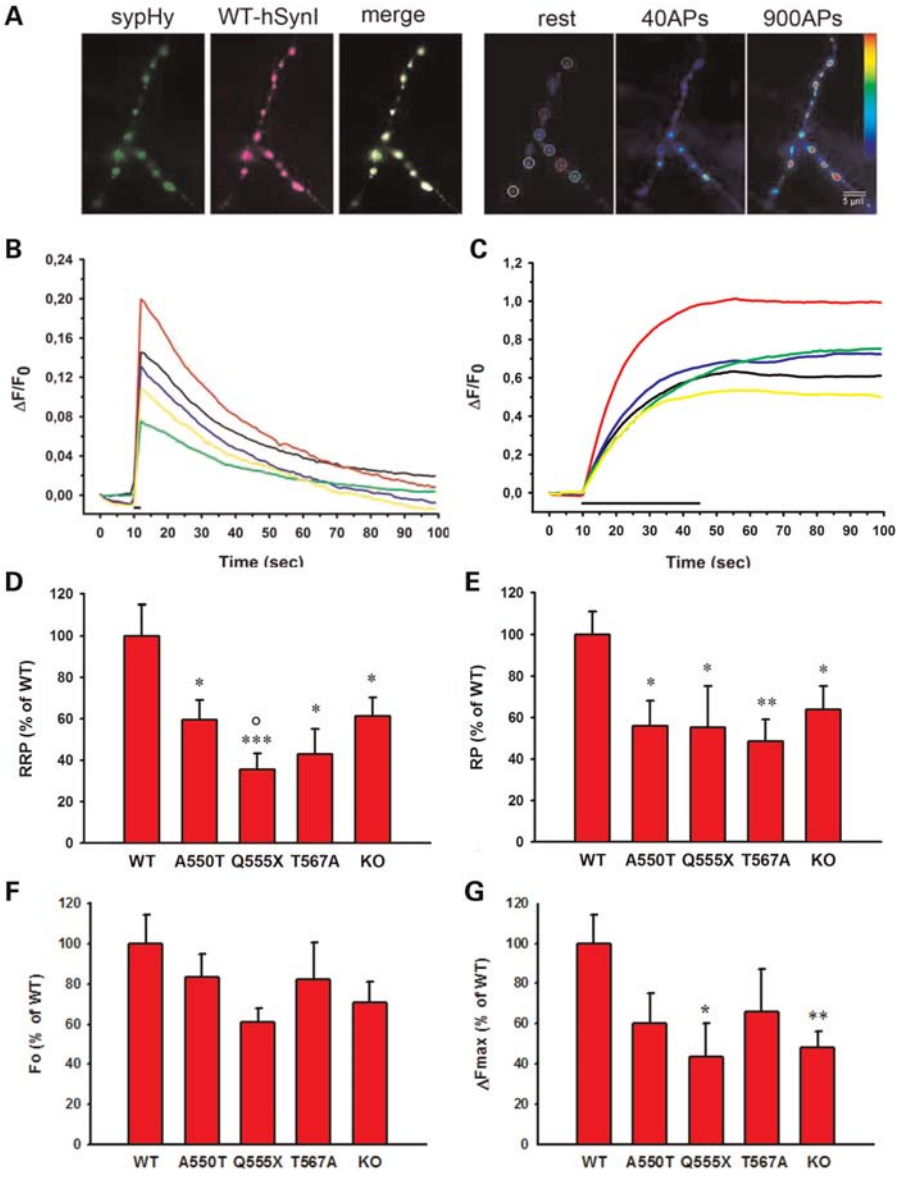

Figure 5. Effects of hSynI mutations on SV pool size and exo-endocytosis in hippocampal neurons expressing synaptop-Hluorin (SypHy). (**A**) Co-localization of SypHy and Cherry-labeled WT-hSynI in presynaptic boutons of SynI KO hippocampal neurons (left) and representative images of SypHy fluorescence detected at rest and in response to sequential stimulation with 40 action potentials (AP) at 20 Hz and 900 AP at 20 Hz in 1 μM bafilomycin (right). Regions of interest (ROIs) of 1.7 μm diameter (circles) corresponding to synaptic boutons are shown. SynI-positive ROIs responding to stimulation were manually selected in a pretrial round and analyzed for fluorescence intensity versus time. (**B** and **C**) Ensemble averages of individual time-courses of SypHy fluorescence in response to field stimulation (horizontal bar) for the determination of RRP (B; 40 AP at 20 Hz) and RP (C; 900 AP at 20 Hz, 1 μM bafilomycin). SynI KO neurons were co-transfected with SypHy and either empty vector (KO, black; $n = 10$ experiments), WT-hSynI (red; $n = 14$ experiments), A550T-hSynI (blue; $n = 13$ experiments), Q555X-hSynI (green; $n = 8$ experiments) or T567A-hSynI (yellow; $n = 8$ experiments). SypHy dequenching was calculated as fractional fluorescence increase over resting levels ($\Delta F/F_0$). (**D** and **E**) The changes in the release from the RRP (D) and the RP (E) are shown by expressing the $\Delta F/F_0$ peak (RRP) or plateau (RP) values evoked by stimulation as a percentage (means ± SEM) of WT-hSynI-transfected neurons. (**F**) Basal levels of SypHy fluorescence (F_0) calculated by averaging the last five frames preceding the stimulation. (**G**) Total SypHy fluorescence (ΔF_{max}) calculated as the difference between the plateau value in NH_4Cl and F_0. Data (means ± SEM) are expressed in percent of WT-hSynI. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ versus WT SynI; $^\circ P < 0.05$ versus SynI KO; one-way ANOVA and Bonferroni's multiple comparison test.

The strong association between ASDs and epilepsy suggests that common mechanisms of synaptic dysfunction may underlie both diseases. In the healthy brain, a balance of excitation and inhibition is essential for all functions, from physiological network activity and oscillations to cognitive processes. On the basis of mouse KO studies, disruption of Syn isoforms is associated with an altered excitatory/inhibitory balance (49,50) and with defects in social and cognitive behavior (43,44). Thus, it is tempting to speculate that, similar to what is reported for NLGNs and NRXNs (42,51), the loss of function brought about by the SynI mutations described here alters the excitatory/inhibitory ratio, with serious consequences in postsynaptic integration, network computation, excitability and activity-dependent plasticity.

Hence, Syns can participate in the synaptic autism pathway (22,41,42), reflecting a disorder of regulation of a family of synaptic genes essential for activity-dependent changes in neuronal function.

MATERIALS AND METHODS

Characterization of epilepsy and ASD phenotypes

Affected individuals gave informed consent, and the study was approved by the ethics committee of the CHUM-Notre-Dame Hospital. Clinical evaluations for the epilepsy phenotype included interview with patients and relatives, standard EEG and brain MRI. Seizure and syndrome classification were made according to the International League Against Epilepsy (1989). In the affected individuals from the EP-08 family, the clinical phenotype consisted of complex partial seizures and rare secondary generalized seizures not otherwise specified. In these individuals, EEG did not reveal interictal epileptic activity, and brain MRI scans were unremarkable except for mild hippocampal atrophy in two individuals. Otherwise, all the affected individuals from our cohort exhibited clinical features compatible with idiopathic partial epilepsy. Standardized Assessment for Diagnosis of Autism (ADI-R and ADOS-G Module-3) was performed in individuals IV-01 and IV-02 and in all patients of our ASD cohort, as described (14). Neuropsychological assessment included the Wechsler adult intelligence scale III and Wechsler intelligence scale for children IV in some individuals. Psychological and psychiatric assessments were performed by neuropsychologists and psychiatrists blinded to the genetic status.

Genotyping and linkage analysis

The microsatellite markers used for scanning the X chromosome and for fine mapping of the linked region were genotyped manually. Allele sizes for each marker were obtained from the Fondation Jean Dausset CEPH database and determined by comparison with the M13mp18 sequence ladder. The marker map positions were based on the sex-averaged maps from Marshfield Medical Research Foundation and UCSC browser. We calculated two-point LOD scores with MLINK from the FASTLINK 3.0P package by using a penetrance of 0.8, a disease allele frequency of 0.00001, a phenocopy frequency of 0.0001 and equal allele frequencies. Under this model, the maximum LOD score for the EP-08 family is 3.23. Modifying the penetrance to 0.7 and phenocopy frequency to 0.006 did not reduce the maximum LOD score significantly.

Screening for mutation

The genomic organization of the human *SYN1* gene was determined by aligning sequences from *SYN1* mRNA (Genbank NM_006950) to the corresponding genomic sequence on chromosome X (Genbank NC_000023). Primers were designed to amplify 400–600 bp fragments from genomic DNA to screen all coding portions of the gene. Portions of the *SYN1* gene were amplified by polymerase chain reaction (PCR) and analyzed by direct sequencing on an ABI3730 automatic sequencer (Applied Biosystems). Primers 5′-TGCCCTTGGGCCGCCAGACCTC-3′ and 5′-CTTGGGGGAAGGGCTTGGCTCAGG-3′ were used to amplify exon 12. For the EP-08 family, we have initially sequenced one affected individual and a control from the family. The $SYN1_{Q555X}$ mutation carrier was assessed by direct sequencing in all affected and non-affected members from the EP-08 family.

Cloning of SYN1 and mutagenesis

Primers were designed to amplify by PCR the complete open reading frame of hSynIa from a human brain cDNA library (Marathon-ready, Clontech) and we subcloned the PCR products into either pcDNA3.1 (Invitrogen), pEGFP or pmCherryC1 (Clontech) vector. The A550T, Q555X and T567A mutants were generated from the wild-type (WT) hSynI cDNA, using site-directed mutagenesis. His-tagged hSynI DE-domains were made using the pRSET vector (Invitrogen).

Biochemical assays

SH3 domain binding. GST or GST-SH3 domain fusion proteins were incubated with extracts of COS-7 cells transiently transfected with pcDNA 3.1 encoding WT or mutant hSynI and the binding was assessed by glutathione–Sepharose pull-down assays (34).

SV binding. SVs were purified from the rat brain, using controlled-pore glass chromatography, and depleted of endogenous Syns. The binding to SVs of His-tagged hSynI DE-domains, extracted from bacterial lysates and affinity-purified using a HisTrap-FF column, was performed using a high-speed sedimentation assay (32).

Phosphorylation. Site-specific phosphorylation of hSynI DE-domains was performed at 30°C for 30 min under the following conditions (in mM): (i) 20 Tris (pH 7.4)/20 $MgCl_2$/2 $MnCl_2$/0.5 dithiothreitol/0.5 Na_3VO_4 plus MAPK/Erk2 (0.1 U/sample); (ii) 16 Tris–HCl (pH 8), 10 $MgCl_2$/0.5 $CaCl_2$/0.07 EGTA/0.1 β-mercaptoethanol plus calmodulin (1 μM)/CaMKII (1 U/sample); (iii) 40 MOPS (pH 7.0)/20 $MgCl_2$ plus cdk5/p25 (0.2 U/sample) in the presence of [^{32}P]-ATP (50 μM; 1–2 μCi/sample). Samples were resolved by SDS–PAGE, and ^{32}P incorporation was quantified by Cherenkov counting of the excised bands.

Primary hippocampal cultures

Offspring of littermates of WT and homozygous SynI KO mice (36) were used in accordance with the guidelines of European Communities (24 November 1986 directive) and National Councils on Animal Care. Hippocampal cells were prepared from SynI KO mouse E17–18 embryos and plated onto ECM gel-coated coverslips (Sigma) at 600 cells/mm^2 and transfected at either 0 or 7 DIV with GFP-tagged hSynI, using lipofectamine 2000 (Invitrogen). For functional experiments, neurons were double-transfected at 9–11 DIV with superecliptic SypHy and Cherry-tagged hSynI. For immunocytochemistry, paraformaldehyde-fixed neurons were labeled

with antibodies to synaptotagmin (1:100; Synaptic Systems), bassoon (1:500; Synaptic Systems) or MAP2 (1:200; Chemicon), followed by Alexa-conjugated secondary antibodies (Invitrogen). Axonal and dendritic lengths were analyzed using the ImageJ software.

Live-cell imaging

Hippocampal neurons were transfected at 11–14 DIV with superecliptic SypHy and Cherry-tagged hSynI. Optical recordings were performed at 17–21 DIV on an Olympus IX-81 microscope with an MT20 Arc/Xe lamp. Time lapses were acquired at 1 Hz for 100 s with an Orca-ER CCD camera (Hamamatsu) and a 60×/1.35 NA objective. After 10 s of baseline acquisition, neurons were electrically stimulated in the presence of 10 μM CNQX/50 μM APV (Tocris), by applying 1 ms current pulses, yielding fields of 10 V/cm. Images were analyzed using the Olympus Cell-R software.

Statistical analysis

We assessed whether an excess of rare variant was found in ASD ($n = 193$) versus control ($n = 291$) chromosomes by using Fisher's exact test. The two-sided *P*-value was considered. All other analyses were carried out by one-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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