Mutations linked to neurodevelopmental disorders, such as intellectual disability (ID), are frequently found in genes that encode for proteins of the excitatory synapse. Transmembrane AMPA receptor regulatory proteins (TARPs) are AMPA receptor auxiliary proteins that regulate crucial aspects of receptor function. Here, we investigate a mutant form of the TARP family member stargazin, described in an ID patient. Molecular dynamics analyses predicted that the ID-associated stargazin variant, V143L, weakens the overall interface of the AMPAR:stargazin complex and impairs the stability of the complex. Knock-in mice harboring the V143L stargazin mutation manifest cognitive and social deficits and hippocampal synaptic transmission defects, resembling phenotypes displayed by ID patients. In the hippocampus of stargazin V143L mice, CA1 neurons show impaired spine maturation, abnormal synaptic transmission and long-term potentiation specifically in basal dendrites, and synaptic ultrastructural alterations. These data suggest a causal role for mutated stargazin in the pathogenesis of ID and unveil a new role for stargazin in the development and function of hippocampal synapses.

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INTRODUCTION

Most of the fast component of excitatory neurotransmission in the central nervous system is mediated by glutamate receptors of the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate type (AMPAR). These receptors are associated with auxiliary proteins that regulate their traffic, gating and pharmacology, increasing receptor functional diversity in the brain [1–3]. Members of the transmembrane AMPAR regulatory protein (TARP) family are widely expressed AMPAR auxiliary subunits [4], and key modulators of AMPAR-mediated transmission. The prototypical TARP stargazin (also known as TARP y2) was discovered in the ataxic stargazer mouse [5], which lacks synaptic AMPARs on cerebellar granule cells [6]. Stargazin interacts with both AMPA receptor subunits and synaptic PDZ-containing proteins such as postsynaptic density protein 95 (PSD95) [7] and this is required for targeting AMPAR to synapses [7–9]. TARPs, including stargazin, couple with the majority of AMPAR complexes in the brain, promote receptor trafficking to the cell surface and their synaptic targeting, and augment their functional properties [1, 2]. Not surprisingly, stargazin regulates baseline synaptic transmission and is also involved in Hebbian and homeostatic forms of synaptic plasticity that are dependent on tightly regulated AMPAR traffic [10–12].

Impaired glutamatergic synaptic transmission and plasticity have been implicated in neurodevelopmental disorders [13]. Evidence from human genetic studies suggests that copy number variation or the presence of rare point mutations in genes encoding proteins of the ionotropic glutamate receptor complex may play a role in the aetiology of these disorders [14–18]. Single nucleotide polymorphisms in the CACNG2 gene encoding stargazin were associated with a subgroup of schizophrenia patients [19], and alterations in the DNA copy number and in the levels of stargazin mRNA were detected in the post-mortem brain of schizophrenia patients [20, 21]. Dysregulated stargazin expression was also found in the dorsolateral prefrontal cortex of patients with bipolar disorder [21], and stargazin polymorphisms were associated with the response to lithium, a frequent treatment for bipolar disorder [21, 22]. A de novo missense mutation in CACNG2 has been identified in a non-syndromic intellectual disability (ID) patient with moderate severity [16]. Taken together, these data point to a possible link between stargazin and the pathogenesis of neurodevelopmental disorders, which has not yet been investigated. Evaluating how human mutations in the stargazin-encoding gene disrupt synaptic function and impact behavior may also provide insight into the physiological role of stargazin.

Here, we investigated the impact of the ID-associated missense V143L mutation in stargazin [16] in the molecular dynamics (MD) of the AMPAR:stargazin complex, in the cell surface diffusion of stargazin and in its ability to traffic AMPAR to the neuronal surface and to the synapse. To evaluate behavioral, neuronal morphology and functional alterations triggered by the stargazin V143L variant, we generated a knock-in (KI) mouse model to express the mutant.
protein. We found that stargazin V143L KI mice display alterations in cognitive and social behavior, along with altered hippocampal spine morphology, associated with synaptic ultrastructural defects. We also found disrupted synaptic transmission and plasticity and aberrant stargazin phosphorylation in stargazin V143L mutant mice.

RESULTS

Intellectual disability-associated stargazin V143L mutation affects the AMPAR:stargazin complex structure

A de novo missense mutation in the CACNG2 gene encoding stargazin was described in a heterozygous 8-year-old male patient with moderate, non-syndromic, intellectual disability [16]. This mutation leads to substitution of valine143 by leucine (p.V143L), a residue in the third transmembrane domain of stargazin that is highly conserved among species (Fig. 1a, c), suggesting a critical role for the function of stargazin. Accordingly, the V143L substitution was predicted to be damaging using PolyPhen-2 [23], SIFT [24] and PROVEAN [25]. Importantly, this variant has not been described in databases collecting sequencing variants for the general population (Genome Aggregation Database or Exome Variant Server).

In order to characterize the effect of the V143L stargazin mutation in the structure and dynamics of the AMPAR:stargazin complex, we used molecular dynamics (MD) simulations. To the best of our knowledge, there are no MD studies available regarding AMPAR:TARP complexes. We applied MD algorithms to predict in silico how the AMPAR:stargazin system responds to a particular perturbation. To this purpose we used homology modeling to construct both the WT and V143L models of the AMPAR:stargazin complex (Fig. 1b–d), based on one of the described cryo-EM structures for the complex [26]. AMPARs are composed of two dimers comprised of A/D and B/C subunits, with the coupled TARP as refer to the GluA structure exhibiting the highest contact surface sites (involving subunits A/D or B/C) [28] (Fig. 1b). We will herein refer to the GluA structure exhibiting the highest contact surface with the coupled TARP as Main GluA and Secondary GluA to the other one. We analyzed the effect of the stargazin V143L mutation at both sites considering different metrics on macromolecular rearrangements such as cross-correlation analysis (CCA) and root mean square deviations (RMSD), and at the interfacial level (solvent accessible surface area - SASA). Free binding energy (∆G\text{bound}) calculations were also performed to assess the mutant effect on the complex binding stability.

Figure 1e reveals the network of correlated/anticorrelated (same/opposite direction) motions between different regions of the AMPAR:stargazin complex structure, which informs on the impact of the stargazin V143L mutation in the overall structural conformation of the macromolecular complex. Overall CCA results indicate that the X site is more prone to conformational rearrangements introduced by the V143L mutation in stargazin than the Y site. RMSD was used to assess protein conformational changes between different time points in the trajectory, and their distribution is shown in Figs. S1 and S2 for all monomers at both sites, in the WT and mutated complexes. V143L stargazin-containing mutated complexes show lower density for lower values than WT complexes, demonstrating a higher flexibility. Furthermore, ∆ASA\text{SASA}\text{V143L} – ∆ASA\text{SASA}\text{WT} tend to be negative, which means that the interface area is larger in the WT complex when compared to V143L. This is particularly relevant for transmembrane domain 1 (M1) of Main GluA and the transmembrane domains (TMD) 3 and 4 of stargazin. The X site shows the greatest differences between mutant and WT stargazin, suggesting that the stargazin V143L mutation especially hinders AMPAR:

The V143L mutation affects the trafficking properties of stargazin

Stargazin plays a role in AMPAR trafficking through the early compartments of the biosynthetic pathway [29], and mediates complexed AMPAR trafficking to the cell membrane, their synaptic stabilization [7, 30] and surface diffusion trapping [8, 31] through binding to PSD95. Given the described roles, we explored the potential effect of the V143L mutation on stargazin’s cell surface diffusion properties. Low-density cortical neurons were co-transfected with plasmids encoding Homer-GFP, for synapse identification, and HA-tagged WT stargazin (Stg\text{WT}) or the V143L stargazin variant (Stg\text{V143L}). We monitored stargazin diffusion by single nanoparticle imaging of HA-stargazin using quantum dots (QDs; Fig. 1f–k). Stargazin V143L particles displayed increased mean square displacement (MSD; Fig. 1h), decreased synaptic residence time (Fig. 1f) and higher diffusion coefficient than Stg\text{WT} (Fig. 1k), suggesting that the V143L mutation renders stargazin more mobile in the plasma membrane.

The ID-associated mutation is located in the third transmembrane domain of stargazin (Fig. 1c), which was shown to be involved in the interaction with AMPAR subunits [32, 33]. Our molecular dynamics analyses indicate that this mutation weakens the interaction of stargazin with the AMPAR complex, in particular in the X site (Fig. 1e and Table 1). We thus hypothesized that stargazin V143L may be defective in trafficking AMPAR to the cell surface and to the synapse. To test this possibility, we used a molecular replacement strategy in which we silenced endogenous stargazin expression in cultured cortical neurons with a specific shRNA [11] and re-introduced either WT stargazin or the V143L variant. We assessed the effect of stargazin depletion and of the expression of the stargazin V143L variant in AMPAR trafficking and synaptic stabilization in cultured neurons (Fig. 1i–n).Cell surface and synaptic expression levels of AMPAR were evaluated by immunolabeling GluA AMPAR subunits using an antibody specific for their extracellular N-terminal region (Fig. 1i). As previously described [11], stargazin silencing led to a decrease on the cell surface (Fig. 1j, m) and synaptic levels of AMPAR (Fig. 1j, n). AMPAR clusters were considered synaptic when colocalizing with PSD95, whose expression was not affected by stargazin silencing (data not shown). In cells co-transfected with stargazin shRNA and WT shRNA-refractory stargazin (KD + Stg\text{WT}), total and synaptic surface levels of GluA were rescued to basal levels. Critically, neuronal transfection of shRNA-refractory stargazin V143L mutant (KD + Stg\text{V143L}) led to a failure in mediating normal AMPAR traffic to the cell surface (Fig. 1j, m) and to the synapse (Fig. 1j, n), showing that the ID-associated mutation impairs stargazin’s role in AMPAR trafficking.

Genetically engineered mice with the stargazin V143L mutation show altered cognitive and social behavior

In order to study the effects of the ID-associated stargazin mutation in vivo, we generated a knock-in (KI) mouse line in which the human mutation was introduced in the mouse Cacng2 gene. Using the gene targeting strategy we targeted the Cacng2 gene to modify the nucleotide in the third exon which was found to be mutated in the ID patient [16] (Fig. S4a). Confirmation of the mutation was performed by Sanger sequencing (Fig. S4b). Heterozygous and homozygous KI mice were viable, did not display gross abnormalities, and did not show spontaneous

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seizures. To determine whether expression of the stargazin V143L variant affects gross brain morphology, we performed Nissl staining in brain coronal slices and compared sections from WT and homozygous stargazin V143L KI (KIVL/VL) mice. As shown in Fig. S4c, no apparent macroscopic defects were visible in the brain of stargazin KIVL/VL animals, suggesting that overall brain morphology is not affected by the stargazin mutation. Moreover, the structural organization of the hippocampus and cortical lamination were preserved (Figs. S4c, f, g).

To assess whether the V143L stargazin mutation affects stargazin protein levels and distribution across the brain, we performed immunolabeling of stargazin in brain coronal and sagittal slices. Stargazin is broadly expressed throughout the mouse brain with high expression levels in the cerebral cortex, hippocampus, and cerebellum [4]. Within the hippocampus, stargazin immunoreactivity was most intense in the stratum oriens of the CA1, CA2 and C3 regions, the stratum lacunosum moleculare of the CA1 and CA2 regions, and particularly in the subiculum...
The ID-associated V143L stargazin mutation weakens the interaction between stargazin and AMPARs, presents altered surface diffusion and elicits defective AMPAR trafficking. a Valine 143 in stargazin is highly conserved among species. b Surface representation of AMPAR:stargazin complex viewed parallel to the membrane (left) and from the extracellular side (right). The extracellular view of the complex (at the membrane level) shows the two different sets of stargazin assembly points (X and Y sites) around AMPAR. Each GluA subunit is colored individually in shades of blue. Stargazin molecules are colored in orange (Y site) or brick (X site). c Side view of the stargazin structure shown as a cartoon with substructures labeled and colored in a spectrum of blue and ligand-binding domain colored in green. LBD, ligand-binding domain; M1-M4, transmembrane domains. d Side view of a GluA2 subunit structure shown as cartoon with substructures labeled and transmembrane domains colored in a spectrum of blue and ligand-binding domain colored in green. LBD, ligand-binding domain; M1-M4, transmembrane domains. e Dynamical cross-correlation maps for the AMPAR:stargazin complex. Top triangle - WT complex, bottom triangle - V143L complex. Substructure annotation was added at the bottom and right of each map for easier reading. CCA goes from -1 (anticorrelated, opposite direction) to 1 (correlated, same direction). Violet boxes highlight the major differences between WT and ID substructures.

Table 1. Free binding energy values for AMPAR:stargazin complexes containing WT or V143L stargazin.

| Site | ΔΔGWT (kcal/mol) | ΔΔGV143L (kcal/mol) | ΔΔG (ΔΔGWT - ΔΔGV143L) | p value |
|------|------------------|---------------------|------------------------|---------|
| Both | -42.98 ± 0.39    | -36.78 ± 0.40       | 6.20 ± 0.55            | <0.0001 |
| X    | -40.02 ± 0.28    | -30.78 ± 0.53       | 9.24 ± 0.60            | <0.0001 |
| Y    | -42.02 ± 0.42    | -41.78 ± 0.51       | 0.24 ± 0.66            | 0.212   |

ΔΔG values were obtained from ΔΔGV143L - ΔΔGWT, and are presented in kcal/mol. Wilcoxon test was used to calculate p value.

(Figs. S4d–f). Stargazin immunoactivity was similar in all genotypes (Figs. S4d–g), indicating that the expression of mutated stargazin does not affect the protein brain-wide distribution and total expression levels. This was also confirmed by western blot analyses, using total lysates from the whole brain, cortex and hippocampus (Figs. S4h–j). The expression levels of stargazin and other TARPs were also assessed by qPCR and no changes were detected in the cortex and hippocampus of Kl mice, compared to WT littermates (Fig. S4k).

Since the V143L stargazin mutation was found in an ID patient [16], we asked whether V143L Kl mice display alterations in motor function, anxiety-like behavior, cognitive and/or social performance that correlate with ID-like symptomatology. We began by assessing motor behavior in the open field test (Fig. S5a) and found that, whereas male stargazin Kl+/VL and Kl−/VL male mice showed comparable the mice traveled and interacted speed to WT male mice (Figs. S5c, e), female stargazin Kl−/VL mice traveled longer distances, and stargazin Kl−/VL female mice showed higher instant speed than WT female mice (Figs. S5b, d), suggesting hyperactivity in female stargazin V143L Kl animals. However, stargazin V143L Kl mice did not display anxiety-like behaviors either in the open field (Fig. S5f) or in the elevated plus maze (Figs. S5g, h), tests, nor did they show depressed-like behavior in the forced swimming test (Figs. S5i, j). Heterozygous stargazin V143L Kl animals failed to alternate above chance level in the T-maze spontaneous alternation test for working memory (Figs. S5k, l).

In an object displacement test for spatial memory evaluation, while WT animals preferred to spend time engaging with the object, neither stargazin Kl+/VL nor Kl−/VL mice showed this preference, and stargazin Kl+/VL mice spent significantly less time exploring the object that was moved when compared to WT animals (Fig. 2a, b). Furthermore, in the contextual fear conditioning test for associative memory, stargazin Kl+/VL mice presented less freezing behavior than WT animals (Fig. 2c, d). These observations suggest that the V143L mutation in stargazin elicits learning and memory impairments. Given the high expression of stargazin in the cerebellum [4], we assessed motor learning of stargazin V143L Kl animals in the rotarod test (Fig. 2e, f). No significant motor abnormalities were displayed by mutant mice in the rotarod test in the first day of the test, but whereas WT and stargazin Kl−/VL mice improved their performance the second day they were placed in the apparatus, stargazin Kl−/VL mice failed to do so, suggesting an impairment in motor learning (Fig. 2f).

Typically, ID patients display social skills, including the will/ability to socially engage with other people. To determine whether stargazin V143L Kl mice display social interaction deficits, we tested these animals in the three-chamber test. Stargazin V143L Kl mice showed preference for a conspecific (Stranger 1 - S1) over an empty cage (E), similarly to WT mice.
**Stargazin V143L KI mice show cognitive and social deficits.** a, b When subjected to the object displacement recognition test, homozygous stargazin V143L (KIVL/VL) mice spent less time exploring the displaced object when compared to their WT counterparts, and did not have preference for the displaced object. Data are presented as mean ± SEM. One-way ANOVA (p = 0.0412) followed by Dunnet’s multiple comparison test, *p < 0.05; one-sample t test to the value of 50%, #p = 0.013. N ≥ 16 (males and females) for all genotypes. c, d Homozygous stargazin V143L knock-in mice presented significantly less freezing behavior than WT counterparts in the contextual fear conditioning test. Data are presented as mean ± SEM. One-way ANOVA (p = 0.0076) followed by Dunnett’s multiple comparison test, *p < 0.05. N ≥ 22 (males and females) for all genotypes. e, f The average time spent on the rotarod on the first day did not significantly vary between genotypes (one-way ANOVA, p = 0.9990). Both WT and heterozygous stargazin V143L (KIVL/VL) animals performed significantly better in the second day, whereas stargazin KIVL/VL mice failed to show motor learning. Ratio paired t test, ****p < 0.0001 for WT mice, ***p = 0.0009 for KIVL/VL mice and p = 0.0522 for KIVL/VL mice. N ≥ 14 for all genotypes (males and females). g Mice were submitted to the three-chamber social interaction paradigm. The time spent approaching the cages, with and without the stranger stimulus mouse, was evaluated for 10 and 5 min, respectively. All animals displayed social preference, but (j, k) stargazin KIVL/VL mice showed no preference for a new stranger mouse in the arena, unlike WT and heterozygous stargazin V143L mice. Data are presented as mean ± SEM (h, j) and median ± IQR (whiskers represent minimum and maximum values) (i, k). Two-way ANOVA (h) p < 0.0001 (S1 vs empty), p > 0.9999 (genotype), p = 0.4107 (interaction); (j) p < 0.0001 (S1 vs S2), p > 0.9999 (genotype), p < 0.0001 (interaction) followed by Sidak’s multiple comparison test, ****p < 0.0001. N ≥ 17 (males and females) for all genotypes. See also Fig. S5.

(For Fig. 2g–i). However, in the presence of a novel social partner (Stranger 2 - S2), contrarily to WT and stargazin KI/VL mice, stargazin KIVL/VL mice did not prefer to interact with the unfamiliar animal (Fig. 2g, j, k). This result suggests a possible deficit in social recognition and/or alterations in the motivation for social novelty. The innate social behavior of nest building was not perturbed in stargazin V143L KI mice (Figs. S5m, n). Together, our results show that the ID-associated mutation in stargazin elicits cognitive and social deficits reminiscent of ID-like symptoms.

**Stargazin V143L mutant mice exhibit early hippocampal synaptic transmission defects**

To assess whether the decrease in surface AMPA receptor levels observed in vitro in neurons expressing stargazin V143L has an impact in glutamatergic transmission in vivo, we performed whole-cell patch-clamp recordings in CA1 pyramidal neurons from acute hippocampal slices of P15–P20 stargazin V143L KI mice, to measure AMPA receptor-mediated miniature excitatory post-synaptic currents (mEPSCs). We found that the frequency of mEPSCs events was significantly decreased in neurons from stargazin KI/VL and KIVL/VL mice compared to WT littermates (Fig. 3a, c). Interestingly, no changes in the amplitude of mEPSCs (Fig. 3a, b) or in the kinetics of these events (Fig. 3a) were observed.

We next investigated the consequences of the ID-associated stargazin mutation in hippocampal functional connectivity and synaptic plasticity by recording field excitatory post-synaptic potentials (fEPSPs) in CA1 basal and apical dendrites while stimulating the Schaffer collateral fibers in the stratum oriens (Fig. 3d) or in the stratum radiatum (Fig. 3e), respectively. First, we tested the impact of the V143L stargazin mutation on synaptic transmission and found decreased fEPSP responses when recording from the stratum oriens (Fig. 3f) but not when recording from the stratum radiatum (Fig. 3g). This suggests that the connectivity to CA1 post-synaptic sites is impaired in basal dendritic synapses but preserved in apical dendritic synapses in the hippocampus CA1 region of stargazin V143L KI animals. No significant alterations were found in the fiber volley amplitude between genotypes,
indicating that there are no gross presynaptic impairments in stargazin V143L KI mice at these synapses (Fig. 3f, g). Indeed, when paired-pulse facilitation, a short-term strengthening of synaptic transmission, was assessed no overt alterations were observed (Figs. S6), further supporting that the presynaptic function is intact in these hippocampal pathways in stargazin V143L KI mice. Finally, we induced long-term potentiation (LTP) in acute hippocampal slices using a theta burst stimulation protocol. Stargazin V143L KI mice showed decreased LTP recorded in basal CA1 synapses (Fig. 3h, j), but normal LTP at apical CA1 synapses (Fig. 3i, k).
Fig. 3 Stargazin V143L KI mice present decreased excitatory synaptic transmission and plasticity in CA1 pyramidal neurons. a Representative traces of mEPSCs recordings and single average event of CA1 pyramidal neurons in acute hippocampal slices from WT, stargazin KIPv/LV and stargazin KIVL/VL mice. b Cumulative probability distribution and average mEPSCs amplitude and (c) frequency plots, showing a reduction in frequency but not amplitude of mEPSCs in stargazin V143L KI mice (P15–P20). Data are presented as mean ± SEM. Kruskal–Wallis (amplitude: \( p = 0.9803 \); frequency: \( p = 0.0003 \)) followed by Dunn’s multiple comparisons test, \( * p < 0.05, \* \* * p < 0.001, \* \* \* \* p < 0.0001 \). To further explore the alterations in spine morphology, we performed ultrastructural analysis of the post-synaptic density (PSD) in hippocampal spines from WT and stargazin V143L KI mice using electron microscopy. Our analysis uncovered a decrease in the length of PSDs from stargazin KIPv/LV and KIVL/VL mice (Figs. 4f, g), as well as a tendency for an increase in the thickness of PSDs from stargazin KIPv/LV mice when compared with WT littermates (Figs. 4f, h), highlighting potential alterations in post-synaptic structure and composition. Moreover, the total levels of PSD95 in the hippocampus of stargazin V143L KI mice were significantly reduced compared to WT littermates (Figs. S8a, b). PSD95 has an important role in silent synapse maturation [35] and PSDs with smaller size and decreased PSD95 content are less stable [36]. The decreased PSD95 levels further support an impairment in spine maturation in the hippocampus of stargazin V143L KI mice. We analyzed the effects of the stargazin V143L mutation in the ultrastructure of PSDs in the cortex (Figs. S8c–e), and also observed a decrease in the length of PSDs in stargazin KIVL/VL mice (Figs. S8c, d). However, in contrast to what was observed in the hippocampus, there was a decrease in the thickness of PSDs from stargazin KIPv/LV mice (Figs. S8c, e). No changes were observed in cortical PSDs total levels of PSD95 (Figs. S8f, g). Together, these data reveal that the stargazin V143L mutation leads to an increase in the density of spines with immature morphology and to ultrastructural changes in post-synaptic compartments, indicating a general spine immaturity state in certain hippocampal subregions of stargazin V143L KI mice. Combined with our functional characterization showing decreased frequency of mEPSC events in CA1 neurons from stargazin mutant mice, as well as decreased connectivity and long-term plasticity in CA1 basal synapses, this strongly suggests that the stargazin V143L mutation perturbs spine maturation and diminishes functional synaptic contacts and plasticity in specific hippocampal subcircuits.
Stargazin phosphorylation and interaction with GluA1 are decreased in stargazin V143L mutant mice

Finally, we determined whether the dendritic spines immaturity (Fig. 4a, b, e) and the decreased PSD length (Fig. 4f, g and S8c, d) found in the brain of stargazin V143L KI animals are accompanied by altered composition of the PSDs. We isolated PSDs from the cerebral cortex of WT, stargazin KI\textsuperscript{VL}/VL and KIVL/VL littermate mice (Fig. S8h) and quantified their content in stargazin, GluA1 and GluA2 AMPAR subunits, as well as PSD95 (Fig. 5a-e). Stargazin expression was decreased in the PSDs of stargazin KI\textsuperscript{VL}/VL and KIVL/VL mice compared to WT mice (Fig. 5a, b), despite not significantly changed total expression levels of stargazin in mutant mice - Fig. S4j). Immunoprecipitation analyses (Fig. 1e), immunoprecipitation of stargazin V143L from the cerebral cortex of stargazin KIVL/VL mice showed decreased co-immunoprecipitation of GluA1, compared with stargazin immunoprecipitated from the cortex of WT littermate mice (Fig. 5f, g), indicating that the V143L mutation in stargazin impairs its interaction with AMPAR subunits in vivo.

The function of stargazin is regulated by the phosphorylation of serine residues in the cytoplasmic C-terminal tail of the protein [12], and these phosphorylation events regulate stargazin interaction with membrane lipids [37], its binding to PSD95 [9, 38], and the diffusional trapping of AMPARs at synaptic sites [31]. The migration pattern of stargazin in denaturing SDS-PAGE conditions correlates with the phosphorylation state of the protein, with phosphorylated stargazin showing slower migration in SDS-PAGE [11, 12]. We noticed that, in PSDs isolated from the cortex of stargazin KIVL/VL mice, stargazin showed faster migration compared to PSDs isolated from WT mice, whereas an intermediate migration pattern was detected in PSDs isolated from heterozygous...
stargazin V143L mice (Fig. 5h, i). To test for altered phosphorylation of mutant stargazin, we treated cortical extracts with λ-phosphatase before PSD purification and found that stargazin bands in PSDs isolated from the cortex of WT or heterozygous stargazin KI+/VL mice shifted to a lower apparent molecular weight, putatively corresponding to the unphosphorylated form of the protein [11, 12] and coincident with the stargazin band in PSDs isolated from untreated cortical extracts from stargazin KIVL/VL mice (Fig. 5j–l). In fact, the gel mobility of the stargazin band in PSDs isolated from stargazin KIVL/VL mice was unchanged by λ-phosphatase treatment (Fig. 5j–l), indicating that the protein is in a dephosphorylated form. These findings are consistent with decreased phosphorylation of V143L stargazin.

DISCUSSION

In this study, we employed molecular dynamics analyses, in vitro and in vivo models to study how an ID-associated mutation in the third transmembrane domain of stargazin impacts the AMPAR: stargazin complex, hippocampal synaptic architecture, synapse function and behavior. Our data suggest that the V143L mutation in stargazin critically affects stargazin interaction with the AMPAR complex, leads to decreased stargazin phosphorylation, decreases AMPAR-mediated synaptic transmission, and contributes to spine immaturity in CA1 hippocampal neurons. A striking aspect of our study is that it reveals not only the pathogenic effect of a mutant form of stargazin associated with disease, but also unveils critical roles for stargazin in regulating synapse structure and function in the hippocampus and in shaping cognitive and social behavior.
Structural analyses highlight that the main interface between AMPAR-stargazin is in the TMD of the complex. This interaction is mainly mediated by TMD3 and TMD4 in stargazin, M1 and M2 in \textit{Main GluA}, and M4 from \textit{Secondary GluA}. Our analysis showed that the V143L mutation impairs the correlated motion between transmembrane regions of stargazin and GluA, especially at M4 of \textit{Secondary GluA}.

The free-energy value is higher for the AMPAR-TARP complex containing stargazin V143L (by \(\sim 14\%\) on the whole complex), particularly at the X site (\(\sim 23\%\) difference, Table 1). Although the theoretical free binding energy values binding for the AMPAR-TARP complex do not consider entropic effects, and assume a stoichiometry of 4 TARPs per AMPAR tetramer, and homomeric AMPAR binding exclusively stargazin, which could be variable in the complex, they still allow us to confidently rank the stability of the different complexes. The higher value determined for the V143L system demonstrates the negative effect of this single point mutation on the overall stability of the protein–protein complex, particularly at the X-site. These predictions were assessed experimentally and are in agreement with the decreased co-immunoprecipitation of GluA1 with the ID-associated variant.

To date, the physiological roles of TARPs have been studied using knock-out mice for the different TARPs, alone or in combination (reviewed in [2]). These analyses have provided crucial insight into partially overlapping although non-redundant functions for different TARPs but are hindered by possible compensatory effects that may arise in the absence of the endogenous proteins. Examining knock-in mouse models expressing mutant forms of stargazin associated with disease has the double advantage of informing on the endogenous role of stargazin, by analyzing the effects of loss of function mutant variants which are still expressed, and on possible pathogenic mechanisms elicited by human stargazin mutations. In this study, we have found that the V143L mutation triggers a striking decrease in the frequency of mEPSCs in hippocampal CA1 pyramidal neurons, and leads to a decrease in evoked synaptic potentials, LTP and spine maturity in CA1 basal dentrites, and to ultrastructural alterations in the post-synaptic compartment. These observations suggest that despite the expression of other TARPs members in the hippocampus, including \(\gamma 3\) and high enrichment in \(\gamma 8\) [4], stargazin is required for normal spine development and for maintaining a full complement of functional synapses, specifically in CA1 basal dentrites. Our results are in line with experiments using stargazer/\(\gamma 8\)-knock-out mice, which showed that AMPAR-mediated transmission in CA1 pyramidal neurons is further reduced, compared to the reduction observed in \(\gamma 8\)-knock-out mice [39], despite the fact that CA1 pyramidal neurons from stargazer mice did not show alterations in the ratio of AMPA to NMDA EPSC amplitudes [40]. The synergistic reduction in AMPAR-mediated transmission in the stargazer/\(\gamma 8\) double knock-out mice implies some degree of functional redundancy for the two TARPs. If mutated stargazin is expressed, its incorporation in AMPAR complexes, even if less efficient than WT stargazin, will thus exert pathogenic effects, as suggested by the reduction in the frequency of mEPSCs and in evoked potentials and spine maturation in basal CA1 dentrites that we observed in stargazin V143L mice. These results are also in agreement with electron microscopy data showing that at Schaffer collateral/commissural synapses in the CA1 hippocampal region the presence of stargazin correlates with higher density of AMPAR expression [41] and thus presumably with the presence of a higher number of functional synapses, and with a recent study showing a high enrichment of stargazin in hippocampal spines [42]. Our data show a specific effect of the stargazin V143L variant in spine maturation in basal dentrites in CA1 neurons, which was not observed in apical dentrites. Spine morphology changes may be secondary to alterations in AMPAR content. Indeed, we detected a decrease in the fEPSPs slope and in LTP in CA1 basal synapses. An alternative is that the stargazin V143L mutation impacts specific interactions that play a role in spine maintenance/maturation, and thus directly impacts spine morphology through changes in the actin cytoskeleton. One example of such a stargazin interactor is Arc/Arg3.1 [43], which regulates spine morphology and structural plasticity through regulation of actin dynamics (reviewed in [44]). Our data at this point do not provide a basis to distinguish between the two possibilities.

Significant change in the frequency, but not in the amplitude, of mEPSCs was detected in CA1 neurons in stargazin V143L mice, which is apparently at odds with previous work showing that stargazin V143L overexpression in cultured cortical neurons leads to decreased frequency and amplitude of mEPSCs [16]. However, one major advantage of analyzing synaptic currents in stargazin knock-in animals is that endogenous levels of expression of mutant stargazin are kept, thus avoiding the over-representation of mutated stargazin in association to AMPAR complexes, and preserving the subcellular distribution of the protein. Curiously, the decrease in the frequency of mEPSCs in CA1 neurons in the stargazin V143L knock-in model was accompanied by a significant decrease in fEPSPs slope and in LTP in CA1 basal synapses but not in CA1 apical synapses. These observations indicate that stargazin has a specific role in maintaining spine structure and synaptic function and plasticity in CA1 basal dentrites, which agrees with the higher expression levels of stargazin in the hippocampal \textit{stratum oriens}, where basal dentrites are located, compared with the \textit{stratum radiatum}. Altogether, our data suggest that, besides the well-described brain region- and cell type-specific roles of TARPs, there may be subfield-specific roles that are determined by the subcellular distribution pattern of different TARPs.

The V143L variant was found to be dephosphorylated in cortical PSDs isolated from homozygous stargazin V143L KI mice, compared to WT PSDs. Phosphorylation of stargazin in its C-terminal region disrupts electrostatic interaction between the membrane and stargazin C-tail [37], promotes the extension of the C-tail into the cytoplasm and binding to PSD95 [38], and triggers diffusional trapping of AMPARs at synaptic sites [31]. Stargazin phosphorylation has been proposed to regulate Hebbian forms of synaptic plasticity [12] and to mediate experience-dependent plasticity and synaptic scaling [10, 11]. The lower level of phosphorylation of stargazin-V143L compared to the WT protein likely underlies its higher membrane diffusion rate at the membrane and its impaired capacity in supporting AMPAR synaptic traffic. The low phosphorylation of stargazin V143L may also determine the sequestration of its C-terminal tail in the plasma membrane and thus impair it from undergoing liquid-liquid phase separation with PSD scaffold proteins [31]. Changes in hippocampal spine maturation and in the ultrastructure of hippocampal PSDs may thus be a consequence of defective stargazin V143L phosphorylation and may be reflected in the decreased number of functional synapses detected in our mEPSC analyses in CA1 hippocampal neurons. While it is likely that the aberrant stargazin V143L phosphorylation contributes to the physiological effects observed, our MD analysis, which does not consider post-translational modifications in stargazin, also suggests compromised function for the V143L stargazin variant.

In this study we found that the V143L mutation in stargazin \(\gamma 1\)/\(\gamma 3\) mice leads to altered spatial memory and associative memory. These alterations in hippocampal-dependent cognitive behavior are likely to be related to the changes in mEPSC frequency, synaptic connectivity and plasticity, in spine maturity and in PSD ultrastructure that we identified in the hippocampus of these mice. We did not detect changes in social interaction in the three chamber test in stargazin V143L mice, but stargazin \(\gamma 1\)/\(\gamma 3\) mice showed impairment in preference for social novelty, suggestive of either a perturbation in social memory or a lack of motivation for social novelty. Stargazin \(\gamma 1\)/\(\gamma 3\) mice also displayed impaired motor learning in the rotorod, pointing to possible functional and...
structural alterations in the cerebellum. Given the elevated expression of stargazin in the cerebellum [4] and its non-redundant functions in cerebellar excitatory synapses in several cerebellum circuits [2, 7, 40], future studies should examine cerebellum circuit-specific dysfunction triggered by the ID-associated stargazin mutation. The cognitive and social behavioral dysfunctions displayed by stargazin V143L mice most likely arise from alterations in a combination of brain circuits, depending on the stargazin expression pattern and its synaptic roles in different cell types. Together, our data provide the first evidence for the causal implication of stargazin in the pathogenesis of neurodevelopmental disorders.

METHODS

Molecular dynamics simulations

The three-dimensional (3D) structure of stargazin was constructed by homology modeling using the MODELLER package [45], the target sequence retrieved from UniProt [46] (Q9Y698) and template used from the GluA2:stargazin complex (PDB-ID: 6DLZ [28]). Molecular Dynamics (MD) simulations of AMPAR-stargazin WT and mutated form (V143L variant) were performed using GROMACS 2018.4 [47] and the CHARMM36 force field [48]. Systems were built using CHARMM-GUI [49, 50] membrane builder with a bilayer membrane of POPC:Cholesterol (9:1 ratio). Root mean square deviations (RMSD) and solvent-accessible surface area (SASA) calculations were performed using the Ca atoms by GROMACS package [45]. The cross-correlation analysis (CCA) was calculated by Bio3D R package [51] for residue-level dynamic analysis using the Ca trajectory. Free-energy calculations were performed using AMBER's MMPBSA.py [52] as implemented in gmx_MMPBSA package [53].

Primary cortical neurons, neuronal transfection and imaging

Primary cultures of rat cortical neurons were prepared from the cortices of E17 Wistar rat embryos, as previously described [54]. Neurons were transfected using a calcium phosphate-mediated transfection protocol [55]. Immunocytochemistry, quantum dots labeling, imaging and analysis were performed blind, and as described [56].

Animal generation and maintenance

Stargazin V143L KI mice were generated by inserting a single nucleotide mutation in the third exon of the Cacng2 gene. The targeting vector was introduced through homologous recombination in R1 cells, as described previously [57]. The imaging, biochemical and behavioral analyses were performed in mice with 8–10 weeks and electrophysiology recordings were performed in 15–20 days-old animals. Both male and female animals were used; in the case where different conclusions were drawn for male and female animals (open field activity), results were plotted separately. All procedures and quantifications were performed by experimentalists blinded to animal genotype. Sample size estimates were based on previous literature. No randomization was applied. Procedures involving animals were performed according to the EU Directive 2010/63/EU guidelines and the experiments were approved by the institutional animal welfare body (ORBEA) and the national competent authority (DGAV).

Behavior analyses

The object displacement test was performed in a 40 × 40 cm open field arena. In the first trial the animals acclimatized to the empty arena for 6 min. In the three following trials, the animals were allowed to explore, for 6 min, two different objects located in a fixed position. In the fifth trial, conducted 24 h later, one of the objects was displaced and the time spent exploring the non-displaced and the displaced object was evaluated. All other behavior tests were performed as described in [58].

Electrophysiology

300 μm acute hippocampal sagittal slices were prepared from WT and stargazin V143L KI mice littersmates, as previously described [58, 59]. Whole-cell voltage-clamp recordings were performed at a holding potential of −80 mV using a Multiclamp 700B amplifier, digitized at 20 kHz with Digidata 1550 A (Molecular Devices Corporation, USA), and acquired using Clampfit 10.7 software (Axon Instruments, USA) [58]. Data were analysed using Clampfit software (Axon Instruments, USA) using a template search method to detect events [60]. EPSPs were evoked by stimulating axons in CA1 stratum oriens or CA1 stratum radiatum at 0.05 Hz using a bipolar electrode and recorded in the same layer. An input-output curve was performed and the stimulation intensity was set to elicit 40–50% of the maximal response. LTP was induced by theta-burst stimulation (TBS; 10 bursts of 4 stimuli at 100 Hz with a burst frequency of 5 Hz) [61]. Fiber volley amplitude and synaptic response slopes were analyzed using Clampfit software. All electrophysiology experiments and analyses were done blind to the genotype.

Labeling, detection and morphological classification of dendritic spines

To achieve sparse labeling of neurons in the hippocampus, we performed tail-vein injections in 4-week-old animals, with 5 μL of AA9V.Syn.eGFP. WPRE.eGFP at a titer of 8.88 × 10^{12} (Penn Vector Core, University of Pennsylvania, Philadelphia, USA). Four weeks post-injection, animals were sacrificed and the brains were collected and processed for neuronal imaging as described [58].

Electron microscopy

Sample preparation and post-synaptic density parameter measurements were performed as previously described in [58].

Biochemistry

Mice were anesthetized with isoflurane and euthanized by decapitation. Tissue lysates and post-synaptic density (PSD) isolations were carried out at 4°C. Cortical lysates were subjected to the PSD isolation protocol previously described in [59]. Immunoprecipitation (IP) of stargazin was performed as previously described [62]. Lambda phosphatase (λ-PP) treatment of cortical PSD samples was performed using the λ-PP treatment kit from New England Biolabs (USA), according to the manufacturer’s instructions.

Statistical analysis

The normality of population distributions was calculated for each experiment by comparison with a theoretical normal distribution using the Shapiro-Wilk normality test. According to this evaluation parametric or non-parametric tests were used, as described in the figure legends. For all tests, p < 0.05 was considered statistically significant. Outliers were identified and removed from the biochemical and behavioral analyses using the Grubbs test. Variance analysis following one-way ANOVA was performed using the Brown-Forsythe test. Analyses were performed using GraphPad 9.0 (Prism, USA). Details concerning number of independent experiments, statistical tests used, and p-values can be found in Table S1. Additional details on the Materials and Methods are available in SI appendix.

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AUTHOR CONTRIBUTIONS
Conceptualization: ALC, JP, ISM, GLC, ASI. Methodology: ALC, JP, GLC. Investigation: GLC, ASI, NB, CAVB, MVR, TR, RM, RPG, BC, SRL, JG, ME. Writing Original Draft: ALC, GLC, ASI, NB, ISM. Funding Acquisition: ALC, JP, ISM; Supervision: ALC, JP, ISM.

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COMPETING INTERESTS
The authors declare no competing interests.

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