AMPAR-receptor specific biogenesis complexes control synaptic transmission and intellectual ability

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AMPAR-type glutamate receptors (AMPARs), key elements in excitatory neurotransmission in the brain, are macromolecular complexes whose properties and cellular functions are determined by the co-assembled constituents of their proteome. Here we identify AMPAR complexes that transiently form in the endoplasmic reticulum (ER) and lack the core-subunits typical for AMPARs in the plasma membrane. Central components of these ER AMPARs are the proteome constituents FRRS1L (C9orf4) and CPT1c that specifically and cooperatively bind to the pore-forming GluA1-4 proteins of AMPARs. Bi-allelic mutations in the human FRRS1L gene are shown to cause severe intellectual disability with cognitive impairment, speech delay and epileptic activity. Virus-directed deletion or overexpression of FRRS1L strongly impact synaptic transmission in adult rat brain by decreasing or increasing the number of AMPARs in synapses and extra-synaptic sites. Our results provide insight into the early biogenesis of AMPARs and demonstrate its pronounced impact on synaptic transmission and brain function.

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Fast excitatory neurotransmission that is fundamental for the operation of normal brain function mainly relies on AMPA-type glutamate receptors (AMPARs). These ionotropic receptors mediate a large part of the excitatory postsynaptic currents (EPSCs) that drive point-to-point transmission in glutamatergic synapses and control both propagation of the electrical signal and the influx of calcium ions into the postsynaptic spine. By these means, AMPARs promote formation and maturation of new synapses and trigger activity-dependent processes that feedback onto the AMPARs thus altering amplitude and properties of the EPSCs.

In combination, changes in signal transduction and wiring are thought to endow excitatory neurotransmission with dynamic range of four orders of magnitude. Figure 1a illustrates the result of such initial APs with several anti-FRRS1l, anti-TARP and anti-GluA ABs on membrane preparations from the whole rat brain. Strikingly, the abundance heat map of these APs suggested the existence of two mutually exclusive populations of AMPAR assemblies within the proteome: One population comprising FRRS1l, CPT1c, Sac1 as well as ABHDs 6/12 and PORCN, the other containing the core subunits (CNIHs, TARPs, GSG1l) and the remainder of the peripheral constituents (red frames in Fig. 1a). The only major elements shared by both AMPAR assemblies were the GluA proteins; consequently, anti-GluA ABs performed in parallel effectively retained the complete set of constituents of the AMPAR proteome (Fig. 1a).

For more detailed investigation of FRRS1l-containing protein assemblies in the rat brain, we next performed two sets of reverse serial AP experiments (termed ‘two-step APs’) that are schematized in Fig. 1b (right panel): in the first AP series, the entire complement of FRRS1l protein, co-assembled with AMPARs or AMPAR-free, was extracted from membrane preparations by a mixture of anti-FRRS1l ABs prior to a target-depleting anti-GluA1-4 AP, and in the second AP series, FRRS1l was entirely affinity-isolated from membrane preparations that have been depleted of all AMPARs via an AP with anti-GluA1-4 ABs (Fig. 1b, right panel, Supplementary Fig. 1a). Quantitative evaluation of the protein amounts determined from nano-LC MS analysis of these serial APs thus provided direct information on (i) the assembly of any proteome constituent with FRRS1l-containing versus FRRS1l-free AMPARs (Fig. 1b, left panel, bars in red and blue, respectively) and (ii) for co-assembly of any proteome constituent with the FRRS1l protein independent of GluA1-4 (Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars). The respective results led to the following major observations: First, FRRS1l assemblies into an average 15–20% of all AMPARs (at steady state) as indicated by the relative amounts of GluA1-4 proteins in the anti-FRRS1l AP (red bars for GluA1-4, Fig. 1b, left two-step AP). Second, 80–85% of AMPARs lack FRRS1l but instead contain the core constituents TARPs, CNIHs and GSG1l (blue bars for GluA1-4 and the core subunits, Fig. 1b, right panel, brown bars).

**Results**

**Proteomic analyses identify distinct AMPAR assemblies.** For assessment of the cell biology of yet uncharacterized AMPAR subunits, we initially set up reverse proteomic analyses combining APs with antibodies (ABs) that target the non-GluA constituents with high-resolution nano-flow tandem mass spectrometry (nano-LC MS/MS) and protein quantification based on calibrated peptide signals (label-free QconCAT). This procedure provides molecular abundance values for all constituents of the AMPAR proteome (Supplementary Data 1) and enables comparison of proteins in different AP samples over a broad dynamic range of four orders of magnitude. Figure 1a illustrates the result of such initial APs with several anti-FRRS1l, anti-TARP and anti-GluA ABs on membrane preparations from the whole rat brain. Strikingly, the abundance heat map of these APs suggested the existence of two mutually exclusive populations of AMPAR assemblies within the proteome: One population comprising FRRS1l, CPT1c, Sac1 as well as ABHDs 6/12 and PORCN, the other containing the core subunits (CNIHs, TARPs, GSG1l) and the remainder of the peripheral constituents (red frames in Fig. 1a). The only major elements shared by both AMPAR assemblies were the GluA proteins; consequently, anti-GluA ABs performed in parallel effectively retained the complete set of constituents of the AMPAR proteome (Fig. 1a).

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**FRRS1l and CPT1c are AMPAR-selective interactors.** In addition to the analyses within the AMPAR proteome, we aimed at a more comprehensive look into interaction partners of FRRS1l by means of APs using four different anti-FRRS1l ABs and two different ABs targeting CPT1c on membrane fractions from the whole rat brain (see Methods section). Preimmunization immunoglobulins G (IgG) as well as FRRS1l-depleted membrane preparations from CPT1c knockout animals served as negative controls defining specificity of (co)-purification (refs 16,21,22; see Methods section). All proteins exceeding the specificity thresholds in at least two different anti-FRRS1l or anti-CPT1c ABs were dubbed bona fide interaction partners of the respective target and summarized in Fig. 2a together with their abundance values (obtained across the different APs). These abundance plots showed that both FRRS1l and CPT1c mutually
co-purified each other at amounts emphasizing their effective co-assembly (close to stoichiometric in anti-FRRS1l APs). Moreover, both target proteins effectively co-purified the same subset of AMPAR proteome constituents, the pore-forming GluA1-4 proteins in anti-FRRS1l and anti-TARP APs highlighted by red boxes. Annotations on the right reflect molecular architecture and/or subcellular localization reported in literature or public databases. (b) Relative amounts of AMPAR constituents determined in two-step APs schematized on the right from CL-47 solubilized membrane fractions of total adult rat brains. Bars in red and blue depict the amount of AMPAR constituents determined in a target-depleting anti-FRRS1l AP (red bars, mean ± s.d. of three measurements) and a subsequent target-depleting anti-GluA AP (blue), each determined as fraction of its summed protein amounts in the two APs. Brown bars illustrate relative amounts of AMPAR constituents (mean ± s.d. of three measurements) in an anti-FRRS1l AP using the flow through of a target-depleting anti-GluA AP as input divided by the summed protein amount determined for any constituent in both APs from rat membranes. Note co-assembly of FRRS1l with GluA1-4 and only a subset of AMPAR proteome constituents.

Interestingly, the amounts of several other AMPAR constituents appeared altered by roughly between 10% and 50% (Fig. 2b). Together, the results of our proteomic analyses established complexes assembled from GluA1-4, FRRS1l, CPT1c and Sac1 as particular AMPAR assemblies that largely differ from the well-known AMPARs in the plasma membrane by lacking the entire set of core constituents. Instead, as CPT1c and Sac1 have been reported as ER proteins23,24, the newly identified population of AMPARs may be localized to this intracellular membrane compartment—provoking the question of its role in AMPAR physiology.

Mutations in the FRRS1L gene cause intellectual disability. In the course of two independent systematic studies analysing cohorts of consanguineous families for genes causative for autosomal recessive intellectual disability, we carried out whole-exome sequencing (WES) followed by appropriate filtering (refs 25,26, see Methods section) on patients and unaffected family members (WES data and identified genes were communicated on the CARID (Consortium of Autosomal Recessive Intellectual Disability) platform). In three independent families from Algeria,
respectively). Decrease of CPT1c, FRRS1l and Sac1 in AMPARs from knockout animals. Dashed line indicating 50% reduction in protein amount is shown for orientation; anti-GluA1-4 APs from brain membrane fractions prepared from both WT and CPT1c knockout mice (normalized to GluA1-4). Note the pronounced decrease of CPT1c, FRRS1l and Sac1 in AMPARs from knockout animals. Dashed line indicating 50% reduction in protein amount is shown for orientation; asterisks denote statistical significance for protein amounts being different between WT and KO (**, ***P values of 0.01 and 0.001 for Students’ t-test, respectively).

Figure 2 | Specific co-assembly of FRRS1l and CPT1c into AMPAR complexes with distinct subunit composition. (a) Amounts of proteins specifically co-purified in APs with four different anti-FRRS1l ABs (upper panel) and two different anti-CPT1c ABs (lower panel) normalized to the amount of purified FRRS1l or CPT1c, respectively (red bar). Data are mean ± s.d. of five (four ABs, one AB mixture, see Methods section) and three (two ABs, one AB mixture) independent APs, respectively. (b) Protein abundance ratios (mean ± s.e.m. of four measurements) determined for the indicated proteome constituents in anti-GluA1-4 APs from brain membrane fractions prepared from both WT and CPT1c knockout mice (normalized to GluA1-4). Note the pronounced decrease of CPT1c, FRRS1l and Sac1 in AMPARs from knockout animals. Dashed line indicating 50% reduction in protein amount is shown for orientation; asterisks denote statistical significance for protein amounts being different between WT and KO (**, ***P values of 0.01 and 0.001 for Students’ t-test, respectively).

Syria and Saudi Arabia (Fig. 3a, Supplementary Table 1), these analyses revealed three homozygous variants in the FRRSIL gene segregating with the disorder and fitting the recessive mode of inheritance suggested by the pedigrees (Fig. 3a): a missense variant leading to a lysine-to-glutamate exchange at residue 321 (NM_014334.3: c.961C>T, p.K321E) in family A, a one-base-pair deletion causing a frameshift and premature stop (NM_014334.2: c.584delT; p.V195E fs*35) in family B and a nonsense mutation resulting in a premature stop at residue 321 (NM_014334.3: c.584delT; p.Q321*) in family C (Fig. 3a,b). Filtering WES data for compound heterozygous variants, X-linked or dominant mutations in the FRRSIL gene led to a similar phenotype in all patients, albeit differences in severity and disease course were observed with the individual mutations. Thus, after achieving independent walking, which all patients of families B and C failed (Fig. 3, Supplementary Table 2).

FRRS1–CPT1c-containing AMPARs are restricted to the ER.

To gain insight into the cell biology of FRRS1l, we next went to heterologous expression, either alone or in combination with the identified partners of the AMPAR proteome, and used confocal fluorescence microscopy, biochemistry and electrophysiology as complementary techniques for analysis (Fig. 4, Supplementary Figs 3–5).

First, we probed complex formation of FRRS1l, CPT1c and Sac1 with GluA1/2 by combinatorial expression and subsequent affinity isolation of the resulting AMPAR assemblies via anti-GluA1/2 or anti-FRRS1l ABs. Western blot-probed gel separations of anti-GluA APs revealed that FRRS1l and CPT1c were efficiently co-purified with GluA1/2 when present together (Fig. 4a), while individual co-purification of either protein was markedly less abundant (Fig. 4a). Similarly, Sac1 displayed weak or no interaction with GluA1/2 alone or together with either FRRS1l or CPT1c but was co-purified in anti-GluA and anti-FRRS1l APs when FRRS1l and CPT1c complexes were strongly restricted speech development and seizures (Fig. 3c and Supplementary Fig. 2).
present for co-assembly (Fig. 4a). In addition, the anti-FRRS1l AP demonstrated robust formation of ternary complexes between FRRS1l, CPT1c and Sac1 (Fig. 4a). Thus, in co-assembled form, FRRS1l and CPT1c promote (or stabilize) their mutual interaction with the AMPAR pore and serve as binding platform for Sac1, in close agreement with the results obtained before from the rat brain (Figs 1 and 2).

Further analysis, by confocal microscopy, revealed another profound effect of FRRS1l–CPT1c interaction: while sole FRRS1l protein was predominantly detected at the plasma membrane (Fig. 4b, upper left panel), CPT1c-assembled FRRS1l appeared entirely re-distributed to intracellular membrane compartments where its staining largely overlapped with those of CPT1c (Fig. 4b, right panels) and the ER-marker calnexin (Supplementary Fig. 3a) or edited GluA2 (Supplementary Fig. 3b). This CPT1c-mediated re-distribution of FRRS1l was also observed with GluA-associated FRRS1l as revealed in a surface biotinylation assay (see Methods section) and by current recordings from outside-out patches: In either approach, FRRS1l was no longer detected in the plasma membrane upon co-expression of CPT1c (Supplementary Fig. 4a,b). Importantly, the redistribution of FRRS1l was specific for the ER-resident CPT1c, as replacing CPT1c with CPT1a, an enzymatically active transamidase that uses S317 as C-terminal -site and the transamidase that uses S317 as C-terminal -site and the transamidase that uses S317 as C-terminal -site and the transamidase that uses S317 as C-terminal -site and the transamidase that uses S317 as C-terminal -site and the transamidase that uses S317 as C-terminal -site and the transamidase that uses S317 as C-terminal -site

As illustrated in Supplementary Fig. 5, western blot analysis of APs and native polyacrylamide gel electrophoresis (BN-PAGE) showed that FRRS1l/CPT1c do not exhibit any subtype preference in their assembly with GluA1-4 (Supplementary Fig. 5a) but exclusively co-assemble with the GluAs into high molecular weight (MW) complexes (Supplementary Fig. 5b). Quantitative analysis by cryo-slicing BN-MS30 in fact showed that these assemblies are formed by about equimolar ratios of GluAs and FRRS1l/CPT1c in line with roughly four FRRS1l/CPT1c complexes co-assembled into each GluA tetramer (Supplementary Fig. 5b).

ER-based processing of FRRS1l was investigated as western blot probing SDS–PAGE separations of brain membranes with anti-FRRS1l revealed two bands with distinct MW (Fig. 4c, left panel). Detailed MS-based sequence analysis of native and recombinant FRRS1l showed that the higher MW band corresponds to the full-length protein (Supplementary Fig. 6a), while the lower MW band is the protein truncated at residue serine 317 (S317) (Fig. 4c, Supplementary Fig. 6a). In addition, mass spectrometry identified S317 as an attachment site for a glycosylphosphatidylinositol (GPI)-anchor through the typical ethanolamine moiety (refs 31,32, Fig. 4c), in line with suggestions from prediction algorithms33. During GPI-anchoring, proteolytic cleavage and side-chain modification are mediated by a GPI transamidase that uses S317 as C-terminal α-site and the hydrophobic domain at the C-terminus of FRRS1l (Fig. 4c, Supplementary Fig. 6b) as a necessary structural cofactor32. Accordingly, FRRS1l is anchored in the ER membrane either via the lipid moiety of the GPI-anchor or via the hydrophobic domain in the C-terminus34. In any case, the particular importance of membrane anchoring for interaction of FRRS1l with the AMPAR pore became evident from further mutagenesis: Deletion of the C-terminus at alanine 318 (A318) entirely

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**Figure 3 | Mutations in FRRS1l identified as disease causing in patients with severe intellectual disability.** (a) Pedigree (and genotypes) of the three different families detailed in the text. Affected patients are indicated by filled symbol; slashes refer to deceased individuals. (b) Schematic representation of the FRRS1l protein (as given in the UniProtKB/Swiss-Prot database) together with the alterations identified in the indicated families and alignment of the primary sequence stretch around lysine 155 (K155) across species. TM is a predicted transmembrane domain. (c) Summary of the clinical features observed in the patients from families A–C. MA denotes mental age, NA means not investigated.
Figure 4 | Assembly of WT and mutant FRRS1l with CPT1c and significance for ER localization and association with GluA proteins. (a) Input and eluates of anti-GluA1 and anti-FRRS1l APs separated by SDS–PAGE and western blot probed for the indicated proteins. Note strong cooperativity of FRRS1l and CPT1c binding to AMPARs. (b) Representative confocal fluorescence images of tsA-201 cells expressing FRRS1l alone (upper left) or together with CPT1a (middle and lower left) or CPT1c (right); fluorescence staining as indicated by the colour coding (with anti-FRRS1l-a, anti-CPT1a or anti-CPT1c). Scale bar is 10 μm. Note marked re-distribution of FRRS1l upon co-expression of CPT1c from plasma membrane to intracellular membranes (FRRS1l staining: Fabricated No. 128). (c) Left: SDS–PAGE separation of membrane preparations from brain and culture cells (expressing the indicated proteins) western blot probed with anti-FRRS1l-a. Marks on the right refer to the distinct MW bands, red denotes FRRS1l with additional mass introduced by the HA-6His tag (Supplementary Fig. 6). Right: CID MS/MS spectrum of the indicated FRRS1l peptide carrying an ethanolamine moiety at S317 as a specific marker for GPI-anchoring (Supplementary Fig. 6). Inset: Scheme highlighting the hydrophobic domain and relevant residues in the C-terminus of FRRS1l. (d) Input and eluates of anti-GluA1/2 APs separated by SDS–PAGE and western blot probed for the indicated proteins. Note the reduction or failure of the mutant FRRS1l proteins to assemble with the GluA proteins.

abolished the robust co-assembly observed with WT FRRS1l (Fig. 4d).

Disease mutations disturb FRRS1l-AMPAR interaction. Finally, we investigated the mutations identified in patients with intellectual disability for their impact on protein expression and assembly of FRRS1l. As shown in Fig. 4d, both truncation mutations, FRRS1l(Q321*) and FRRS1l(V195E fs*35), failed co-assembly with GluA1/A2 (Fig. 4d) and also appeared less abundantly expressed (or less stable) than the WT protein. In contrast, the K155E substitution mutant was still able to interact with GluA1/A2, albeit less effectively than WT FRRS1l (Fig. 4d).

Together, the results obtained from heterologous expression in culture cells demonstrated robust complex formation of FRRS1l and CPT1c, as well as their co-assembly with GluA tetramers in the ER. This co-assembly with the AMPAR pore and/or the stability of the FRRS1l protein is largely affected by the mutations identified in patients with intellectual disability.

FRRS1l-containing AMPARs in brain neurons localize to the ER. The subcellular distribution of FRRS1l seen in transfected culture cells was entirely recapitulated in hippocampal neurons as detected by confocal microscopy and pre-embedding immunogold EM in respective slice preparations of the rat brain. Thus immuno-fluorescence staining of FRRS1l closely overlapped with that of the ER-marker calnexin (Fig. 5a), and gold particles coupled to anti-FRRS1l were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b). In more detail, 96.1% of all immunoparticles were almost exclusively detected at ER membranes, but not over the Golgi complex, and only very rarely at/or close to the plasma membrane (Fig. 5b).
containing AMPARs, we used patch-clamp recordings from various types of hippocampal neurons in brain slices combined with manipulation of protein levels for FRRS1l or CPT1c. The latter was achieved with lentiviruses that were stereotactically administered to P6/7 rats (ref. 35, see Methods section) to promote either knockdown of protein expression via target-specific short hairpin RNAs (sh-FRRS1l, sh-CPT1c, Fig. 6a, for efficiency and specificity of protein knockdown: lower inset and Supplementary Fig. 7) or (over)expression of exogenous protein. Figure 6a illustrates EPSCs mediated by AMPARs in single synapses of hilar neurons in the hippocampus under control conditions and after virus-directed manipulations. The respective EPSCs were measured by the paired-bouton recording technique in postsynaptic mossy cells (MCs) in response to single action potentials elicited in presynaptic mossy fibre boutons (MFBs) by brief current pulses (Fig. 6a, upper inset35). Under control conditions (untransduced MCs), the MFB–MC EPSCs that are predominantly carried by CNIH2-containing GluA1/A2 AMPARs35 exhibited large amplitudes with a mean value

Figure 6 | Alterations in EPSCs upon knockdown of FRRS1l in individual MFB–MC synapses. (a) Representative action potential and EPSC traces determined by paired bouton recordings (upper inset) in hippocampal slices from MFB–MC synapses of an uninfected MC (control, left) or MCs transduced with sh-FRRS1l (middle) or sh-CPT1c (right). Current and time scaling as indicated. Grey lines are mono-exponential fits to the decay phase yielding time constants of 10.4 ms (control), 10.1 ms (sh-FRRS1l) and 10.1 ms (sh-CPT1c). Inset: Confocal fluorescence image of an uninfected (lower) and a sh-FRRS1l (fluorescence of GFP marker) transduced (upper) MC used for recordings (filled with biocytin, red fluorescence); framed images depict anti-FRRS1l staining of the two cells. Scale bar is 10 μm. (b) Summary plots of amplitudes (left) and decay time constants of the EPSCs determined in experiments as in a. Squares represent mean ± s.e.m. of the experiments shown.
(± s.e.m.) of 578.1 ± 57.2 pA (n = 15; Fig. 6a, b). Virus-driven knockdown of either FRRS1l or CPT1c, however, significantly reduced the amplitude to about 60% (Fig. 6a, b; values of 370.3 ± 47.7 pA (n = 11) and 331.6 ± 47.3 pA (n = 13) for sh-FRRS1l and sh-CPT1c, respectively; P < 0.01, Mann–Whitney U-test), while leaving the time course of the current decay unaffected (values for τ_{decay} of 11.3 ± 0.4, 11.4 ± 0.6 and 11.2 ± 0.5 ms for control, sh-FRRS1l and sh-CPT1c, respectively). Moreover, the paired-pulse ratios (at 50 Hz) determined in all MFB–MC pairs were similar in untransduced controls and virus-infected MCs (mean (± s.e.m.) of 146.0 ± 6.6% (n = 15, control), 147.2 ± 7.4% (n = 11, sh-FRRS1l) and 145.1 ± 6.0% (n = 13, sh-CPT1c), indicating that knockdown of FRRS1l and CPT1c did not affect the probability of transmitter release from the presynapse.

Similar results as for single MFB–MC synapses were obtained from EPSCs evoked by spontaneous action potentials in the large number of synapses forming onto individual MCs. As demonstrated by distribution histograms, sh-FRRS1l shifted the EPSC amplitudes towards smaller values, while overexpression of FRRS1l resulted in a shift in the opposite direction (Fig. 7a). When compared to untransduced controls, sh-FRRS1l and sh-CPT1c reduced the EPSC amplitudes to values of 0.68 and 0.64, respectively, while exogenous FRRS1l expression increased the EPSC amplitude to 1.32 (Fig. 7b; P < 0.01, Mann–Whitney U-test). In neither case was the time course of the EPSCs affected by these manipulations (values for τ_{decay} (mean ± s.e.m.) of 112.2 ± 0.2, 104.2 ± 0.3, 111.2 ± 0.3 and 107.2 ± 0.5 ms for control, sh-FRRS1l, sh-CPT1c and FRRS1l overexpression, respectively). Determination of miniature EPSCs in control MCs and MCs transduced with sh-FRRS1l or sh-CPT1c corroborated the amplitude effects (respective values (mean ± s.e.m.) of 22.7 ± 1.8 pA (n = 5), 17.8 ± 1.4 pA (n = 4) and 18.1 ± 0.3 pA (n = 4) for control, sh-FRRS1l and sh-CPT1c, respectively) and suggested that the observed reduction in EPSC amplitude results from a reduced number of functional AMPARs in the postsynapse.

The specificity of the observed knockdown effects on AMPARs was probed in two further sets of experiments: First, by applying either target-unrelated 'control' sh-RNAs or additional sh-RNAs directed against alternative sequences on the FRRS1l or CPT1c mRNAs, and second, by comparing the EPSC components mediated by AMPARs and NMDA (N-methyl-D-aspartate)-type glutamate receptors (NMDARs) in sh-RNA transduced and control MCs. As shown in Fig. 7b, no alterations in the EPSC amplitude were observed in MCs virally transduced with sh-RNAs targeting TARPs 2 and 8 that were not detected in MCs35 did not change the EPSC amplitude. In contrast, transduction of MCs with the additional sh-RNAs directed against FRRS1l (sh-FRRS1l-b, -c) or CPT1c (CPT1c-b) resulted in a decrease in EPSC...
amplitude (Supplementary Fig. 8) similar to that observed before (Fig. 7b). The AMPA/NMDA ratio determined for the dual component EPSCs recorded at 40 mV (Supplementary Fig. 9) were reduced by approximately 45% in MCs transduced with sh-FRRS1l or sh-CPT1c compared to uninfected control MCs, indicating a selective effect of the protein knockdown on AMPARs (AMPA/NMDA ratio: 1.12 ± 0.07, n = 9 for control MCs, 0.57 ± 0.04, n = 10 and 0.65 ± 0.07, n = 9 for sh-FRRS1l- and sh-CPT1c-transduced MCs, respectively).

Next, we probed the effects of sh-RNA-mediated knockdown of FRRS1l and CPT1c and of FRRS1l (over)expression in other types of hippocampal neurons and investigated their significance for AMPARs in extra-synaptic localization. As illustrated in Fig. 7b, the decrease and increase in EPSC amplitude observed with sh-FRRS1l/sh-CPT1c and FRRS1l (over)expression was not restricted to MCs but was similarly observed in interneurons of the hilar region, as well as in CA3 pyramidal cells (Fig. 7b, middle and right panels, Supplementary Fig. 8). The impact of FRRS1l/CPT1c knockdown on extra-synaptic AMPARs was tested by dual recordings in MCs as schematized in Fig. 7c (inset): After recording spontaneous EPSCs in whole-cell configuration, the patch-pipette placed on the cell soma was excised into outside-out configuration for fast agonist-application experiments measuring currents through the somatic AMPARs. Representative current traces of such experiments recorded in the same MC together with the summary bar graphs illustrate a concomitant reduction by 40–50% of both EPSCs and somatic AMPAR currents as a consequence of protein knockdown by sh-FRRS1l and sh-CPT1c (Fig. 7c,d).

Together, these results indicated that FRRS1l–CPT1c complexes, although strictly localized to intracellular ER membranes, are able to profoundly affect the amplitude of the EPSCs by controlling the number of AMPARs in both synapses and extra-synaptic sites of the plasma membrane.

**Discussion**

We identified FRRS1l and CPT1c as key components of distinct assemblies of AMPARs that are exclusively localized to ER membranes and lack the inner core subunits characteristic for AMPAR complexes at the plasma membrane. These assemblies likely represent an early step in AMPAR biogenesis and markedly affect the amplitude of AMPAR-mediated fast EPSCs. Their significance for brain function is reflected by the loss-of-function phenotype with intellectual disability and epilepsy.

For unbiased analysis of complex assemblies encoded by the AMPAR proteome, we used a reverse proteomic approach with non-GluA ABs and (absolute) protein quantification based on high-resolution MS and calibrated peptide signals (Fig. 1a). This approach revealed two subsets of constituents that are mutually exclusive for large parts except for the pore-forming GluA proteins and established a population of AMPAR assemblies with FRRS1l and CPT1c as key components. Subsequent work using two-step and multi-epitope APs (Figs 1b and 2), biochemistry as well as EM and fluorescence microscopy established several key features of FRRS1l–CPT1c-containing AMPARs (Figs 2–5): They (i) lack the known core subunits TARPs, CNIHs and GSG1l, (ii) represent roughly 15–20% of all (steady state) AMPAR assemblies in the rodent brain, (iii) they are exclusively localized to ER membranes through the ER-resident CPT1c, and (iv) they do not exhibit region specificity in distribution across regions of the brain.

Important to note, both FRRS1l and CPT1c display pronounced selectivity for assembly with AMPARs (over very few other mostly ER-based proteins) as indicated by our comprehensive proteomic analysis (Fig. 2a). This selectivity contrasts the interactions reported for other ER-localized constituents of the AMPAR proteome, including ABHDs and PORCN.

The significance of FRRS1l–CPT1c-containing AMPAR assemblies for the cell physiology became evident in functional recordings from various types of hippocampal neurons (Figs 6 and 7). Knockdown of FRRS1l and CPT1c (Figs 6 and 7) and exogenous (over)expression of FRRS1l (Fig. 7) caused a profound decrease or increase of EPSC amplitudes, respectively, by altering the number of AMPARs in the surface membrane of synaptic and extra-synaptic sites. Similarly, decreased mEPSC amplitudes were also reported for cultured hippocampal neurons from CPT1c knockout animals as a consequence of posttranscriptional action(s) of CPT1c (different from de-palmitoylation) impacting surface expression of AMPARs.

In fact, these results are complemented by two additional (interesting) observations derived from QconCAT-based protein quantification and correlation analyses: first, the protein amounts of AMPAR core subunits, typical for the receptors at the plasma membrane, are reduced proportionally to the amount of FRRS1l in experiments with several independent sh-RNAs (Fig. 8a, left panel), and second, correlation of FRRS1l amounts was maximal with the sum of protein amounts determined for the core subunits (rather than with the amounts of the individual subunits or total AMPARs; Fig. 8a, right panel). Both observations together with their mutual exclusiveness (Fig. 1) strongly suggest that all surface AMPARs (containing TARPs, CNIHs and/or GSG1l) emerge from FRRS1l–CPT1c-containing AMPAR assemblies in the ER.

In the context of a neuron, our results may be combined into a scheme (Fig. 8b), where FRRS1l–CPT1c assemblies act as a general ‘catalyst’ of AMPAR biogenesis. As a first step in AMPAR biogenesis, newly synthesized GluA tetramers (GluA\(_{\text{tetra}}\); Fig. 8b) co-assemble with (up to four) FRRS1l–CPT1c complexes (Fig. 4, Supplementary Fig. 5) thus ‘priming’ them for interaction with the auxiliary proteins CNIHs, TARPs or GSG1l. Binding of these subunits to GluA\(_{\text{tetra}}\)-FRRS1l/CPT1c generates a short-lived transitional complex (‘priming of assembly’ in Fig. 8b) that has two main consequences: First, it leads to rapid dissociation of FRRS1l/CPT1c, and, second, it establishes stable heteromultimers of GluA and CNIH/TARP subunits. While these stable AMPAR complexes proceed towards the ER-exit sites and are finally delivered to the plasma membrane (through the secretory pathway), FRRS1l/CPT1c complexes remain in the ER, ready for re-association with new GluA\(_{\text{tetra}}\) and thus re-entering the catalysis cycle. In this model, FRRS1l/CPT1c complexes operate as classical catalysts driving GluA-CNITH/TARP assembly that can also occur in their absence, albeit with less efficiency. As a consequence of this mechanism, the number of AMPARs at the plasma membrane can be effectively regulated as seen in knockdown and overexpression experiments (Figs 6 and 7). It is noteworthy that the presented model does not preclude any additional factors described for ER exit of GluA and/or GluA-CNITH/TARP complexes\(^{40,41}\) nor does it rule out additional regulatory processes occurring in the secretory pathway or along vesicle exocytosis/endocytosis. Importantly though, the latter cannot compensate for the disruption of the ER-priming complex (as induced by sh-RNA-mediated knockdown of FRRS1l or CPT1c or by the disease mutations in FRRS1l, Figs 6–8).

Our experimental results have some additional noteworthy implications. First, they emphasize the potential significance of functionally uncharacterized, including purely intracellular, constituents of the AMPAR proteome for assembly and function of this key component of excitatory synaptic transmission. Second, they demonstrate that the proteome constituents do not assemble randomly, but rather in ‘groups’ as shown here for
The significance of FRRS1l–CPT1c-containing AMPARs in the context of the whole brain is reflected by both the phenotype of CPT1c knockout mice presenting with impaired spatial learning, reduced muscular strength and hypoaactivity\cite{23,24}, but in particular by the severe clinical features observed in patients carrying homozygous mutations in the FRRS1l gene (Fig. 3; refs 27,28). All of these patients exhibited markedly impaired brain function with severe intellectual disability and epilepsy.

A few of these features may be reconciled by the properties of FRRS1l expression and the results of its knockdown in adult rats (mimicking the lack of FRRS1l-GluA complex; Figs 6 and 7). Thus a decrease in EPSC amplitudes in most (if not all) excitatory glutamatergic synapses is expected to impact both synaptogenesis and signal transmission and, consequently, the processes underlying synaptic plasticity involved in skill learning and memory formation. Furthermore, the reduced EPSCs should, via imbalance between excitatory and inhibitory synaptic transmission, promote the onset of seizures\cite{43,44}. And finally, the regression phenotype observed in patients of families B and C might correlate with the pronounced upregulation observed for FRRS1l expression during postnatal development\cite{15}, while the milder phenotype segregating with the K155E mutation (family A) was accompanied by a less impaired interaction of FRRS1l with GluA\textsubscript{1x} (Fig. 4d).

In conclusion, our work provides de novo assignment of cellular function(s) to FRRS1l–CPT1c complexes, previously uncharacterized constituents of the AMPAR proteome, that impact receptor biogenesis and fast excitatory synaptic transmission. Our work emphasizes not only the importance of the AMPAR proteome but also necessitates further extensive research to understand its individual components in terms of overall AMPAR biology and its significance for encoding the molecular framework underlying the operation and dynamics of excitatory neurotransmission in the brain.

**Methods**

**Molecular biology.** The CDNAs were used all verified by sequencing and had the following GenBank (www.ncbi.nlm.nih.gov/genbank) accession numbers: M386801.1 (GluA1f, flip variant of GluA1), NM_017261.2 (GluA2f), NM_053551 (TARP-2), NM_001025132 (CNIH-2), AF357970.1 (CPT1c), NM_014334 (FRRS1l), and BC117725.1 (Sac1).

**Biochemistry and cell biology.** Affinity purification from brain membranes. Plasma-membrane-enriched protein fractions were prepared from freshly isolated WT rat or mouse brains (pools of 10–20 animals) or from three mouse brains of CPT1c knockout animals\cite{23,24}, as previously described\cite{16}, and solubilized with buffers CL-47 and CL-91 (Logopharm GmbH, Germany) for 30 min on ice (at 1 mg protein per ml). After clearing by ultracentrifugation (150,000 g, 10 min), solubilisates were incubated with the respective immobilized ABs. After 2 h of incubation and two brief washes, proteins were eluted, shortly run on SDS–PAGE gels and silver-stained. Lanes were cut into two sections (high and low MW) and digested with sequencing-grade modified trypsin (Promega, Mannheim, Germany). Peptides were extracted and prepared for MS analysis as described\cite{23,24}. The following ABs were used: anti-GluA1 (Millipore, #AB1504, 1:1,000), anti-GluA2 (NeuroMab, #75-002, 1:1,000), anti-GluA2/3 (Millipore, #07-598, 1:1,000), anti-GluA3 (Synaptic Systems, #182203, 1:1,000), anti-GluA4 (Millipore, #AB1508, 1:1,000), rabbit anti-FRRS1l-a, anti-FRRS1l-b (epitope: rat FRRS1l aa 47-66, AB generation by AbFrontiers, South Korea, 1:1,000, 15 μg), anti-CPT1c-a (Santa Cruz Biotechnology, sc-139479, 1:1,000, 15 μg), anti-CPT1c-b\cite{15}, anti-TARP-a (anti-TARP-y2.3; Millipore, #07-577, 15 μg), anti-TARP-b (anti-TARP-y2.4,8; NeuroMab, #12-370, 15 μg), and IgG rabbit (Millipore, #12-370, 15 μg). Two anti-FRRS1l and anti-CPT1c APs used mixtures of ABs (anti-FRRS1l-a,b,c; anti-CPT1c-a,b).

**Target-depleted solubilisates as source for anti-FRRS1l-c APs.** Rat membrane proteins (1.5 ml) solubilized with CL-47 and CL-91 (1 mg ml\textsuperscript{-1}) were incubated for 2 h with 120 μg of a mixture of immobilized anti-FRRS1l-a and anti-CPT1c-b. Thereafter, ABs were removed and the supernatant, deprived of at least 95% of FRRS1l protein, was subsequently incubated for 2 h with anti-FRRS1l-c ABs. After brief washing, proteins were eluted with SDS buffer without dithiothreitol. These
eluates served as a negative control (specificity determination) in APs with the respective AB (see ‘Protein quantification’).

**Two-step APs.** Rat and mouse brain solubilates (0.5 mL, concentration 1 mg mL⁻¹) were incubated for 2 h with a mixture of immobilized FRRS1l-targeting ABs (anti-FRRS1l-a, -b, -c; 80 μg). Subsequently, the supernatants were subjected to APs with a mixture of anti-GluA1-4 ABs (see above, 80 μg); bound proteins were eluted in reverse order: An AP using the anti-GluA1-4 AB mix (80 μg) followed by an AP with the mixture of anti-FRRS1l ABs (80 μg). Efficiency of the APs was verified by western blot analysis of SDS–PAGE–resolved samples taken before and after each incubation step (Supplementary Fig. 1). The eluates were processed for MS analysis as described above. APs shown in Fig. 1 were performed 2–4 times.

**AMPARs in neuronal cultures after FRRS1l knockdown.** Primary cortical neurons were prepared from rats at E18 and cultured essentially as described. Cells were transfected with lentiviruses targeting ABs (anti-FRRS1l-a, -b, -c, -d, -f; 80 μg). Efficiency of the lentiviruses was estimated by Western blot analysis of SDS–PAGE–resolved samples taken before and after infection. The blots (Fig. 4) have been cropped for presentation. Full size images are presented in Supplementary Fig. 6.

**Reconstitution of protein complexes.** The indicated proteins were expressed in transiently transfected tsA-201 cells that had been cultured according to manufacturer’s instructions (Thermo Fisher Scientific, Germany) and a nominal resolution of 15,000 (full-width at half-maximum) at 400 p.p.m. and a scan range of 3–3,000 m/z. Up to 10 data-dependent CID fragment ion spectra (isolation width 1 m/z with wideband activation) per cycle were acquired in the ion trap with a target value of 10,000 (maximum injection time 200 ms for complex mixtures and 400 ms for gel bands) with dynamic exclusion (exclusion duration 30 s; exclusion mass width ± 2 p.p.m., preview mode for FTMS master scans, charge state screening, monoisotopic precursor selection and charge state rejection (unassigned charge states and for trypsin-digested samples also charge state 1) enabled. For highly reliable peptide identification in excised FRRS1l-containing gel bands, additional high-resolution CID fragment ion spectra of z-lytic peptide-digested samples were acquired in the Orbitrap analyser with a target value of 50,000 (maximum injection time 500 ms) and a nominal resolution of 15,000 (full-width at half-maximum at m/z 400, centrring at m/z 200). The mass spectrometric data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD006413 and 10.6019/PXD006413.

**Protein identification.** LC-MS/MS data were extracted using ‘mconvert.exe’ (part of ProteoWizard; http://proteowizard.sourceforge.net/, version 3.0.6906).

**Protein quantification.** Analyses were performed in triplicate using a modified UniProtKB/Swiss-Prot entry (release 2017_01 for Figs 1b, 2b and 8a, release 2015_09 or newer for remaining analyses; all rat, mouse and human entries, as well as sp|P02769, sp|P00766 and sp|P00761, supplemented with the TrEMBL/NCBI entries tr|D3ZVQ3, tr|D4A4M0, tr|M0RB53, tr|D4A0X1 and XP_008765687.1 for missing AMPAR constituents) using Mascot 2.0.8 (Matrix Science, UK). Initially, preliminary searches with high peptide mass tolerance (± 50 p.p.m.) were performed. After linear shift mass recalibration using in-house developed software, peptide mass tolerance was reduced to ± 5 p.p.m. for final searches. Fragment mass tolerance was set to ± 0.8 Da (on trap MS/MS spectra). One missed trypsin cleavage and common variable modifications (i.e., N-terminal acetylation and oxidation) were accepted for peptide identification. Significance threshold was set to P < 0.05. Proteins identified by only one specific MS/MS spectrum or representative contaminations such as keratins or immunoglobulins were eliminated.

**Primary sequence analysis.** Peak lists extracted from the LC-MS/MS/MS data of the FRRS1l protein band were further searched against the human UniProtKB/Swiss-Prot entries, the heterologous FRRS1l and C1C1c sequences (see above) and a series of C-terminally truncated FRRS1l sequences (truncation after P316, S317, A318, A319, Y320, P321, T322, F333, S342 or S525 to promote Mascot identification of the C-terminal peptide of the truncated protein in the lower MW band). For high-resolution MS/MS spectra acquired in the Orbitrap analyser, the fragment mass tolerance was reduced to ± 20 m.u. One missed trypsin cleavage and up to five missed WaLP/MaLP cleavages (after A, T or V/A, F, L, M, T or V, respectively) were allowed for the respective digests. Singular peptide matches with poor Mascot identification scores or expect values were manually checked before the respective peptide sequences were integrated in the coverage shown in Supplementary Fig. 6.

**Protein quantification.** Label-free quantification of proteins was based on peak volumes (PVs = peptide m/z signal intensities integrated over time) of peptide features as described previously. Peptide feature extraction was done with MaxQuant (http://www.maxquant.org/48, version 1.4) with integrated effective mass calibration. Features were then aligned between different LC-MS/MS runs and assigned to peptides with retention time tolerance ± 1 min and mass tolerance: ± 1.5 p.p.m. using an in-house developed software. The resulting peptide PV tables formed the basis for protein quantification (molecular and relative abundance in Figs 1c, 2c and Supplementary Figs 1b and 5c).

**Molecular abundances of AMPAR constituents** (Fig. 1a) were determined from protein profiles calibrated with a label-free QconCAT method. Protein and AMPAR protein profiles were integrated with the respective peptide sequences calibrated with a label-free QconCAT method15,16. First, the peptide PV data for each protein were filtered for outliers and false-positive assignments using a recently developed correction-based method. For each peptide, the PVs were then normalized to their maximum over all AP data sets yielding relative protein profiles, ranked for each protein by pairwise Pearson correlation. The medians of at least 2–7 or, for larger proteins, the 50% best...
correlating protein-specific peptide profiles were used to calculate the relative abundance values of each protein profile as described in ‘Protein profiles’30. These protein profiles were scaled to best fit the peptide calibration values obtained from MS analyses of fusion protein standards15,16 to obtain molecular abundance values (arbitrary units) for each protein. These values were finally normalized to the molecular abundance of the primary target protein(s) in each AP data set to obtain the relative abundance associated with the target. For two proteins in which included proteins for which QconCAT standards were not available) were estimated from abundance values (spec) values determined as the sum of all assigned and protein isoform-specific PVs divided by the number of MS-accessible protein isoform-specific amino acids49.

For in silico quantitative proteomics in two different groups of samples (replicate measurements in Figs 1b, 2a and 8a and Supplementary Fig. 1b), protein ratios (rPVs) were calculated after normalization of each AP data set to the (relative) abundance of its primary target (that is, molecular abundance of total AMPA receptors (Fig. 1a) and relative abundance of all AMPA subunits (Fig. 8a) as follows: For the two-step APs (Fig. 1b and Supplementary Fig. 1b), rPVs were calculated for each first replicate AP versus the averaged PVs of the second replicate APs using the TopCorr method49. The relative amount (F) of the protein found in the first AP was determined by dividing each rPV value by the sum of (rPV + 1) and given as mean ± s.e.m. The fraction of the protein in the second AP was defined as (1 – F). Protein ratios in Figs 2a and 8a were determined from the protein profiles (determined as described above); for Fig. 2b, reference was the mean of four APs from WT, and for Fig. 8b, reference was the mean of four APs from control sh-RNA-transfected cultures. A two-sided Student’s t-test was performed to determine significance of the observed changes. For the specificity threshold, which were determined in previous work40, in Figs 1a and 2b, the histograms of all proteins detected in the respective AP versus control49. Proteins were considered specifically co-purified with FRRS1l when (i) rPV (WT rat versus IgG)/threshold(versus IgG) was >1 in experiments with two independent ABs and (ii) the control purification using a target protein-specific antibody (obtained for anti-FRRS1l-e) showed rPV (WT rat versus the biochemical decontrol)/threshold(versus decontrol) >1. Similarly, specificity of co-purification with CPT1c was determined based on criteria (i) rPV ratio (WT rat versus IgG)/threshold(versus IgG) >1 for all experiments (two independent CPT1c ABs) done from WT rat and WT mouse compared to IgG, and (ii) rPV ratio (WT mouse versus the CPT1c knockout)/threshold(versus knockout control) >1 for both ABs.

Electrophysiology. In vivo stereotactic injection. The distinct lentiviruses were injected into Wistar rats 6–7 days after birth (P6–P7). Animals were anesthetized by injection of a ketamine/doriden mixture and mounted in a Kopf stereotaxic instrument (Life Technologies, USA). Recordings were performed 10–18 days following virus injection. All experiments were in accordance with national and institutional guidelines and approved by the Animal Care Committee Freiburg according to the Tiererschutzgesetz (AZ. G-12/17).

Generation of lentivirus. The lentivirus driving sequence of sh-RNAs or FRSS1l protein were generated as described in ref. 15. Briefly, oligonucleotides targeting rat FRSS1l (5’-TTGGGATCTGTCATTGG-3’, sh-FRSSL1; 5’-GCGAAGCCTGTAATGACCAAAA-3’, sh-FRSSL1-b; 5’-TTGGGATCTGTCATTGG-3’, sh-FRSSL1-c; 5’-GCCAGGCTGTAATGACCAAAA-3’, sh-FRSSL1-d; 5’-GGTAATTTAACCCGCACTAACAA-3’, sh-FRSSL1-e; 5’-GCCAACAGCTGTCATTGAA-3’, sh-FRSSL1-f), rat TARP-8 (5’-CGGAGGACACGACGAAAA-3’) and rat TARP-2 (5’-CGGAGGACACGACGAAAA-3’) were annealed with the Ion AmpliSeq Exome Kit. The DNA Libraries were pooled, barcoded and sequenced using an Ion Torrent Proton sequencer. Variant calling and filtering strategies were as described55. Briefly: (i) common genetic variants based on available public databases (ExAc, EVS, 1000GP) were excluded, (ii) remaining variants were evaluated for all inheritance patterns (autosomal-dominant due to a de novo mutation, autosomal-recessive due to homogenous or compound heterozygous mutations and X chromosome recessive inheritance), and (iii) variants segregating with the symptoms and predicted to impact the protein structure (missense, splicing, insertions or deletions and so on) were selected. From this, variants observed in >2% in our in-house database (>4000 exomes) were excluded and the remaining candidate variants were checked for quality, pathogenicity and phenotype as mentioned above. Conventional Sanger sequencing confirmed candidate variants; segregation of these variants with the disease was assessed for all available family members.

Immunohistochemistry. After fixation with paraformaldehyde, transverse hippocampal slices (60 μm thick) were obtained from the brains of 3–4-week-old Wistar rats. Brain slices and tsa-201 cells (culturing and transfection as above) were blocked with 6% normal goat serum in 0.1 M phosphate buffer. Proteins were immunodetected after permeabilization with 0.1% Triton X-100 using the following target-specific primary ABs: anti-FRRS1l-a, anti-FRSSL1-c, anti-VGlut1 (NeuroMab, #73-066), anti-CPT1c (sc-39340, Santa Cruz Biotechnology), anti-CPT1c (sc-393070, Santa Cruz Biotechnology), and anti-Calnexin (ab140818, Abcam). Mitochondria were detected with the mitotracker Deep Red FM (Molecular Probes, USA). All other secondary antibodies were conjugated to Cy2, Cy3, Cy5, Cy7, Alexa488 and Alexa555. (Molecular Probes, USA) were incubated for 1 h. Brain slices and coverslips were mounted in Fluor Save reagent (Calbiochem). All experiments in Figs 4 were done three times as independent transfections; immunostainings in slices (Fig. 5) were performed with two different animals. For verification of protein knockdown by sh-RNA transfection, cells were filled with whole-cell recording with 0.1% biocytin (Molecular Probes, USA) added to the intracellular solution. After recordings, slices were fixed overnight at 4°C in 0.1 M phosphate buffer containing 4% parafomaldehyde. Immunofluorescence was analysed using a confocal laser-scanning microscope (LSM 710 meta, Zeiss). Confocal images were acquired with a Plan-Apochromat 40×/1.3 N.A. and 63×/1.4 N.A. oil objectives (Zeiss). Line scans were done with the Zen 2012 SP1 software (Zeiss, Germany).

Electronic microscopy. For pre-embedding immunogold labeling of FRSS1l, perfused tissues from two Wistar rats were prepared as described previously56,57. Sections (50 μm) from the CA1 area of the hippocampus were cryo-protected and freeze-thawed, then incubated in 20% normal goat serum (NGS; Vector Laboratories) and incubated with anti-FRSSL1 (2.6 μg ml⁻¹) diluted in 1% NGS. After rinsing in 1% NGS for 1 h, sections were incubated with goat anti-rabbit secondary AB (Fab fragment, diluted 1:100) coupled to 1.4 nm gold nanoparticles (Nanoprobes, Stony Brook, NY), made up in TBS containing 1% NGS, overnight at 4°C. After washes in TBS, sections were washed in double-distilled water followed by silver enhancement of gold particles with an HQ Silver Kit (Nanoprobes, USA) for 10 min. After several washes in phosphate buffer (PBS), sections were treated with 0.02% OIO, in PBS and double-distilled water and then contrasted in 1% uranyl acetate for 40 min. Subsequently, they were dehydrated in a series of ethanol and propylene oxide and flat embedded in epoxy resin (Durcupan ACM; Fluka, Switzerland). After polymerization, ultrathin sections (70 nm) were cut using an ultramicrotome (Reichert Ultracut E; Leica, Vienna, Austria) and analysed in an electron microscope (Philips CM10).

In the present study, the authors have successfully demonstrated the utility of quantitative proteomics in assessing the relative abundance of proteins in different conditions. This approach allows for a more accurate and comprehensive understanding of protein expression patterns and can be particularly valuable in identifying potential biomarkers or therapeutic targets.

For the analysis of protein abundance changes, the authors utilized quantitative proteomics techniques, which involve the comparison of protein expression levels across different experimental conditions. This was achieved by using a combination of shotgun proteomics and targeted approaches, such as mass spectrometry-based quantification.

The authors selected specific proteins of interest, such as AMPA receptors and FRSS1l, using sequence-specific antibodies. These antibodies were designed to recognize unique epitopes within the protein sequence, enabling the specific detection and quantification of the corresponding protein.

The results indicated significant changes in protein expression levels, both in cell culture experiments and in vivo studies. These changes were observed at the protein level, suggesting that these proteins play critical roles in the processes being studied.

Overall, the study provides valuable insights into the complex interplay of protein expression and function, highlighting the potential of quantitative proteomics as a powerful tool for understanding biological systems.
were synthesized as sense-antisense hairpins, subcloned into pSuper (OligoEngine) and then transfected to viral vectors (FUGW) equipped with enhanced GFP. For exogenous (over)expression, FRS11 was subcloned into a double promoter lentivector (System Biosciences, #CD511B-1) with c-opGFP as expression marker. Lentiviruses were generated by transfecting 29A-201 cells with transfer (pFUGW) and packaging (pSVS and pPA.G) vectors. The medium was collected after 72 h and purified on aartificial cerebrospinal fluid providing stock solutions with a titer of $1 \times 10^{10}$ ml$^{-1}$.

Slice preparation. Transverse 300-μm-thick hippocampal slices were cut from the brains of 3–4-week-old Wistar rats, as described. Hippocampal slices were cut in ice-cold sucrose-containing physiological saline using a commercial vibratome (VT1000S, Leica Microsystems). Slices were incubated at 35°C, transferred to a recording chamber and superfused with physiological saline at room temperature. Cells and subcellular compartments (MFBs) were visualized by infrared differential interference contrast (IR-DIC) video-microscopy using an Axio examiner microscope (Zeiss, Germany) equipped with a 63× water-immersion objective coupled to an epifluorescence system.

**Cellular and subcellular patch-clamp recording.** Patch pipettes were pulled from borosilicate glass (Hilgenberg, Germany; outer diameter, 2 mm; wall thickness, 0.7 mm for presynaptic recordings and 0.5 mm for somatic recordings). When filled with internal solution, they had resistances of 0.7 MΩ for presynaptic recordings and 0.5 MΩ for somatic recordings. When coupled to an epi-fluorescence system.

**Data availability.** The mass spectrometry proteomics data generated during this study are available via ProteomeXchange with identifier PXD006413; any other data supporting the findings of this study is available from the corresponding authors upon reasonable request.

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Author contributions

J.S., U.S., L.C., R.A.J. and B.F. conceived the project. All authors performed experiments and analysed data. R.A.J., L.C. and B.F. wrote the manuscript with support from all authors.

Additional information

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