Epilepsy and intellectual disability linked protein Shrm4 interaction with GABABRs shapes inhibitory neurotransmission

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Shrm4, a protein expressed only in polarized tissues, is encoded by the KIAA1202 gene, whose mutations have been linked to epilepsy and intellectual disability. However, a physiological role for Shrm4 in the brain is yet to be established. Here, we report that Shrm4 is localized to synapses where it regulates dendritic spine morphology and interacts with the C terminus of GABAB receptors (GABABRs) to control their cell surface expression and intracellular trafficking via a dynein-dependent mechanism. Knockdown of Shrm4 in rat severely impairs GABABR activity causing increased anxiety-like behaviour and susceptibility to seizures. Moreover, Shrm4 influences hippocampal excitability by modulating tonic inhibition in dentate gyrus granule cells, in a process involving crosstalk between GABABRs and extrasynaptic δ-subunit-containing GABAARs. Our data highlights a role for Shrm4 in synaptogenesis and in maintaining GABABR-mediated inhibition, perturbation of which may be responsible for the involvement of Shrm4 in cognitive disorders and epilepsy.
The actin-binding proteins Shroom (Shrm) play an important role in cytoskeletal organization and consist of an N-terminal PDZ domain, a central Apx/Shrm Domain 1 (ASD1) and a C-terminal ASD2 domain. There are four evolutionarily conserved Shrm proteins (Shrm1–4) that are localized to polarized tissues, including neurons. Of these, only Shrm4 lacks the actin-targeting ASD1 motif, and the role of its PDZ domain is unknown. Murine Shrm4 possesses putative binding sites for EVH1 (poly-proline rich domain), a PDZ (SNF binding motif) and a stretch of glutamine and glutamate residues preceding the C-terminal ASD2 motif that is unique to Shrm4, but not other family members. Shrm4 is ubiquitously expressed throughout embryonic and adult murine brains and also binds to F-actin in non-neuronal cells.

The importance of Shrm4 is illustrated by two de novo balanced X-chromosomal translocations, which disrupts the KIAA1202 gene (Xp11.2) that encodes for Shrm4. In addition, a balanced X-chromosomal translocations, which disrupts the disability (ID) and increased susceptibility to seizures. Recent pathogenic missense mutation was identified in an unrelated gate heterodimers comprising GABA B1 (GABA B1) and R2 but not other family members. Shrm4 is ubiquitously expressed neurologically in several conditions is unknown. Indeed, the role of Shrm4 in the brain is also known, but given the pathological profile, it may regulate GABA-mediated inhibition. GABA activates ionotropic GABA A receptors (GABA A Rs) 14 to control inhibition, which is important for synaptic plasticity. The importance of these receptors is emphasized during dysfunction, which occurs in different neurological diseases.

Here, we report that Shrm4 interacts with GABA A Rs to facilitate trafficking to dendrites using a dynin-dependent mechanism. For cell surface expression, GABA A Rs are obligate heterodimers comprising GABA A R1 (GABA A R1) and R2 (GABA A R2) subunits that mediate long-lasting synaptic inhibition. However, the motor-protein-dependent trafficking of these receptors is not fully understood.

We found that loss of Shrm4 compromises GABA A R delivery to postsynaptic compartments, impairs GABA A R-mediated K + currents and GABA A R-mediated tonic inhibition in the hippocampus, and reduces dendritic spine density altering the composition of synaptic proteins resulting in increased anxiety-like behaviour and susceptibility to seizures in rats. Our study suggests a possible underlying mechanism by which Shrm4 may be involved in epilepsy and ID.

**Results**

Shrm4 is an interacting partner of GABA A Rs. We first investigated the subcellular localization of Shrm4 in cultured rat hippocampal neurons at 18 days in vitro (DIV) by immunostaining and found colocalization with presynaptic (Bassoon) and postsynaptic markers of excitatory (PSD-95) and inhibitory (GABA A β3) synapses (Supplementary Fig. 1a). By using electron microscopy and post-embedding immunogold methods with an anti-Shrm4 antibody, gold nanoparticles were identified in presynaptic and postsynaptic areas and along dendrites (Supplementary Table 1). Biochemical fractionation of adult rat brain hippocampi and cortices revealed enrichment of Shrm4 in the postsynaptic density (PSD) fraction further confirming its presence at synapses (Supplementary Fig. 1c).

To explore the role(s) of Shrm4 in neurons, we searched for binding partners using yeast two-hybrid (Y2H) screening. The PDZ domain of Shrm4 (residues 1–91; Fig. 1a) was selected as the bait to screen against an adult human brain cDNA library, as this domain participates in protein–protein interactions. Twenty positive cDNA clones were isolated; six of these encoded a 100 amino acids stretch of the C-terminal tail of GABA B1 present in both splice variants B1a and B1b, which differ by the inclusion of two Sushi domains in the N terminus only in GABA B1a (refs 12,14) (Fig. 1a). The interaction between Shrm4 and the GABA B1 C-tail was crucially reliant on the Shrm4-PDZ domain (PDZ domain 1–91: ++::ΔPDZ 91–1,492: negative; ΔASD2 1–1,213: ++::Δ; Fig. 1a). We confirmed this interaction using rat brain lysates and found that endogenous Shrm4 co-precipitates with GABA A R1/β (Fig. 1b). GABA A R1 antibody specificity was confirmed by western blots from GABA A R1/β and GABA A B1 knockout mice (Supplementary Fig. 1d). Moreover, direct stochastic optical reconstruction microscopy (dSTORM) of 14DIV hippocampal neurons revealed that Shrm4 and GABA A Rs co-cluster in neurons (Fig. 1c). We used immunoprecipitation to determine the minimal region of the GABA A 91 C-tail that interacts with Shrm4 in HEK293 cells expressing Shrm4-GFP, GABA A B1 and GABA A B2 cDNAs with differing C terminal (Fig. 1f). This revealed that R859LITTRG EWQSEA in the C-tail of GABA A B1 interacted with the Shrm4-PDZ domain (Fig. 1f). To confirm this interaction, we produced a cell-permeable peptide fragment (Tat-peptide) encompassing the Shrm4-GABA A B1 binding site on GABA A B1 (859–870). We verified the efficiency of this peptide in pull-down experiments with GST-PDZ incubating different concentration of Tat control or Tat-859–870 with lysates of HEK293 cells overexpressing GABA A B1–GFP and GABA A B2 (Supplementary Fig. 1f) and a dose-dependence was observed. We then incubated lysates from HEK293 cells overexpressing GABA A B1a and GABA A B2 with this peptide before GST pull-down using Shrm4-PDZ or its mutant form (K26G27/AA). Consistent with previous results, co-precipitation of GABA A B1 was reduced after preincubation with the Tat-peptide compared with its scrambled control (Fig. 1g).

These results reveal a new association between the PDZ domain of Shrm4 and residues 859–870 of the GABA A B1 C terminus.

Shrm4 regulates the surface expression of GABA A Rs. Given that the C-tail of GABA A B1 is important for trafficking to the cell surface, we next examined whether Shrm4 regulates GABA A R trafficking. We designed two shRNAs that specifically targeted rat (shRNA#1 and #2) and human (shRNA#2) Shrm4 transcripts. To test their effectiveness as Shrm4 silencers, HEK293 cells were transfected with human HA-Shrm4 cDNA (Rescue) with or without each shRNA. Both shRNAs reduced Shrm4 expression while co-transfection of a rescue construct with shRNA#1 restored expression (Supplementary Fig. 2a,b). We extended this approach to neurons, transfecting Scrambled, shRNA#1, shRNA#2 or Rescue constructs at 8DIV for later analysis of surface levels of GABA A B1 at 18DIV. Interestingly, both shRNAs reduced surface levels of GABA A B1 that can be rescued by the re-expression of Shrm4 (Fig. 1h, full neurons in Supplementary Fig. 2c). As an additional control, we tested the effect of knocking down Shrm3 and found no effect on GABA A B1 surface expression indicating a unique role played by Shrm4 in GABA A B1 trafficking (Supplementary Fig. 2d). We next used lentiviral delivery to corroborate our immunostaining results. For this we used cell surface biotinylation assays for GABA A Rs (Fig. 1i). Shrm4 knockdown reduced surface expression of GABA A Rs without...
Figure 1 | Shrm4 interaction with GABA\(_{\beta}\)Rs modulates cell surface expression. (a) Representations of Shrm4 and GABA\(_{\beta}\)R subunit 1a and 1b interacting domains. The PDZ domain of Shrm4 (1-91 aa) was used as bait for Y2H screening on adult human brain cDNA library. Twenty positive clones were isolated; six encoded for a 100 aa stretch of the GABA\(_{\beta}\)R subunit 1 C-terminal tail (both isoforms (1a and 1b)). The PDZ domain and ADSD2 constructs interact with the C-terminal tail of GABA\(_{\beta}\)1 (++) while APDZ truncated construct does not (−). TMD: Transmembrane domains. (b) Co-immunoprecipitation experiments on adult rat brain extracts using anti-Shrm4 antibody show Shrm4 and GABA\(_{\beta}\) association (full blot in Supplementary Fig. 11). (c) (Left) dSTORM imaging of GABA\(_{\beta}\)R (red) and Shrm4 (green) on 1DIV rat cultured hippocampal neurons. (Right) Shrm4 and GABA\(_{\beta}\)R puncta co-cluster as evidenced by cross-correlation analysis. Error-bars are s.e.m.; number of regions = 29, number of fields = 3. Scale bar, 0.4 μm. (d) GST pull-down experiments on 18DIV rat cultured hippocampal neurons (1) and rat brain (2) extract show that Shrm4-PDZ domain (GST-PDZ; aa 1-91) pulls down both GABA\(_{\beta}\)A isoforms whereas (2) mutant GST-PDZ (AA) does not (full blot in Supplementary Fig. 11). (e) Co-immunoprecipitation experiments on HEK293 cells expressing HA-Shrm4 and GABA\(_{\beta}\)2-Flag, showing that Shrm4 does not associate with GABA\(_{\beta}\)2 in absence of GABA\(_{\beta}\)1 (full blot in Supplementary Fig. 11). (f) Scheme of truncated GABA\(_{\beta}\)R constructs: deletion 1: Δ859–961; deletion 2: Δ922–961; and deletion 3: Δ870–961 with co-immunoprecipitation results from HEK293 cells expressing Shrm4-GFP and each of the GABA\(_{\beta}\)R constructs. GFP-Shrm4 immunoprecipitates full-length GABA\(_{\beta}\)1a, deletion 2, and deletion 3, but not deletion 1 (full blot in Supplementary Fig. 11). (g) (Top) GST pull-down experiments using GST-PDZ or mutant GST-PDZ (AA) on lysates of HEK293 cells overexpressing GABA\(_{\beta}\)A2 and GABA\(_{\beta}\)A3 incubated with Tat-control peptide or Tat-859–870 peptide corresponding to the previously identified minimal GABA\(_{\beta}\)2-Shrm4 binding region. (Bottom) Histograms showing GABA\(_{\beta}\)A2 mean intensity normalized to Tat-control peptide ± s.e.m. n = 3; **P = 0.046; t-test (full blot in Supplementary Fig. 11). (h) Surface immunostaining for GABA\(_{\beta}\)R in rat cultured hippocampal neurons at 18DIV transfected with scrambled, shRNA#1, shRNA#2 or rescue constructs at 8DIV. Scale bar, 15 μm. Histograms show mean ± s.e.m. n = 5–15. Scrambled versus shRNA#1 *P = 0.0153; Scrambled versus shRNA#2 *P = 0.0153; One-way ANOVA–Mann-Whitney. (i) GABA\(_{\beta}\)R cell surface biotinylation from 18DIV rat cultured hippocampal neurons infected with scrambled#1 (Scr#1) or shRNA#1 (shRNA#1) at 8DIV and western blot. Shrm4 silencing reduces GABA\(_{\beta}\)R surface expression, but does not change total signal for GABA\(_{\beta}\)1 or surface (and total) signal for GABA\(_{\beta}\)1 α1 subunit. Histograms show mean ± s.e.m.; n = 4; **P = 0.0069; t-test (full blot in Supplementary Fig. 11).

affecting the total expression of GABA\(_{\beta}\)Rs or the total and surface expression of GABA\(_{\beta}\)R α1 subunits used as negative controls (Fig. 1i). Thus, Shrm4 is important for GABA\(_{\beta}\)1 trafficking in neurons.

Shrm4 regulates synaptic structure and protein composition. Since Shrm4 colocalizes with PSD-95 and Bassoon, we investigated if its knockdown affected excitatory synapses in rat pyramidal hippocampal neurons. In these excitatory neurons, expressing scrambled or shRNA#1 before synaptogenesis (8DIV) did not alter the branching or dendritic diameters at 18DIV (Fig. 2a). We therefore refined our analysis and studied dendritic spines, where defects are commonly linked to ID21. First, we investigated the expression levels of PSD-95, AMPA receptor subunit GluA2 and presynaptic markers Bassoon and Synapsin,
Figure 2 | Shrm4 is key mediator of dendritic spine formation and morphology. (a) Sholl analyses were performed on 18DIV rat hippocampal neurons transfected at 8DIV with GFP-coexpressing knockdown shRNA#1 or scrambled#1. No difference in dendritic arborization and diameter was observed between the two conditions. Scale bar, 10 μm. (b) Images of rat hippocampal neurons transfected at 8DIV with Shrm4 scrambled#1, shRNA#1 or rescue constructs and immunostained for PSD-95, Bassoon, GluA2 and Synapsin post-synaptogenesis at 18DIV. Scale bar, 10 μm. (c) Numbers of fluorescent puncta/15 μm dendrite (left panel) and mean fluorescence intensity of synaptic markers (right panel). The effect of Shrm4 silencing could be rescued by coexpressing wild-type Shrm4 cDNA resistant to knockdown by shRNA#1 (Data normalized to scrambled#1 controls) Histograms show mean ± s.e.m.; see Supplementary Table 2 for statistical tests. (d) Normalized spine density per 20 μm of hippocampal neurons transfected at 8DIV for Shrm4 knockdown and rescue. See Supplementary Table 2 for statistical tests. (e) Spine head width and (f) length for scrambled, knockdown and rescue cells. See Supplementary Table 2 for statistical tests. (g) Percentages of mushroom, stubby and thin spikes for scrambled, knockdown and rescue cells. Histograms show mean ± s.e.m.; see Supplementary Table 2 for statistical tests. (h) (Left) Images of rat hippocampal neurons transfected at 8DIV with scrambled, GABAβ1 knockdown shRNA or rescue constructs and analysed at 18DIV. (Right) Histogram showing normalized spine density per 20 μm of hippocampal neurons transfected at 8DIV for scrambled, GABAβ1 knockdown and rescue; Histograms show mean ± s.e.m.; n = 6–8 cells; Scrambled versus shRNA **P = 0.0056; shRNA versus rescue *P = 0.0108, one-way ANOVA-Mann-Whitney. Scale bars, 10 μm. (i) (Left) Images of 17DIV rat hippocampal neurons transfected at 5DIV with pSuper and treated from 8DIV to 13DIV with a Tat control or Tat-859–870 peptide. (Right) Histogram showing spine density per 40 μm dendrite of 17DIV-treated hippocampal neurons; n = 10; ***P = 0.0001; t-test. Scale bar, 10 μm. (j) Representative Ca²⁺ signals from hippocampal neurons expressing dsRed-coexpressing knockdown shRNA#1 or scrambled#1 along with GCaMP6 fast under basal conditions in modified Krebs solution (in mM: 140 NaCl, 2.5 CaCl₂, 4.7 KCl, 11 glucose, and 5 HEPES, pH 7.4). Scale bar, 5 μm. (k) Representative Ca²⁺ signals imaged from single dendrites (grey) and averaged traces (black) using GCaMP6f. (l) Maximum and averaged ΔF/F₀ values of Ca²⁺ signals in scrambled#1 and shRNA#1 condition. (m) Time course of averaged ΔF/F₀ values and single exponential fits (red) of Ca²⁺ signals in scrambled#1 and shRNA#1 condition. (n) Mean rise times and (o) decay τ of Ca²⁺ signals in scrambled#1 and shRNA#1 condition n = 10–15; histograms are means ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001; t-test.

using immunofluorescence (Fig. 2b, full neurons shown in Supplementary Fig. 3). The numbers and intensities of fluorescent puncta for all four markers were reduced in Shrm4-silenced neurons and rescued by HA-Shrm4 (Fig. 2c). The decrease occurred in parallel with a reduction in spine density and this was also rescued by HA-Shrm4 (Fig. 2d). These results were replicated by silencing via shRNA#2 (Supplementary Fig. 4a). Finally, to attribute these changes to Shrm4 specific knockdown, we silenced Shrm3 and found no changes to dendritic spine density as shown in Supplementary Fig. 2d.

Shrm4 silencing also had profound consequences on spine morphology reducing spine length, without affecting spine head width (Fig. 2e,f) while increasing the number of stubby spines, compared with scrambled controls (Fig. 2g). These effects were reversed using the rescue construct discounting non-specific effects of the shRNA (Fig. 2e–g). Interestingly, no changes in spine density were observed with Shrm4 knockdown after synaptogenesis at 12DIV (Supplementary Fig. 4b) suggesting a role for Shrm4 in synaptogenesis or in synapse maintenance.

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To assess whether Shrm4 silencing reduced the density of spines via down-regulation of dendritic GABA<sub>R</sub>Rs, we transfected hippocampal neurons with GABA<sub>B1</sub> shRNA, or scrambled shRNA<sup>20</sup> or GABA<sub>B1</sub> shRNA together with shRNA-insensitive GABA<sub>B1b</sub> cDNA before synaptogenesis (8DIV), for processing at 18DIV for immunofluorescence. GABA<sub>B1</sub> knockdown reduced GABA<sub>B1b</sub> immunofluorescence (Supplementary Fig. 5a), and similar to Shrm4 silencing, also reduced the density of spines, which was rescued by shRNA-insensitive GABA<sub>B1b</sub> cDNA (Fig. 2h).

Then, we evaluated the specific involvement of Shrm4–GABA<sub>B1</sub>Rs interaction on spine density by applying the Tat-859–870 peptide to hippocampal cultured neurons from 8DIV to 13DIV during the synaptogenesis peak. This treatment was sufficient to induce a reduction in spine density similar to that observed in Shrm4 or GABA<sub>B1</sub> knockdown condition demonstrating that the interaction between the two proteins is necessary for normal spine development (Fig. 2i). Interestingly, silencing of Shrm4 also resulted in changes in spontaneous Ca<sup>2+</sup> transients (Fig. 2j,k), assessed using GCaMP6f, without a change in mean amplitudes of spikes (Fig. 2f) in hippocampal neurons. In particular, we observed slower rise (Fig. 2m,n) and decay times (Fig. 2m,o) of Ca<sup>2+</sup> transients in Shrm4-knockdown neurons, which could have important consequences for spine morphology. Moreover, these defects were not rescued by the concomitant overexpression of GABA<sub>B1</sub>ΔC mutant demonstrating the dependency of these effects on GABA<sub>B1</sub>Rs surface expression (Supplementary Fig. 5b).

Thus, Shrm4 is localized at excitatory synapses and plays a crucial role in determining the morphology and molecular structure of dendritic spines most likely by modulating the surface expression of GABA<sub>B1</sub> receptors.
Figure 3 | Shrm4 links GABA<sub>R</sub> to the dynein/dynactin complex. (a) Immunofluorescence of GABA<sub>R</sub> intensity in the cell body of 18DIV cultured hippocampal neurons transfected with Shrm4 shuffled, knockdown (shRNA#1) or rescue constructs at 8DIV. All data in bar graphs throughout are means ± s.e.m.; n = 10, 10, 15 *P = 0.0057; F(2,32) = 6.094, one-way ANOVA-Mann–Whitney. (b) Co-immunoprecipitation experiments on brain extracts using polyclonal anti-Shrm4 show that Shrm4, GABA<sub>B</sub>a, GABA<sub>B</sub>b, and DIC are associated. By contrast, KIF5A is not present in this complex. (c) Co-immunoprecipitation of both GABA<sub>B</sub>a isoforms in HEK293 cells transfected with Shrm4-V5 and GABA<sub>B</sub>a-myc (top) or GABA<sub>B</sub>b-myc (bottom). GABA<sub>B</sub>a/GABA<sub>B</sub>b and the endogenous DIC co-immunoprecipitated with Shrm4-V5. (d) Endogenous DIC co-immunoprecipitates with expressed HA-Shrm4 in HEK293 cells in the absence of GABA<sub>B</sub>a or GABA<sub>B</sub>b subunits. Monoclonal antibodies (anti-V5, anti-myc and anti-DIC) were used for western blot. (e) Western blot from in vitro pull-down assay of purified Histidine-tagged DIC using GST-PDZ and mutant GST-PDZ (AA). The Shrm4-PDZ domain is able to directly bind DIC even when its common binding site is mutated. Ponceau Red staining of the blot has been presented at the bottom. (f) GST pull-down experiments on brain extracts show that the Shrm4-PDZ domain pulls down DIC while the mutant GST-PDZΔ414 does not. Ponceau red staining of the blot has been presented at the bottom (full blot in Supplementary Fig. 11). (g) Conventional (top left) and super-resolution direct stochastic optical reconstruction microscopy (dSTORM) (bottom left) imaging of Tubulin-ATTO 488 (shown in red) and Shrm4-Alexa 647 (shown in green). Shrm4 positive puncta are localized along microtubule-positive filaments, as evidenced in the details of the super-resolution image (top right). The super-resolution data has been quantified by cross-correlation analysis. The positive, higher than 1 cross-correlation, indicates co-clustering of the two fluorescent signals. The positive, higher than 1 cross-correlation, indicates co-clustering of the two fluorescent signals. (h) Schematic illustrating unilateral local injection of AAV5-shRNA (left hemisphere) and AAV5-shRNA (right hemisphere) into rat brain CA1 hippocampus, with time line for recovery and experiments. (i) Western blots and histograms showing monoclonal anti-DIC immunoprecipitation from scrambled and shRNA lysates of infected hippocampus extracts (n = 6 rats). Co-precipitated GABA<sub>B</sub>a levels were normalized to DIC immunoprecipitation levels and the normalized percentage of GABA<sub>B</sub>a co-precipitation was calculated. Histograms show mean ± s.e.m.; *P < 0.05, t-test.
Furthermore, we verified that Shrm4 knockdown was not altering GABA$_{B1}$/GABA$_{B2}$ dimerization$^{32}$. We therefore co-transfected GABA$_{B1}$-myc and GABA$_{B2}$-flag with either shRNA#1 or its scrambled control. This was followed by immunostaining for the receptors. The analysis revealed high correlation in both scrambled and Shrm4 knockdown conditions excluding dysfunctional receptor dimerization (Supplementary Fig. 8b).

To address whether the reduction in dendritic GABA$_{B}$Rs was a consequence of decreased transport, fluorescence recovery after photobleaching (FRAP) was applied to cultured hippocampal neurons expressing GABA$_{B1}$-RFP with GFP-shRNA#1 or GFP-p150-cc1. For both, fluorescence recovery of GABA$_{B1}$-RFP was significantly delayed compared with neurons expressing the GFP control. Only an increased half-time for fluorescence recovery, without affecting the mobile fraction (plateau reached after 350 s), was observed in Shrm4-silenced and GFP-p150-cc1-expressing neurons, suggesting that transport of dendritic receptors, occurring either intracellularly or at the surface level, was severely impaired (Fig. 4c).

These results suggest that Shrm4 mediates dendritic transport of the GABA$_{B}$Rs heterodimer via its interaction with GABA$_{B1}$ and DIC.
Physiological role of Shrm4 in vitro and in vivo. GABA<sub>B</sub>Rs activate G protein-coupled inwardly-rectifying K<sup>+</sup> channels, generating slow inhibitory postsynaptic currents (IPSCs)\(^5\). As Shrm4 regulates GABA<sub>B</sub>R dendritic cell surface number, we examined K<sup>+</sup> currents induced by the GABA<sub>B</sub>R agonist baclofen (10–100 μM) on 14DIV hippocampal neurons transfected at 7DIV with either GFP-coexpressing knockdown shRNA#1 or scrambled#1 or p150-cc1. Peak K<sup>+</sup> current densities (pA/pF) were lower in Shrm4-silenced and dynemin-inhibited neurons compared with scrambled controls (Fig. 4d), consistent with a reduced number of dendritic GABA<sub>B</sub>Rs. This reduced current was reversed by expressing shRNA-insensitive Shrm4 (Supplementary Fig. 9a). By contrast, K<sup>+</sup> currents activated by metabotropic glutamate receptors were unaffected by Shrm4 silencing and dynemin inhibition (Supplementary Fig. 9b). GABA<sub>B</sub>R-mediated miniature IPSCs (mIPSCs) were also unaffected by Shrm4 silencing (Supplementary Fig. 9c), indicating that Shrm4 and dynemin/dynactin selectively regulate GABA<sub>B</sub>R-mediated responses.

To assess if Shrm4 silencing affected the neurophysiology of GABA<sub>B</sub>Rs, we injected AAV5-scrambled (left hemisphere) and AAV5-shRNA#1 (right hemisphere) into the hippocampal CA1 region of 3 months-old rats\(^3\) (Fig. 4e). Input/output currents were measured to evaluate the basal excitatory transmission and we did not observe any statistically significant difference between the two conditions (Supplementary Fig. 10a). We also recorded field excitatory postsynaptic potentials (fEPSPs) in the apical dendritic layer of CA1 3 weeks later by inducing long-term potentiation (LTP) or long-term depression (LTD) via Schaffer collateral stimulation. LTP and LTD induction and maintenance were unaffected (Supplementary Fig. 10b,c) consistent with the unchanged PSD-95 intensity in brain slices from injected animals (Supplementary Fig. 10d). The apparent contradiction with the defect observed in dendritic spine number can be explained by noting that these animals were injected at three months of age, when the peak of synaptogenesis has passed. We know that Shrm4 knockdown in mature neurons did not affect spine number (Supplementary Fig. 5b), whereas it is still able to impair GABA<sub>B</sub> trafficking (Supplementary Fig. 4b) as shown by the decreased polarity index.

Whole-cell K<sup>+</sup> currents evoked by baclofen in the CA1 of acute slices from injected animals were significantly reduced by Shrm4 knockdown compared with scrambled controls (Fig. 4f). The injection of AAV5-shRNA#2 induced similar reductions in K<sup>+</sup> current confirming the specificity of our results. Thus, Shrm4 silencing reduces functional GABA<sub>B</sub>R responses in vitro and in vivo, in accord with a reduced number of surface dendritic GABA<sub>B</sub>Rs.

Shrm4 silencing in vivo affects hippocampal tonic inhibition. Recent evidence suggests that GABA<sub>B</sub>R activation enhances the conductance of extrasynaptic δ subunit-containing GABA<sub>B</sub>Rs of dentate gyrus granule cells (DGGCs)\(^{34-36}\). As Shrm4 silencing reduced GABA<sub>B</sub>R activity, we explored if this affected tonic inhibition in DGGCs.

Animals were injected with AAV5-scrambled (left hemisphere) and AAV5-knockdown (right hemisphere) shRNAs into the DG (Fig. 5a). Whole-cell recordings 3 weeks later from DGGCs in acute slices\(^{35,36}\) subject to scrambled shRNA revealed that GABA (5 μM) increased the bicuculline-sensitive baseline current and current noise variance (Fig. 5b). For cells expressing Shrm4-shRNA#1, both current and noise variance were reduced compared with controls (Fig. 5b). As tonic inhibition in DGGCs relies on δ subunit-containing GABA<sub>B</sub>Rs\(^5\) and can be regulated by GABA<sub>B</sub>Rs, we considered whether the reduced tonic current was a direct consequence of a reduction in surface GABA<sub>B</sub>Rs. Repeating these experiments in the presence of the GABA<sub>B</sub>R antagonist CGP54628, showed similar reduction in tonic current and noise variance evident in the knockdown condition without CGP pre-treatment (Fig. 5c). These data suggested that reducing cell surface GABA<sub>B</sub>Rs has no direct effect on tonic inhibition, which is more likely due to an indirect impairment of δ subunit-containing GABA<sub>B</sub>Rs. This was confirmed using the agonist THIP at δ subunit-selective concentrations (3 μM) and as expected, the mean current and noise variance were reduced in DGGCs in the hemisphere carrying the Shrm4 knockdown compared with the control hemisphere (Fig. 5d). To exclude off-target effects of shRNA#1, we injected a second knockdown construct, AAV5-shRNA#2, which produced identical effects on THIP-induced currents (Fig. 5e). By contrast, GABA<sub>B</sub>R-mediated mIPSCs in DGGCs were unaffected (Supplementary Fig. 10e), indicating that Shrm4 specifically regulates δ subunit-containing GABA<sub>B</sub>R-mediated responses.

Interestingly, co-immunoprecipitation using brain extracts and monoclonal anti-GABA<sub>B</sub>R δ subunit revealed that GABA<sub>B</sub>Rs and δ subunit-containing GABA<sub>B</sub>Rs co-associate. By contrast, the synaptic GABA<sub>B</sub>R γ2 subunit\(^37\) was absent (Fig. 5f). Thus, by controlling postsynaptic GABA<sub>B</sub>Rs, Shrm4 is also able to regulate tonic inhibition mediated by δ subunit-containing GABA<sub>B</sub>Rs.

In vivo Shrm4 silencing causes behavioural deficits. A role for GABA<sub>B</sub>Rs in anxiety\(^{38}\) and epilepsy\(^{14,39}\) is well-known. GABA transmission has been also linked to neurodevelopmental disorders such as autism spectrum disorders (ASD)\(^{40,41}\). Furthermore, GABA<sub>B</sub>R agonist application has been proposed as therapeutic strategy for social deficits, repetitive behaviours and other aspects of ASD in different mouse models\(^42\). To understand if reducing GABA<sub>B</sub>R numbers and tonic inhibition by Shrm4 knockdown has behavioural implications, we injected rats bilaterally with either Shrm4 knockdown (AAV5-shRNA#1 or AAV5-shRNA#2), or AAV5-scrambled shRNA (AAV5-scrambled#1 or AAV5-scrambled#2) and extensively analysed the behaviour of these animals (Fig. 6a). Locomotor activity of the injected animals was unaffected allowing us to perform subsequent behavioural analyses without locomotor bias (Fig. 6b).

The elevated plus maze (EPM) and the marble-burying test measured anxiety levels\(^43\) whereas social behaviours were evaluated in a three-chamber apparatus\(^44\) and in the tube test for aggressivity\(^45\). AAV5-knockdown-shRNA-injected rats (with either shRNA#1 or shRNA#2) exhibited increased anxiety and impaired social behaviour (Fig. 6c–f). In the EPM, Shrm4-knockdown rats made fewer open-arm entries (Fig. 6c) and spent less time in open arms compared with AAV5-scrambled shRNA-injected controls (Fig. 6c). The total number of arm entries was unaffected by AAV5-knockdown-shRNA, confirming that locomotion was unaffected.

In the marble-burying test, animals injected with AAV5-shRNA#1 buried a higher number of marbles and spent less time before burying compared with AAV5-scrambled controls confirming an increased anxiety level (Fig. 6d). Social behaviours of animals injected with AAV5-shRNA#1 were also found impaired with reduced time spent close to a stranger naive animal (Fig. 6e, sociability) and to a second new stranger animal (Fig. 6e, social novelty) compared with AAV5-scrambled controls. These animals also showed greater aggression in the tube test in terms of percentage of wins versus the control-injected animals (Fig. 6f).

We then assessed involvement of Shrm4’s deficiency in epilepsy by pentylenetetrazole (PTZ) administration to evaluate...
seizure sensitivity and recorded electro-encephalograms (EEG) to measure spontaneous electrical activity (Fig. 7a).

Electrical activity evaluated for 24 h in freely moving awake animals showed a significant spontaneous spike activity in all the AAV5-shRNA1 injected rats compared with the AAV5-scrambled1 (Fig. 7b,c) suggesting an increased general excitability.

In fact, following PTZ injections (i/p 45 mg kg−1), the latency to the first seizure was reduced and the seizure duration was longer for knockdown shRNA-injected animals (Fig. 7d). The severity of the response to PTZ was also increased with greater number of tonic-clonic seizures (Fig. 7d). This indicates that in vivo Shrm4 silencing in CA1 produces defects in anxiety, social behaviour and susceptibility to seizures. Similar effects on seizure susceptibility were obtained when we injected rats intraperitoneally with Tat-859–870 peptide administering PTZ 12 h after (Fig. 7e,f) demonstrating that the disruption of Shrm4–GABABRs interaction was responsible of these effects.

These phenotypes parallel the defects in GABAβR trafficking and transmission observed in vitro and in vivo in Shrm4-silenced hippocampal neurons.

Discussion
Several neurodevelopmental IDs including autism and Fragile X syndrome are characterized by a reduction of GABABR expression levels, and treatment with GABABR agonists have been reported to improve susceptibility to seizures and social and cognitive behaviour. In this study, we have characterized an interaction between the ID-linked protein Shrm4, GABAβRs and dynnein motor protein. The disruption of this complex leads to dysfunction of GABAβRs cell surface targeting and subsequent reduction of signalling efficacy, and this has interesting parallels with these neurodevelopmental disorders.

We have characterized physiological roles for Shrm4 by discovering a new interaction between its PDZ domain and the
GABAB C terminus, a region important for GABABRs trafficking\textsuperscript{19,20}. Only one other PDZ domain-containing protein, Mupp1, is known to interact with GABABRs, but its physiological role remains unknown\textsuperscript{21}. The localization of Shrm4 to microtubule-positive filaments in dendrites suggests its interaction with GABABRs is potentially critical for delivering these receptors to membrane-delimited signalling domains. Consistent with this, using shRNA silencing to reduce Shrm4 levels we observed decreased GABABR in dendrites, causing their accumulation in the soma. This most likely involved dysfunctional GABABR transport to the dendrites, a process driven by microtubule-based motor proteins: kinesins and dyneins. In this regard, we found that Shrm4 directly binds the dynein intermediate chain with a distinct portion of the PDZ domain (that is, the β1 and β2 strands) allowing for the simultaneous binding of Shrm4 with both GABAB\textsubscript{1a} and DIC. In fact we found that GABAB\textsubscript{R} and the dynein intermediate chain co-associate, and significantly, dynein inhibition reduced GABAB\textsubscript{R} transport to the dendrites, but had no effect on trafficking into axons. Therefore, both dynein and Shrm4 are required to target GABAB\textsubscript{R} to dendrites.

We know that Shrm4 can associate with both GABAB\textsubscript{1a} and GABAB\textsubscript{1b}, and that Shrm4 silencing reduced the levels of both isoforms in the dendrites. However, GABAB\textsubscript{1a} is preferentially sorted to axons under physiological conditions\textsuperscript{22} being targeted via its Sushi domains in pre-Golgi ER or ERGIC\textsuperscript{23}. We surmised that only GABAB\textsubscript{1a} subunits that escape axonal targeting in pre-Golgi compartments can then associate with Shrm4 in the Golgi to be re-directed (like GABAB\textsubscript{1a}) to dendrites\textsuperscript{24}.

On the basis of this premise, we propose that Shrm4, similar to the ID protein PQBP1, functions as an adaptor protein\textsuperscript{25} for intracellular trafficking of cargo, by binding the C termini of GABAB\textsubscript{1a} and also dynein to target these receptors to dendrites. This is further supported by our finding that in vivo Shrm4 silencing reduced the association between GABAB\textsubscript{R}s and dynein and differs from other post-translational modifications that influence the kinetics and pharmacological properties of GABAB\textsubscript{R}s\textsuperscript{12}.

Our biochemical and imaging data strongly suggest that Shrm4 and dynein play crucial roles in inhibitory transmission. Shrm4 silencing or dynein/dynactin inhibition specifically reduced baclofen-activated K\textsuperscript{+} currents due to dysfunctional GABAB\textsubscript{R}s.
trafficking and depletion of surface dendritic GABA$_A$Rs without affecting GABA$_A$R-mediated mIPSCs and glutamate-mediated K$^+$ currents.

Depleting postsynaptic GABA$_A$Rs in vivo increased seizure susceptibility, as noted for GABA$_{A_b1}^+$ knockout mice\cite{56}; the weaver mouse (with mutated GIRK2 channel), and the Girk2$^{-/-}$ null mouse, all of which are characterized by a significant loss of GABA$_A$R-mediated inhibition\cite{53,37}. Thus it is not surprising that Shrm4 silencing, or the disruption of Shrm4–GABA$_A$Rs interaction, are associated with augmented neuronal excitability, as showed by the increase in spontaneous spikes recorded by EEG and in seizures susceptibility after PTZ injection.

GABA$_A$R involvement in anxiety-related disorders remains unclear. While GABA$_{A_{bi}}^{-/-}$ knockouts show increased anxiety\cite{56}, mice lacking either GABA$_{B_{ta1}}$ or GABA$_{B_{tb1}}$ isoforms are not anxious\cite{40}, possibly due to isoform compensation. However, hippocampal Shrm4 silencing was associated with anxiogenic behaviour, and as this impairs trafficking of both GABA$_A$R isomers to dendrites, the increased anxiety is likely due to a reduction in postsynaptic GABA$_A$R numbers. Social behaviours also appeared to be impaired on Shrm4 knockdown; this is not surprising since GABA transmission has been linked to ASD symptoms\cite{40–42}. In addition, down-regulating GABA$_A$Rs by Shrm4 silencing surprisingly affected tonic inhibition. We found GABA$_A$Rs and $\delta$ subunit-containing GABA$_A$Rs co-associated, which is consistent with their extrasynaptic localization in the molecular layer of the DG\cite{59,60}. By silencing Shrm4, tonic inhibition mediated by $\delta$ subunit-containing GABA$_A$Rs is impaired, which could also contribute towards increased seizures, a feature noted previously for Gabrd$^{-/-}$ mice\cite{61}.

Defects in dendritic spine density and shape are established pathological correlates of X-linked ID\cite{21,62} and Shrm4 silencing, before synaptogenesis, reduced spine density, length and affected their pre- and postsynaptic molecular composition. Shrm4 binds

Figure 7 | Shrm4 silencing in vivo increases neuronal excitability. (a) Experimental design for investigating behavioural effects of Shrm4 silencing in rat hippocampal CA1. Rats were injected bilaterally with either AAV5-scrambled#1 or AAV5-shRNA#1 and tested after 3 weeks. (b) EEG recording in basal condition—two representative traces (4 h recording) of basal cerebral activity in freely moving awake rats are shown. The traces of the AAV5-shRNA#1 rat showed abnormalities in term of presence of spikes of high amplitude compared with AAV5-scrambled#1 rat. The traces of the remaining AAV5-shRNA#1 rats showed similar patterns (data not shown). (c) Quantification shows higher number of spikes and an increased number of animals showing spikes in AAV5-shRNA#1-injected rats compared with AAV5-scrambled#1 rats. (number of spikes/hour: $n = 5, 5; **P < 0.0001$; t-test; animals displaying spikes: $n = 5, 5; ***P < 0.0001$; t-test). (d) PTZ injection (45 mg kg$^{-1}$ i.p.) significantly decreased the mean latency to the first seizure and increased the mean duration of seizures and number of tonic-clonic (Racine scale 5) seizures evaluated for 30 min in AAV5-shRNA#1 compared with AAV5-scrambled#1-injected rats (latency to the first seizure: $n = 8, 9$; **$P = 0.0201$; t-test; time spent in seizures: $n = 8, 9$; ***$P = 0.0214$; t-test; number of tonic-clonic seizures: $n = 9, 9$; **$P = 0.0069$; t-test). (e) Experimental design for investigating the effect of intraperitoneal injection of the Tat-859-870 (active) peptide compared with the Tat control (inactive) on susceptibility to seizures. (f) The injection of the Tat-859-870 given 12 h before the test significantly increased the susceptibility to PTZ-induced seizures (45 mg kg$^{-1}$ i.p.) in terms of reduced latency to the 1st seizure (**$P < 0.001$, $n = 3$; t-test) and increased time spent in seizures (**$P = 0.0057$, $n = 3$; t-test) even if the number of seizures was not different. All histograms show mean ± s.e.m.; **$P < 0.001$; $n = 3$; t-test.)

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F-actin and can influence actin remodelling in non-neuronal cells and F-actin dynamic is crucial for synaptogenesis and spine plasticity. Although Shrm4 could regulate the spine cytoskeleton, this is considered unlikely because Shrm4 silencing after maturation has no effect on spine morphology, even though dendritic GABAAR trafficking caused by Shrm4 silencing. Indeed, GABAAR silencing before synaptogenesis, or the inhibition of Shrm4–GABAB interaction with Tat-859–870, induced a similar reduction in spine density. In addition, given GABAARs modulate dendritic Ca²⁺ signals, by inhibiting voltage-gated Ca²⁺ channels, perturbation in Ca²⁺ signalling due to altered trafficking of GABAARs could also underlie spine defects.

In conclusion, our data identify Shrm4 as an important protein for synaptogenesis and for maintaining the inhibitory equilibrium mediated by the GABAARs and extracellular 8 subunit-containing GABAARs. The consequences of disrupting Shrm4 expression are severe and manifest by increased anxiety, social behaviours impairments and a predisposition towards epilepsy.

Methods

Animals. Experimental procedures were performed in accordance with the European Communities Council Directive (86/809/EEC) on the care and use of animals and the UK Animals (Scientific Procedures) Act 1986, and were approved by the CNR Institute of Neuroscience.

cDNA, shRNA constructs and cell-permeable peptide. The human KIAA1202 open reading frame 1 was subcloned into pcDNA5/V5-HisB (Invitrogen) to obtain Shrm4-pEGFP-C1 (Clontech) to obtain GFP-Shrm4; and PTTL-HA to obtain HA-Shrm4 (gift from Vera Lachaise). Flag-tagged GABAARα2, myc-tagged full-length GABAARβ1a, GABAARβ1b–Δ859–961, GABAARβ2–961 and GABAARβ3–Δ780–961 were cloned into the pRfKs plasmid for expression in HEK293 cells to identify the minimal GABAARβ1 sequence that interacts with Shrm4 (ref 23).

In vitro experiments. pC-myc-GABAARα2 and pC-myc-GABAARβ1a constructs were used to investigate the distribution of GABAAR α and β isomers in cultured hippocampal neurons, GABAARβ1a-mRFP (generous gift by Andre’s Couve, University of Chile) and GABAB1a–mRFP (generous gift by Andre’s Couve, University of Chile) were used to track postsynaptic activated NMDARs and glutamatergic synapses, respectively. 

Immunofluorescence and surface staining. Cultured hippocampal neurons were fixed in 4% paraformaldehyde/4% sucrose for 4 h at room temperature and incubated in GDB1X solution (2 mM MgCl₂ and 0.1 mM CaCl₂) neurons were fixed for 10 min at room temperature in 4% paraformaldehyde/4% sucrose without permeabilization. Coverslips were then washed, and incubated with Alexa 488 (1:400, Invitrogen), Alexa 555 (1:400, Invitrogen) or Cy5 (1:200, Jackson Immunoresearch) secondary antibodies for 1 h at room temperature. Confocal images were obtained as described previously.

Cytoskeletal imaging. Confocal images were acquired with an LSM510 Meta confocal microscope (Carl Zeiss; gift from F. Monzino) and a × 63 objective (numerical aperture 1.4) with sequential acquisition setting, at 1,280 × 1,024 pixels resolution.

Western blots. Proteins were transferred from gels onto nitrocellulose membranes followed by incubation with the following primary antibodies at room temperature for 2–3 h in 5% milk at the stated dilutions: Shrm4 (1:200, used previously), GABAARα1, GABAARβ1a, GluA2, MBL International, GABAARβ1, Millipore, HA (1:1,000, Invitrogen) and GABAARβ2 (1:1,000, gift of B.B. Kibsa) (1:1,000, Abcam, Kif5B (1:1,000, gift of F. Navone) and Kif5C (1:1,000, Abcam) (all raised in rabbit); and α-tubulin (1:400, Sigma), GABAAR Receptor 1 (1:2,000, Abcam), GABAARβ6 subunits (1:500, ROCKLAND), V5 (1:1,000, Invitrogen), myc (1:2,000, MBL, Synaptic Systems), Synaptophysin (1:500, NeuroMab), Dic (1:1,000, Abcam) (raised in mouse); and GABAARβ2 (1:2,000, Abcam) raised in guinea pig. After rinsing, primary antibodies were revealed by incubation at room temperature for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (both 1:2,000 from GE Healthcare) and immunoreactive bands on blots were visualized by enhanced chemiluminescence (GE Healthcare).
Image data were Z series projections of about 6–10 images, each averaged four times and taken at depth intervals of 0.75 μm.

**Quantification of GABAB fluorescence intensity.** Images were quantified as previously described in ref. 72. Images were acquired using a ×63 objective and averaged intensity of signals in proximal axon and primary dendrites were measured in ImageJ. Brevican, a proteoglycan present in the growth cone was used to identify the beginning of the axon and confirmed axonal identification. To prevent selection bias during quantification, the axon and dendritic segments were selected in one channel (20 times) and quantified in another channel (GABABRs). A third channel was used to identify the axon (Brevican). The axon and dendritic signals were measured in segments of the same size. To control for background signals, we measured the intensity near the axon or dendrite (same segment size) and subtracted the random fluorescence intensity in these regions. The dendrite intensity was quantified by using the Peakfinder plugin (Mathworks) and quantified in the other channel. To control for background signals, we measured the intensity near the axon or dendrite (same segment size) and subtracted the random fluorescence intensity in these regions. The dendrite intensity was quantified by using the Peakfinder plugin (Mathworks) and quantified in the other channel.

**Calcium imaging and analysis.** Ca^{2+} transients were imaged at 20 Hz in KREBS using GCamp6 in hippocampal neurons transfected at DIV with scrambled, Shrm4 knockdown and GABAB1AC constructs along with cDNAs for GCaMP6f and mGFP4. Ca^{2+} transients were normalized to baseline fluorescence (ΔF/F) to obtain values of ΔF/F. Peaks were detected in Matlab using the Peakfinder plugin (Nathanael Yoder, Mathworks). Ca^{2+} transients less than ×3 the signal-to-noise ratio were excluded from the analysis.

**dSTORM and analysis of cross-correlation.** Super-resolution localization imaging was carried out by direct stochastic optical reconstruction microscopy (dSTORM)73. Briefly, the fluorescent molecules of a sample prepared according to a standard immunofluorescence protocol (see above) were induced to blink on and off by modifying the chemical composition of the medium. If few molecules fluoresce at a given time, such that the diffusion limited spots corresponding to individual fluorophores are well separated, the position of each molecule can be quantified with higher accuracy than the resolution limit74. By acquiring multiple images of the same field (tens to hundreds of thousands), and storing the position of the localized molecules at each frame it is therefore possible to obtain an image of the sample with resolution higher than the diffusion limit. dSTORM was performed on an optical setup based on a Leica SR GSD-3D (Leica Microsystems, Erlangen, Germany) super-resolution microscope equipped with a ×160 1.43 NA objective, an Andor iXon Ultra-897 EM-CCD sensor and three (405 nm 30 mW, 488 nm 300 mW and 642 nm 500 mW) solid state lasers. The sample was mounted on the stage and the medium was substituted before acquisition with a mix of glucose oxidase (560 μg/ml), calFacing (400 μg/ml) and Cysteine HCl (100 mM) in TN buffer with 10% glucose w/v at pH 6 to induce blinking of the fluorophores75. Alexa 647 and Atto 488 were imaged sequentially, starting from the red channel. 30,000 images were collected for each channel with 5–20 ms exposure times and an increasing ramp of 405 nm laser intensity, with powers between 0 and 0.8 mW, to reactivate the molecules in long-lived dark states. The super-resolution images were reconstructed using the proprietary analysis software, Discarding all the detected events with less than 20 photons/pixel for Alexa 647 and 40 photons for Atto 488. To quantify the co-clustering between the two labelled proteins, the list of particles detected for each channel were used to compute the spatial cross-correlation between the two signals76, using previously published routines based on Fast Fourier transformation written in Matlab77. Briefly flat cross-correlation curves are representative of no co-clustering between the two labelled proteins, while curves that significantly differ from 1 indicate that the two proteins cluster together. For each condition, the cross-correlation was evaluated on ~30 randomly chosen square regions of 2 μm sides. For visualization purposes, super-resolution images were rendered as a 2D histograms with pixel sizes equal to 20 nm.

**Y2H screening.** For Y2H experiments, a fragment corresponding to the Shrm4 N-terminal PDZ domain (aa 1–91) was cloned in-frame with the GAL4 DNA-binding domain of the pGKB7 vector and used as bait to screen an adult human brain CDNA library (Clontech, Matie and Plate Library). Positive yeast clones grew on plates containing X-GAL and Aurorobin HIT (DFO/AXA plates) and expressed all four reporter genes: HIS3, ADE2, AUR1C and MEL1 under the control of three distinct Gal4-responsive promoters. CDNA plasmids from positive clones were recovered with the Easy Yeast plasmid isolation kit and transformed into DH5 E. coli grown on ampicillin plates, followed by sequencing.

**Cell surface biotinylation assay.** Hippocampal neuron membrane proteins were biotinylated using membrane-impermeable sulfo-NHS-SS-biotin (0.3 mg/ml 1, Pierce) in KREBS at 37°C. The neurons were then washed with saline (TBS) supplemented with 0.1 mM CaCl2, 1 mM MgCl2 and 50 mM glucose at 37°C, and rinsed with TBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 (without glycerine) on ice, followed by lysis in extraction buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% SDS, and protease inhibitors). Lysates were boiled for 5 min with biotinylated and proteinase K-treated proteins were precipitated with a 1:1000 mixture of beads (Dynabeads, Invitrogen). The beads were washed with lysis buffer, boiled for 5 min in sample buffer, filtered, and the proteins in solution separated by SDS–PAGE for western blotting.

**Live-cell internalization assay.** GABAB-R internalization in live hippocampal neurons was investigated as described previously76,79. Neurons at 14–21DIV expressing GABAB-R heterodimers (R1α with hongulartoxin (BTX) binding site alone) and GABAB-Rs along with R2) and either GFP-shRNA or GFP-scrambled-shRNA were incubated in 1 mM d-tubocurarine for 2 min at room temperature followed by 3 μg ml^{-1} BTX-AF555 (which attaches to the R1a binding site) for 10 min at room temperature. Receptor internalization was then followed as decay in surface fluorescence at 30–32°C under the confocal microscope.

**Live-cell imaging and FRAP.** For live-cell imaging, neurons co-transfected with GABAB-R–GFP together with either GFP, Shrm4-shRNA or GFP-p150-cc1, were placed in an incubator with imaging medium at 37°C and 3% CO2 mounted on a Zeiss LSM510 Meta confocal microscope. Neurons expressing Shrm4-shRNA or scrambled shRNA were imaged with the 458 nm laser; neurons expressing GABAB-R–mRFP were imaged with the 543 nm laser.

**FRAP experiments** were performed following Fossett et al.80 on GABAB-R–GFP, positive dendrites; regions of interest (ROI) on dendrites were defined and a pre-blinking image acquired at the start. The ROI was next bleached by scanning 30 times with 405 and 458 nm lasers until no fluorescence signal was detectable. Fluorescence signal recovery was imaged over 10 min, and normalized to the total fluorescence change of the pre-blinking ROI, which was verified as constant over time.

The images were analysed with ImageJ and the results analysed with Prism (GraphPad).

**Computational modelling.** The structural models of GABAB1R and GABAB2R were developed by comparative modelling using MODELLER81. We first modelled the 591–918 sequence GABAB1R comprising the seven-helix bundle, the intracellular and extracellular loops, and the C terminus up to the end of the coiled–coil region (879–918 sequence) by using the crystal structure of the metabotropic glutamate receptor 5 (mGlur5; PDB 5CCG) as a template. One hundred models were generated by randomizing all the Cartesian coordinates of standard residues in the initial model. Extra-helical restraints were imposed to the following portions of the receptor: 766–772 and 879–918, corresponding, respectively, to the N terminus of helix 5 (H5) and the C terminal coiled-coiled helix. In the model selected according to a corroborated procedure82, the 887–918 portion of the C-terminal helix was replaced with the corresponding helix from the crystallographic coiled–coil heterodimer (PDB: 4PAS). The best selected model of the GABAB1R served as a template to build the seven-helix bundle and the loops of the GABAB2R. The best model among the 100 produced was docked onto the structural model of GABAB1R following an already described procedure83. The receptor orientation corresponding to an H1–H1, H7–H7 dimer, the most compatible with the C terminal coiled–coil heterodimer, was employed for model completion by adding the C terminus. In this respect, the C-terminal helix was extracted from the crystallographic heterodimer (PDB: 4PAS), whereas the segment connecting the C-terminal end of H7 and the coiled-coiled helix was built by means of the loop routine in MODELLER.

The information from our in vitro experiments were employed to drive the docking between the 859–870 GABAB1R and the J2 strand of Shrm4-PDZ and between the J1 strand of Shrm4-PDZ (PDB 2EDP) and the 128–135 strand of IC2 in complex with dimer Lc8 of dynamine (PDB: 2PGI). The two strands were docked so as to form an extension of the antiparallel sheet between IC2 and LC8.

**Injection of adenovirus virus constructs.** Five to seven-weeks-old male Sprague Dawley rats, kept under 12h light/dark conditions, with ad libitum food and water, were anaesthetized with zoletil100 (0.1 ml per 100 g) and xylazine (0.1 ml per 100 g) and secured in a Kopf stereotaxic frame. A 26 gauge stereotaxic frame was inserted into each CA1 (ref. 22). AAV5-shRNA#1 (or #2) or AAV5-scrambled-shRNA#1 (or #2) (Penn Vector Core, University of Pennsylvania, USA) were injected directly into the CA1 (coordinates AP: −3.8 mm relative to Bregma; L: ±1–2 mm; DV: −2 mm from skull; Paxinos and Watson, 1985) or into the Dentate Gyrus (coordinates AP: −3.6 mm relative to Bregma; L: 3.5/−1 mm; DV: −3.5 mm from skull; Paxinos and Watson, 1985). Zoanthus sp. green fluorescence protein was also incorporated into the AAV construct to report injection coordinates and volumes. The animals were allowed to recover for 3 weeks before experimentation.

**Whole-cell patch-clamp recording of GIRK currents.** Current densities of G protein–coupled inwardly rectifying K+ (GIRK) channel conductance in response to GABAB activation by baclofen, or mGlur activation by glutamate, were recorded from hippocampal neurons in culture at 12–16DIV, using whole-cell patch-clamp electrophysiology as described previously. Neurons were transfected at 7DIV with shRNA, scrambled shRNA or the coiled-coil domain of dynamin (GFP-p150-cc1, (Penn Vector Core, University of Pennsylvania, USA)).
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For whole-cell patch-clamp electrophysiological recordings on dentate gyrus granule cells, coronal hippocampal slices (thickness, 250–300 μm) were prepared and incubated first for 40 min at 36 °C and then for 30 min at room temperature in oxygenated (95% O2/5% CO2) ACSF. Slices were transferred to a recording chamber perfused with ACSF at 33 °C temperature at a rate of about 2 ml min⁻¹.

Tonic GABAergic currents and mIPSCs were recorded at a holding potential of ~25 mV in the presence of kynurenic acid (3 mM) with the calcium chloride internal solution (above) supplemented with 5 mM QX-314 (lidocaine N-ethyl bromide, only for tonic currents).

Access resistance was between 10 and 20 MΩ if it changed by >20% during the recording, the recording was discarded. For tonic currents recordings, after a baseline period of 2–5 min, GABA (5 mM) or THIP (3 mM) was added to the ACSF to increase the tonic component of the GABAergic transmission and to measure modification in extrasynaptic GABAₐ receptor subunits composition. At the end of the experiments bicuculline (20 mM) was added to block all GABAergic currents. For the recording of mIPSCs, lidocaine (500 mM) was added in the external solution and recordings were performed as previously described for culture experiments. Analysis was performed offline with Clampfit 10.1 software. CGP 54628, the selective GABAₐ antagonist, was used at the final concentration of 5 μM.

Behavioural tests. Behavioural tests were carried out 3 weeks after rats were injected bilaterally with either Shrm4 AAV5-shRNA#1 (or #2) or AAV5-scrambled shRNA (or #2). The rats were maintained in 2 per cage post injections before experiments were performed. Behavioural tests were carried out in the following order: a 7-day gap of a week before tests: spontaneous motor activity, elevated plus maze, marble-burying, EEG and PTZ-induced seizures. Ten animals per condition were submitted to the behavioural tests for 3 consecutive weeks and during the fourth and fifth weeks, animals of each condition were divided in two subgroups: one submitted to PTZ-induced seizures and the other to EEG. Behavioural experiments were carried out during the light phase of the light/dark cycle between 1000 hours and 1400 hours, and performed by trained observers blind to treatment.

Spontaneous motor activity test. Motor measurements were taken in AAV5-shRNA#1 (or #2) or AAV5-scrambled shRNA#1 (or #2) bilaterally injected rats. Spontaneous motor activity was evaluated as previously described in an activity cage with the following dimensions: 43 cm long × 43 cm wide × 32 cm high (Ugo Basile, Varese, Italy), placed in a sound attenuating room. The cage was fitted with two parallel horizontal infrared beams 2 cm off the floor. Cumulative horizontal movements were counted every 10 min for 30.

Elevated plus maze test. The elevated plus maze procedure was used as described previously to investigate anxiety-related behaviours in rats. The apparatus consisted of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) at right angles, extending from a central platform (10 cm × 10 cm). The apparatus was placed 50 cm above the floor in the centre of a small quiet room in dim light (about 30 lux). Testing was conducted during the early light phase (0930–13.30 hours) of the cycle. After 20 min adaptation to the new surroundings, an animal was placed on the central platform facing an open arm. The number of open- and closed-arm entries, and time spent in open arms were recorded over 5-min periods.

Marble-burying test. This test of anxiety was used according to ref. 85. The test was conducted in a 43 cm × 26 cm × 22 cm box cage with 5 cm of fresh hardwood chip bedding. Each animal was habituated for 15 min to the cage. Then, an array of 24 standard marbles was arranged uniformly over the surface. Individual subjects were placed again in the test cage for 15 min. The number of marbles buried and the latency to the first burying was recorded. A marble was scored as buried if more than two-thirds of it was covered with sawdust. New bedding was used for each animal and marbles were cleaned with 10% acetic acid solution between animals. The same subjects were used for this test and in the elevated plus maze and were tested in a counterbalanced order.

Sociability and social novelty test. Social behaviour was carried out in a three-chamber apparatus according to Leite et al.86 The apparatus was an acrylic rectangular box divided into three compartments of equal size (35 cm high, 50 cm width, 50 cm deep) provided with doors. The sociability test was divided in three sequential phases of 10 min each. During the habituation period, the test rat was placed in the middle chamber for 10 min while the rat was free to explore the three compartments. Each of the two sides contained an identical empty wire cage. In the sociability phase, an unfamiliar rat (stranger 1) of the same strain, sex and weight was enclosed in one of the wire cages and the time spent in each compartment with the object or social stimulus was measured. In the social novelty phase, a new unfamiliar rat (stranger 2) was enclosed into the wire cage in the opposite compartment and the time spent in each compartment was measured. Before the injection of the social stimulus, each test rat was habituated in the central chamber. The test was videotaped and the time spent in each compartment was measured offline.
Tube test. The tube test is a well-known testing paradigm designed to measure social hierarchies, and thus is relevant when investigating social dominance in mice, but it can be adapted to rats. This test measures dominant/submissive behaviour without allowing them to fight and injure each other. Rats were initially habituated to the testing apparatus, which consisted of a 10 cm (diameter) by 50 cm (length) transparent plastic tube, of sufficient size to allow one but not two rats to move through the tube. Over two consecutive days, rats were allowed to run through the tube on eight occasions, with alternate trials in which the entry and exit ends were switched. Competition trials involved simultaneously releasing two competing rats into opposite ends of the tube. The individual rat that was able to travel forwards through the tube to exit the other side ‘won’ and was deemed dominant; the rat that retreated was considered subordinate. The number of wins (%) on the total number of competitions was measured.

Pentylenetetrazole-induced seizures. Animals (5 per condition) previously injected (CA1) with AAV5-shRNA#1 or scrambled AAV5-shRNA#1 were injected with pentylenetetrazole (45 mg·kg⁻¹, i.p., Sigma, St. Louis, MO, USA). Seizures were monitored for 30 min after administration and rated on the Racine scale (1972) (score: 0, no change; I, myoclonic jerks; II, minimum seizures, with convulsive wave through body; III, fully-developed minimal seizures, clonus of head muscles and forelimbs, righting reflex present; IV, major seizures (generalized without tonic phase); V, generalized tonic-clonic seizures. The latency (s) to the first seizure and total time spent in seizures (IV and V scale) were recorded.

Electrocencephalogram recording. AAV5-scrambled#1 and AAV5-shRNA#1 rats were anaesthetized with an i.p. injection of chloral hydrate dissolved in saline and given at a volume of 10 ml·kg⁻¹. Under anaesthesia (450 mg·kg⁻¹, body weight of chloral hydrate; i.p., Sigma-Aldrich), all the rats were placed in a stereotaxic instrument and four silver–silver chloride ball electrodes were fixed epidurally with dental acrylic cement, as described in detail elsewhere68, on the right and left of the parieto-occipital cortex according to the Paxinos and Watson brain atlas, 2 mm anterior, 2 mm lateral from the midline and 3 mm posterior from the bregma. The four electrodes, and one inserted into the nasal bone and used as ground, were connected to a microconnector attached to the rat’s head with dental cement (New Galetti e Rossi, Milan, Italy). Animals were treated with cetirizine (50 mg·kg⁻¹, i.p.) for three days. One week after electroencephalogram placement the rats were allowed to acclimatize themselves to a sound-attenuated Faraday chamber for 1 h a day for 3 days. Then, each freely moving, awake rat was anaesthetized with chloral hydrate (i.p. 15 mg·kg⁻¹) and other organs.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

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Author contributions
J.Z., E.M. and M.P. conceived the original idea and wrote the manuscript. J.Z. and E.M. designed and performed the majority of the experiments. S.H. and L.M. designed and performed electrophysiological experiments. A.L., C.H. and P.V. designed and performed biochemical experiments. D.M. designed and performed dSTORM experiments. L.B. designed and performed electron microscopy experiments. M.F. designed and performed time-lapse experiment. L.P. and D.B. designed and performed behavioural tests. M.S., M.F., J.H., V.K., F.F., C.S., B.B., S.B. and T.G.S. provided useful comments and review the manuscript. M.P. coordinated the entire project and obtained the main source of fundings.

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