Missense mutation of Fmr1 results in impaired AMPAR-mediated plasticity and socio-cognitive deficits in mice

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Fragile X syndrome (FXS) is the most frequent form of inherited intellectual disability and the best-described monogenic cause of autism. CGG-repeat expansion in the FMR1 gene leads to FMR1 silencing, loss-of-expression of the Fragile X Mental Retardation Protein (FMRP), and is a common cause of FXS. Missense mutations in the FMR1 gene were also identified in FXS patients, including the recurrent FMRP-R138Q mutation. To investigate the mechanisms underlying FXS caused by this mutation, we generated a knock-in mouse model (Fmr1R138Q) expressing the FMRP-R138Q protein. We demonstrate that, in the hippocampus of the Fmr1R138Q mice, neurons show an increased spine density associated with synaptic ultrastructural defects and increased AMPA receptor-surface expression. Combining biochemical assays, high-resolution imaging, electrophysiological recordings, and behavioural testing, we also show that the R138Q mutation results in impaired hippocampal long-term potentiation and socio-cognitive deficits in mice. These findings reveal the functional impact of the FMRP-R138Q mutation in a mouse model of FXS.
The formation of functional synapses in the developing brain is fundamental to establishing efficient neuronal communication and plasticity, which underlie cognitive processes. In the past years, synaptic dysfunction has clearly emerged as a critical factor in the etiology of neurodevelopmental disorders including Autism Spectrum disorder (ASD) and Intellectual Disability (ID). X-linked ID (XLID) accounts for 5–10% of ID patients and is caused by mutations in genes located on the X chromosome. The Fragile X Syndrome (FXS) is the most frequent form of inherited XLID and the first monogenic cause of ASD with a prevalence of 1:4000 males and 1:7000 females. The majority of FXS patients exhibit mild-to-severe ID associated with significant learning and memory impairments, Attention Deficit Hyperactivity Disorder (ADHD), and autistic-like features2,3. To date, no effective therapeutic strategies are available.

FXS generally results from a massive expansion of the trinucleotide CGG (> 200 repeats) in the 5′-UTR region of the FMR1 gene leading to its transcriptional silencing and consequently, the lack of expression of the encoded Fragile X Mental Retardation Protein (FMRP)4,5. FMRP is an RNA-binding protein that binds a large subset of mRNAs in the mammalian brain and is a key component of RNA granules. These granules transport translationally-repressed mRNAs essential for the synaptic function along axons and dendrites1,3. Neuronal activation triggers the local translation of these critical mRNAs at synapses allowing spine maturation and elimination, which are essential processes to shape a functional neuronal network in the developing brain.

Accordingly, the lack of FMRP expression in FXS patients and Fmr1 knockout (Fmr1-KO) animal models leads to a pathological increase in immature dendritic protrusions due to a failure in the synaptic maturation and/or elimination processes6. These defects correlate with significant alterations in glutamatergic a-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated synaptic plasticity, including Long-Term Depression (LTD) and Potentiation (LTP)1,7. Consequently, these defects lead to learning and memory deficits and underlie the abnormal socio-emotional behaviors in Fmr1-KO mice1,7.

While the CGG-repeat expansion is the most frequent cause of FXS, other mutagenic mechanisms have been reported, including deletions, promoter variants, missense, and nonsense mutations. To date, more than 120 sequence variants have been identified in the FMR1 gene. However, only three missense mutations (I304N, G266E, and R138Q) have been functionally studied and showed an association with the etiology of FXS8-13. Among them, the R138Q mutation is of particular interest since it has been identified in three unrelated individuals presenting clinical traits of FXS. The first male patient sequenced displayed ID, anxiety, and seizures11,13, while the second presented the classical features of FXS including ID, ADHD, seizures, and ASD14. Interestingly, a female with mild ID and attention deficits was recently identified bearing the R138Q mutation15.

The R138Q mutation does not affect the ability of FMRP to bind polyribosomes and repress the translation of specific target mRNAs13. In addition, the intracellular perfusion of a short N-terminal version of FMRP-R138Q in Fmr1-KO CA3 hippocampal neurons failed to rescue the action potential broadening, suggesting a functional alteration of the FMRP-R138Q truncated mutant form13. However, the cellular and network alterations underlying the phenotype described in FMRP-R138Q FXS patients remain to be elucidated. Here, we have engineered a knock-in mouse model expressing the recurrent missense R138Q mutation in FMRP (Fmr1R138Q). We demonstrate that Fmr1R138Q mice exhibit postsynaptic alterations in the hippocampus, including an increased dendritic spine density, AMPAR-mediated synaptic plasticity defects associated with severe impairments in their socio-cognitive performances.

**Results**

Fmr1 mRNA and FMRP protein levels in Fmr1R138Q mice. To assess the pathophysiological impact of the recurrent R138Q mutation in vivo, we generated a specific knock-in mouse line expressing the R138Q FXS mutation using classical homologous recombination in murine C57BL/6 embryonic stem (ES) cells (Fig. 1a). The R138Q coding mutation was introduced in exon 5 by changing the CGA arginine codon into aCAA nucleotide triplet coding for a glutamine (R138Q: c.413G > A). The integrity of the FXS mutation in the Fmr1R138Q mice was confirmed by genomic DNA sequencing (Fig. 1a). Fmr1R138Q mice were viable, showed a standard growth and normal fertility and mortality rates (Supplementary Fig. 1).

The expression pattern of the FMRP protein is developmentally regulated16. To assess whether the R138Q mutation affects the developmental profile of FMRP, we compared the FMRP protein levels in the brain of WT and Fmr1R138Q mice at different postnatal days (PND) (Fig. 1b). As expected from the literature1,5, the FMRP protein level peaked at PND10–15 (WT PND15: 1.33 ± 0.246) and then significantly decreased in the adult brain of WT mice (WT PND90: 0.441 ± 0.078). FMRP-R138Q protein levels showed a similar pattern in the developing Fmr1R138Q brain (Fig. 1b; Fmr1R138Q PND15: 1.300 ± 0.303; Fmr1R138Q PND90: 0.491 ± 0.072), indicating that the R138Q mutation does not alter the protein expression of the pathogenic FMRP.

Since FXS is an RNA-binding protein regulating the local translation of a large number of mRNAs important to the synaptic function, we compared the total levels of a subset of its target mRNAs in PND90 WT and Fmr1R138Q male littermates. There were no apparent macroscopic defects in the Fmr1R138Q brain and the structural organization of the hippocampus was preserved (Fig. 1d).

**Fmr1R138Q mice show an increase in hippocampal spine density.** FMRP is essential to proper spine elimination and maturation3. A hallmark of the classical FXS phenotype is a pathological excess of long thin immature dendritic protrusions12, resulting from a failure in postsynaptic maturation and/or elimination processes. To understand if the R138Q mutation impacts spine maturation and/or elimination, we analyzed the morphology and density of dendritic spines in the Fmr1R138Q hippocampus (Fig. 2). We first used attenuated Sindbis viral particles in WT and Fmr1R138Q cultured hippocampal neurons at 13 days in vitro (13 DIV) to express free GFP and outline the morphology of dendritic spines18,19. We then compared the density and morphology of dendritic spines 20 h post transduction (Fig. 2a). Interestingly, while the length of dendritic spines was similar for both genotypes (WT: 1.563 ± 0.0717 μm; Fmr1R138Q: 1.521 ± 0.06193 μm), Fmr1R138Q neurons displayed a significant increase in spine density compared to WT neurons (WT: 6.06 ± 0.126 spines per 10 μm; Fmr1R138Q: 8.16 ± 0.207 spines per 10 μm).

We also evaluated the characteristics of dendritic spines in the CA1 region of the hippocampus of PND90 WT and Fmr1R138Q male littermates using Golgi-Cox staining (Fig. 2b; Supplementary Fig. 2). While there was no difference in spine length and width, Fmr1R138Q hippocampal neurons displayed a significant increase in spine density both in basal (Fig. 2b; WT: 8.096 ± 0.232 spines per 10 μm; Fmr1R138Q: 10.62 ± 0.167 spines per 10 μm) and apical dendrites (Supplementary Fig. 2; WT: 8.996 ±
0.189 spines per 10 μm; Fmr1<sup>R138Q</sup>: 9.96 ± 0.152 spines per 10 μm). Taken together, these data indicate that the R138Q mutation rather impairs the elimination of dendritic spines than their maturation given that dendritic spines are morphologically similar in WT and Fmr1<sup>R138Q</sup> brains.

To go deeper into the characterization of dendritic spines, we performed ultrastructural analyses of WT and Fmr1<sup>R138Q</sup> hippocampi using transmission electron microscopy (TEM). Stereological analyses pointed out a significant increase in the density of excitatory synapses in the Fmr1<sup>R138Q</sup> hippocampus (Fig. 2c; WT: 1.371 ± 0.129 synapses per μm<sup>3</sup>; Fmr1<sup>R138Q</sup>: 2.187 ± 0.161 synapses per μm<sup>3</sup>) in agreement with the Golgi-Cox staining data (Fig. 2b, Supplementary Fig. 2). Interestingly, while there was no difference in the length of the postsynaptic densities (PSD; WT: 232.63 ± 6.26 nm; Fmr1<sup>R138Q</sup>: 222.73 ± 5.64 nm), the PSD thickness in Fmr1<sup>R138Q</sup> hippocampal neurons was largely reduced (Fig. 2c; WT: 44.70 ± 0.99 nm; Fmr1<sup>R138Q</sup>: 34.96 ± 0.76 nm). We also measured a significant increase in the density of synaptic vesicles in Fmr1<sup>R138Q</sup> hippocampal presynaptic termini (Fig. 2c; WT: 120.48 ± 4.65 vesicles per μm<sup>2</sup>; Fmr1<sup>R138Q</sup>: 148.97 ± 4.25 vesicles per μm<sup>2</sup>). Altogether, these data reveal that the R138Q mutation leads to ultrastructural alterations both in the pre- and postsynaptic compartments.

Increased surface-expressed AMPAR levels in Fmr1<sup>R138Q</sup> mice.

To characterize the effect of the R138Q mutation on the composition of synapses, we compared the total protein levels of several pre- and postsynaptic proteins in brain homogenates prepared from WT and Fmr1<sup>R138Q</sup> male littermates (Fig. 3a; Supplementary Fig. 3). Interestingly, we measured a significant increase in the total amount of the GluA1 AMPAR subunit in the Fmr1<sup>R138Q</sup> brain (GluA1<sup>Fmr1R138Q</sup>: 1.45 ± 0.12 vs WT). All the other proteins investigated in the Fmr1<sup>R138Q</sup> brain, including proteins involved in the trafficking of AMPAR, showed levels similar to their WT littermates (Fig. 3a; GluA2<sup>Fmr1R138Q</sup>: 1.16 ± 0.18; PSD95<sup>Fmr1R138Q</sup>: 1.13 ± 0.12; GRIP1<sup>Fmr1R138Q</sup>: 1.27 ±...
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Figure 2. The *Fmr1*<sup>R138Q</sup> hippocampus exhibits increased dendritic spine density and ultrastructural alterations. **a** Confocal images of secondary dendrites from GFP-expressing WT and *Fmr1*<sup>R138Q</sup> KI cultured hippocampal neurons. Scale bar, 5 μm. Box plots indicate median (middle line), 25<sup>th</sup>, 75<sup>th</sup> percentile (box), and min to max values (whiskers) obtained for spine density and length in WT and *Fmr1*<sup>R138Q</sup> neurons. N = 48-54 neurons for ~1400-2200 spines analyzed per genotype from six biologically independent experiments. Two-tailed Mann-Whitney test. ***p < 0.0001. **b** Representative images of Golgi-stained basal secondary dendrites of CA1 hippocampal neurons from PND90 WT and *Fmr1*<sup>R138Q</sup> KI littermates. Histograms show the density of spines, spine length and width from WT and *Fmr1*<sup>R138Q</sup> CA1 secondary dendrites. Error bars represent the mean ± s.e.m. N = 30 neurons per genotype from three biologically independent experiments (1500-2000 spines analyzed per genotype). Two-sided Mann-Whitney test; ***p < 0.0001. **c** Representative EM images of pre- and postsynaptic (*) elements in CA1 synapses of PND90 WT and *Fmr1*<sup>R138Q</sup> hippocampal E endosomes, SV synaptic vesicles, Arrowheads, postsynaptic densities (PSD). Scale bar, 100 nm. Box plots indicate median (line), 25<sup>th</sup>, 75<sup>th</sup> percentile (box), and min to max values (whiskers) for PSD length and thickness, the density of synapses and synaptic vesicles in WT and *Fmr1*<sup>R138Q</sup> CA1 hippocampal neurons. Approximately 130 PSD (length and thickness), 60 presynaptic boutons, and 350 μm<sup>2</sup> of total surface area (synapse density) per genotype were analyzed from three independent sets of the experiment. Unpaired t test. ns not significant. ***p = 0.0003; ****p < 0.0001. Source data are provided as a Source Data file.

To further explore the AMPAR defects in the *Fmr1*<sup>R138Q</sup> brain, we compared the levels of surface-expressed GluAl in DIV15 WT and *Fmr1*<sup>R138Q</sup> cultured hippocampal neurons (Fig. 3b). Using surface-immunolabelling assays with specific anti-GluAl antibodies, we showed that the surface levels of GluAl in *Fmr1*<sup>R138Q</sup> neurons were significantly increased (Fig. 3b; Mean surface GluAl intensity, WT: 1 ± 0.087; *Fmr1*<sup>R138Q</sup>: 1.42 ± 0.13), with a higher density of surface GluAl-containing clusters (Fig. 3b; Surface cluster density, WT: 1 ± 0.049; *Fmr1*<sup>R138Q</sup>: 1.22 ± 0.06). Since AMPARs are often concentrated in dendritic spines, this finding corroborates the increased number of dendritic spines measured in *Fmr1*<sup>R138Q</sup> neurons (Fig. 2). We further confirmed the significant increase in GluAl surface expression in *Fmr1*<sup>R138Q</sup> neurons using cell surface biotinylation assays (Fig. 3c; GluAl *Fmr1*<sup>R138Q</sup>: 1.85 ± 0.19 vs WT). Interestingly, while there was no alteration in the total levels of GluA2 in the *Fmr1*<sup>R138Q</sup> brain (Fig. 3a), the surface expression of GluA2 was significantly higher in cultured *Fmr1*<sup>R138Q</sup> hippocampal neurons (Fig. 3c; GluA2 *Fmr1*<sup>R138Q</sup>: 1.50 ± 0.09 vs WT).

We then examined the surface expression of AMPARs in acute hippocampal slices using B53-crosslinking assays (Fig. 3d). Consistent with the above data, the surface expression of both GluAl and GluA2 was also significantly increased in the *Fmr1*<sup>R138Q</sup> hippocampus (Fig. 3d, Lanes +B53; GluAl *Fmr1*<sup>R138Q</sup>: 1.76 ± 0.21 vs WT; Lanes +B53; GluA2 *Fmr1*<sup>R138Q</sup>: 1.58 ± 0.17 vs WT). Altogether, these data clearly indicate that the R138Q mutation leads to increased surface levels of both GluAl and GluA2 in vitro and in vivo.

**Altered synaptic transmission in the *Fmr1*<sup>R138Q</sup> hippocampus.**

We showed that the recurrent FXS R138Q missense mutation leads to an increase in AMPAR surface expression. To assess whether this increase occurs at least in part synthetically, we performed super-resolution STimulated Emission Depletion (STED) microscopy on surface-labeled GluAl or GluA2 in WT and *Fmr1*<sup>R138Q</sup> cultured hippocampal neurons (Fig. 4a-d). We first measured the mean fluorescence intensity from surface-expressed GluAl and GluA2 at Homer1-labeled postsynaptic sites (Fig. 4a-c). We found that the mean surface GluAl fluorescence intensity per synapse is significantly increased in *Fmr1*<sup>R138Q</sup> neurons (Fig. 4c, WT: 797 ± 28; *Fmr1*<sup>R138Q</sup>: 1013 ± 34), whereas the fluorescence associated with surface-expressed GluA2 is reduced (Fig. 4c, WT: 1373 ± 34; *Fmr1*<sup>R138Q</sup>: 1148 ± 29). At the postsynapse, AMPARs are organized in 80–90 nm nanodomains facing presynaptic glutamate release sites for efficient synaptic transmission. Thus, we compared the mean number of nanodomains containing surface-expressed GluAl and GluA2 in WT and *Fmr1*<sup>R138Q</sup> hippocampal neurons (Fig. 4a, b, d). Consistent with the literature, we measured a density of ~2–2.1 nanodomains per spine for both surface-associated GluAl and GluA2 in WT neurons (Fig. 4d, WT sGluAl: 2.03 ± 0.107; WT sGluA2: 2.12 ± 0.099 nanodomains per spine). Interestingly, in *Fmr1*<sup>R138Q</sup> spines there was a significant increase in the mean
number of both surface-associated GluA1 and GluA2 nanodomains (Fig. 4d, Fmr1R138Q sGluA1: 2.582 ± 0.106; Fmr1R138Q sGluA2 2.554 ± 0.078). These data indicate that the missense R138Q mutation leads to a significant increase in the number of postsynaptic nanodomains containing AMPARs. However, while the mean fluorescence associated with surface-expressed GluA1 is also increased in hippocampal Fmr1R138Q synapses, the synaptic fluorescence level from surface-labeled GluA2 is decreased revealing that the upregulation of surface GluA2 measured in biochemical experiments is rather due to its extrasynaptic increase. This indicates that the FXS mutation not only impacts the surface expression of both GluA1 and GluA2 but also differentially perturbs their synaptic targeting, trafficking, and nanoscale organization.

Finally, to better understand whether the increase in surface-expressed AMPARs measured in the Fmr1R138Q hippocampus (Figs. 2, 3, and 4a–d) is associated with alterations in glutamatergic transmission, we performed whole-cell patch-clamp recordings in CA1 neurons from hippocampal slices of PND90 WT and Fmr1R138Q littermates (Fig. 4e–k). We showed that the amplitude of AMPAR-mediated miniature Excitatory PostSynaptic Currents (mEPSCs) is significantly enhanced in Fmr1R138Q mice (Fig. 4e, f; WT: 17.09 ± 0.539 pA; Fmr1R138Q: 18.97 ± 0.405 pA). Interestingly, we did not measure any significant differences in the frequency of mEPSCs (Fig. 4g, h; WT: 0.178 ± 0.040 Hz; Fmr1R138Q, 0.143 ± 0.025 Hz) or the kinetics of these events (Fig. 4i, j) between the two genotypes.

Altogether the data from the above experiments (Figs. 2–4) revealed that the R138Q FXS mutation leads to important pre- and postsynaptic alterations resulting in synaptic transmission deficits in the Fmr1R138Q hippocampus.

Impaired long-term potentiation in the Fmr1R138Q hippocampus. We next investigated the consequences of the R138Q mutation in hippocampal plasticity. Since the level of surface-expressed AMPARs is enhanced in the Fmr1R138Q hippocampus, we wondered whether the induction of LTP could trigger a further increase in synaptic AMPARs (Fig. 5 and Supplementary Fig. 4). To test this hypothesis, we first combined surface immunolabeling assays with the chemical induction of LTP (cLTP) on WT and Fmr1R138Q hippocampal neurons (Fig. 5a, b). In line with the literature, the level of surface GluA1 was significantly increased upon cLTP in WT neurons (Mean surface GluA1 intensity, WT: 1.427 ± 0.129 vs basal; Surface cluster density, WT: 1.308 ± 0.085 vs basal) whereas the surface level of GluA1 in Fmr1R138Q neurons was unexpectedly decreased (Mean surface GluA1 intensity, Fmr1R138Q: 0.6354 ± 0.08647 vs basal; Surface cluster density, Fmr1R138Q, 0.7455 ± 0.08227 vs basal).
We confirmed these results using surface biotinylation assays in WT and Fmr1R138Q hippocampal neurons in basal and cLTP-induced conditions (Supplementary Fig. 4a–c). The cLTP treatment triggered the increase in both GluA1 and GluA2 surface expression in WT neurons (WT GluA1 cLTP: 1.578 ± 0.107; WT GluA2 cLTP: 1.203 ± 0.008). In contrast, the surface levels of AMPARs upon cLTP were not increased but as above, rather decreased in Fmr1R138Q hippocampal neurons (Fmr1R138Q GluA1 cLTP: 0.490 ± 0.073; Fmr1R138Q GluA2 cLTP: 0.899 ± 0.157).

In addition to the data obtained on hippocampal cultures, we performed BS3-crosslinking assays and showed that the induction...
of cLTP in Fmr1R138Q hippocampal slices led to a significant reduction in the levels of surface-expressed AMPARs (Fig. 5c–e, Fmr1R138Q-GluA1 cLTP: 0.706 ± 0.08 vs basal; Supplementary Fig. 4d–f, Fmr1R138Q-GluA2 cLTP: 0.860 ± 0.05 vs Fmr1R138Q WT), further confirming that the R138Q mutation impairs the AMPAR trafficking. As expected, the cLTP treatment was able to increase the surface levels of AMPARs in hippocampal slices from WT littermate brains (Fig. 5c, e, WT GluA1 cLTP: 3.691 ± 0.675 vs basal; Supplementary Fig. 4d–f, WT GluA2 cLTP: 1.953 ± 0.315 vs WT basal).

Altogether, these data indicate that the hippocampal plasticity is severely impaired in the Fmr1R138Q mice. It has been demonstrated that the LTP induction promotes a reorganization of the AMPAR nanodomains. Thus, to further assess the impact of the R138Q mutation in cLTP-activated neurons, we performed super-resolution STED microscopy on surface-labeled GluA1 in cLTP-treated WT and Fmr1R138Q hippocampal cells (Fig. 5f–h). As expected, the mean fluorescence intensity per synapse (Fig. 5f, g; WT basal: 797 ± 28; WT cLTP: 1076 ± 35), as well as the density of the postsynaptic nanodomains (Fig. 5f, h; WT basal: 2.023 ± 0.107; WT cLTP: 2.783 ± 0.09 nanodomains per spine) containing the surface-expressed GluA1 subunits were both significantly increased in WT cLTP-treated neurons. In contrast, Fmr1R138Q neurons exhibited a significant decrease in the mean postsynaptic surface GluA1 fluorescence intensity upon cLTP (Fig. 5f, g; Fmr1R138Q basal: 1013 ± 34; Fmr1R138Q cLTP: 890 ± 30) while the mean density of surface GluA1-containing nanodomains remained unchanged (Fig. 5f, h; Fmr1R138Q sGluA1 basal: 2.582 ± 0.05; Fmr1R138Q sGluA1 cLTP: 2.599 ± 0.040 nanodomains per spine). These data thus reveal that the R138Q mutation also impairs the synaptic reorganization of AMPARs upon cLTP in Fmr1R138Q cultured hippocampal neurons, further confirming that the activity-dependent trafficking of these receptors is impaired in the Fmr1R138Q mice. Altogether, these data indicate that, contrary to WT neurons, the induction of cLTP does not promote any increase in the surface nor the synaptic levels of AMPARs in the Fmr1R138Q hippocampus. Therefore, to determine if the expression of the FMRP-R138Q mutant also physiologically impacts the AMPAR-mediated responses, we induced LTP by high-frequency stimulation (HFS) in acute WT and Fmr1R138Q hippocampal slices and recorded the postsynaptic responses in CA1 neurons (Fig. 5i–k). First, we tested the impact of the R138Q mutation on the CA3 to CA1 synaptic transmission and did not find any significant differences with the WT responses in Input/Output curves established following the stimulation of the Schaffer collaterals (Supplementary Fig. 5). This indicates that the connectivity between the pre- and postsynaptic sites is preserved in the Fmr1R138Q hippocampus. Next, we found that the induction of LTP by HFS was evoked as expected in the WT hippocampus (WT LTP: 157.2 ± 6.52% vs basal) but was drastically reduced in Fmr1R138Q male littermates (Fmr1R138Q LTP: 122.4 ± 10.12% vs basal), in line with the impaired cLTP seen in both biochemical and imaging experiments (Fig. 5a–h).

In addition, we did not measure any significant difference in the mean fiber volley (FV) slope between genotypes (Fig. 5i–k), suggesting that the impaired LTP in the Fmr1R138Q hippocampus rather arises from postsynaptic impairments than from presynaptic alterations.

Fmr1R138Q mice display ID- and ASD-like features. The R138Q mutation has been identified in both male and female patients. Since the mutation affects the surface levels of AMPARs and directly impacts synaptic plasticity in the hippocampus, we investigated whether male and female Fmr1R138Q mice display altered cognitive and/or social performances (Fig. 6). To avoid possible pitfalls due to an impact of the R138Q mutation on locomotion, we first tested PND40–45 mice using the open field test to detect any potential motor defects. We did not measure any significant differences in the number of crossings between the two genotypes demonstrating that there is no motor alteration in the Fmr1R138Q mice (Supplementary Fig. 6).

Communication skills are altered in some FXS patients and Fmr1-KO mice. We therefore compared the ultrasonic vocalization (USV) profile in PND7 WT and Fmr1R138Q pups removed from the nest and found a ~50% decrease in the number of USVs in Fmr1R138Q compared to WT animals in both genders (Fig. 6a, b; Fmr1R138Q male: 75.36 ± 17.71 USVs; WT male: 147.4 ± 14.06 USVs; Fmr1R138Q female: 79.88 ± 35.68 USVs; WT female: 163.4 ± 31.49 USVs). These data indicate that the R138Q mutation leads to severe communicative deficits in infant Fmr1R138Q mice.

Next, we evaluated the cognitive performance in PND40–45 males and females with the novel object recognition test (Fig. 6c, d). We found a profound deficit for both genders as the Fmr1R138Q mice spent significantly less time than WT animals exploring the novel object (Fmr1R138Q male: 51.18 ± 3.26%; WT male: 71.16 ± 3.72%; Fmr1R138Q female: 45.27 ± 4.37%; WT female: 66.42 ± 3.31%). In addition, the Fmr1R138Q mice were spending significantly more time sniffing the old object (Fmr1R138Q male: 27.27 ± 3.85 s; WT male: 16.46 ± 2.46 s; Fmr1R138Q female: 19.22 ± 3.13 s; WT female: 9.77 ± 0.98 s), thus showing a lower discrimination index (Discrimination index Fmr1R138Q male: 2.36 ± 0.65%; WT male: 42.31 ± 7.45%; Discrimination index Fmr1R138Q female: −9.45 ± 8.73%; WT female: 32.84 ± 6.63%). These data indicate that the R138Q mutation substantially alters the cognitive function in the Fmr1R138Q mice.

About 25% of FXS patients present ASD traits, including social avoidance and decreased social skills. Therefore, to evaluate the impact of the R138Q mutation in ASD-like behaviors, we tested sociability in both male and female WT and Fmr1R138Q mice using the three-chamber test (Fig. 6e, f). As expected, WT animals from both sexes spent significantly more time sniffing the novel mouse rather than the empty cage. We showed that both the time spent sniffing the social stimulus and the ability to discriminate...
between the novel mouse and the empty cage was dramatically reduced in Fmr1R138Q males (Fmr1R138Q male sniffing time: 42.45 ± 13.84%; WT male: 80.48 ± 4.39%; Discrimination index Fmr1R138Q male: −15.10 ± 27.69%; WT male: 60.96 ± 8.78%). However, there was no significant difference between the WT and Fmr1R138Q female mice (Fmr1R138Q female sniffing time: 63.21 ± 9.25%; WT female: 69.29 ± 10.69%; Discrimination index Fmr1R138Q female: 26.41 ± 18.51%; WT female: 38.58 ± 21.37%).

Altogether, our behavioral data are in line with our biochemical and physiological data and reveal that Fmr1R138Q mice display important communicative, cognitive, and social deficits.
Fig. 5 LTP is impaired in Fmr1R138Q mice. a Secondary dendrites from TTX-treated WT and Fmr1R138Q 15 DIV neurons stained for MAP2-positive microtubule (green) and surface-expressed GluA1 (red) in basal conditions and upon cLTP induction. Bar, 20 μm. b Boxplots indicate median (line), 25th, 75th percentile (box), and min–to-max values (whiskers) obtained for surface GluA1 intensity and cluster density in WT and Fmr1R138Q neurons in control and cLTP conditions. Values were normalized to their basal conditions. N = 39–42 neurons per genotype from four independent experiments. Two-tailed Mann–Whitney test. *p = 0.0103; **p = 0.0053 (WT sGluA1 intensity); ***p = 0.0034 (KI sGluA1 intensity); ****p = 0.0036 (KI sGluA1 cluster). c, d Immunoblots showing the surface expression of GluA1 in basal and cLTP-induced conditions in PND90 TTX-treated WT (c) and Fmr1R138Q (d) hippocampal slices using BS3-crosslinking assays. Control Tubulin immunoblot is included to control the absence of intracellular BS3-crosslinking. e The surface/intracellular ratio in the WT was set to 1 and Fmr1R138Q values were calculated respective to the WT. Bars show the mean ± s.e.m. N = 7 independent experiments. Two-tailed ratio t test. *p = 0.0358, **p = 0.0004. f STED images of surface-expressed GluA1 (STED, green) in postsynaptic Homer1 sites (confocal, red) of cLTP induced WT and Fmr1R138Q hippocampal neurons. Scale bar, 500 nm. g, h Box plots indicate median (middle line), 25th, 75th percentile (box), and min to max values (whiskers) obtained for the postsynaptic surface-associated GluA1 fluorescent intensity (g) and nanocluster density (h) computed from STED imaging data in basal and cLTP-treated WT and Fmr1R138Q neurons. N = 13–17 (WT) and 15–18 (Fmr1R138Q) neurons were analyzed from three independent experiments. g, h Unpaired t test. ***p < 0.0001, **p < 0.0079. ns not significant. Values for control surface GluA1 in (f–h) are taken from Fig. 4 (c, d) since these experiments were performed in parallel. i Schematic diagram of the stimulating and recording areas in the mouse hippocampus. j fEPSPs were recorded at CA1 synapses on hippocampal slices from P35–42 WT and Fmr1R138Q littermates in basal conditions and upon LTP induction by high-frequency stimulation (HFS, 3 x 100Hz, 1 s). k Histograms show the mean ± s.e.m. of fiber volley (FV) and fEPSP slopes from 12–16 neurons per genotype in four independent experiments. Unpaired t test with Welch’s correction. ns not significant; ***p = 0.0092. Source data are provided as a Source Data file.

Discussion

Synaptic transmission and/or plasticity defects have been clearly linked to the development of many, if not all, neurological disorders. Therefore, a better understanding of the pathways underlying these alterations is essential to develop strategies to rescue the identified dysfunctions and design innovative targeted therapies to treat these diseases. Here, we generated and characterized a novel mouse model for FXS expressing the recurrent R138Q missense mutation in the FMRP protein. We show that the R138Q mutation leads to an increase in spine density, alterations in both the pre- and postsynaptic organization, and an impaired LTP in the Fmr1R138Q hippocampus. The consequence of this plasticity defect is an abnormal socio-cognitive behavior in Fmr1R138Q mice that resembles the ID and ASD-like traits described in FXS patients bearing the R138Q mutation.

To date, only two studies have provided some insights into the impact of the R138Q mutation on neuronal function13,23. FMRP is known to participate in the regulation of AMPAR trafficking17, which is critical to maintaining the synaptic function. Alpatov and colleagues23 investigated the impact of the R138Q mutation on the basal trafficking of AMPAR and reported that the exogenous expression of FMRP-R138Q does not impact the constitutive endocytosis of AMPAR in an Fmr1-KO background. Here, we revealed an altered surface expression of both GluA1 and GluA2 AMPAR subunits within the postsynaptic membrane of the Fmr1R138Q hippocampus, participating in an LTP impairment in these mice. We also identified that the R138Q mutation leads to alterations in the postsynaptic organization of AMPAR-containing nanodomains (Fig. 4). Nanoscale scaffolding domains at the PSD are essential to organize and concentrate AMPARs to allow an efficient synaptic transmission20,24. These 80-nm AMPAR nanodomains facing presynaptic glutamate release sites are dynamic and cLTP induction promotes their nanoscale reorganization20,22,24. Importantly, the calcium-dependent function of AMPARs also relies on the presence of edited GluA2 subunits at the synapse, which confers a low Ca2+ permeability to GluA2-containing heteromers (calcium impermeable, CI-AMPARs). Conversely, GluA1 homomers, which lack the GluA2 subunit, are permeable to Ca2+ and refer as CP-AMPARs25,27. In basal conditions, the majority of AMPAR assemblies in the adult hippocampus contain edited Ca2+-impermeable GluA2 subunits, which is essential to maintaining a low intracellular Ca2+ concentration. In the Fmr1R138Q hippocampal synapses, there is an excess of GluA1 subunits associated with a decrease in the overall amount of surface-expressed GluA2 at the synapse (Fig. 4a–d). This likely reflects a different AMPAR subunit composition in Fmr1R138Q synapses, potentially favoring the formation of CP-AMPARs. The recruitment of CP-AMPARs to postsynaptic sites plays a key role in the initial phase of LTP22,25,26. Interestingly, while we measured an overall synaptic increase in CP-AMPARs upon cLTP induction in WT neurons, this event is compromised in the Fmr1R138Q hippocampus (Fig. 5g). Thus, it is tempting to speculate that the enhanced expression of synaptic CP-AMPARs in basal conditions in Fmr1R138Q neurons likely impairs the recruitment of additional GluA1-containing CP-AMPARs that are necessary to initiate the LTP. Furthermore, we showed here that, contrary to the WT synapse, there is no increase in GluA1-containing nanodomains upon LTP in Fmr1R138Q neurons (Fig. 5h), which could also participate in the impaired postsynaptic response measured in the Fmr1R138Q hippocampus.

The Fmr1R138Q postsynaptic compartment also presents important ultrastructural alterations and displays an increase in the density of presynaptic neurotransmitter vesicles and a significant reduction of the PSD thickness (Fig. 2). This could lead to defective synaptic responses including altered diffusive properties and/or anchoring of AMPARs within the postsynaptic membranes, which is critical to recruiting AMPARs at the hippocampal synapse upon LTP induction28,29.

Altogether, these data uncover a previously unsuspected postsynaptic impact of the R138Q mutation leading to both basal and activity-dependent AMPAR trafficking defects in the Fmr1R138Q hippocampus.

The second study investigating the functional impact of the R138Q mutation reported that the exogenous overexpression of a truncated (FMRP1-298) version of the FMRP-R138Q mutant fails to rescue action potential (AP) broadening in Fmr1-KO neurons13, which correlates with an increased presynaptic release30. However, the presynaptic release per se was not assessed in this work. The authors also showed that the R138Q mutation disrupts the interaction of the short FMRP1-298 form with the β4 subunit of BK channels, thus underlying AP duration13,30. To conclude, they hypothesized that an alteration of the presynaptic function is likely responsible for the ID and seizures exhibited by the first FXS R138Q patient13. Importantly, BK channels are localized both at pre- and postsynaptic sites31. Consequently, alterations in FMRP/BK channel interaction may not only be linked to a
**Fig. 6 Fmr1R138Q** mice show communication deficits and socio-cognitive alterations. 

**a** Schematic of the isolation-induced ultrasonic vocalizations (USVs) test. Compared to WT animals, Fmr1R138Q KI mice (PND7) emit ~50% less USVs when removed from the nest. Histogram shows the mean values ± s.e.m. of USVs in WT and Fmr1R138Q males and females. WT, N = 13 males, 11 females; Fmr1R138Q, N = 10 males, 8 females. *p = 0.028. 

**b** Schematic of the object recognition test used to assess the cognitive domain. Quantification shows the mean values ± s.e.m. of time sniffing the new object (%), the old object (%), and the discrimination index (%) for both PND40–45 WT and Fmr1R138Q males and females. WT, N = 10 males, 9 females; Fmr1R138Q, N = 10 males, 10 females. *p = 0.011 (male), *p = 0.028 (female), ***p < 0.001.

**c** Scheme of the three-chamber test used to assess sociability. Histograms show the mean percentage ± s.e.m. of discrimination index and time sniffing the stimulus mouse for both PND40–45 WT and Fmr1R138Q males and females. WT, N = 7 males, 8 females; Fmr1R138Q, N = 7 males, 8 females. *p = 0.017. Two-way ANOVA with genotype and sex as factors followed by Newman-Keuls post-hoc test for individual group comparisons were computed for all behavioral studies. Source data are provided as a Source Data file.
presynaptic impairment as suggested by the authors but also to the postsynaptic defects unraveled in the present work.

In the present study, we also demonstrated that the R138Q mutation leads to an increase in the density of synapses in the Fmr1R138Q hippocampus. Unexpectedly, the frequency of mEPSCs, which usually correlates with synaptic density, is not altered (Fig. 4e–k). Recently, it was reported that the FMRP-R138Q mutant fails to restore activity-dependent bulk endocytosis (ADBE) in Fmr1-KO neurons, perhaps through the loss of BK channel interaction32. Our EM measurements also revealed a significant increase in the density of presynaptic vesicles in Fmr1R138Q hippocampal termini (Fig. 2c). This ultrastructural observation could potentially result from an impaired presynaptic glutamate release or a disruption in the exocytosis/recycling process of the synaptic vesicles. This may in turn directly impact the neurotransmitter release and, together with the identified postsynaptic alterations, may mask the effects on the mEPSC frequency in the Fmr1R138Q hippocampus.

The hippocampal plasticity acts on several brain functions that impact a wide range of behavioral responses. We found that the R138Q mutation leads to altered cognitive performances in both male and female Fmr1R138Q mice using the novel object recognition (NOR) test (Fig. 6c, d), likely due to the impaired hippocampal LTP in these mice. Indeed, while it is known that the perirhinal cortex is critical to object recognition memory assessed with the NOR test, it has been proposed that the perirhinal cortex and the hippocampus play complementary roles in the NOR task33,34. In particular, at the start of the training session, object information is stored in the perirhinal cortex and when a threshold amount of information is acquired, object information is transferred to the hippocampus and becomes strong object memory. If the threshold is not reached, then the information remains perirhinal cortex dependent as a weak object memory34. Thus, it is likely that the impaired LTP observed in the Fmr1R138Q mice participates in the altered cognitive performances observed during the NOR test.

Here, we identified strong social deficits in male Fmr1R138Q mice (Fig. 6f, g). It has been demonstrated that the hippocampal formation is also involved in different components of the social repertoire, including social memory, adaptation to new social contexts, and the maladaptive social behavior observed across several psychiatric disorders35. While the hippocampal plasticity defects in the Fmr1R138Q mice may not completely explain the social deficits observed in males, it is likely they contribute, at least partially, to such abnormal behavior. Interestingly, we did not measure any significant impairment in the social behavior of female Fmr1R138Q mice using the three-chamber test (Fig. 6e, f). Sex differences in the function of the hippocampus including the hippocampal neuronal morphology and synaptic plasticity have been observed in rodents36. For instance, estrogen may interfere and play a protective role in the Fmr1R138Q social phenotype37. Indeed, several preclinical, as well as clinical studies reported a neuroprotective role of estrogens in a number of neurodevelopmental disorders38–43, including autism39,40. However, while estrogens may play a protective role contributing to the normal behavior of the Fmr1R138Q female mice in the three-chamber test, on the basis of the current behavioral data we cannot exclude that Fmr1R138Q female mice still display defects in their social repertoire. Such impairments may be either more subtle in females than in males or revealed by behavioral tasks other than the three-chamber test.

Since most of the knowledge on FXS derives from studies using Fmr1-KO models, it is important to compare the phenotype reported in these models with our data on the Fmr1R138Q mice. Because the R138Q mutation occurs in the FMR1 gene, it has been unequivocally associated with the development of an FXS-like pathology. It is important to stress here that the three unrelated FMRP-R138Q patients show markedly variable phenotypes11,13–15, indicating that the same mutation leads to different clinical features ranging from mild symptoms to a full, complex classical FXS phenotype. Even if some of the defects measured in Fmr1R138Q animals are different from those measured in Fmr1-KO mice, it is important to notice that they rely on alterations in the same cellular processes, including synaptic elimination and AMPAR-mediated synaptic function, which likely underlie the socio-emotional and cognitive deficits. For instance, an increased spine density and immaturity have been consistently reported in the Fmr1-KO hippocampus18,19,44,45. While we also measured an increase in spine density in the Fmr1R138Q hippocampus, we did not notice any modification in the overall maturity of the dendritic protrusions. In addition, while the surface levels of AMPARs are significantly increased in the Fmr1R138Q hippocampus, it is rather decreased in different brain regions of the Fmr1-KO mouse46–49 including the hippocampus. However, alterations in postsynaptic AMPAR surface expression result in impaired basal synaptic transmission in both models49 (Fig. 4). Finally, we have shown that the NMDAR-mediated LTP is severely impaired in the Fmr1R138Q hippocampus. While LTP impairments have also been reported in the Fmr1-KO hippocampus50,51, the molecular mechanisms underlying these defects in Fmr1R138Q mice are likely different from those in the Fmr1-KO background. We also showed that Fmr1R138Q mice display reduced social interaction in the three-chamber test, which is reminiscent of autistic-like features. Fmr1R138Q mice are also unable to discriminate between a familiar and a novel object demonstrating that learning and memory processes are impaired in these animals. Similar cognitive and socio-emotional deficits have been reported in the Fmr1-KO mice in the same behavioral tasks52,53. Altogether, our data indicate that different mutations in the FMR1 gene engage distinct molecular mechanisms leading to similar pathological conditions. Therefore, the identification of additional FMRP-R138Q patients will undoubtedly help to shed light on the phenotypic and mechanistic similarities and differences with the classical FXS pathology.

From a therapeutic point of view, it might be of interest to target the AMPARs in the Fmr1R138Q brain. The excess of available AMPARs in the Fmr1R138Q brain may correlate with the intractable seizures observed in the first-reported patient carrying the R138Q mutation13. The FDA-approved anti-epileptic AMPAR antagonist Perampanel54 could be used to reduce the activity of these glutamate receptors and treat the epileptic manifestations in FXS patients carrying the R138Q mutation. Preclinical studies are now required to first investigate the epileptic activity in the Fmr1R138Q mouse line and then determine if Perampanel can correct the altered AMPAR function, as well as the socio-cognitive behaviors in these mice.

Generating preclinical models for brain disorders is an essential step toward the development of efficient therapies to treat human diseases. In this context, the Fmr1R138Q mouse line certainly represents a unique preclinical model to test the efficacy of new molecules and/or the repurposing of existing FDA-approved drugs to correct the altered pathways identified in these mice. This may also lead in the near future to the development of clinical studies to assess the potential benefits of these drugs in FXS patients. Therefore, gaining insights into this complex neurodevelopmental disorder may lead to innovative therapeutic strategies for these particular cases of FXS. Nevertheless, since AMPAR alterations as well as synaptic defects have been linked to other neurodevelopmental disorders, these approaches might be extended to classical FXS patients and more generally to patients presenting ID and ASD in which the AMPAR pathway is altered.
Primary neuronal cultures. The protocol to prepare primary neuronal cultures from mouse embryos was approved by the National Animal Care and Ethics Committee (Project reference APAF1 #18648-2019111115666). Hippocampal neuron cultures were prepared from WT and Fmr1R138Qembryonic (E15.5) C57BL/6 mice. Neurons were plated in Neurobasal medium (Invitrogen, France) supplemented with 2% B27 (Invitrogen), 0.5 mM glutamine, and penicillin/streptomycin (Osmye) on 60-mm dishes or 16-mm glass coverslips (VWR) pre-coated with Matrigel (BD Biosciences). Neurons (600,000 cells per 60-mm dish or 80,000 cells per 16-mm coverslip) were then used at 14–15 DIV.

Sindbis virus production and neuronal transduction. Attenuated Sindbis virus particles (SIVp5; Invitrogen) were generated using a pCMV5-1 plasmid and the MRR packaging cell line. Neurons were co-cultivated with Sindbis virus pseudotyped with the MRR virus. Neurobasal medium (Invitrogen, France) supplemented with 2% B27 (Invitrogen), 0.5 mM glutamine, and penicillin/streptomycin (Osmye) on 60-mm dishes or 16-mm glass coverslips (VWR) pre-coated with Matrigel (BD Biosciences). Neurons (600,000 cells per 60-mm dish or 80,000 cells per 16-mm coverslip) were then used at 14–15 DIV.

Biocytin assays. Live Tetrodotoxin (TTX, 0.5 μM)-treated control or Fmr1R138Q hippocampal neurons (14–15 DIV) were surface biotinylated for 10 min at 4°C on ice using the membrane impermeant Sulfo-NHS-SS-Biotin (Pierce, 0.3 mg mL−1 in PBS). After three washes in ice-cold PBS, neurons were incubated with NH4Cl (50 mM in PBS) for 5 min at 4°C to quench the remaining available NHS groups. After three washes in PBS, biotin particles were introduced in the culture medium and incubated for 48 h at 37°C. After washing, neurons were fixed in 4% formaldehyde in PBS for 1 h and then permeabilised by sonication. Cells were incubated with avidin–biotin complex (Vector Labs) overnight at 4°C. Neurons were then washed and incubated with streptavidin–HRP (Vector Labs) for 1 h at RT. After washing, neurons were incubated with 3,3-diaminobenzidine (DAB) substrate (Sigma) for 5 min, washed, and mounted in crystal mountant (Lab-Tek, USA).

BS3-crosslinking assays. Hippocampi from WT and Fmr1R138Q male littersates were included in agar. Two hundred fifty-micrometre-thick hippocampal slices were pretreated in ice-cold oxygenated (5% CO2, 95% O2) sucrose solution (2.5 mM KCl, 1.25 mM NaH2PO4, 10 mM MgSO4, 0.5 mM CaCl2, 26 mM NaHCO3, 11 mM glucose, 234 mM sucrose) for 30 min at 35°C. Hippocampal slices were then mounted on a PerkinElmer (USA) cryostat and transduced with Fmr1R138Q pseudotyped viruses. Neurons were allowed to recover for 1 h at 35°C before fixation with 4% PFA in PBS for 15 min. After fixation, slices were washed with PBS and permeabilised with a solution of 0.1% BSA, 0.1% Triton X-100 in PBS. Subsequently, slices were washed again with PBS and blocked with 1% BSA for 1 h at RT. Slices were then incubated with primary antibodies overnight at 4°C. Primary antibodies: mouse anti-FMRP18 1 μg/mL (DSHB, Clone 2F5-1); Rabbit anti-GAPDH antibody 1/25000 (Sigma). Secondary antibodies (Jackson ImmunoResearch) were used at 1/500 dilution. Finally, slices were washed with PBS and mounted in FluorSave (Millipore). The experiments were repeated at least three times with different batches of slices.

Immunoblotting. Protein extracts were isolated from SIV-transduced neurons using a buffer containing 1% Triton X-100. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (BioTrace NT, Pall, USA). Blots were blocked with 5% milk in Tris-buffered saline and incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with secondary antibodies (1:5000 dilution) for 1 h at RT. Antibody activity was detected using chemiluminescence (Pierce). The bands were quantified using ImageJ software. For quantification, densitometry analysis was performed using ImageJ software. Protein levels were normalised to GAPDH as an internal control.
acquired on a Fusion FX7 system (Vilber Lourmat). Full-size blots for cropped gels are shown in the Source data file.

**Immunolocalization of surface-expressed GluA1 AMPAR subunits**

Live TXR-treated WT and Fmr1<sup>−/−</sup> hippocampal neurons (14–15 DIV) were incubated with a mouse monoclonal anti-N-terminal GluA1 antibody (1:100; Merk-Millipore #MAB2683) for 10 min at RT. After three washes, the neurons were quickly fixed in PBS containing 3.7% formaldehyde and 5% sucrose for 5 min at RT. Cells were then thoroughly rinsed and incubated with a non-permeabilizing blocking solution (0.2% BSA, 0.2% Triton X-100, and 5% HS) for 1 h at RT, with the appropriate secondary antibodies (Thermofisher/Invitrogen #A-21202 and #A-21203; 1:200) conjugated to Alexa488 or 594 as indicated in PBS containing 0.2% BSA. Neurons were then further fixed for 10 min in PBS containing 3.7% formaldehyde and 5% sucrose to stabilize the surface-associated secondary antibodies. All steps were performed in PBS containing 0.2% BSA, 0.2% Triton X-100, and 5% HS. Neurons were incubated with guinea pig anti-MAP2 (1:1000; Synaptic systems #188004) antibodies overnight at 4 °C. Cells were then washed three times in PBS and incubated with the indicated secondary Alexa- conjugated antibodies for 1 h at RT, mounted with Mowiol (Sigma), and stored at −20 °C until confocal examination. The surface GluA1 intensity which represents the fluorescence associated with the GluA1 antibody labeling was then measured, as well as the density of surface GluA1 clusters, which rather correlates with the distribution of the surface-expressed AMPARs at synapses along dendrites since GluA1 is generally concentrated to dendritic spines.

For the imaging, the TXR-treated neurons were incubated with either a mouse monoclonal anti-terminal GluA1 (1/40, Merk-Millipore #MAB2683) or GluA2 (1/30, Merk-Millipore #MAB397) antibodies and treated or not for cLTP as above. To visualize the surface-expressed subunits, neurons were labeled with the appropriate secondary antibodies conjugated to the StarRed dye (Abberior GmbH STED1001; 1/100) prior to the permeabilization step and staining for Homer1 with a rabbit anti-Homer1 (1/500, Synaptic System). Coverslips were mounted in Abberior Mount Solid Antifade (Abberior GmbH, Göttingen). STED images were acquired using a Leica SP8 STED XLe (Leica Microsystems, Nanterre), at 400 Hz through a 100X/1.4 NA Oil objective using the Leica LAS X software. Z-stack of confocal images of Homer1 immunolabelled with Alexa546-labeled secondary antibodies were obtained by a laser excitation at 561 nm and combined with a STED image acquisition of surface GluA1 or GluA2 immunostained with a StarRed Fluorophore excited at 633 nm and depleted at 775 nm (20–30% of power). Z-stack of 2D STED images had a 20 × 20 × 200 nm voxel size and all images were deconvolved. In ice-cold dissecting solution (234 mM sucrose, 2.5 mM KCl, 0.5 mM NaCl, 10 mM MgCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, and 11 mM D-glucose) oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at pH 7.4. Slices were then incubated in a recording chamber perfused with 2.5 ml min<sup>−1</sup> aCSF (119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 1.3 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, and 11 mM D-glucose) oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at pH 7.4. Slices were first incubated for 1 h at 37 °C in aCSF (119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 1.3 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, and 11 mM D-glucose) oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at pH 7.4. Slices were then further stained for 1 h at RT with the appropriate secondary antibodies (Thermofisher/Invitrogen #A-21202 and #A-21203; 1:200) conjugated to Alexa488 or 594 as indicated in PBS containing 0.2% BSA. Neurons were then fixed for 10 min in PBS containing 3.7% formaldehyde and 5% sucrose to stabilize the surface-associated secondary antibodies. All steps were performed in PBS containing 0.2% BSA, 0.2% Triton X-100, and 5% HS. Neurons were incubated with guinea pig anti-MAP2 (1:1000; Synaptic systems #188004) antibodies overnight at 4 °C. Cells were then washed three times in PBS and incubated with the indicated secondary Alexa- conjugated antibodies for 1 h at RT, mounted with Mowiol (Sigma), and stored at −20 °C until confocal examination. The overall surface GluA1 intensity which are shown in the Source data file.

**Slice preparation and LTP electrophysiological recordings**

WT and Fmr1<sup>−/−</sup> male littermate (PND35–42) brains were incubated for 4 min in ice-cold sucrose cutting solution (19.99 mM KCl, 260 mM NaHCO<sub>3</sub>, 11.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM sucrose, 220 mM glucose, 2.0 mM CaCl<sub>2</sub>, 6 mM MgCl<sub>2</sub>) gassed with carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>; pH 7.4 at 37 °C). Sagittal hippocampal slices (350 µm) were then incubated in standard artificial cerebrospinal fluid (aCSF; 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 10 mM glucose, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>). Slices were allowed to recover at 35 °C for 45 min, continuously oxygenized with carbogen, and then for 45 min at RT. Recordings were performed in ACSF continuously oxygenized with carbogen at 33–34 °C in a submerged recording chamber perfused at a low rate (1.8 ml min<sup>−1</sup>). Glass microelectrodes (tip diameter 5–10 µm; resistance: 0.2–0.3 Ω) were filled with aCSF. Field recordings were performed using MultiClamp 700B amplifier (Molecular Devices, Foster City, CA, USA) and Clampfit software. Schaffer collateral inputs (CA1) were stimulated using a bipolar electrode and recorded in the stratum radiatum of the CA1. A baseline of 10 min was recorded in the current-clamp mode with a single stimulation at 0.1 Hz every 10 s. LTP was induced by high-frequency stimulation (3 × 100 Hz for 1 s, 30 s inter burst), and transferred at 0.1 Hz stimulation rate for 50 min. The analysis was performed using the Clampfit software (Molecular Device). Along with the 6 Hz stimulation, fiber volley (FV) and synaptic response slopes were calculated and normalized to the baseline levels. Experiments were done blind to the genotype. To assess for synaptic changes, the EPSP/FV slope ratio was calculated and averaged for 1-min time periods. Quantification and statistical comparisons were computed by comparing the EPSP/FV ratios obtained at 40–50 min after induction to the baseline level.

**Behavioral tasks.** All experiments were performed between 9:00 a.m. and 4:30 p.m. Both male and female WT and Fmr1<sup>−/−</sup> mice were tested. The experiments were approved by the Italian Ministry of Health (Rome, Italy; Authorization number: 87-2019-PR) and performed in agreement with the ARRIVE guidelines, with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU. All behavioral experiments were performed and scored blind to the genotype. Data are expressed as mean ± s.e.m.

**Locomotor activity.** At PND40–45, during the habituation session of the novel object recognition test, locomotor activity was calculated using a grid that divides the arena into equally sized squares and that is projected over the recordings. The number of line crossings made by the animal was quantified to assess its motor activity.

**The isolation-induced ultrasonic vocalizations (USVs) test.** The test was performed as previously described. Briefly, each pup (PND7) was individually removed from the nest and placed into a black Plexiglas arena, located inside a sound-attenuating and temperature-controlled chamber. USVs from the pups were detected for 3 min by a microphone (Avisoft Bioacoustics, Germany) sensitive to frequencies between 10 and 250 kHz and fixed at 10 cm above the arena. Pup auxiliary temperature was measured before and after the test by a digital thermometer. The emission of USVs was analyzed using Avisoft Recorder software (Version 5.1).

**Novel object recognition test.** The novel object recognition test was performed at PND40–45. The test consisted of three phases: habituation, training, and test. In the habituation phase, the animals were allowed to explore an empty arena (a Plexiglas arena measuring 40 × 40 cm<sup>2</sup>) for 5 min. Twenty-four hours later, on
the training trial, each mouse was individually placed into the arena containing two identical objects (A1 and A2), equidistant from each other, and allowed to explore the objects for 10 min. After 1 h, during the test phase, one copy of the familiar object (A3) and a new object (B) was placed in the same location as during the training trial. The time spent exploring each object was recorded for 5 min. The discrimination index was calculated as the difference in time exploring the novel and the familiar objects, expressed as the percentage ratio of the total time spent exploring both objects.62

Three-chamber test. This test was performed at PND40–45. The apparatus was a rectangular three-chamber box, with two lateral chambers (20.5 (length) × 41 (width) × 22.5 (height) cm) connected to a central chamber (20 (length) × 41 (width) × 22.5 (height) cm). Each lateral chamber contained a small Plexiglas cylindrical cage. Each experimental mouse was individually allowed to explore the central compartment for 5 min with both doors closed. Next, each experimental mouse was individually allowed to explore the apparatus for 10 min and then confined in the central compartment. An unfamiliar stimulus animal was confined in a cage located in one chamber of the apparatus, while the cage in the other chamber was left empty. Both doors to the side chambers were then opened, allowing the experimental animal to explore the apparatus for 10 min. The percentage of time spent in the social approach (sniffing the stimulus animal) and the discrimination index were scored using the Observer XT, version 12.0 software (Noldus Information Technology, The Netherlands). The discrimination index was calculated as the difference in time exploring the stimulus and the empty cage, expressed as the percentage ratio of the total time spent exploring both the stimulus and empty cage.

Data manipulation and statistical analysis. Statistical analyses were calculated using GraphPad Prism (v7.0; GraphPad Software, Inc) or Sigma Plot (v13.0; Systat Software, Inc, USA) software. All data are expressed as mean ± s.e.m. Unpaired t test (Figs. 2c, 3a, 3c, d, k and Supplementary Figs. 1b and 3j), Ratio t test (Figs. 3c, d, b, h and Supplementary Fig. 4) or non-parametric Mann–Whitney test (Figs. 2a, 2b, 3b, 5e and supplementary Fig. 2) were used to compare medians of two data sets. Statistical significance for multiple comparison data sets was computed two-way ANOVA with Sidak correction (Figs. 2a, 3b, 5e and Supplementary Fig. 2) were analyzed by two-way ANOVA including genotype and treatment as main factors, followed by Sidak’s multiple comparisons test. Statistical analyses were calculated using GraphPad Prism (v7.0; GraphPad Software, Inc) or Sigma Plot (v13.0; Systat Software, Inc, USA) software. All data are expressed as mean ± s.e.m. Unpaired t test (Figs. 2c, 3a, 3c, d, k and Supplementary Figs. 1b and 3j), Ratio t test (Figs. 3c, d, b, h and Supplementary Fig. 4) or non-parametric Mann–Whitney test (Figs. 2a, 2b, 3b, 5e and supplementary Fig. 2) were used to compare medians of two data sets. Statistical significance for multiple comparison data sets was computed two-way ANOVA with Sidak’s post test (Fig. 1b). Behavioral data (Fig. 6 and Supplementary Fig. 4) were analyzed by two-way ANOVA including genotype and sex as factors with a Newman–Keuls post hoc test for individual group comparisons. Normality for all groups was verified using the Shapiro–Wilk test. For electrophysiological data, distributions were analyzed by a Kolmogorov–Smirnov test (Fig. 4). *p < 0.05 was considered significant.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. The B6N Fmr1<sup>–/–</sup> line is available to the scientific community via the Mouse Clinical Institute (ICs, Illkirch, France; http://www.ics-mci.fr/en/), or the INFRAFRONTIER consortium (the European Research Infrastructure for phenotyping and archiving of model mammalian genomes (https://www.infrafrontier.eu/)). Source data are provided with this paper.

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