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1 **Metformin ameliorates core deficits in a Fragile X syndrome mouse model**

2

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8 Fragile X syndrome is the leading monogenic cause of ASD. Trinucleotide repeats in the  
9 *FMRI* gene abolish FMRP protein expression, leading to hyperactivation of ERK and  
10 mTOR signaling, upstream of mRNA translation. Here we show that metformin, the  
11 most widely used anti-type 2 diabetes drug, rescues core phenotypes in *FmrI*<sup>-y</sup> mice and  
12 selectively normalizes Erk signaling, Eif4e phosphorylation and the expression of  
13 Mmp9. Thus, metformin is a potential FXS therapeutic.

14 Dysregulated mRNA translation is linked to core pathologies diagnosed in the Fragile X  
15 neurodevelopmental Syndrome (FXS), such as social and behavior problems, developmental  
16 delays and learning disabilities<sup>1,2</sup>. In the brains of FXS patients and knockout mice (*FmrI*<sup>-y</sup>;  
17 X-linked *FmrI* deletion in male mice), loss of Fragile X mental retardation protein (FMRP)  
18 results in hyperactivation of the mammalian/mechanistic target of rapamycin complex 1  
19 (mTORC1) and the extracellular signal-regulated kinase (ERK) signaling pathways<sup>1,2</sup>.  
20 Consistent with increased ERK activity, eukaryotic initiation factor 4E (eIF4E)  
21 phosphorylation is elevated in the brain of FXS patients and *FmrI*<sup>-y</sup> mice, thereby promoting  
22 translation of the mRNA encoding for matrix metalloproteinase 9 (MMP-9), which is elevated  
23 in the brains of both FXS patients and the *FmrI*<sup>-y</sup> mice<sup>1-5</sup>. In accordance with these findings,  
24 knockout of *Mmp9* rescues the majority of phenotypes in *FmrI*<sup>-y</sup> mice. MMP-9 degrades  
25 components of the extracellular matrix, including proteins important for synaptic function and  
26 maturation, which are implicated in FXS and autism spectrum disorders (ASD). Recent  
27 observations indicate that metformin, a first-line therapy for type 2 diabetes, imparts  
28 numerous health benefits beyond its original therapeutic use, such as decreased cancer risk  
29 and improved cancer prognosis<sup>6</sup>. Metformin inhibits the mitochondrial respiratory chain  
30 complex 1, leading to a decrease in cellular energy state and thus activation of the energy  
31 sensor AMP-activated protein kinase (AMPK)<sup>6</sup>. Several AMPK-independent activities of  
32 metformin have also been reported<sup>7,8</sup>. Since metformin suppresses translation by inhibiting

33 mTORC1 and ERK pathways, we reasoned that metformin could have beneficial therapeutic  
34 effects in *Fmr1*<sup>-y</sup> mice<sup>9</sup>.

35

36 Adult (8-12 weeks old) wild-type (WT) and *Fmr1*<sup>-y</sup> mice were injected intraperitoneally (i.p.)  
37 with metformin (200 mg/kg/day, a concentration previously used in preclinical studies<sup>8</sup>) or  
38 vehicle for 10 days (**Fig. 1a**). Metformin, as previously reported<sup>10</sup>, crosses the blood brain  
39 barrier (BBB), achieving lower concentrations in brain than plasma after acute and chronic  
40 injection (**Supplementary Figs. 1 and 2**). Twenty-four hours after the last injection of  
41 metformin, mice were subjected to a social novelty test. Vehicle-treated *Fmr1*<sup>-y</sup> mice were  
42 impaired in the preference for social novelty, showing no preference for interaction with the  
43 novel (stranger 2) over the original social stimulus (stranger 1; **Fig. 1b,c**). Metformin  
44 treatment restored the impaired preference of *Fmr1*<sup>-y</sup> mice for the novel stranger mouse, thus  
45 rescuing the social deficit. Next, we examined the effect of metformin on repetitive behavior,  
46 a core characteristic of FXS patients that is recapitulated in *Fmr1*<sup>-y</sup> mice as increased self-  
47 grooming<sup>1,11</sup>. Metformin reversed the increased grooming in *Fmr1*<sup>-y</sup> mice (**Fig. 1d**) and  
48 decreased the number of grooming bouts (**Fig. 1e**) measured 24 hours after the last injection.  
49 Prolonged exposure to metformin is required to rescue behavioral deficits since one- and five-  
50 day treatments of *Fmr1*<sup>-y</sup> mice failed to correct the core FXS phenotypes (**Supplementary**  
51 **Figs. 3 and 4**). We tested several other behavioral phenotypes including audiogenic seizures,  
52 hyperactivity and cognitive function in *Fmr1*<sup>-y</sup> mice; we observed no cognitive impairment in  
53 *Fmr1*<sup>-y</sup> mice. Ten-day treatment with metformin reduced the incidence of seizures but did  
54 not impact hyperactivity (**Supplementary Figs. 5 and 6**).

55

56 Neurons from FXS patients and *Fmr1*<sup>-y</sup> mice exhibit aberrant spine morphology<sup>1,11</sup>. We  
57 observed spine dysmorphogenesis in *Fmr1*<sup>-y</sup> mice as evidenced by increased density of

58 dendritic spines in CA1 hippocampal pyramidal neurons, along with fewer mature stubby and  
59 mushroom spines, and an increased number of immature filopodia-like spines (**Fig. 1f,g,h**).

60 **Ten-day metformin** administration corrected the dendritic abnormalities in *Fmr1*<sup>-/-</sup> mice (**Fig.**  
61 **1f,g,h**).

62

63 *Fmr1*<sup>-/-</sup> mice also display exaggerated group 1 mGluR-dependent LTD<sup>1,12</sup>. **Ten-day**  
64 metformin treatment rescued exaggerated LTD (**Fig. 1i,j,k**) in *Fmr1*<sup>-/-</sup> mice, as well as  
65 restored excitatory synaptic activity to WT levels in hippocampal slices of *Fmr1*<sup>-/-</sup> mice  
66 (**Supplementary Fig. 7**).

67

68 A hallmark of post-adolescent FXS male patients and *Fmr1*<sup>-/-</sup> mice is macroorchidism<sup>11,12</sup>.  
69 **Ten-day** metformin administration also led to a partial reduction in testicular weight in *Fmr1*<sup>-/-</sup>  
70 mice (**Fig. 2a**).

71

72 *Fmr1*<sup>-/-</sup> mice exhibit **elevated** mRNA translation<sup>1,12</sup>. Consistent with previous studies<sup>1,12,13</sup>,  
73 basal levels of protein synthesis were elevated in *Fmr1*<sup>-/-</sup> mice and **ten-day** metformin  
74 treatment reduced the excessive translation (**Fig. 2b**).

75

76 ERK and mTOR signaling pathways are hyperactivated in *Fmr1*<sup>-/-</sup> mice<sup>1,2,12,13</sup>. **Ten-day**  
77 metformin treatment restored the levels of **phosphorylated mitogen-activated protein kinase**  
78 (**Mapkk** encoding Mek; p-MEK), p-ERK, p-eIF4E, and MMP-9 in prefrontal cortex and  
79 hippocampus (**Fig. 2c-j**), whereas the levels of p-S6 remained elevated in the hippocampus of  
80 metformin-treated *Fmr1*<sup>-/-</sup> mice (**Supplementary Figs. 8a,b and 9**). Similarly, **ten-day**  
81 metformin treatment rescued increased p-ERK in the striatum, but not in the cerebellum  
82 (**Supplementary Fig. 10a,b**) of *Fmr1*<sup>-/-</sup> mice, and affected specific known synaptic FMRP

83 targets, MAP2 and synapsin, with no effect on eEF2 and PUM2 levels<sup>14</sup> (Supplementary  
84 Fig. 11). Apart from the brain, p-ERK was increased in the liver, but not in gonads  
85 (Supplementary Fig. 10c,d) of *Fmr1*<sup>-y</sup> mice. Ten-day metformin treatment did not rescue the  
86 increased ERK phosphorylation in the liver (Supplementary Fig. 10d), suggesting the  
87 implication of other pathways<sup>12</sup> or endocrine regulation outside the brain of *Fmr1*<sup>-y</sup> mice.

88

89 Ten-day metformin administration did not activate Ampk in the prefrontal cortex and  
90 hippocampus of *Fmr1*<sup>-y</sup> mice, as evidenced by the lack of increased phosphorylated Ampk (p-  
91 AMPK), and of its downstream substrates p-Acc1, p-Tsc2, p-Raptor, and p-Braf (Ser729) in  
92 metformin-treated mice (Supplementary Figs. 8c-k and 9a). These findings are consistent  
93 with previous reports showing that chronic metformin administration does not increase p-  
94 AMPK in the brain<sup>15,16</sup>. It is not immediately clear why ten-day metformin administration  
95 does not increase p-AMPK in the brain, however, in accordance with previous studies<sup>17,18</sup>, a  
96 single injection of 200 mg/kg, i.p. metformin induced a transient increase in p-AMPK  
97 (Supplementary Fig. 1c). A plausible explanation for the change in ERK signaling following  
98 chronic metformin treatment is due to the rescue of elevated expression of Braf and Craf in  
99 *Fmr1*<sup>-y</sup> mice (Supplementary Fig. 9)<sup>19</sup>.

100

101 Presently, there is no cure for FXS or ASD, and recently completed clinical trials in teenagers  
102 or adults with FXS are not promising<sup>20</sup>. Our data show that metformin, the most widely used  
103 anti-diabetic FDA-approved drug for patients aged 10 years and older, corrects most  
104 phenotypic deficits in the adult FXS mouse model. Thus, metformin, whose long-term safety  
105 and tolerability are extensively documented in clinical practice, is one of the very few  
106 compounds that can be promptly repurposed as an FXS therapeutic for patients aged 10 years  
107 and older. Moreover, our data are in accordance with a recent finding that metformin

108 treatment corrects circadian and cognitive deficits in a *Drosophila* Fragile X model<sup>21</sup>.  
109 Importantly, we present a potential molecular mechanism for metformin in FXS by showing  
110 that chronic metformin treatment corrects enhanced Raf/Mek/Erk signaling and Mmp9  
111 expression in *Fmr1*<sup>-y</sup> mice (Fig. 2 and Supplementary Fig. 9). Similarly, lovastatin, a drug  
112 that downregulates ERK signaling, also rescued audiogenic seizures, exaggerated mGluR-  
113 LTD, and decreased general protein synthesis in *Fmr1*<sup>-y</sup> mice<sup>13</sup>. Metformin, however, corrects  
114 a broader range of phenotypes than lovastatin. Combining these findings bolster the critical  
115 role of aberrant ERK activity in engendering FXS-like phenotypes in FXS. Since *Mmp9*  
116 mRNA translation is stimulated by eIF4E phosphorylation and knockout of *Mmp9* reversed  
117 abnormal phenotypes in *Fmr1*<sup>-y</sup> mice<sup>1,5</sup>, it is highly likely that the rescue by metformin is  
118 selectively mediated via ERK/eIF4E-dependent normalization of MMP-9 expression in the  
119 brain, providing a very strong mechanistic avenue for the action of metformin. We cannot  
120 exclude a yet unidentified, peripherally-mediated rescue mechanism, given the known  
121 inhibition of gluconeogenesis by metformin or altering the gut microbiota<sup>22</sup>. Such peripheral  
122 phenotypes are linked to autism, intellectual disability and FXS, and have been shown to  
123 affect brain plasticity<sup>23</sup>.

124

125

126 **METHODS**

127 Methods and any associated references are available in the online version of the paper.

128

129 **SUPPLEMENTARY INFORMATION**

130 Supplementary Information is available in the online version of the paper.

131

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138

139 **AUTHOR CONTRIBUTIONS**

140 I.G., A.K. and J.P. designed the experiments, performed data analysis and wrote the  
141 manuscript. I.G., A.K., J.P., A.A-V, E.F., R.C., V.S., T.P., A.N., S.W., S.M.J., C.C., E.A.M.  
142 and C.G.G. designed and carried out experiments. A.S., V.T.T., I.A.G. and K.G. assisted with  
143 experiments. K.N. supervised the project. J-C.L., C.G.G. and N.S. supervised the project,  
144 designed experiments and edited the manuscript. All authors revised the manuscript.

145

146 **COMPETING FINANCIAL INTERESTS**

147 The authors declare no competing financial interest.

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175

176

177 **FIGURE LEGENDS**

178

179 **Figure 1** Chronic metformin treatment corrects social deficit, repetitive behavior, aberrant  
180 dendritic spine morphology and exaggerated LTD in *Fmr1*<sup>-/-</sup> mice. **(a)** Metformin or vehicle  
181 was injected i.p. over 10 days (200 mg/kg/day) followed by analysis of social behavior,  
182 grooming, dendritic spine morphology, and LTD. Preference for social novelty was assessed  
183 in the three-chamber social interaction test by measuring time spent with the novel social  
184 stimulus (stranger 2 (S2)) or the previously encountered mouse (S1) **(b)**; and time spent in  
185 each chamber **(c)**. Vehicle-treated WT (n = 10) and *Fmr1*<sup>-/-</sup> (n = 10), and metformin-treated  
186 WT (n = 9) and *Fmr1*<sup>-/-</sup> (n = 12). **(d)** Self-grooming test with total time spent grooming and  
187 **(e)** total number of grooming bouts. Vehicle-treated WT (n = 10) and *Fmr1*<sup>-/-</sup> (n = 10), and  
188 metformin-treated WT (n = 8) and *Fmr1*<sup>-/-</sup> (n = 12). **(f)** Golgi-cox staining of CA1 dendritic  
189 spines in metformin or vehicle-injected WT and *Fmr1*<sup>-/-</sup> mice. Scale bar: 2  $\mu$ m. **(g)**  
190 Quantification of spine density, measured as the number of spines per 10  $\mu$ m and **(h)** spine  
191 subtype analysis (S/M = spiny/mushroom; F = filopodial) presented as a fraction of total  
192 spines for each subtype (n = 4 in each group). mGluR-LTD was measured in CA1 in response  
193 to DHPG (50  $\mu$ M for 10 min) in slices prepared from **(i)** vehicle-treated WT (n = 9) and *Fmr1*<sup>-/-</sup>  
194 (n = 17) mice, and **(j)** metformin-treated WT (n = 9) and *Fmr1*<sup>-/-</sup> (n = 15) mice. **(k)** fEPSP  
195 slope during the last 10 minutes of recording. All values are shown as mean  $\pm$  s.e.m. \*\*\*P <  
196 0.001, \*\*P < 0.01, \*P < 0.05 versus all other groups; N.S., not significant; calculated by two-  
197 way ANOVA with Tukey's *post hoc* test.

198

199 **Figure 2** Chronic metformin treatment corrects macroorchidism, increased translation and  
200 reduces the phosphorylation of upstream eIF4E effectors. **(a)** Mean testicular weight of

201 vehicle- and metformin-treated WT and *Fmr1*<sup>-y</sup> mice. Vehicle-treated WT (n = 6) and *Fmr1*<sup>-y</sup>  
202 (n = 6), and metformin-treated WT (n = 6) and *Fmr1*<sup>-y</sup> (n = 7). **(b)** Western blots of lysates  
203 from hippocampal slices incubated with puromycin to measure basal rates of protein  
204 synthesis.  $\beta$ -tubulin was used as a loading control. Puromycin incorporation is presented as  
205 percentage change relative to vehicle-treated WT slices (n = 7 in each group). Representative  
206 western blots of lysates from vehicle- and metformin-treated WT and *Fmr1*<sup>-y</sup> mice and  
207 quantification of phosphorylation and total levels of **(c)** MEK, **(d)** ERK, **(e)** eIF4E and **(f)**  
208 MMP-9 in prefrontal cortex and **(g)** MEK, **(h)** ERK, **(i)** eIF4E and **(j)** MMP-9 in  
209 hippocampus. GAPDH was used as a loading control. For quantification, the phospho-protein  
210 signal was normalized first against total protein, and then presented relative to vehicle-treated  
211 WT (n = 6 in each group, n = 5 for MMP-9 in prefrontal cortex). All values **(a-h)** are shown  
212 as mean  $\pm$  s.e.m. \*\*\*P < 0.001, \*\*P < 0.01, \*P < 0.05 versus all other groups; N.S., not  
213 significant; calculated by two-way ANOVA with Tukey's *post hoc* test.

214

215

216

217 **ONLINE METHODS**

218 **Knockout mice and metformin administration.** *Fmr1*<sup>-y</sup> (the *Fmr1* gene is on the X mouse  
219 chromosome, thus male animals have a -/y genotype; y corresponds to Y mouse  
220 chromosome), and WT mice on C57BL/6J background (Jackson Laboratories) were  
221 previously described<sup>21</sup>. Food and water were provided *ad libitum* and mice were kept on a 12-  
222 h light/dark cycle (7:00-19:00 light period). After weaning at postnatal day 21, mice were  
223 group housed (maximum of five per cage) by sex. Cages were maintained in ventilated racks  
224 in temperature (20-21°C) and humidity (~55%) controlled rooms. Standard corncob bedding  
225 was used for housing (Harlan Laboratories Inc.).

226 All animals received a 10-day chronic treatment with metformin (200 mg/kg/day,  
227 intraperitoneal injection) or vehicle (saline), except when indicated otherwise. Injecting  
228 groups were randomized over all cages.

229 All procedures were in compliance with the Canadian Council on Animal Care guidelines and  
230 were approved by McGill University and Université de Montréal.

231

232 **Three-chamber sociability and preference for social novelty tests.** The apparatus consisted  
233 of three Plexiglas chambers: the central chamber (36 cm x 28 cm x 30 cm) was divided from  
234 two side chambers (each chamber: 29 cm x 28 cm x 30 cm) by Plexiglas walls, as previously  
235 described (Stoelting Co.)<sup>24,25</sup>. Each side was accessible to the mouse from the center through a  
236 doorway covered by a removable sliding door. A camera was mounted above the apparatus to  
237 record testing. The test consisted of 3 phases: habituation, sociability, and preference for  
238 social novelty. In the first part, three-month old male mice were placed in the middle chamber  
239 and were allowed to explore all three empty chambers for 10 min. After this period of  
240 habituation, mice were gently guided back to the center chamber of the apparatus and the  
241 sliding doors to access the remaining two chambers were closed. In the second part of the test,

242 an unfamiliar mouse (stranger 1) was placed into one of the two remaining side chambers,  
243 enclosed in a wire cage to ensure that only the test mouse could initiate social interaction. An  
244 empty wire cage, identical to the wire cage housing stranger 1, was placed in the  
245 corresponding spot on the other side chamber. The side doors were then opened  
246 simultaneously to allow the test mouse to explore the three-chamber apparatus for 10 min to  
247 assess sociability. At the end of the 10 min period the test mouse was gently guided to the  
248 central chamber and sliding doors were closed. In the final part of the test, a new unfamiliar  
249 mouse (stranger 2) was placed in the previously empty wire cage, and the test mouse could  
250 explore the three chambers for an additional 10 min to assess preference for social novelty.  
251 Stranger mice consisted of age- and sex-matched C57BL/6J mice that were group-housed (4  
252 per cage) and were used in a counterbalanced way. The empty wire cages were alternated  
253 between side chambers for different test mice. Stranger 1 and stranger 2 mice always came  
254 from different home cages. Mice were tested in the morning during the light cycle. Time spent  
255 directly sniffing, defined as the time the test mouse spent in direct nose contact with wire  
256 cages, time spent in each chamber, and the number of transitions into the chambers, were  
257 scored manually. Data was scored in a blind to genotype manner, and if possible by a third  
258 party, using a stopwatch. Statistical analysis included mixed ANOVA with a Tukey's *post*  
259 *hoc* test for multiple comparisons.

260

261 **Self-grooming test.** The setup consisted of a new Plexiglas cage equal in size to the home  
262 cage, containing approximately 1 cm of bedding material but no nesting material. A camera  
263 was placed vertically in front of the cage for recording. *Fmr1*<sup>-y</sup> and WT mice (3 month old  
264 males) were placed in a new Plexiglas cage and allowed to explore for 20 minutes. The first  
265 10 minutes of the experiment were considered as the habituation phase, followed by the final  
266 10 minutes which were used to acquire self-grooming data. Total time spent grooming and the

267 total number of grooming bouts was used to analyze grooming behavior. Data was manually  
268 scored in a blind to genotype manner, and if possible by a third party, using a stopwatch. All  
269 measures were analyzed with a two-way ANOVA with Tuckey's *post hoc* test.

270

271 **Audiogenic seizures.** Mice (male, P21-P24) were chronically injected for 10 days with  
272 metformin (200 mg/kg) or vehicle prior to experimentation. Mice were individually  
273 habituated in an isolated, sound insulated behavioral chamber made of transparent plastic (28  
274 x 17 x 16 cm outside dimensions) for 2 min and were subjected to a 130 dB acoustic stimulus  
275 using a personal alarm (Vigilant) for 2 min, where the incidence of wild running, tonic-clonic  
276 seizures, and status epilepticus were recorded.

277

278 **Open-field exploration.** Animals (male, 8-12 weeks old) were first habituated to the dimly lit  
279 experimental room (~15 lux) for 30 min and then individually placed in an illuminated clear  
280 Plexiglas chamber (40 x 40 x 40 cm, ~1200 lux) with a white floor. Animals were allowed to  
281 explore freely for 10 min following an initial 1 min habituation phase. Total path length, as a  
282 measure of hyperactive behavior, was calculated using ANY-maze.

283

284 **Light-dark transition test.** The test apparatus was composed of two adjacent chambers  
285 connected by a small opening: a dark enclosed chamber made of black Plexiglas (20 x 40 x 40  
286 cm) and a chamber with three clear Plexiglas walls with an open top. Mice (male, 8-12 weeks  
287 old) were placed into the "light" side (~390 lux) and allowed to explore freely for 10 min. An  
288 entry was defined as the mouse placing all 4 feet into each zone.

289

290 **Morris water maze and reversal learning.** Chronic metformin (200 mg/kg) or vehicle  
291 (saline) administration started 5 days prior to training and lasted throughout the whole course

292 of testing, for a total of 10 days. The circular water maze pool was 100 cm in diameter. The  
293 water was maintained at 22-23°C and made opaque by addition of white tempera. The  
294 platform was 10 cm in diameter. Mice (male, 8-12 weeks old) were handled daily for 3 days  
295 before the start of the experiment. During the experiment, mice were trained three times per  
296 day with an inter-trial interval of 30 min over five consecutive days (Day 1-5). Each trial was  
297 a maximum of 120 s, or until the mouse found the platform. If the mouse did not find the  
298 platform in the assigned time, it was guided to the platform and stayed there for 10 s before  
299 being returned to the home cage. For the probe test (Day 6), the platform was removed and  
300 each mouse was allowed to swim for 30 s. For the reversal learning paradigm, in which the  
301 hidden platform was relocated to the opposite quadrant (Day 6-7), mice received the same  
302 training procedure as described before. The platform was removed for the probe test of the  
303 reversal learning (Day 8) to assess spatial retention. The experiment was recorded with a  
304 video tracking system (HVS Image) whereby latency to reach the platform during acquisition  
305 and time spent in target quadrant during the probe trials was determined.

306

307 **Contextual fear conditioning.** During acquisition (5 min), two foot shocks of 0.7 mA for 1 s  
308 separated by 60 s were administered after an initial 2-min period of context exploration.  
309 Twenty-four hours after training, mice (male, 8-12 weeks old) were tested for contextual fear  
310 memory in the same context for 5 min, as assessed by the percentage of total time spent  
311 freezing in the conditioning context. Behavioral scoring was carried out for a 5-min period, in  
312 5-s intervals, assigning animals as either ‘freezing’ or ‘not freezing’. Freezing (%) indicates  
313 the number of intervals where freezing was observed, divided by the total number of 5 s  
314 intervals.

315

316 **Novel object recognition.** On day one, mice (male, 8-12 weeks old) were first habituated for  
317 15 min in a square testing arena (40 x 40 cm) followed by 15 min in an opaque box before  
318 being returned to their home cages. On day two and three, mice were put back in the arena for  
319 15 min and presented with two identical objects (familiar) within specific areas (counter-  
320 balanced locations of objects). Mice were allowed to freely explore the arena and objects,  
321 followed by 15 min in an opaque box and then returned to their home cages. On day four, one  
322 of the objects (used for days two and three) was replaced with a third object (novel object)  
323 and the mice were allowed to explore the environment for 15 min. Time spent exploring each  
324 object was recorded. Object exploration was defined as the time spent interacting with an  
325 object, when the mouse was sniffing and touching the object. Total exploration time was  
326 quantified as the time interacting with both objects. To assess preferential attention to an  
327 object, a discrimination index was calculated  $(t_{\text{novel}} - t_{\text{familiar}})/(t_{\text{novel}} + t_{\text{familiar}})$ . A positive index  
328 represents a preference for the novel object.

329

330 **Western blot and antibodies.** The brain tissue (3 month old males) was homogenized in  
331 RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium  
332 deoxycholate, 5 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 10 mM NaF, 1 mM  $\beta$ -  
333 glycerophosphate, 1 mM sodium orthovanadate) containing protease inhibitors (Roche).  
334 Protein extracts were heat denatured and resolved by SDS-PAGE or gradient precast  
335 (ThermoFisher Scientific). Following electrophoresis, proteins were transferred to  
336 nitrocellulose membranes and western blotting was performed. Membranes were stripped in  
337 25 mM glycine-HCl pH 2.0, 1% SDS for 30 min at room temperature, followed by washing in  
338 TBS-T before re-probing. Immunoreactivity was detected by enhanced chemiluminescence  
339 (plus-ECL; Perkin Elmer Inc.) after exposure to an X-Ray film (Denville Scientific Inc.).



340 Quantification of immunoblots was performed using ImageQuant 5.2. Values were  
341 normalized against GAPDH.

342 The following antibodies were used: eIF4E (610270, BD Transduction Laboratories);  
343 phospho-eIF4E (NB-100-79938, Novus Biologicals); ERK (sc-93, Santa Cruz); phospho-  
344 ERK (4370, Cell Signaling); MEK1/2 (4694, Cell Signaling); phospho-MEK1/2 (9154, Cell  
345 Signaling); FMRP (4317, Cell Signaling); MMP-9 (TP221, Torrey Pines); AMPK (2532, Cell  
346 Signaling); phospho-AMPK (2535, Cell Signaling); ACC1 (4190, Cell Signaling); phospho-  
347 ACC1 (11818, Cell Signaling); S6 (2217, Cell Signaling); phospho-S6 (2215, Cell Signaling);  
348 TSC2 (4308, Cell Signaling); phospho-TSC2 (1387, Cell Signaling); Raptor (2280, Cell  
349 Signaling); phospho-Raptor (2083, Cell Signaling); c-Raf (53745, Cell Signaling); b-Raf  
350 (ab33899, Abcam); phospho-b-Raf S729 (ab124794, Abcam); phospho-b-Raf S602 (PA5-  
351 38412, Thermo Fisher Scientific); Synapsin (5297, Cell Signaling); eEF2 (2332, Cell  
352 Signaling); MAP2 (ab5392, Abcam); PUM2 (A300-202A, Bethyl Laboratories); GAPDH (sc-  
353 32233, Santa Cruz);  $\beta$ -actin (A5441, Sigma); secondary anti-mouse and anti-rabbit (GE  
354 Healthcare). GAPDH (sc-32233, Santa Cruz); secondary anti-mouse and anti-rabbit (GE  
355 Healthcare). For statistical analysis of western blots results we used two-way ANOVA with  
356 Tukey's *post hoc* test, and one-way ANOVA with Tukey's *post hoc* test (p-AMPK in the  
357 hippocampus, single injection metformin experiment).

358

359 **LTD recordings.** For analysis of hippocampal LTD, male 31- to 34-day-old wildtype or  
360  $Fmr1^{-/y}$ , treated with either saline or metformin (as described above) were used. After  
361 obtaining hippocampal slices (400  $\mu$ m thickness), CA1 and CA3 hippocampal regions were  
362 isolated by a surgical excision and incubated for 2 h at 32°C in oxygenated artificial cerebral  
363 spinal fluid for recovery (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 2 mM  
364  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 26 mM  $\text{NaHCO}_3$ , and 10 mM glucose). Later, slices were placed in a

365 recording chamber at 27–28°C and perfused with ACSF for an additional 30 min. A glass  
366 electrodes (2–3 MΩ) was filled with ACSF and gently placed on CA1 stratum radiatum to  
367 record field EPSPs (fEPSPs), evoked by stimulation of Schaffer collaterals. The stimulating  
368 concentric bipolar tungsten electrode was placed in the mid-stratum radiatum proximal to the  
369 CA3 region to deliver 0.1 ms pulses at 0.033 Hz. The intensity was adjusted to evoke fEPSPs  
370 with 60% maximal amplitude. mGluR-LTD was induced by perfusing a group I mGluR  
371 agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG; 50 μM; Tocris Bioscience) for 10 min in  
372 ACSF. fEPSPs were recorded for a total of 60 min after induction onset. Slope measurements  
373 were performed on digitized analog recordings using the Clampfit analyze function, between  
374 10% and 90% of maximal fEPSP amplitude during an epoch defined by constant cursor  
375 placements. This setting excluded fibre volley and population spikes. Data was then analyzed  
376 using two-way ANOVA with Tukey's *post hoc* test.

377

378 **Miniature EPSC recordings.** Organotypic hippocampal slices were prepared from WT and  
379 *Fmr1*<sup>-y</sup> mice (postnatal day 4-6). The brain was removed and dissected in Hanks' balanced  
380 salt solution (Invitrogen)-based medium. Corticohippocampal slices (400 μm thick) were  
381 obtained with a McIlwain tissue chopper (Campden Instruments). Slices were placed on  
382 Millicell culture plate inserts (Millipore) and incubated in OptiMem (Invitrogen)-based  
383 medium in a humidified atmosphere of 5% CO<sub>2</sub> and 95% O<sub>2</sub> at 37°C.

384 Experiments were performed after 14-20 days in culture. Cultures were treated with  
385 metformin (50 μM) or vehicle (Optimem media) for 4-5 days before electrophysiology  
386 experiments which were performed blinded to treatment. Whole-cell recordings were obtained  
387 from CA1 pyramidal neurons using borosilicate pipettes (3–6 MΩ) filled with intracellular  
388 solution containing (in mM) 132 CsMeSO<sub>3</sub>, 8 CsCl, 0.6 EGTA, 10 diNa-phosphocreatine, 10  
389 HEPES, 4 ATP-Mg<sup>2+</sup>, 0.4 GTP-Na (pH 7.25-7.30 with CsOH, 275-280 mOsmol).

390 Spontaneous miniature EPSCs (mEPSCs) were recorded in the presence of TTX (5 nM;  
391 Abcam) in ACSF containing (in mM) 124 NaCl, 2.5 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>,  
392 2 MgSO<sub>4</sub>, 10 D-Glucose, (pH 7.37-7.41 with NaCl, 295-305 mOsm). Recordings were  
393 obtained using a Multiclamp 700 A amplifier and a 1440 A Digidata acquisition board  
394 (Molecular Devices). Signals were low-pass-filtered at 2 kHz, digitized at 20 kHz and stored  
395 on a PC. mEPSCs were recorded in whole cell voltage-clamp at a holding potential of -70 mV  
396 and identification of mEPSCs was confirmed by application of CNQX (10 μM). Access  
397 resistance was routinely monitored and recordings were only included if <30 MΩ and with  
398 variation <25% over the recording period. For analysis, mEPSC traces were filtered at 2.8  
399 kHz (Bessel filter) using pClamp10 software (Molecular Devices) and miniature events were  
400 analyzed using MiniAnalysis (Synaptosoft). Two-way ANOVA with Tukey's *post hoc* test  
401 was used to assess statistical significance.

402

403 **Analysis of neuronal morphology by Golgi-Cox Staining.** Four male mice per genotype  
404 and treatment (3 months old) were used for morphological analysis. Rapid GolgiKit (FD  
405 NeuroTechnologies) was used for the staining procedure according to the manufacturer's  
406 instructions. Briefly, whole brains were isolated from each animal, rinsed once in Milli-Q  
407 water and quickly immersed into impregnation solution (A+B), stored at room temperature in  
408 the dark for three weeks. 120 μm sections were cut, processed, and mounted following the  
409 protocol provided with the kit. Hippocampal sections were imaged on a confocal microscope  
410 (LSM710, Zeiss). Apical dendrites of five pyramidal neurons from the hippocampal CA1 area  
411 per animal were analyzed. To measure spine density on apical shaft dendrites, the number of  
412 spines on each successive 25 μm segment was counted starting at the soma and continuing to  
413 the end of the dendrite. Densities for each segment and for each neuron were pooled to get an  
414 average spine density per animal; the difference between genotypes was analyzed by two-way

415 ANOVA with Tukey's *post hoc* test. For each neuron, the spine morphology was determined  
416 by the first 10 spines in every 25  $\mu\text{m}$  bin along the apical shaft. Spines were assigned one of  
417 the five morphological categories based on published methods<sup>24-26</sup>; A:Thin, B:Stubby,  
418 C:Mushroom, D:Filopodia, E:Branched. Chi-Square analysis was used to compare the  
419 distribution of spines in these categories between genotypes. For statistical analysis, we used  
420 two-way ANOVA with Tukey's *post hoc* test.

421

422 **Measurement of *de novo* protein synthesis.** To assess whether metformin corrects increased  
423 translation in *Fmr1*<sup>-y</sup> mice, we measured *de novo* protein synthesis in hippocampal slices  
424 using the SUNSET puromycin incorporation assay<sup>24,27</sup>. Transverse hippocampal slices (400  
425  $\mu\text{m}$ ) were prepared from 5-6 week old mice and allowed to recover for at least 3 h. Puromycin  
426 labeling was performed as described<sup>24,27,28</sup>. Briefly, the slices were incubated with puromycin  
427 (Sigma, 5  $\mu\text{g}/\text{ml}$  in ACSF) for 45 min and then processed for western blotting, as described  
428 before, using an anti-puromycin antibody. Slices processed in parallel but not incubated with  
429 puromycin served as an unlabeled control. Protein synthesis was determined by measuring  
430 total lane signal from 15-250 kDa and subtracting unlabeled protein control. Signals were  
431 quantified using ImageJ, normalized to  $\beta$ -tubulin and presented as percentage change relative  
432 to control. For statistical analysis of western blots results, we used two-way ANOVA with  
433 Tukey's *post hoc* test.

434

435 **Metformin Bioanalysis, LC-MS/MS.** WT mice on C57BL/6J background (Charles River  
436 Laboratories, 8-10 weeks old males) were used for the study. Food and water were provided  
437 *ad libitum* and mice were kept on a 12-h light/dark cycle (7:00-19:00 light period). For  
438 pharmacokinetic study, the mice received a single dose of metformin (200 mg/kg, i.p.) and the  
439 plasma and brain tissues were collected at 0, 0.5, 1, 2, and 4 h after drug administration. For

440 the dose-response study, the mice were treated for 10 days with 25, 50, 100, or 200 mg/kg/day  
441 (i.p.), and the plasma and brain tissues were collected 24 h after the last injection. Brain tissue  
442 homogenate and plasma concentration of metformin was determined by protein precipitation  
443 and liquid chromatography with mass spectrometric detection (LC-MS/MS). Metformin  
444 powder (Sigma), was used to prepare a 1.00 mg/mL solution in DMSO adjusting for salt  
445 factor as applicable. Calibration spiking solutions were prepared at 10.0, 20.0, 50.0, 100, 200,  
446 500, 1000, 2000, 5000, 10000, 20000, 50000, and 100000, ng/mL in DMSO from the primary  
447 stock solution. Plasma and brain tissue samples were quickly collected and stored at -70°C.  
448 Brain samples, and blank tissues were homogenized with 3 parts distilled water per g of tissue  
449 for a final processing dilution factor of 4-fold. The resultant blank tissues were utilized for  
450 matrix calibration standards, which were prepared the same day of analysis, on ice at 0.5, 1.0,  
451 2.5, 5.0, 10.0, 25.0, 50.0, 100, 250, 500, 1000, 2500, and 5000 ng/mL; by spiking blank brain  
452 tissue homogenate and plasma matrices at 1:20 with appropriate metformin spiking solution.  
453 Subsequently, an aliquot of the matrix samples, matrix calibration standards, and matrix  
454 blanks were taken and protein precipitated by the addition Labetalol in 100% Acetonitrile  
455 (1:4). The resultant matrix samples, matrix calibration standards, and matrix blanks were  
456 vortexed for 1 min and centrifuged for 10 min at 3300 rpm at 4°C. Then 100 µL of the  
457 resultant supernatant was transferred into a clean 96-well plate and diluted with aqueous  
458 solution (1:1). All matrices; plasma and brain tissue, were processed independently and in  
459 discrete batches containing appropriate matrix study samples, matrix calibration standards,  
460 and matrix blanks respectively. The analysis for each discrete batch was performed on a  
461 LC-MS/MS system: AB Sciex QTRAP 6500, with a Shimadzu Nexera UPLC system utilizing  
462 a ZIC-HILIC 2.1 x 50 mm analytical column, 3.5 µm pore size. An injection volume of 1.5  
463 µL was utilized for all samples and standards, with a flow rate of 1.0 mL/min. The Mobile  
464 Phases consisted of the following: Mobile Phase A – 10 mM Ammonium Acetate in Water,

465 Mobile Phase B – 0.1% Formic Acid (v/v) in Acetonitrile. Mass Spectrometry data was  
466 generated with positive Electrospray Ionization (ESI+) using multiple reaction monitoring  
467 (MRM) of the following transitions: Metformin 130.324/60.100 Da and Labetalol (IS)  
468 329.200/311.200 Da. Subsequent least squares linear regression was performed on matrix  
469 calibration standards and the matrix sample concentrations were interpolated from the  
470 appropriate matrix curve. All dilution factors were accounted for in final sample data with  
471 concentration of metformin expressed in ng/mL and ng/g for plasma and brain tissue samples,  
472 respectively.

473

474 **Statistical analysis.** Experimenters were blinded to the genotype and treatment during testing  
475 and scoring. To decide the sample size in our behavioral, electrophysiological, imaging, and  
476 biochemical experiments, we followed the standard sample sizes used in similar experiments  
477 in each of the relevant fields in the literature. The sample sizes in our behavioral studies were  
478 based on Figure 5b in Mogil et al.<sup>26</sup>. All experimental n numbers are individual animals unless  
479 otherwise stated – technical replicates of some western blots were carried out. All data are  
480 presented as mean ± s.e.m. Statistical significance was set at 0.05. Statistical results, along  
481 with tests used (one-way ANOVA, two-way ANOVA, and mixed ANOVA), are summarized  
482 in **Supplementary Table 1**. SPSS (IBM), Statistica (Statsoft), Sigmaplot (Systat Software  
483 Inc.) and Graphpad Prism (Graphpad Software) were used for statistical analysis.  
484 **Supplementary Table 1** outlines the statistics used for each figure.

485

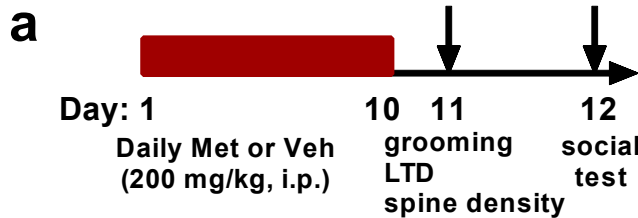
486 **Data-availability statements.** The data supporting the findings of this study are available  
487 from the corresponding author upon request.

488

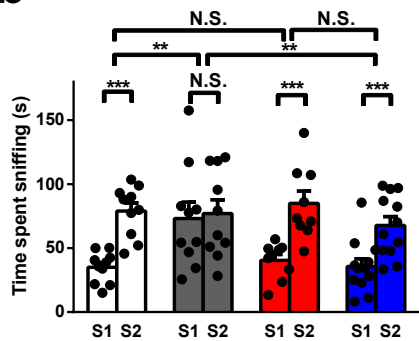
489 24 Gkogkas, C.G. et al. *Cell Rep* **9**, 1742-1755 (2014).

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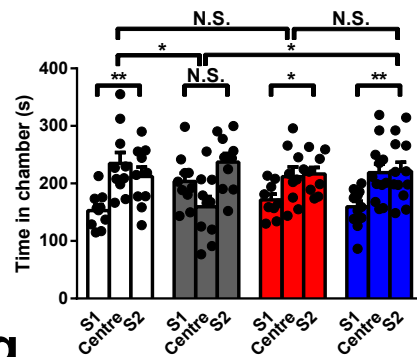
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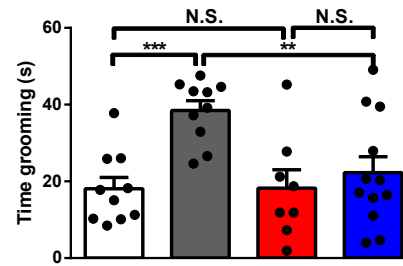
**b Preference for social novelty**



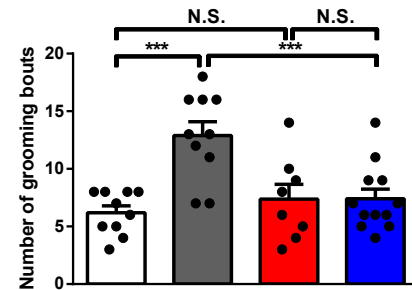
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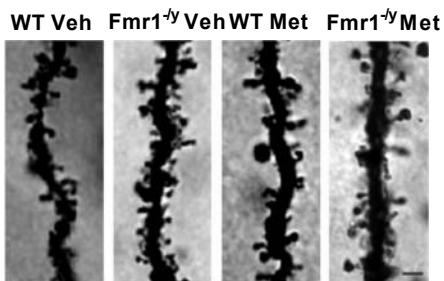
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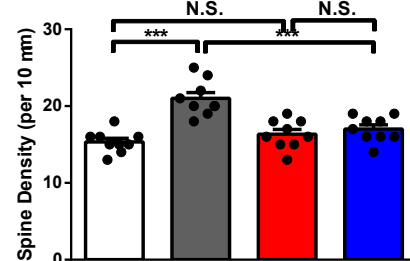
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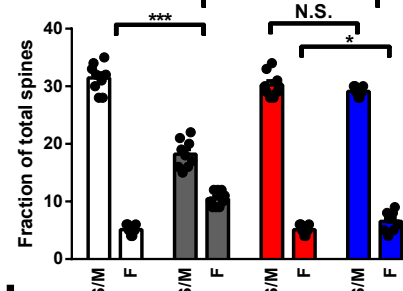
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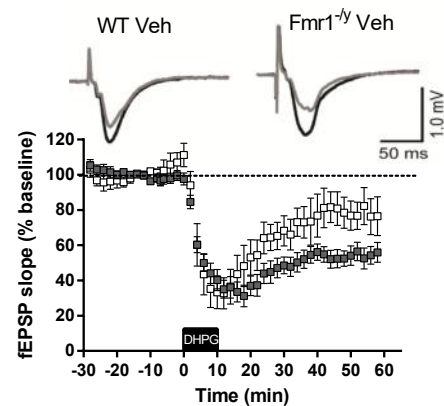
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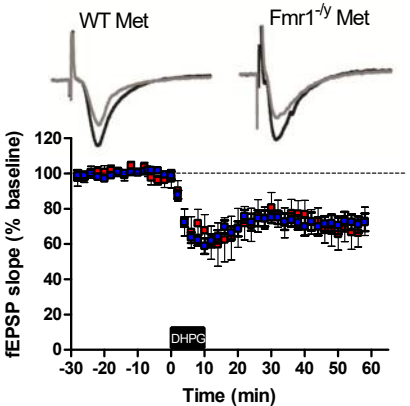
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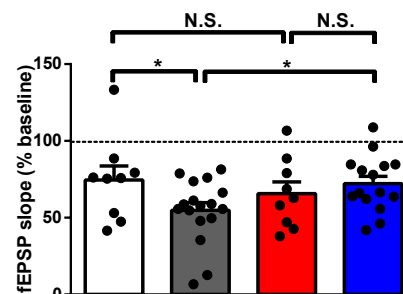
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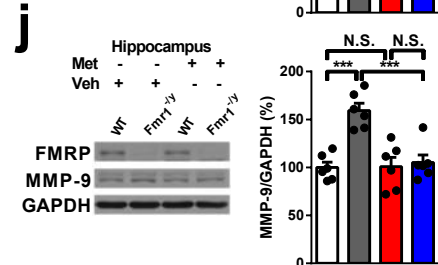
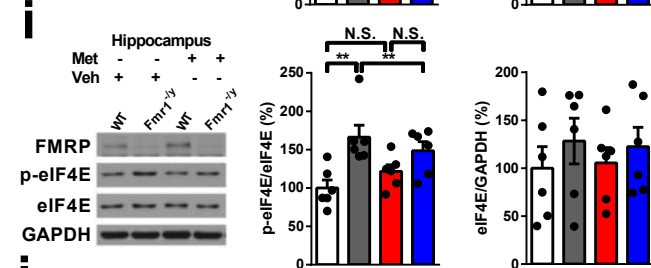
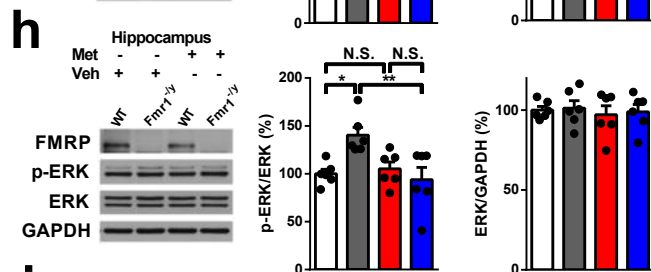
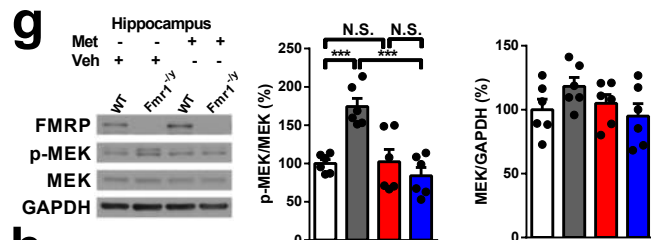
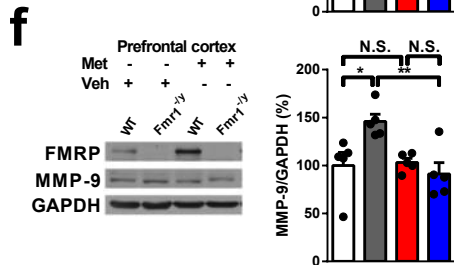
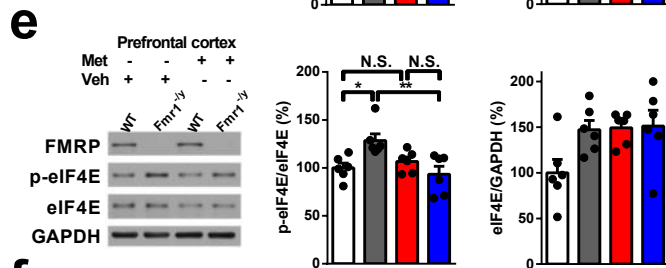
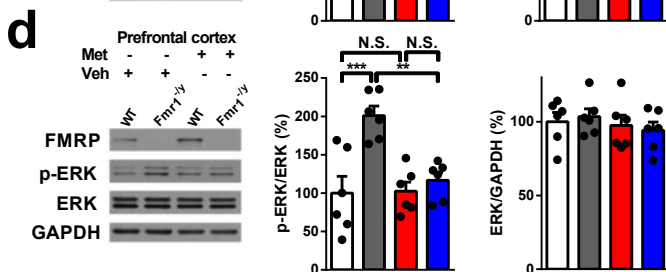
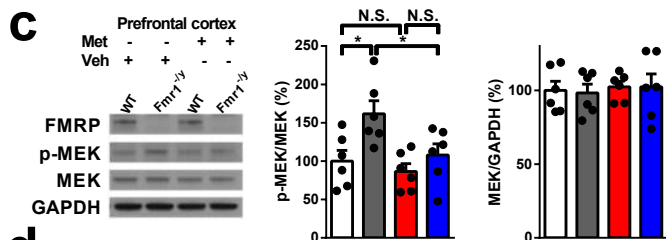
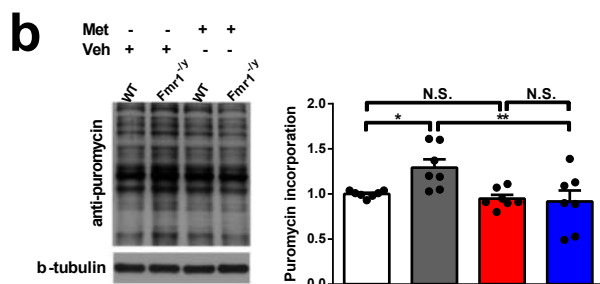
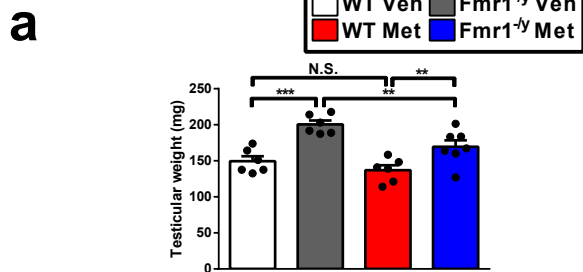
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**k**

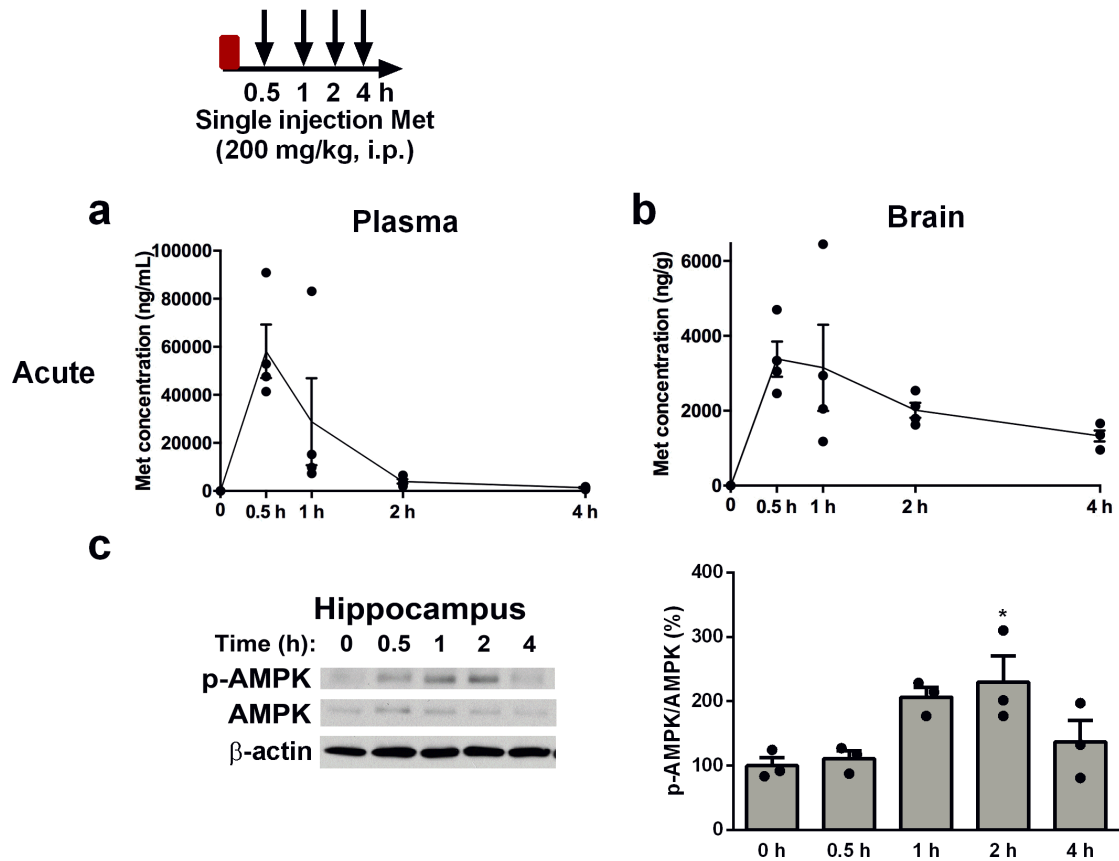




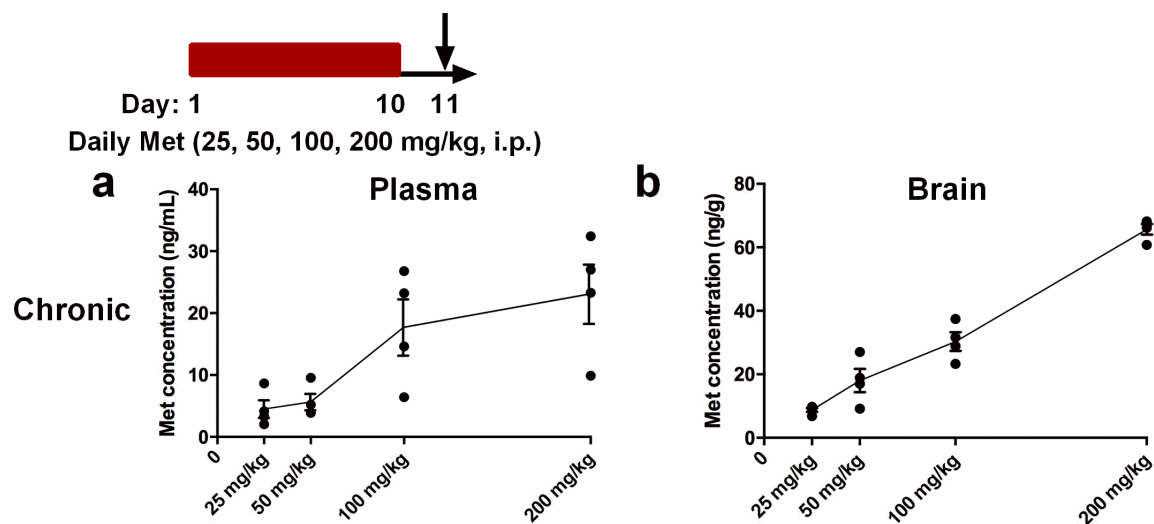


## SUPPLEMENTARY INFORMATION

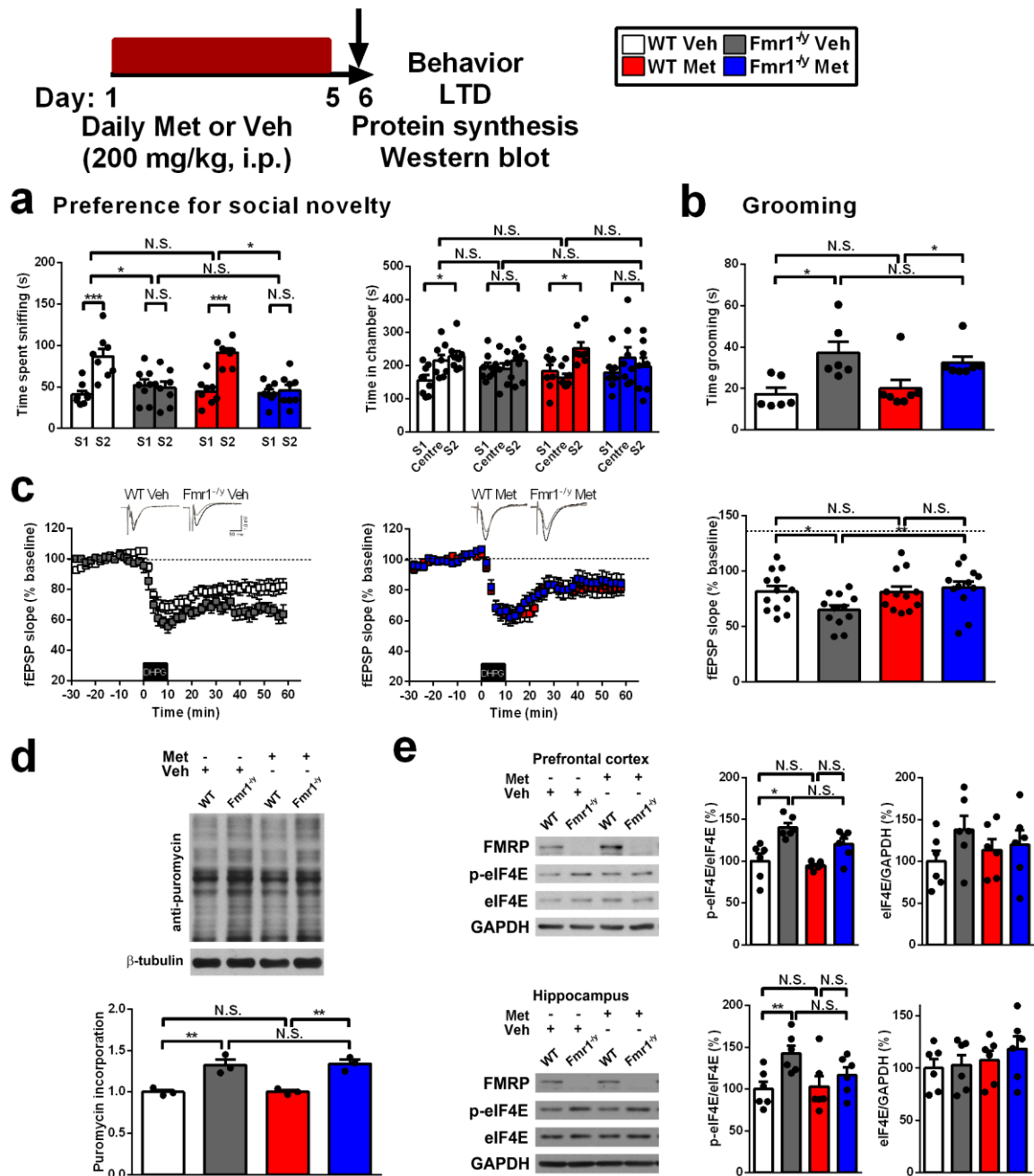
### SUPPLEMENTARY FIGURES



**Supplementary Figure 1** Pharmacokinetic study and AMPK activation *in vivo* after acute metformin (200 mg/kg) treatment. The concentrations of metformin were measured by LC-MS/MS in the plasma (**a**), and brain (**b**) at different time points (0, 0.5, 1, 2, and 4 h) after a single metformin injection (200 mg/kg, i.p.) (n = 4 in each group). (**c**) Representative immunoblots of hippocampal lysates from vehicle- and metformin-treated WT and *Fmr1*<sup>-/-</sup> mice probed for total and phosphorylated AMPK (n = 3 in each group).  $\beta$ -actin was used as loading control. For quantification, the phospho-protein signal was normalized first against total protein, and then presented relative to vehicle-treated WT. Values are shown as mean  $\pm$  s.e.m. \*P < 0.05, versus all other groups; calculated by one-way ANOVA with Tukey's *post hoc* test.

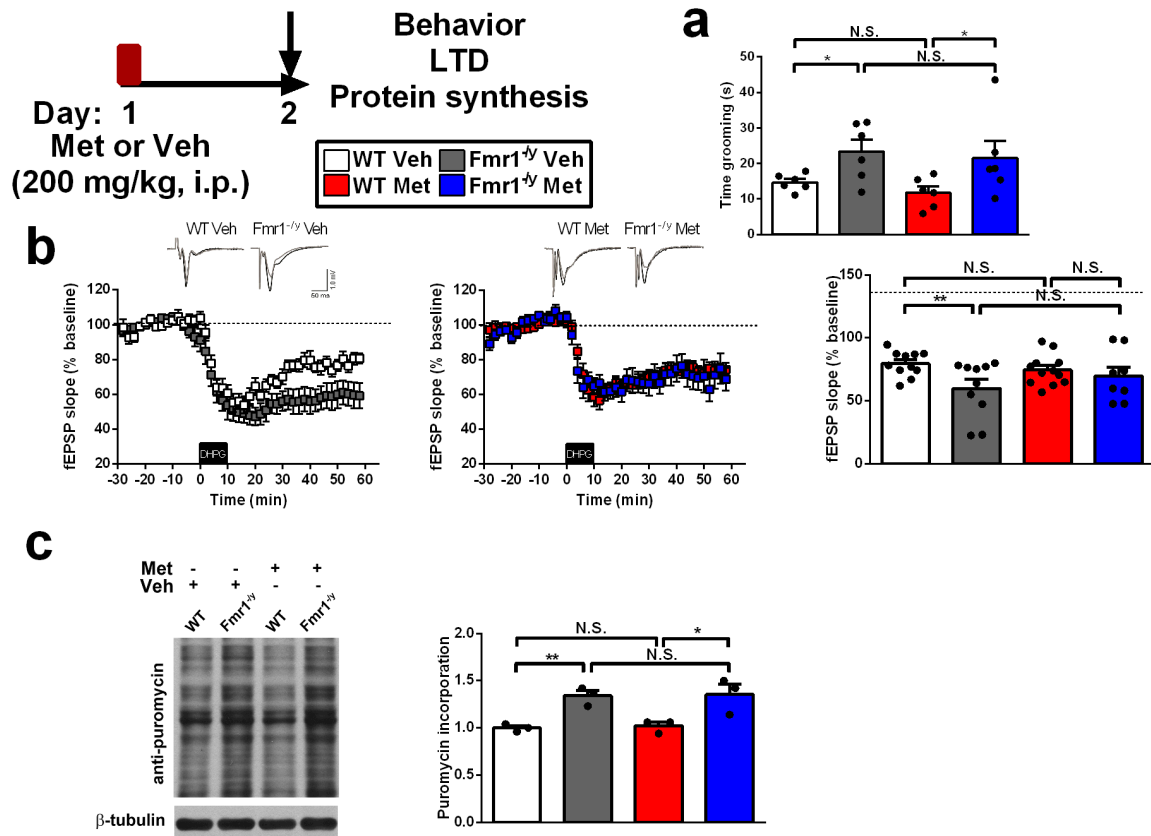


**Supplementary Figure 2** Metformin concentrations *in vivo* in plasma and brain after a 10-day chronic metformin treatment. The concentrations of metformin were measured by LC-MS/MS in the plasma (**a**), and brain (**b**) 24 h after last metformin injection (25, 50, 100, and 200 mg/kg, i.p.) (n = 4 in each group).

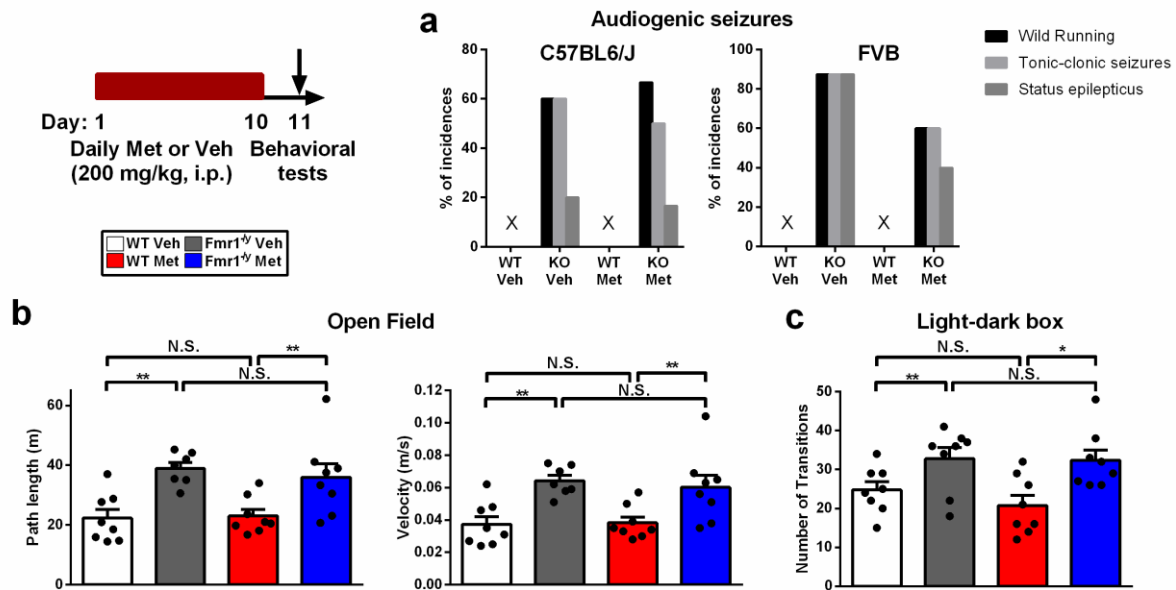


**Supplementary Figure 3** Five-day metformin treatment (200 mg/kg/day, i.p.) does not rescue impaired social and grooming behavior, general translation, and phosphorylated eIF4E in *Fmr1*<sup>-/-</sup> mice. **(a)** Preference for social novelty was assessed in the three-chamber social interaction test by measuring time spent with the novel social stimulus (stranger 2 (S2)) or the previously encountered mouse (S1) and time spent in each chamber; calculated by two-way mixed ANOVA with Tukey's *post hoc* test (n = 8 mice for each group). **(b)** Self-grooming test with total time spent grooming; calculated by two-way ANOVA with Tukey's *post hoc* test. Vehicle-treated WT (n = 6) and *Fmr1*<sup>-/-</sup> (n = 6) mice, and metformin-treated WT (n = 7) and *Fmr1*<sup>-/-</sup> (n

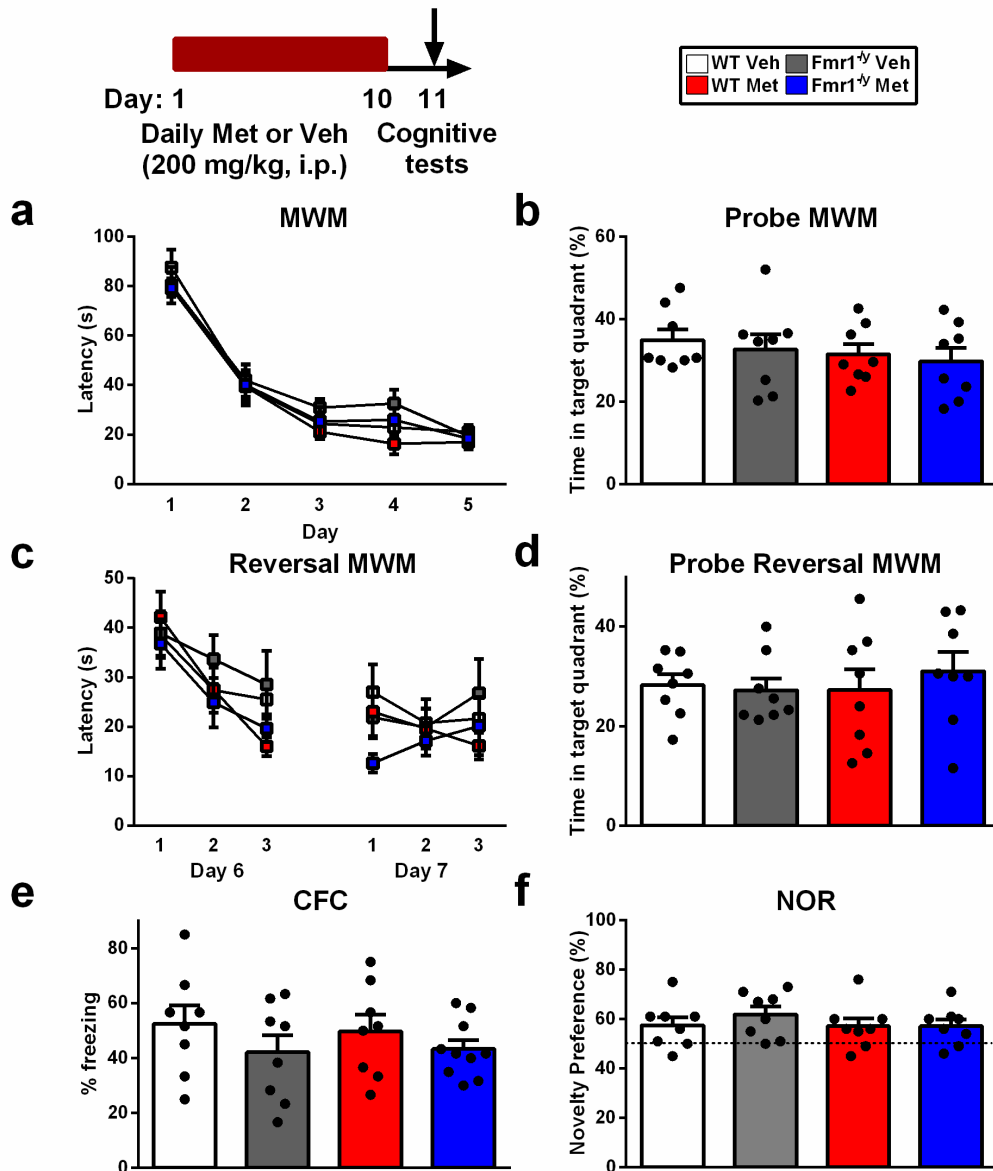
= 7) mice. (c) Time-plots of mGluR-LTD measured in CA1 in response to DHPG (50  $\mu$ M for 10 min) in slices prepared from vehicle-treated WT (n = 13) and *Fmr1*<sup>-/-</sup> (n = 12) mice, and metformin-treated WT (n = 12) and *Fmr1*<sup>-/-</sup> (n = 12) mice. Quantification (right) of mGluR-LTD during the last 10 min of recording. Exaggerated mGluR-LTD in metformin-treated *Fmr1*<sup>-/-</sup> mice was rescued. (d) Western blots of lysates from hippocampal slices incubated with puromycin to measure basal rates of protein synthesis and  $\beta$ -tubulin as a loading control. Puromycin incorporation is presented as percentage change relative to vehicle-treated WT slices (n = 3 in each group). (e) Representative immunoblots and blot quantification of total and phosphorylated eIF4E in prefrontal cortex and hippocampus from vehicle- and metformin-treated WT and *Fmr1*<sup>-/-</sup> mice (n = 6 in each group). GAPDH was used as a loading control. For quantification, the phospho-protein signal was normalized first against total protein, and then presented relative to vehicle-treated WT. All values are shown as mean  $\pm$  s.e.m. \*\*\*P < 0.001, \*\*P < 0.01, \*P < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.



**Supplementary Figure 4** One-day metformin treatment (200 mg/kg/day, i.p.) did not rescue increased grooming, exaggerated LTD, and increased general translation in *Fmr1*<sup>-/-</sup> mice. **(a)** Self-grooming test with total time spent grooming; calculated by two-way ANOVA with Tukey's *post hoc* test. *n* = 6 mice for each group. **(b)** Time-plots of mGluR-LTD measured in CA1 in response to DHPG (50  $\mu$ M for 10 min) in slices prepared from vehicle-treated WT (*n* = 11) and *Fmr1*<sup>-/-</sup> (*n* = 10) mice, and metformin-treated WT (*n* = 12) and *Fmr1*<sup>-/-</sup> (*n* = 8) mice. Quantification (right) of mGluR-LTD slope during the last 10 min of recording. **(c)** Western blots of lysates from hippocampal slices incubated with puromycin to measure basal rates of protein synthesis and  $\beta$ -tubulin as a loading control. Puromycin incorporation is presented as percentage change relative to vehicle-treated WT slices (*n* = 3 in each group). All values are shown as mean  $\pm$  s.e.m. \*\**P* < 0.01, \**P* < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.

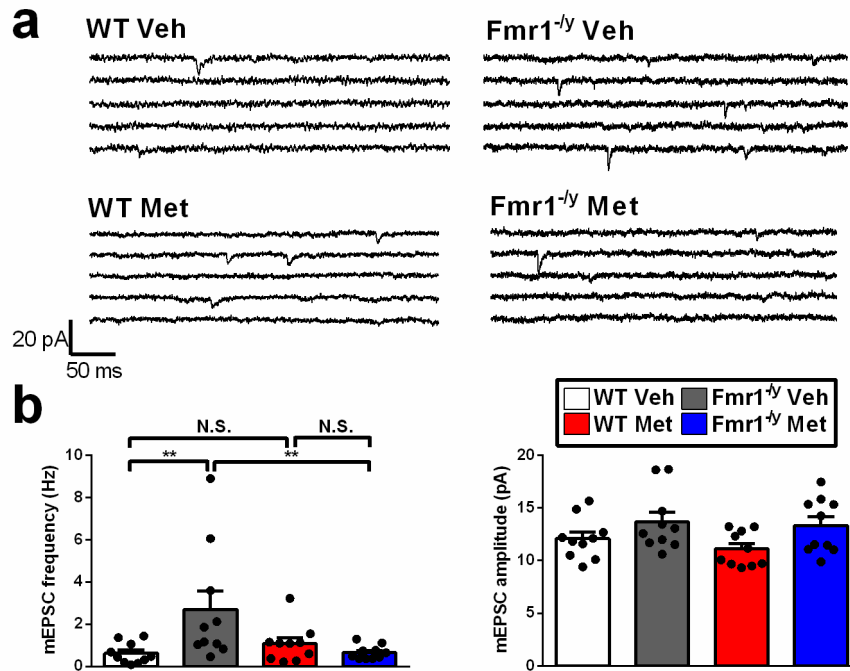


**Supplementary Figure 5** Effect of chronic metformin treatment (200 mg/kg/day, 10 days, i.p.) in *Fmr1*<sup>-y</sup> and WT mice on audiogenic seizures and hyperactivity. Audiogenic seizures (**a**) were tested on *Fmr1*<sup>-y</sup> and WT mice in a C57BL6/J (**a, left panel**) (vehicle-treated WT (n = 2) and *Fmr1*<sup>-y</sup> (n = 5) mice, and metformin-treated WT (n = 6) and *Fmr1*<sup>-y</sup> (n = 6) mice) and FVB background (**a, right panel**) (vehicle-treated WT (n = 7) and *Fmr1*<sup>-y</sup> (n = 8) mice, and metformin-treated WT (n = 7) and *Fmr1*<sup>-y</sup> (n = 10) mice), and were scored for wild running, tonic-clonic seizures and status epilepticus. Vehicle- and metformin-treated WT animals did not show any seizures. Metformin-treated *Fmr1*<sup>-y</sup> mice showed reduced occurrence of audiogenic seizures. Vehicle-treated *Fmr1*<sup>-y</sup> mice displayed increased activity in the open field test (**b**) for the path length (**b, left panel**) and velocity (**b, right panel**) (vehicle-treated WT (n = 8) and *Fmr1*<sup>-y</sup> (n = 7) mice, and metformin-treated WT (n = 8) and *Fmr1*<sup>-y</sup> (n = 8) mice), as well as an increased number of transitions in the light-dark box (**c**) compared to vehicle-treated WT mice (n = 8 in each group). Ten days metformin treatment did not rescue hyperactivity in *Fmr1*<sup>-y</sup> mice. Values are shown as mean ± s.e.m. \*\*P < 0.01, \*P < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.

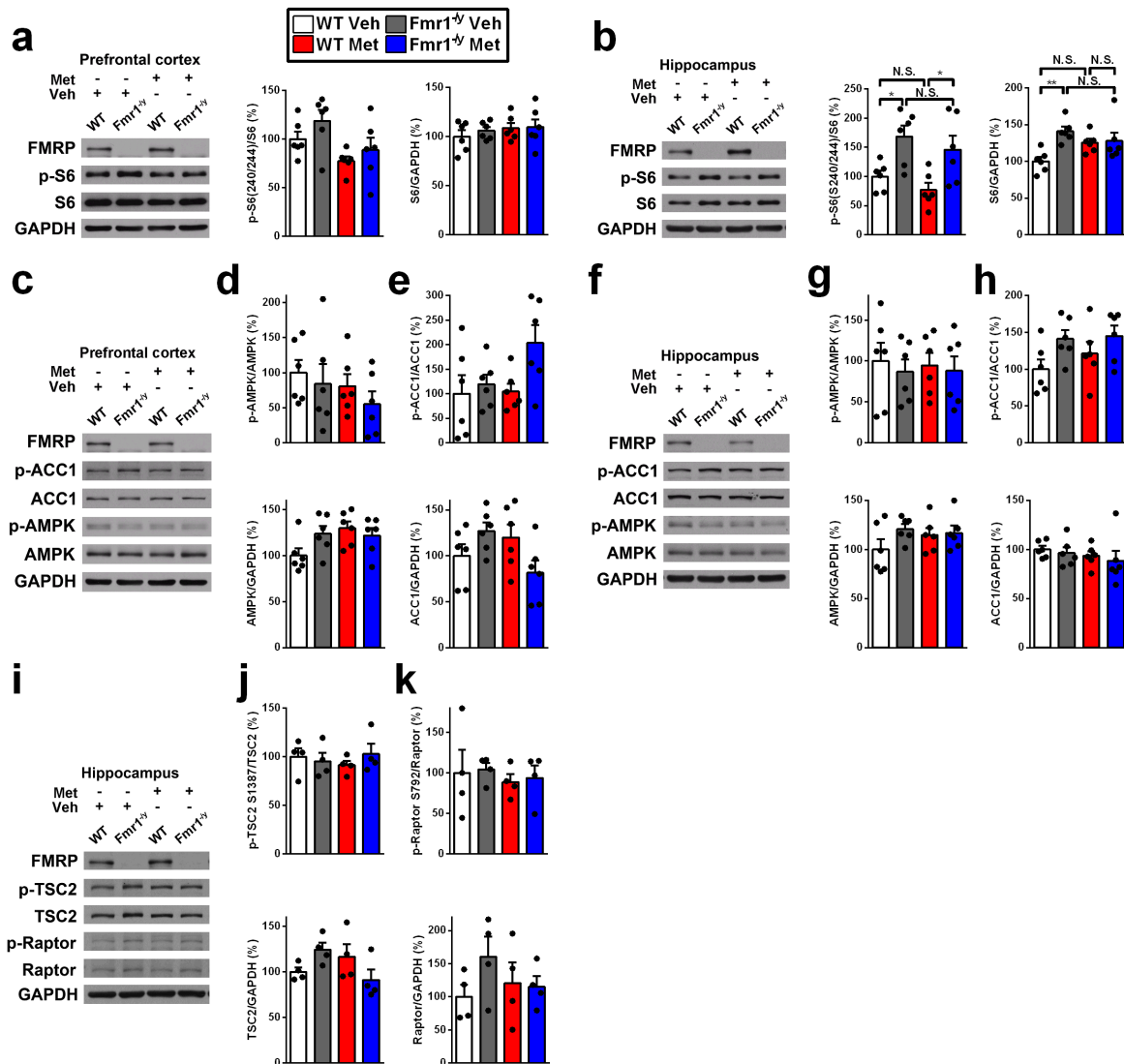


**Supplementary Figure 6** Three behavioral tasks to study cognition in 10-day metformin-treated (200 mg/kg, i.p.) *Fmr1*<sup>-/-</sup> and WT mice (C57BL6/J). Vehicle-treated *Fmr1*<sup>-/-</sup> mice did not show a significant cognitive impairment compared to vehicle-treated WT mice in spatial learning during the 5-day acquisition (**a**), probe trial (**b**), acquisition during the 2-day reversal learning (3 trials per day) (**c**), and probe trial of reversal learning (**d**) in the Morris water maze (MWM) (n = 8 in each group). In the contextual fear conditioning (CFC) task (**e**), no significant difference in % of freezing behavior was observed between metformin- and vehicle-treated *Fmr1*<sup>-/-</sup> and WT mice. Vehicle-treated WT (n = 8) and *Fmr1*<sup>-/-</sup> (n = 8) mice, and metformin-treated WT (n = 8) and *Fmr1*<sup>-/-</sup> (n = 10) mice. In the novel object recognition (NOR) task (**f**), no difference in % of preference for novelty was observed between all the tested groups (n = 8 in each group).



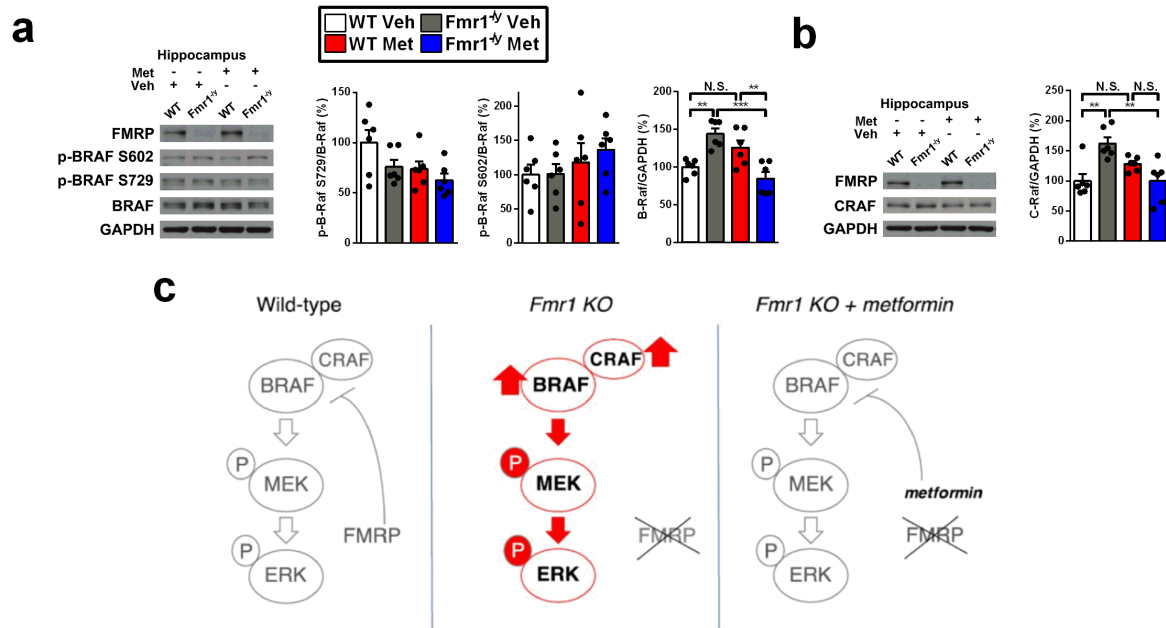


**Supplementary Figure 7** Metformin restores excitatory synaptic activity in *Fmr1*<sup>-/-</sup> mice. **(a)** Representative traces of mEPSCs from pyramidal cells in hippocampal slice cultures from WT and *Fmr1*<sup>-/-</sup> mice treated with vehicle or 50  $\mu$ M metformin for 4-5 days prior to recording. **(b)** Bar graphs showing that metformin treatment corrected the increase in mEPSC frequency in vehicle-treated *Fmr1*<sup>-/-</sup> neurons ( $2.71 \pm 0.87$  Hz) as compared to WT neurons ( $0.62 \pm 0.16$  Hz), with no effect on mEPSC amplitude. \*\* $P < 0.01$ ; N.S., not significant; two-way repeated measures ANOVA with Tukey's *post hoc* test;  $n = 10$  recordings per group. All values are shown as mean  $\pm$  s.e.m.

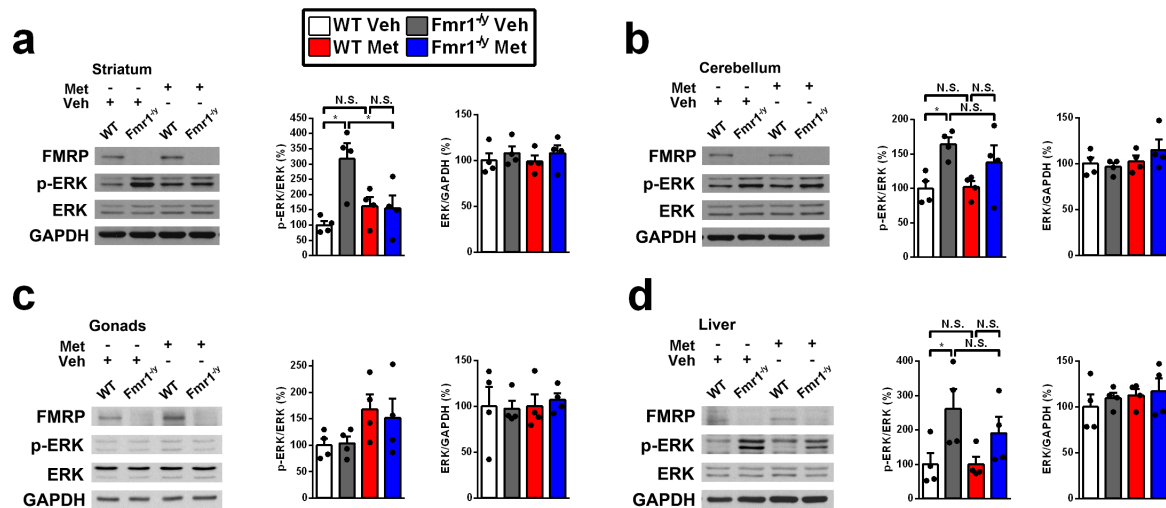


**Supplementary Figure 8** Chronic metformin treatment failed to reduce phosphorylation of S6 (S240/244), AMPK, ACC1, TSC2 and Raptor in *Fmr1<sup>-y</sup>* mice. Representative immunoblots and blot quantification of prefrontal cortex (**a**) and hippocampal (**b**) lysates from vehicle- and metformin-treated WT and *Fmr1<sup>-y</sup>* mice probed for total and phosphorylated S6 (S240/244) ( $n = 6$  in each group). Representative immunoblots of prefrontal cortex (**c**) and hippocampal (**f**) lysates from vehicle- and metformin-treated WT and *Fmr1<sup>-y</sup>* mice probed for total and phosphorylated AMPK, and ACC1, and quantification of total and phosphorylated levels of (**d**) AMPK and (**e**) ACC1 in the prefrontal cortex, and (**g**) AMPK, and (**h**) ACC1 in the hippocampus ( $n = 6$  in each group). (**i**) Representative immunoblots of hippocampal lysates from vehicle- and metformin-treated WT and *Fmr1<sup>-y</sup>* mice probed for total and phosphorylated TSC2 and Raptor, and quantification of total and phosphorylated levels of (**j**) TSC2 and (**k**) Raptor ( $n = 4$  in each group). GAPDH was used as a loading control. For quantification, the phospho-protein signal was normalized first against total protein, and then presented relative to

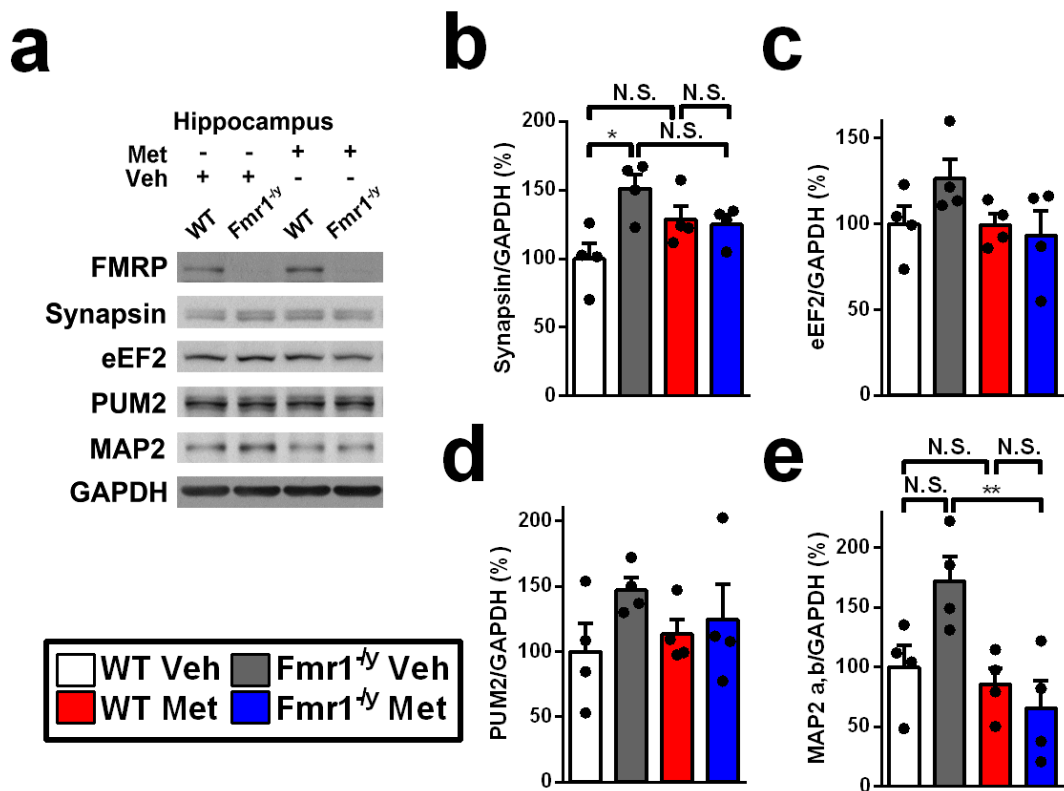
vehicle-treated WT. Values are shown as mean  $\pm$  s.e.m. \*\*P < 0.01, \*P < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.



**Supplementary Figure 9** Chronic metformin treatment reduced total B-Raf and C-Raf proteins in the hippocampus of *Fmr1<sup>-/-</sup>* mice. Representative immunoblots and blot quantification of total and phosphorylated B-Raf (**a**), and total C-Raf (**b**) from vehicle- and metformin-treated WT and *Fmr1<sup>-/-</sup>* mice ( $n = 6$  in each group). GAPDH was used as a loading control. For quantification, the phospho-protein signal was normalized first against total protein, and then presented relative to vehicle-treated WT. Values are shown as mean  $\pm$  s.e.m. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test. (c) Proposed action of chronic metformin treatment to reduce hyperactivated ERK signaling in the brain of *Fmr1<sup>-/-</sup>* mouse.

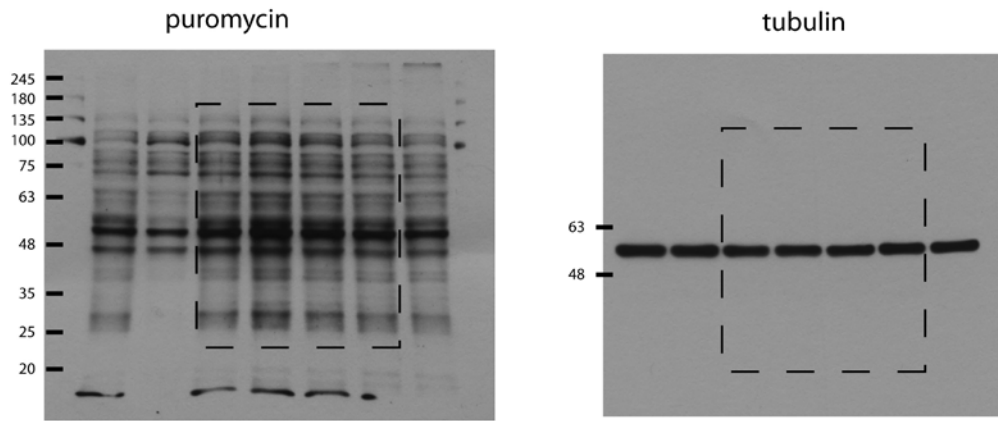


**Supplementary Figure 10** Chronic metformin treatment reduced phosphorylated ERK in the striatum, but not in cerebellum, gonads and liver of *Fmr1<sup>-/-</sup>* mice. Representative immunoblots and blot quantification of total and phosphorylated ERK in the striatum (a), cerebellum (b), gonads (c), and liver (d) from vehicle- and metformin-treated WT and *Fmr1<sup>-/-</sup>* mice (n = 4 in each group). GAPDH was used as a loading control. For quantification, the phospho-protein signal was normalized first against total protein, and then presented relative to vehicle-treated WT. Values are shown as mean  $\pm$  s.e.m. \*P < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.

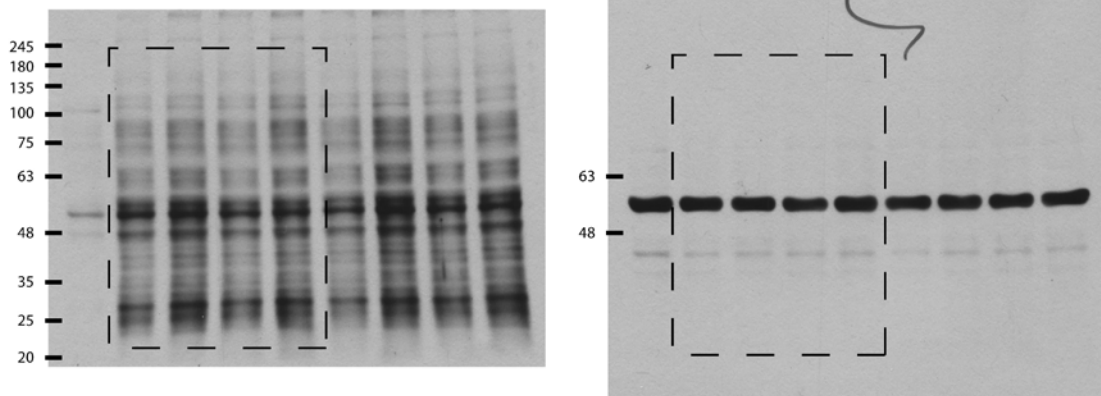


**Supplementary Figure 11** Chronic metformin treatment reduced levels of FMRP targets, synapsin and MAP2 a,b in the hippocampus of *Fmr1<sup>-/-</sup>* mice. Representative immunoblots (a) and blot quantification of synapsin (b), eEF2 (c), PUM2 (d) and MAP2 a,b (e) in the hippocampus from vehicle- and metformin-treated WT and *Fmr1<sup>-/-</sup>* mice (n = 4 in each group). GAPDH was used as a loading control. For quantification, the protein signal was normalized first against loading control, and then presented relative to vehicle-treated WT. Values are shown as mean  $\pm$  s.e.m. \*\*P < 0.01, \*P < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.

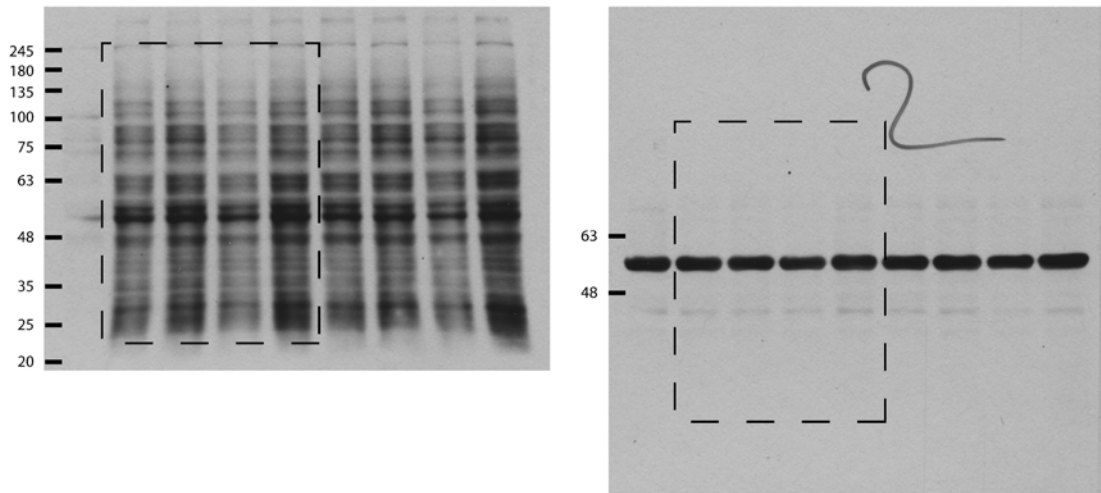
Fig. 2b



Supp. Fig. 3d



Supp. Fig. 4c



**Supplementary Figure 12** Original images of representative western blots of *de novo* protein synthesis

Fig. 2c

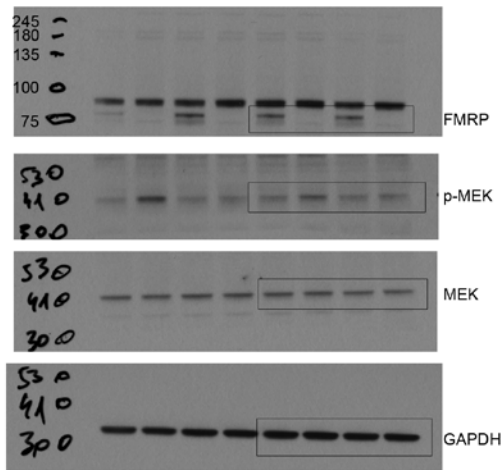


Fig. 2f

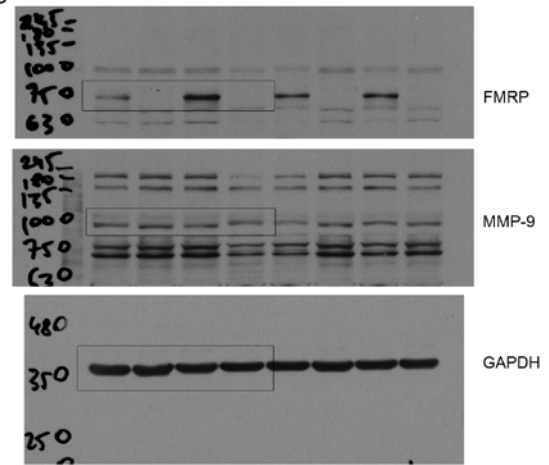


Fig. 2d

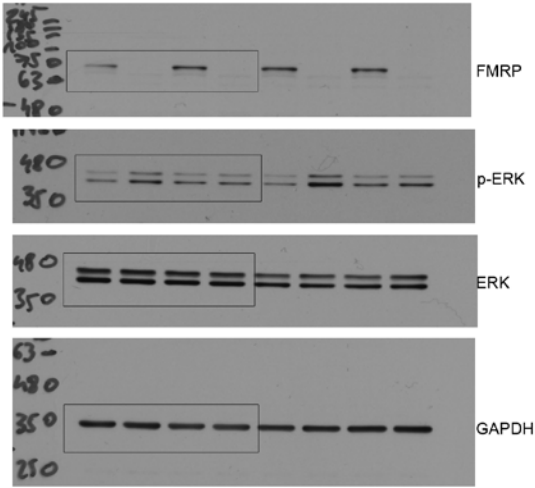


Fig. 2g

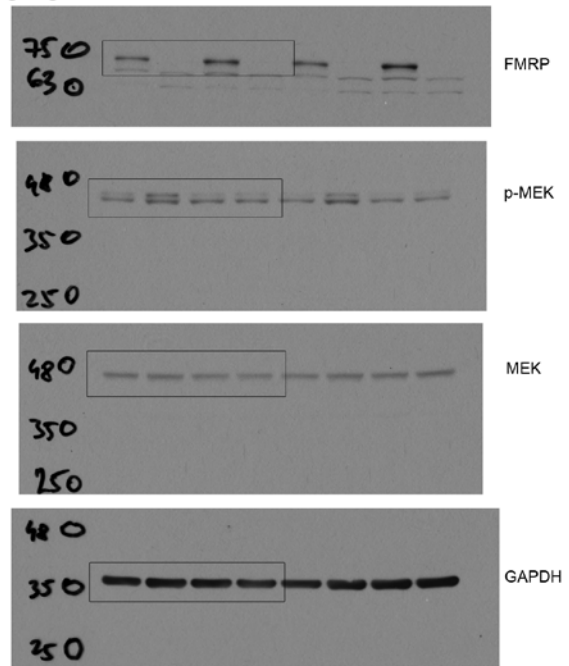


Fig. 2e

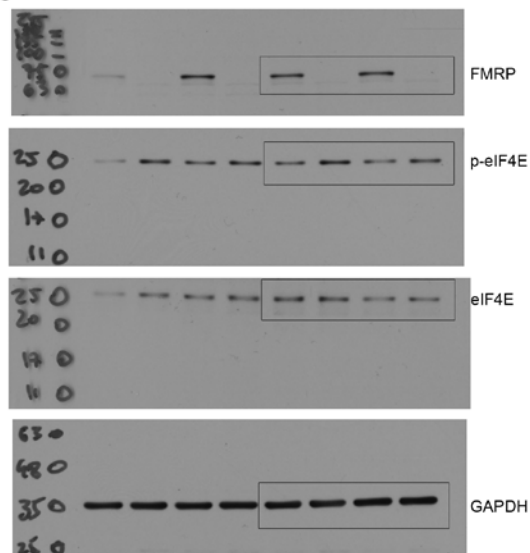
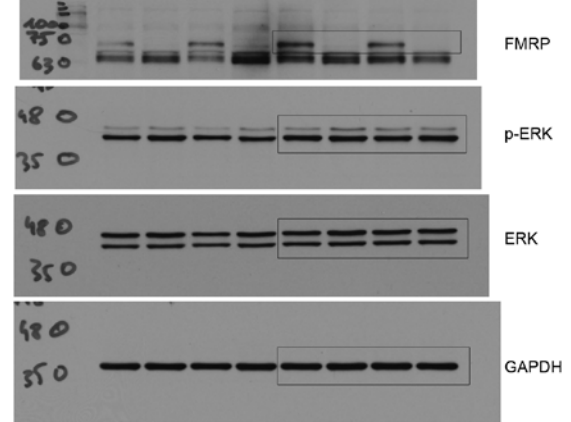


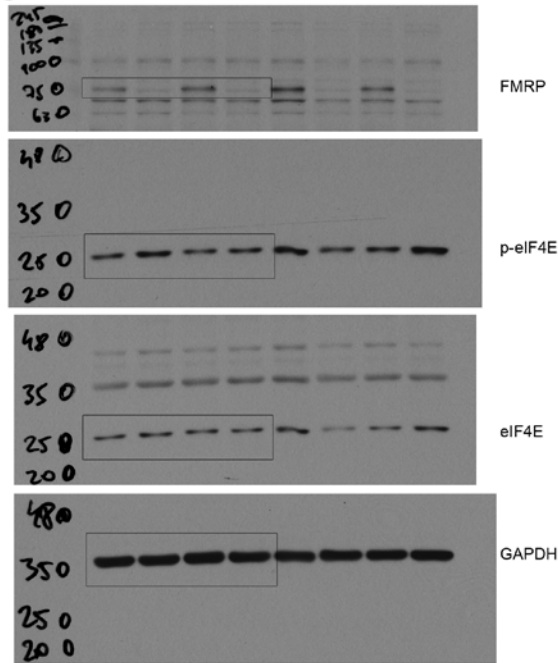
Fig. 2h



Supplementary Figure 13 Original images of representative western blots in Fig. 2



Fig. 2i



Supp. Fig. 3e

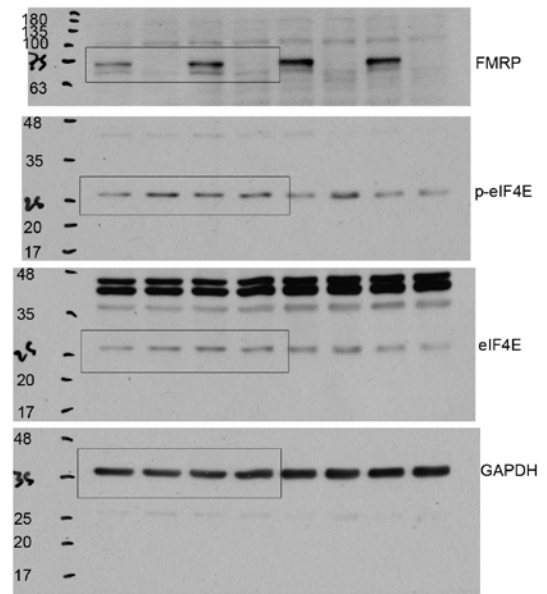
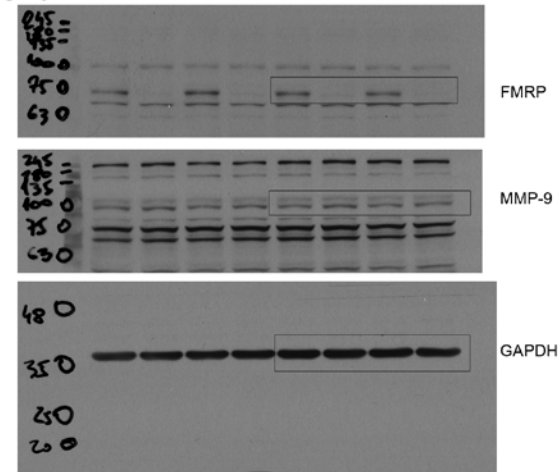
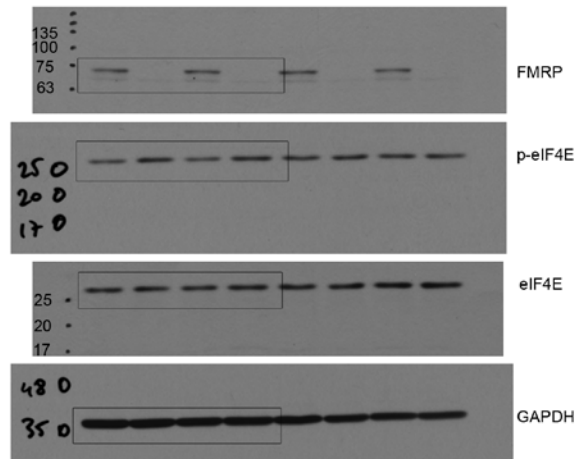


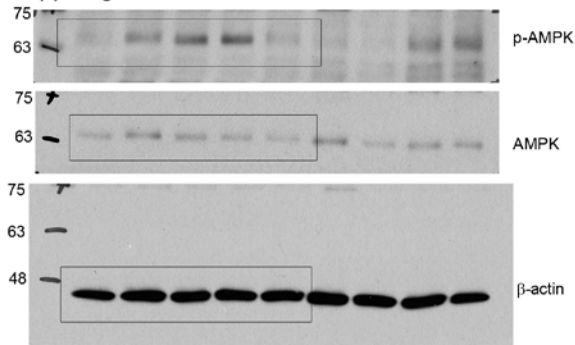
Fig. 2j



Supp. Fig 3e (lower panel)

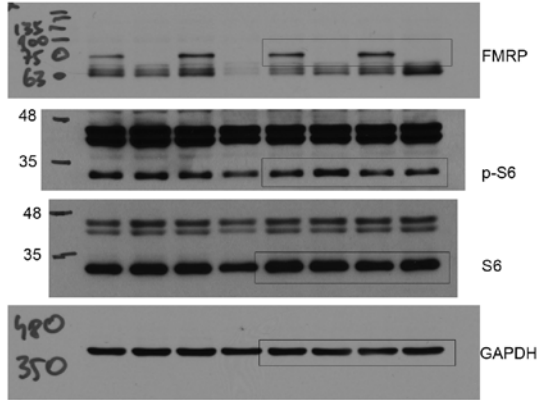


Supp. Fig 1c

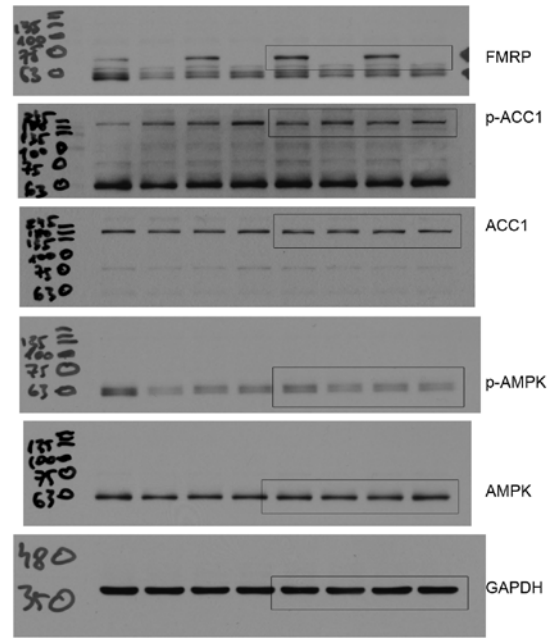


**Supplementary Figure 14** Original images of representative western blots in Fig. 2, and Supplementary Figs. 1 and 3

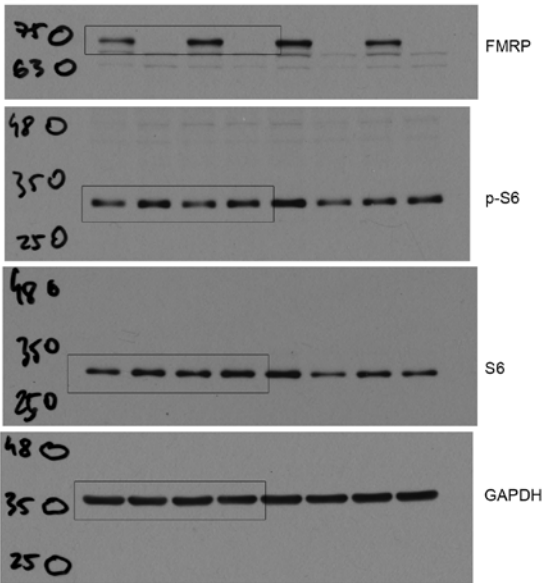
Supp. Fig. 8a



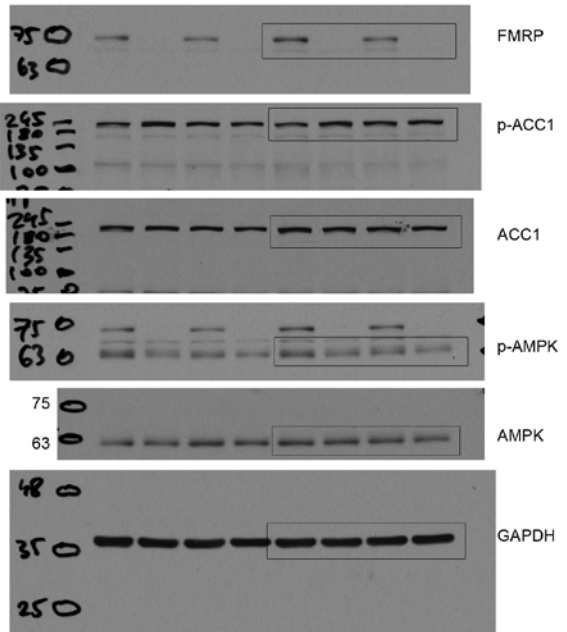
Supp. Fig 8c



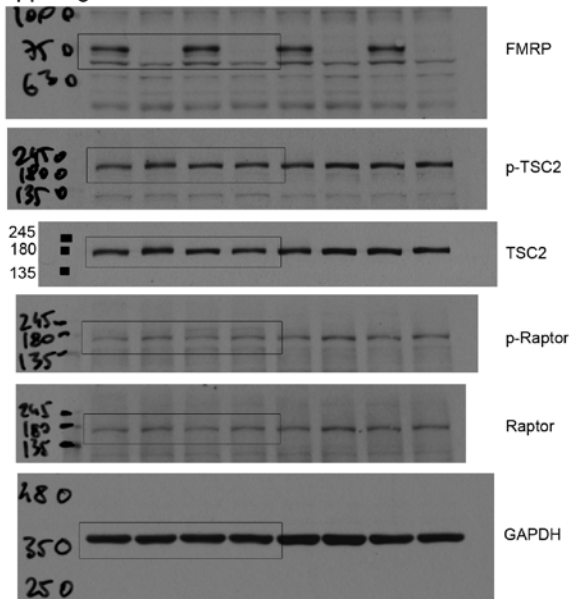
Supp. Fig 8b



Supp. Fig. 8f

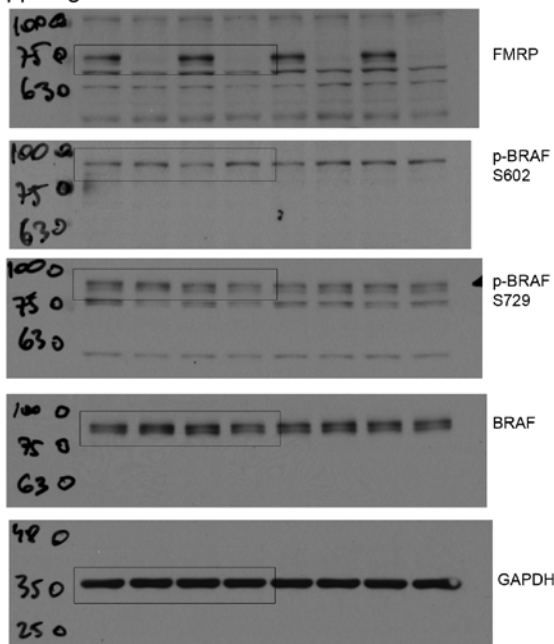


Supp. Fig. 8i

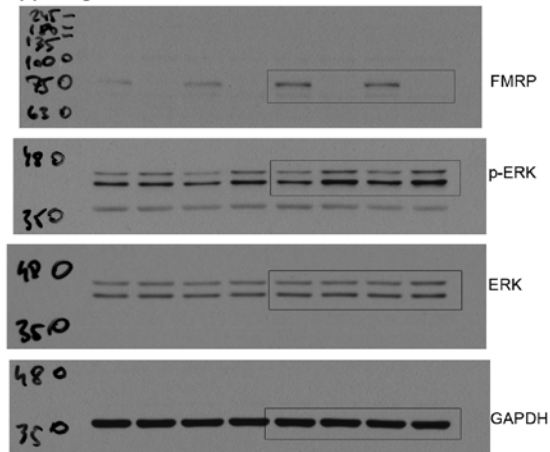


Supplementary Figure 15 Original images of representative western blots in Supplementary Fig. 8

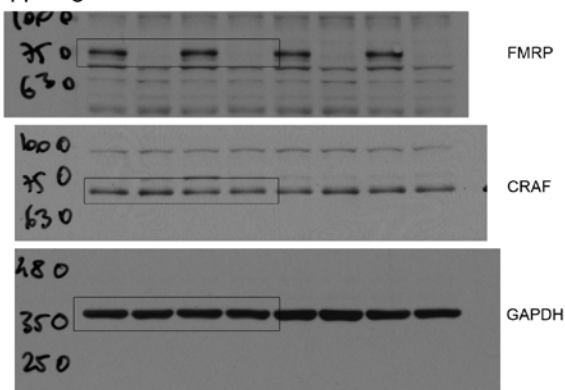
Supp. Fig. 9a



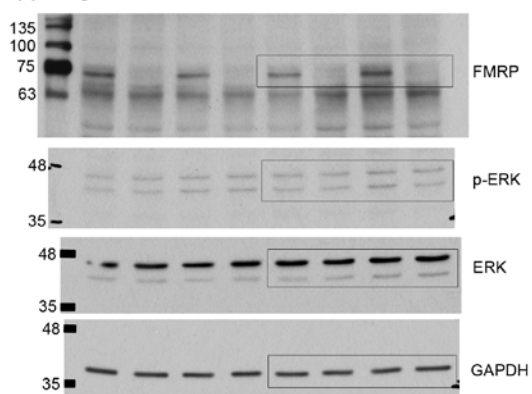
Supp. Fig. 10b



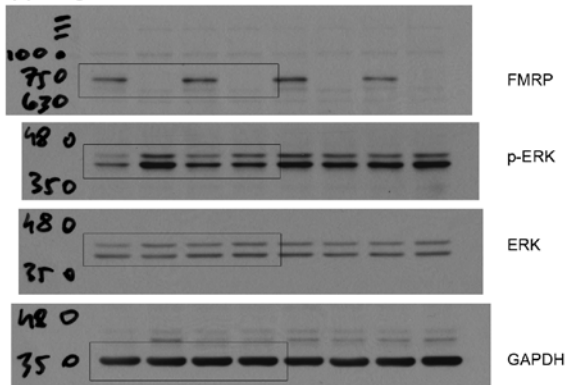
Supp. Fig. 9b



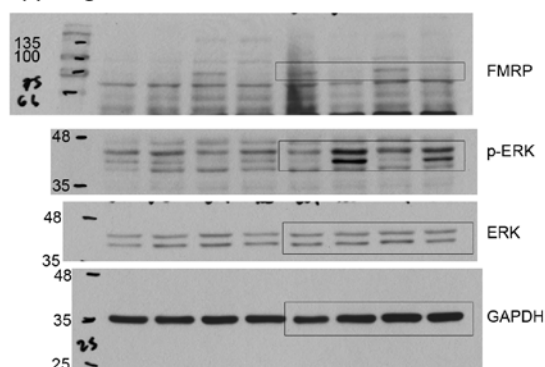
Supp. Fig. 10c



Supp. Fig. 10a

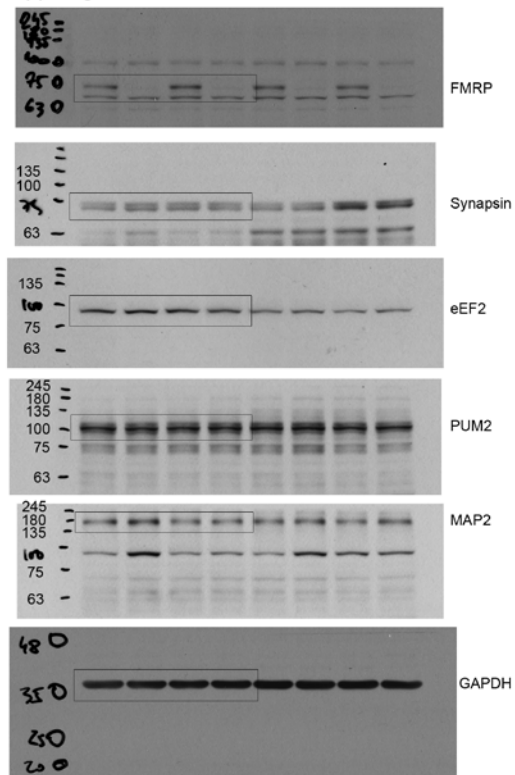


Supp. Fig. 10d



**Supplementary Figure 16** Original images of representative western blots in Supplementary Figs. 9 and 10

Supp. Fig. 11a



**Supplementary Figure 17** Original images of representative western blots in Supplementary Fig. 11

Supplementary Table 1. Statistical analysis for Figures 1-2 and Supplementary Figures 1-11.

\*Supplementary Figures data (mean ± s.e.m.) is available upon request.

Figure and nr of animals or cells used	Statistical analysis	Post hoc tests	Mean ± s.e.m.
<p><b>1b, c:</b> Preference for social novelty</p> <p>WT Veh (n = 10)</p> <p>Fmr1<sup>-/-</sup> Veh (n = 10)</p> <p>WT Met (n = 9)</p> <p>Fmr1<sup>-/-</sup> Met (n = 12)</p>	<p>Two-way mixed ANOVA</p> <p><i>Time sniffing</i></p> <p>Chamber: F(1,37) = 48.173, p &lt; 0.001</p> <p>Group: F(3,37) = 2.329, p = 0.090</p> <p>Chamber x Group: F(3,37) = 4.392, p = 0.010</p> <p><i>Time in chamber</i></p> <p>Chamber: F(1,37) = 25.241, p &lt; 0.001</p> <p>Group: F(3,37) = 3.600, p = 0.022</p> <p>Chamber x Group: F(3,37) = 0.456, p = 0.715</p>	<p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, p = 0.006</p> <p>WT Met vs KO Met, p = 0.971</p> <p>WT Veh vs WT Met, p = 0.963</p> <p>KO Veh vs KO Met, p = 0.005</p> <p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, p = 0.013</p> <p>WT Met vs KO Met, p = 0.968</p> <p>WT Veh vs WT Met, p = 0.997</p> <p>KO Veh vs KO Met, p = 0.017</p>	<p>S1:</p> <p>WT Veh 35 ± 4</p> <p>KO Veh 73 ± 13</p> <p>WT Met 40 ± 5</p> <p>KO Met 36 ± 6</p> <p>S2:</p> <p>WT Veh 79 ± 6</p> <p>KO Veh 77 ± 11</p> <p>WT Met 85 ± 10</p> <p>KO Met 68 ± 7</p> <p>Centre:</p> <p>WT Veh 235 ± 19</p> <p>KO Veh 159 ± 18</p> <p>WT Met 212 ± 17</p> <p>KO Met 219 ± 15</p> <p>S2:</p> <p>WT Veh 212 ± 16</p> <p>KO Veh 237 ± 15</p> <p>WT Met 217 ± 11</p> <p>KO Met 222 ± 15</p>
<p><b>1d, e:</b> Grooming</p> <p>WT Veh (n = 10)</p> <p>Fmr1<sup>-/-</sup> Veh (n = 10)</p> <p>WT Met (n = 8)</p> <p>Fmr1<sup>-/-</sup> Met (n = 12)</p>	<p>Two-way ANOVA</p> <p><i>Time</i></p> <p>Genotype: F(1,36) = 10.662, p = 0.002</p> <p>Treatment: F(1,36) = 4.613, p = 0.039</p> <p>Genotype x Treatment: F(1,36) = 4.782, p = 0.035</p> <p><i>Number of bouts</i></p> <p>Genotype: F(1,36) = 11.672, p = 0.002</p> <p>Treatment: F(1,36) = 4.767, p = 0.036</p> <p>Genotype x Treatment: F(1,36) = 11.386, p = 0.002</p>	<p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, p &lt; 0.001</p> <p>WT Met vs KO Met, p = 0.455</p> <p>WT Veh vs WT Met, p = 0.979</p> <p>KO Veh vs KO Met, p = 0.003</p> <p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, p &lt; 0.001</p> <p>WT Met vs KO Met, p = 0.977</p> <p>WT Veh vs WT Met, p = 0.428</p>	<p>WT Veh 18.1 ± 3.0</p> <p>KO Veh 38.5 ± 2.5</p> <p>WT Met 18.2 ± 4.8</p> <p>KO Met 22.3 ± 4.1</p> <p>WT Veh 6.2 ± 0.6</p> <p>KO Veh 12.9 ± 1.2</p> <p>WT Met 7.4 ± 1.3</p>

		KO Veh vs KO Met, p < 0.001	KO Met 7.3 ± 0.9
<b>1g, h: Spine density</b>	Two-way ANOVA		
WT Veh (n = 4)	<i>Nr of spines</i>	<i>Post hoc</i> Tukey's test:	
Fmr1 <sup>-/-</sup> Veh (n = 4)	Genotype: F(1,32) = 26.741, p < 0.001	WT Veh vs KO Veh, p < 0.001	WT Veh 15.3 ± 0.5
WT Met (n = 4)	Treatment: F(1,32) = 6.000, p = 0.02	WT Met vs KO Met, p = 0.447	KO Veh 21.0 ± 0.8
Fmr1 <sup>-/-</sup> Met (n = 4)	Genotype x Treatment: F(1,32) = 16.667, p < 0.001	WT Veh vs WT Met, p = 0.257	WT Met 16.3 ± 0.6
		KO Veh vs KO Met, p < 0.001	KO Met 17.0 ± 0.6
	<i>Mushroom</i>	<i>Post hoc</i> Tukey's test:	
	Genotype: F(1,32) = 108.587, p < 0.001	WT Veh vs KO Veh, p < 0.001	WT Veh 31.4 ± 0.8
	Treatment: F(1,32) = 49.390, p < 0.001	WT Met vs KO Met, p = 0.262	KO Veh 18.2 ± 0.8
	Genotype x Treatment: F(1,32) = 77.527, p < 0.001	WT Veh vs WT Met, p = 0.218	WT Met 30.2 ± 0.7
		KO Veh vs KO Met, p < 0.001	KO Met 29.1 ± 0.3
	<i>Filopodial</i>	<i>Post hoc</i> Tukey's test:	
	Genotype: F(1,32) = 71.558, p < 0.001	WT Veh vs KO Veh, p < 0.001	WT Veh 5.1 ± 0.3
	Treatment: F(1,32) = 23.558, p < 0.001	WT Met vs KO Met, p = 0.016	KO Veh 10.4 ± 0.4
	Genotype x Treatment: F(1,32) = 23.558, p < 0.001	WT Veh vs WT Met, p = 1.000	WT Met 5.1 ± 0.3
		KO Veh vs KO Met, p < 0.001	KO Met 6.6 ± 0.6
<b>1k: LTD</b>	Two-way ANOVA		
WT Veh (n = 9)	Genotype: F(1,46) = 1.075, p = 0.305	<i>Post hoc</i> Tukey's test:	
Fmr1 <sup>-/-</sup> Veh (n = 17)	Treatment: F(1,46) = 0.457, p = 0.503	WT Veh vs KO Veh, p = 0.032	WT Veh 75 ± 9
WT Met (n = 9)	Genotype x Treatment: F(1,46) = 4.222, p < 0.046	WT Met vs KO Met, p = 0.480	KO Veh 55 ± 5
Fmr1 <sup>-/-</sup> Met (n = 15)		WT Veh vs WT Met, p = 0.393	WT Met 66 ± 8
		KO Veh vs KO Met, p = 0.028	KO Met 72 ± 5
<b>2a: Testicle weight</b>	Two-way ANOVA		
WT Veh (n = 6)	Genotype: F(1,21) = 32.605, p < 0.001	<i>Post hoc</i> Tukey's test:	
Fmr1 <sup>-/-</sup> Veh (n = 6)	Treatment: F(1,21) = 8.846, p = 0.007	WT Veh vs KO Veh, p < 0.001	WT Veh 149 ± 6
WT Met (n = 6)	Genotype x Treatment: F(1,21) = 1.596, p = 0.220	WT Met vs KO Met, p = 0.004	KO Veh 200 ± 5
Fmr1 <sup>-/-</sup> Met (n = 7)		WT Veh vs WT Met, p = 0.248	WT Met 137 ± 6
		KO Veh vs KO Met, p = 0.006	KO Met 170 ± 8
<b>2b: De novo protein synthesis</b>	Two-way ANOVA		
WT Veh (n = 7)	Genotype: F(1,24) = 2.454, p = 0.130	<i>Post hoc</i> Tukey's test:	
Fmr1 <sup>-/-</sup> Veh (n = 7)	Treatment: F(1,24) = 6.198, p = 0.02	WT Veh vs KO Veh, p = 0.021	WT Veh 1.00 ± 0.01
WT Met (n = 7)	Genotype x Treatment: F(1,24) = 3.782, p = 0.064	WT Met vs KO Met, p = 0.792	KO Veh 1.29 ± 0.09
Fmr1 <sup>-/-</sup> Met (n = 7)		WT Veh vs WT Met, p = 0.704	WT Met 0.95 ± 0.04
		KO Veh vs KO Met, p = 0.005	KO Met 0.92 ± 0.12
<b>2c-j: Western blot</b>	Two-way ANOVA		
For all data except for MMP-9 in prefrontal cortex:	<i>Prefrontal cortex</i>	<i>Post hoc</i> Tukey's test:	
	<i>p-MEK/MEK</i>	WT Veh vs KO Veh, p = 0.027	WT Veh 100 ± 14
WT Veh (n = 6)	Genotype: F(1,20) = 8.693, p = 0.008	WT Met vs KO Met, p = 0.705	KO Veh 162 ± 17

Fmr1 <sup>-/-</sup> Veh (n = 6)	Treatment: F(1,20) = 5.660, p = 0.027	WT Veh vs WT Met, p = 0.904	WT Met 86 ± 10
WT Met (n = 6)	Genotype x Treatment: F(1,20) = 2.016, p = 0.171	KO Veh vs KO Met, p = 0.014	KO Met 108 ± 15
Fmr1 <sup>-/-</sup> Met (n = 6)			
	<i>MEK/GAPDH</i>		WT Veh 100 ± 6
	Genotype: F(1,20) = 0.021, p = 0.887		KO Veh 98 ± 6
	Treatment: F(1,20) = 0.250, p = 0.623		WT Met 102 ± 4
	Genotype x Treatment: F(1,20) = 0.017, p = 0.896		KO Met 102 ± 9
	<i>p-ERK/ERK</i>	<i>Post hoc</i> Tukey's test:	
	Genotype: F(1,20) = 15.141, p < 0.001	WT Veh vs KO Veh, p < 0.001	WT Veh 100 ± 22
	Treatment: F(1,20) = 7.636, p = 0.012	WT Met vs KO Met, p = 0.905	KO Veh 201 ± 12
	Genotype x Treatment: F(1,20) = 8.609, p = 0.008	WT Veh vs WT Met, p = 0.999	WT Met 103 ± 12
		KO Veh vs KO Met, p = 0.003	KO Met 117 ± 10
	<i>ERK/GAPDH</i>		WT Veh 100 ± 6
	Genotype: F(1,20) = 0.000, p = 0.997		KO Veh 103 ± 5
	Treatment: F(1,20) = 0.956, p = 0.340		WT Met 98 ± 7
	Genotype x Treatment: F(1,20) = 0.320, p = 0.578		KO Met 94 ± 6
	<i>p-eIF4E/eIF4E</i>	<i>Post hoc</i> Tukey's test:	
	Genotype: F(1,20) = 1.404, p = 0.250	WT Veh vs KO Veh, p = 0.023	WT Veh 100 ± 5
	Treatment: F(1,20) = 5.023, p = 0.036	WT Met vs KO Met, p = 0.458	KO Veh 129 ± 7
	Genotype x Treatment: F(1,20) = 10.899, p = 0.003	WT Veh vs WT Met, p = 0.877	WT Met 107 ± 5
		KO Veh vs KO Met, p = 0.004	KO Met 93 ± 8
	<i>eIF4E/GAPDH</i>		WT Veh 100 ± 15
	Genotype: F(1,20) = 3.583, p = 0.073		KO Veh 147 ± 10
	Treatment: F(1,20) = 4.255, p = 0.052		WT Met 149 ± 7
	Genotype x Treatment: F(1,20) = 3.050, p = 0.096		KO Met 151 ± 17
WT Veh (n = 5)	<i>MMP-9/GAPDH</i>	<i>Post hoc</i> Tukey's test:	
Fmr1 <sup>-/-</sup> Veh (n = 5)	Genotype: F(1,16) = 2.891, p = 0.108	WT Veh vs KO Veh, p = 0.023	WT Veh 100 ± 14
WT Met (n = 5)	Treatment: F(1,16) = 6.638, p = 0.020	WT Met vs KO Met, p = 0.830	KO Veh 146 ± 8
Fmr1 <sup>-/-</sup> Met (n = 5)	Genotype x Treatment: F(1,20) = 8.415, p = 0.010	WT Veh vs WT Met, p = 0.996	WT Met 103 ± 4
		KO Veh vs KO Met, p = 0.007	KO Met 91 ± 12
	<i>Hippocampus</i>	<i>Post hoc</i> Tukey's test:	
	<i>p-MEK/MEK</i>	WT Veh vs KO Veh, p < 0.001	WT Veh 100 ± 5
	Genotype: F(1,20) = 6.175, p = 0.022	WT Met vs KO Met, p = 0.667	KO Veh 174 ± 11
	Treatment: F(1,20) = 15.178, p < 0.001	WT Veh vs WT Met, p = 0.999	WT Met 102 ± 16
	Genotype x Treatment: F(1,20) = 16.846, p < 0.001	KO Veh vs KO Met, p < 0.001	KO Met 84 ± 11
	<i>MEK/GAPDH</i>		WT Veh 100 ± 8
	Genotype: F(1,20) = 0.265, p = 0.612		KO Veh 118 ± 7
	Treatment: F(1,20) = 1.298, p = 0.268		WT Met 105 ± 7
	Genotype x Treatment: F(1,20) = 3.077, p = 0.095		KO Met 95 ± 10

	<p><i>p-ERK/ERK</i></p> <p>Genotype: <math>F(1,20) = 2.749, p = 0.113</math></p> <p>Treatment: <math>F(1,20) = 5.618, p = 0.028</math></p> <p>Genotype x Treatment: <math>F(1,20) = 8.715, p = 0.008</math></p> <p><i>ERK/GAPDH</i></p> <p>Genotype: <math>F(1,20) = 0.109, p = 0.744</math></p> <p>Treatment: <math>F(1,20) = 0.350, p = 0.561</math></p> <p>Genotype x Treatment: <math>F(1,20) = 0.004, p = 0.950</math></p> <p><i>p-eIF4E/eIF4E</i></p> <p>Genotype: <math>F(1,20) = 16.194, p &lt; 0.001</math></p> <p>Treatment: <math>F(1,20) = 18.973, p &lt; 0.001</math></p> <p>Genotype x Treatment: <math>F(1,20) = 3.989, p = 0.059</math></p> <p><i>eIF4E/GAPDH</i></p> <p>Genotype: <math>F(1,20) = 0.739, p = 0.400</math></p> <p>Treatment: <math>F(1,20) = 0.769, p = 0.391</math></p> <p>Genotype x Treatment: <math>F(1,20) = 3.866, p = 0.063</math></p> <p><i>MMP-9/GAPDH</i></p> <p>Genotype: <math>F(1,20) = 16.803, p &lt; 0.001</math></p> <p>Treatment: <math>F(1,20) = 11.770, p = 0.003</math></p> <p>Genotype x Treatment: <math>F(1,20) = 12.526, p = 0.002</math></p>	<p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, <math>p = 0.019</math></p> <p>WT Met vs KO Met, <math>p = 0.797</math></p> <p>WT Veh vs WT Met, <math>p = 0.976</math></p> <p>KO Veh vs KO Met, <math>p = 0.006</math></p> <p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, <math>p = 0.002</math></p> <p>WT Met vs KO Met, <math>p = 0.494</math></p> <p>WT Veh vs WT Met, <math>p = 0.366</math></p> <p>KO Veh vs KO Met, <math>p = 0.001</math></p> <p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, <math>p &lt; 0.001</math></p> <p>WT Met vs KO Met, <math>p = 0.978</math></p> <p>WT Veh vs WT Met, <math>p = 0.999</math></p> <p>KO Veh vs KO Met, <math>p &lt; 0.001</math></p>	<p>WT Veh <math>100 \pm 5</math></p> <p>KO Veh <math>140 \pm 8</math></p> <p>WT Met <math>105 \pm 7</math></p> <p>KO Met <math>94 \pm 13</math></p> <p>WT Veh <math>100 \pm 2</math></p> <p>KO Veh <math>101 \pm 5</math></p> <p>WT Met <math>97 \pm 6</math></p> <p>KO Met <math>99 \pm 5</math></p> <p>WT Veh <math>100 \pm 5</math></p> <p>KO Veh <math>166 \pm 16</math></p> <p>WT Met <math>122 \pm 9</math></p> <p>KO Met <math>149 \pm 12</math></p> <p>WT Veh <math>100 \pm 22</math></p> <p>KO Veh <math>128 \pm 24</math></p> <p>WT Met <math>105 \pm 16</math></p> <p>KO Met <math>122 \pm 20</math></p> <p>WT Veh <math>100 \pm 6</math></p> <p>KO Veh <math>159 \pm 8</math></p> <p>WT Met <math>101 \pm 10</math></p> <p>KO Met <math>105 \pm 8</math></p>
<p><b>Supplementary 1a, b:</b></p> <p>Pharmacokinetics</p> <p>0 h (n = 4)</p> <p>0.5 h (n = 4)</p> <p>1 h (n = 4)</p> <p>2 h (n = 4)</p> <p>4 h (n = 4)</p>	<p>One-way ANOVA</p> <p><i>Plasma</i></p> <p>Time: <math>F(4,15) = 6.964, p = 0.002</math></p> <p><i>Brain</i></p> <p>Time: <math>F(4,15) = 5.936, p = 0.005</math></p>	<p><i>Post hoc</i> Tukey's test:</p> <p>0 vs 0.5, <math>p = 0.005</math></p> <p>0.5 vs 1, <math>p = 0.241</math></p> <p>0.5 vs 2, <math>p = 0.008</math></p> <p>0.5 vs 4, <math>p = 0.006</math></p> <p><i>Post hoc</i> Tukey's test:</p> <p>0 vs 0.5, <math>p = 0.006</math></p> <p>0.5 vs 1, <math>p = 0.998</math></p> <p>0.5 vs 2, <math>p = 0.463</math></p> <p>0.5 vs 4, <math>p = 0.131</math></p>	*
<p><b>Supplementary 1c:</b></p> <p>Western blot</p> <p>0 h (n = 3)</p> <p>0.5 h (n = 3)</p> <p>1 h (n = 3)</p>	<p>One-way ANOVA</p> <p><i>Hippocampus</i></p> <p>p-AMPK/AMPK</p> <p>Time: <math>F(4,10) = 5.072, p = 0.017</math></p>	<p><i>Post hoc</i> Tukey's test:</p> <p>0 vs 0.5, <math>p = 0.998</math></p> <p>0 vs 1, <math>p = 0.089</math></p> <p>0 vs 2, <math>p = 0.033</math></p> <p>0 vs 4, <math>p = 0.845</math></p>	



2 h (n = 3) 4 h (n = 3)			
<b>Supplementary 2a, b:</b> Dose-response  25 mg/kg (n = 4) 50 mg/kg (n = 4) 100 mg/kg (n = 4) 200 mg/kg (n = 4)	One-way ANOVA <i>Plasma</i> Time: $F(3,12) = 7.017, p = 0.006$  <i>Brain</i> Time: $F(3,12) = 98.789, p < 0.001$	<i>Post hoc</i> Tukey's test: 25 vs 50, $p = 0.996$ 25 vs 100, $p = 0.078$ 25 vs 200, $p = 0.012$  <i>Post hoc</i> Tukey's test: 25 vs 50, $p = 0.091$ 25 vs 100, $p < 0.001$ 25 vs 200, $p < 0.001$	
<b>Supplementary 3a:</b> Preference for social novelty  WT Veh (n = 8) Fmr1 <sup>-y</sup> Veh (n = 8) WT Met (n = 8) Fmr1 <sup>-y</sup> Met (n = 8)	Two-way mixed ANOVA <i>Time sniffing</i> Chamber: $F(1,28) = 30.489, p < 0.001$ Group: $F(3,28) = 5.016, p = 0.007$ Chamber x Group: $F(3,28) = 10.210, p < 0.001$  <i>Time in chamber</i> Chamber: $F(1,28) = 8.850, p = 0.006$ Group: $F(3,28) = 1.735, p = 0.183$ Chamber x Group: $F(3,28) = 0.964, p = 0.424$	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, $p = 0.046$ WT Met vs KO Met, $p = 0.011$ WT Veh vs WT Met, $p = 0.928$ KO Veh vs KO Met, $p = 0.821$  <i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, $p = 0.646$ WT Met vs KO Met, $p = 0.252$ WT Veh vs WT Met, $p = 0.372$ KO Veh vs KO Met, $p = 0.694$	
<b>Supplementary 3b</b> 5-day metformin grooming  WT Veh (n = 6) Fmr1 <sup>-y</sup> Veh (n = 6) WT Met (n = 7) Fmr1 <sup>-y</sup> Met (n = 7)	Two-way ANOVA <i>Time grooming</i> Genotype: $F(1,20) = 14.904, p < 0.001$ Treatment: $F(1,20) = 0.004, p = 0.947$ Genotype x Treatment: $F(1,20) = 1.020, p = 0.325$	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, $p = 0.005$ WT Met vs KO Met, $p = 0.037$ WT Veh vs WT Met, $p = 0.536$ KO Veh vs KO Met, $p = 0.427$	
<b>Supplementary 3c</b> 5-day metformin LTD  WT Veh (n = 13) Fmr1 <sup>-y</sup> Veh (n = 12) WT Met (n = 12) Fmr1 <sup>-y</sup> Met (n = 12)	Two-way ANOVA Genotype: $F(1,45) = 1.642, p = 0.207$ Treatment: $F(1,45) = 3.643, p = 0.063$ Genotype x Treatment: $F(1,45) = 4.232, p = 0.045$	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, $p = 0.022$ WT Met vs KO Met, $p = 0.590$ WT Veh vs WT Met, $p = 0.916$ KO Veh vs KO Met, $p = 0.008$	
<b>Supplementary 3d</b> 5-day metformin <i>De novo</i> protein synthesis  WT Veh (n = 3) Fmr1 <sup>-y</sup> Veh (n = 3) WT Met (n = 3)	Two-way ANOVA Genotype: $F(1,8) = 48.306, p < 0.001$ Treatment: $F(1,8) = 0.048, p = 0.832$ Genotype x Treatment: $F(1,8) = 0.023, p = 0.883$	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, $p = 0.006$ WT Met vs KO Met, $p = 0.005$ WT Veh vs WT Met, $p = 1.000$ KO Veh vs KO Met, $p = 0.993$	

Fmr1 <sup>-y</sup> Met (n = 3)			
<b>Supplementary 3e</b> 5-day metformin Western blot	Two-way ANOVA <i>Prefrontal cortex</i> <i>p-eIF4E/eIF4E</i> Genotype: F(1,20) = 27.037, p < 0.001 Treatment: F(1,20) = 4.028, p = 0.058 Genotype x Treatment: F(1,20) = 1.376, p = 0.254  <i>Hippocampus</i> <i>p-eIF4E/eIF4E</i> Genotype: F(1,20) = 7.664, p = 0.012 Treatment: F(1,20) = 1.243, p = 0.278 Genotype x Treatment: F(1,20) = 1.936, p = 0.179	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.001 WT Met vs KO Met, p = 0.045 WT Veh vs WT Met, p = 0.934 KO Veh vs KO Met, p = 0.144  <i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.037 WT Met vs KO Met, p = 0.766 WT Veh vs WT Met, p = 0.997 KO Veh vs KO Met, p = 0.315	
WT Veh (n = 6) Fmr1 <sup>-y</sup> Veh (n = 6) WT Met (n = 6) Fmr1 <sup>-y</sup> Met (n = 6)			
<b>Supplementary 4a</b> 1-day metformin grooming	Two-way ANOVA <i>Time grooming</i> Genotype: F(1,20) = 12.461, p = 0.002 Treatment: F(1,20) = 1.279, p = 0.271 Genotype x Treatment: F(1,20) = 0.009, p = 0.924	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.019 WT Met vs KO Met, p = 0.025 WT Veh vs WT Met, p = 0.473 KO Veh vs KO Met, p = 0.396	
WT Veh (n = 6) Fmr1 <sup>-y</sup> Veh (n = 6) WT Met (n = 6) Fmr1 <sup>-y</sup> Met (n = 6)			
<b>Supplementary 4b</b> 1-day metformin LTD	Two-way ANOVA Genotype: F(1,37) = 5.636, p = 0.023 Treatment: F(1,37) = 0.196, p = 0.661 Genotype x Treatment: F(1,37) = 2.008, p = 0.165	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.009 WT Met vs KO Met, p = 0.512 WT Veh vs WT Met, p = 0.465 KO Veh vs KO Met, p = 0.223	
WT Veh (n = 11) Fmr1 <sup>-y</sup> Veh (n = 10) WT Met (n = 12) Fmr1 <sup>-y</sup> Met (n = 8)			
<b>Supplementary 4c</b> 1-day metformin <i>De novo</i> protein synthesis	Two-way ANOVA Genotype: F(1,8) = 37.400, p < 0.001 Treatment: F(1,8) = 0.075, p = 0.791 Genotype x Treatment: F(1,8) = 0.014, p = 0.909	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.009 WT Met vs KO Met, p = 0.012 WT Veh vs WT Met, p = 0.992 KO Veh vs KO Met, p = 0.999	
WT Veh (n = 3) Fmr1 <sup>-y</sup> Veh (n = 3) WT Met (n = 3) Fmr1 <sup>-y</sup> Met (n = 3)			
<b>Supplementary 5b</b> Open Field	Two-way ANOVA <i>Path length</i> Genotype: F(1,26) = 22.131, p < 0.001 Treatment: F(1,26) = 0.053, p = 0.819 Genotype x Treatment: F(1,26) = 0.213, p = 0.648  <i>Velocity</i>	<i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.001 WT Met vs KO Met, p = 0.006 WT Veh vs WT Met, p = 0.867 KO Veh vs KO Met, p = 0.640  <i>Post hoc</i> Tukey's test:	
WT Veh (n = 8) Fmr1 <sup>-y</sup> Veh (n = 7) WT Met (n = 8) Fmr1 <sup>-y</sup> Met (n = 8)			

	<p>Genotype: <math>F(1,26) = 21.530, p &lt; 0.001</math></p> <p>Treatment: <math>F(1,26) = 0.025, p = 0.875</math></p> <p>Genotype x Treatment: <math>F(1,26) = 0.118, p = 0.733</math></p>	<p>WT Veh vs KO Veh, <math>p = 0.002</math></p> <p>WT Met vs KO Met, <math>p = 0.006</math></p> <p>WT Veh vs WT Met, <math>p = 0.893</math></p> <p>KO Veh vs KO Met, <math>p = 0.733</math></p>	
<p><b>Supplementary 5c</b></p> <p>Light-dark box</p> <p>WT Veh (n = 8)</p> <p>Fmr1<sup>-y</sup> Veh (n = 8)</p> <p>WT Met (n = 8)</p> <p>Fmr1<sup>-y</sup> Met (n = 8)</p>	<p>Two-way ANOVA</p> <p>Genotype: <math>F(1,28) = 14.337, p &lt; 0.001</math></p> <p>Treatment: <math>F(1,28) = 0.713, p = 0.406</math></p> <p>Genotype x Treatment: <math>F(1,28) = 0.489, p = 0.490</math></p>	<p><i>Post hoc</i> Tukey's test:</p> <p>WT Veh vs KO Veh, <math>p = 0.038</math></p> <p>WT Met vs KO Met, <math>p = 0.004</math></p> <p>WT Veh vs WT Met, <math>p = 0.285</math></p> <p>KO Veh vs KO Met, <math>p = 0.919</math></p>	
<p><b>Supplementary 6a-d</b></p> <p>Morris Water Maze</p> <p>WT Veh (n = 8)</p> <p>Fmr1<sup>-y</sup> Veh (n = 8)</p> <p>WT Met (n = 8)</p> <p>Fmr1<sup>-y</sup> Met (n = 8)</p>	<p>Two-way mixed ANOVA</p> <p><i>Acquisition MWM</i></p> <p>Group: <math>F(3,112) = 0.892, p = 0.458</math></p> <p>Day: <math>F(4,112) = 111.265, p &lt; 0.001</math></p> <p>Group x Day: <math>F(12,112) = 0.495, p = 0.914</math></p> <p>Two-way ANOVA</p> <p><i>Probe MWM</i></p> <p>Genotype: <math>F(1,28) = 0.417, p = 0.524</math></p> <p>Treatment: <math>F(1,28) = 1.094, p = 0.305</math></p> <p>Genotype x Treatment: <math>F(1,28) = 0.009, p = 0.923</math></p> <p>Two-way mixed ANOVA</p> <p><i>Acquisition reversal MWM</i></p> <p>Group: <math>F(3,140) = 1.973, p = 0.141</math></p> <p>Trial: <math>F(5,140) = 10.687, p &lt; 0.001</math></p> <p>Group x Trial: <math>F(15,140) = 0.586, p = 0.882</math></p> <p>Two-way ANOVA</p> <p><i>Probe reversal MWM</i></p> <p>Genotype: <math>F(1,28) = 0.173, p = 0.681</math></p> <p>Treatment: <math>F(1,28) = 0.183, p = 0.672</math></p> <p>Genotype x Treatment: <math>F(1,28) = 0.554, p = 0.463</math></p>		
<p><b>Supplementary 6e</b></p> <p>CFC</p> <p>WT Veh (n = 8)</p> <p>Fmr1<sup>-y</sup> Veh (n = 8)</p> <p>WT Met (n = 8)</p> <p>Fmr1<sup>-y</sup> Met (n = 10)</p>	<p>Two-way ANOVA</p> <p>Genotype: <math>F(1,26) = 1.127, p = 0.298</math></p> <p>Treatment: <math>F(1,26) = 1.001, p = 0.326</math></p> <p>Genotype x Treatment: <math>F(1,26) = 0.006, p = 0.937</math></p>		



<p>WT Veh (n = 4) Fmr1<sup>-y</sup> Veh (n = 4) WT Met (n = 4) Fmr1<sup>-y</sup> Met (n = 4)</p> <p>WT Veh (n = 4) Fmr1<sup>-y</sup> Veh (n = 4) WT Met (n = 4) Fmr1<sup>-y</sup> Met (n = 4)</p>	<p><i>S6/GAPDH</i> Genotype: F(1,20) = 7.909, p = 0.011 Treatment: F(1,20) = 0.584, p = 0.454 Genotype x Treatment: F(1,20) = 6.149, p = 0.022</p> <p><i>p-AMPK/AMPK</i> Genotype: F(1,20) = 0.305, p = 0.587 Treatment: F(1,20) = 0.013, p = 0.910 Genotype x Treatment: F(1,20) = 0.034, p = 0.856</p> <p><i>p-ACCI/ACCI</i> Genotype: F(1,20) = 5.626, p = 0.028 Treatment: F(1,20) = 0.835, p = 0.371 Genotype x Treatment: F(1,20) = 0.397, p = 0.536</p> <p><i>p-TSC2/TSC2</i> Genotype: F(1,12) = 0.184, p = 0.676 Treatment: F(1,12) = 0.005, p = 0.947 Genotype x Treatment: F(1,12) = 0.915, p = 0.358</p> <p><i>p-Raptor/Raptor</i> Genotype: F(1,12) = 0.076, p = 0.788 Treatment: F(1,12) = 0.384, p = 0.547 Genotype x Treatment: F(1,12) = 0.000, p = 0.983</p>	<p><i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.006 WT Met vs KO Met, p = 0.995 WT Veh vs WT Met, p = 0.133 KO Veh vs KO Met, p = 0.626</p> <p><i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.180 WT Met vs KO Met, p = 0.615 WT Veh vs WT Met, p = 0.698 KO Veh vs KO Met, p = 0.997</p>	
<p><b>Supplementary 9a, b:</b> Western blot</p> <p>WT Veh (n = 6) Fmr1<sup>-y</sup> Veh (n = 6) WT Met (n = 6) Fmr1<sup>-y</sup> Met (n = 6)</p>	<p>Two-way ANOVA <i>Hippocampus</i> <i>b-Raf/GAPDH</i> Genotype: F(1,20) = 0.044, p = 0.836 Treatment: F(1,20) = 4.555, p = 0.045 Genotype x Treatment: F(1,20) = 29.514, p &lt; 0.001</p> <p><i>p-b-Raf S729/ b-Raf</i> Genotype: F(1,20) = 3.925, p = 0.061 Treatment: F(1,20) = 4.900, p = 0.039 Genotype x Treatment: F(1,20) = 0.539, p = 0.471</p> <p><i>p-b-Raf S602/ b-Raf</i> Genotype: F(1,20) = 0.253, p = 0.620</p>	<p><i>Post hoc</i> Tukey's test: WT Veh vs KO Veh, p = 0.004 WT Met vs KO Met, p = 0.007 WT Veh vs WT Met, p = 0.124 KO Veh vs KO Met, p &lt; 0.001</p>	

	<p>Treatment: <math>F(1,20) = 1.872, p = 0.186</math>  Genotype x Treatment: <math>F(1,20) = 0.195, p = 0.663</math></p> <p><i>c-Raf/GAPDH</i></p> <p>Genotype: <math>F(1,20) = 2.498, p = 0.130</math>  Treatment: <math>F(1,20) = 2.616, p = 0.121</math>  Genotype x Treatment: <math>F(1,20) = 17.981, p &lt; 0.001</math></p>	<p><i>Post hoc</i> Tukey's test:  WT Veh vs KO Veh, <math>p = 0.003</math>  WT Met vs KO Met, <math>p = 0.267</math>  WT Veh vs WT Met, <math>p = 0.278</math>  KO Veh vs KO Met, <math>p = 0.003</math></p>	
<p><b>Supplementary 10a-d:</b>  Western blot</p> <p>WT Veh (n = 4)  Fmr1<sup>-y</sup> Veh (n = 4)  WT Met (n = 4)  Fmr1<sup>-y</sup> Met (n = 4)</p>	<p>Two-way ANOVA</p> <p><i>Striatum</i></p> <p><i>p-ERK/ERK</i></p> <p>Genotype: <math>F(1,12) = 6.057, p = 0.030</math>  Treatment: <math>F(1,12) = 3.793, p = 0.075</math>  Genotype x Treatment: <math>F(1,12) = 6.325, p = 0.027</math></p> <p><i>Cerebellum</i></p> <p><i>p-ERK/ERK</i></p> <p>Genotype: <math>F(1,12) = 11.142, p = 0.006</math>  Treatment: <math>F(1,12) = 0.644, p = 0.438</math>  Genotype x Treatment: <math>F(1,12) = 0.923, p = 0.356</math></p> <p><i>Gonads</i></p> <p><i>p-ERK/ERK</i></p> <p>Genotype: <math>F(1,12) = 0.093, p = 0.765</math>  Treatment: <math>F(1,12) = 5.606, p = 0.035</math>  Genotype x Treatment: <math>F(1,12) = 0.266, p = 0.625</math></p> <p><i>Liver</i></p> <p><i>p-ERK/ERK</i></p> <p>Genotype: <math>F(1,12) = 11.427, p = 0.005</math>  Treatment: <math>F(1,12) = 0.245, p = 0.629</math>  Genotype x Treatment: <math>F(1,12) = 0.729, p = 0.410</math></p>	<p><i>Post hoc</i> Tukey's test:  WT Veh vs KO Veh, <math>p = 0.019</math>  WT Met vs KO Met, <math>p = 1.000</math>  WT Veh vs WT Met, <math>p = 0.977</math>  KO Veh vs KO Met, <math>p = 0.036</math></p> <p><i>Post hoc</i> Tukey's test:  WT Veh vs KO Veh, <math>p = 0.044</math>  WT Met vs KO Met, <math>p = 0.374</math>  WT Veh vs WT Met, <math>p = 0.999</math>  KO Veh vs KO Met, <math>p = 0.611</math></p> <p><i>Post hoc</i> Tukey's test:  WT Veh vs KO Veh, <math>p = 0.048</math>  WT Met vs KO Met, <math>p = 0.326</math>  WT Veh vs WT Met, <math>p = 0.994</math>  KO Veh vs KO Met, <math>p = 0.777</math></p>	
<p><b>Supplementary 11b-e</b>  Western blot</p> <p>WT Veh (n = 4)  Fmr1<sup>-y</sup> Veh (n = 4)  WT Met (n = 4)  Fmr1<sup>-y</sup> Met (n = 4)</p>	<p>Two-way ANOVA</p> <p><i>Hippocampus</i></p> <p><i>Synapsin/GAPDH</i></p> <p>Genotype: <math>F(1,12) = 5.891, p = 0.032</math>  Treatment: <math>F(1,12) = 0.019, p = 0.891</math>  Genotype x Treatment: <math>F(1,12) = 7.966, p = 0.015</math></p> <p><i>eEF2/GAPDH</i></p>	<p><i>Post hoc</i> Tukey's test:  WT Veh vs KO Veh, <math>p = 0.014</math>  WT Met vs KO Met, <math>p = 0.992</math>  WT Veh vs WT Met, <math>p = 0.210</math>  KO Veh vs KO Met, <math>p = 0.280</math></p>	

Genotype:  $F(1,12) = 0.851, p = 0.374$   
Treatment:  $F(1,12) = 2.357, p = 0.151$   
Genotype x Treatment:  $F(1,12) = 2.183, p = 0.165$

*PUM2/GAPDH*

Genotype:  $F(1,12) = 2.457, p = 0.143$   
Treatment:  $F(1,12) = 0.060, p = 0.810$   
Genotype x Treatment:  $F(1,12) = 0.907, p = 0.360$

*MAP2/GAPDH*

Genotype:  $F(1,12) = 1.876, p = 0.196$   
Treatment:  $F(1,12) = 10.052, p = 0.008$   
Genotype x Treatment:  $F(1,12) = 5.782, p = 0.033$

*Post hoc* Tukey's test:

WT Veh vs KO Veh,  $p = 0.083$   
WT Met vs KO Met,  $p = 0.882$   
WT Veh vs WT Met,  $p = 0.947$   
KO Veh vs KO Met,  $p = 0.009$