Metformin ameliorates core deficits in a mouse model of fragile X syndrome

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

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Fragile X syndrome is the leading monogenic cause of ASD. Trinucleotide repeats in the *FMR1* gene abolish FMRP protein expression, leading to hyperactivation of ERK and mTOR signaling, upstream of mRNA translation. Here we show that metformin, the most widely used anti-type 2 diabetes drug, rescues core phenotypes in *Fmr1*[-/y] mice and selectively normalizes Erk signaling, Eif4e phosphorylation and the expression of Mmp9. Thus, metformin is a potential FXS therapeutic.

Dysregulated mRNA translation is linked to core pathologies diagnosed in the Fragile X neurodevelopmental Syndrome (FXS), such as social and behavior problems, developmental delays and learning disabilities[1,2]. In the brains of FXS patients and knockout mice (*Fmr1*[-/y]; X-linked *Fmr1* deletion in male mice), loss of Fragile X mental retardation protein (FMRP) results in hyperactivation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) and the extracellular signal-regulated kinase (ERK) signaling pathways[1,2]. Consistent with increased ERK activity, eukaryotic initiation factor 4E (eIF4E) phosphorylation is elevated in the brain of FXS patients and *Fmr1*[-/y] mice, thereby promoting translation of the mRNA encoding for matrix metalloproteinase 9 (MMP-9), which is elevated in the brains of both FXS patients and the *Fmr1*[-/y] mice[1-5]. In accordance with these findings, knockout of *Mmp9* rescues the majority of phenotypes in *Fmr1*[-/y] mice. MMP-9 degrades components of the extracellular matrix, including proteins important for synaptic function and maturation, which are implicated in FXS and autism spectrum disorders (ASD). Recent observations indicate that metformin, a first-line therapy for type 2 diabetes, imparts numerous health benefits beyond its original therapeutic use, such as decreased cancer risk and improved cancer prognosis[6]. Metformin inhibits the mitochondrial respiratory chain complex 1, leading to a decrease in cellular energy state and thus activation of the energy sensor AMP-activated protein kinase (AMPK)[6]. Several AMPK-independent activities of metformin have also been reported[7,8]. Since metformin suppresses translation by inhibiting

33   mTORC1 and ERK pathways, we reasoned that metformin could have beneficial therapeutic

34   effects in *Fmr1*[-/y] mice[9].

35

36   Adult (8-12 weeks old) wild-type (WT) and *Fmr1*[-/y] mice were injected intraperitoneally (i.p.)

37   with metformin (200 mg/kg/day, a concentration previously used in preclinical studies[8]) or

38   vehicle for 10 days (**Fig. 1a**). Metformin, as previously reported[10], 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*[-/y] 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*[-/y] 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*[-/y] mice as increased self-

47   grooming[1,11]. Metformin reversed the increased grooming in *Fmr1*[-/y] 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*[-/y] 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*[-/y] mice; we observed no cognitive impairment in

53   *Fmr1*[-/y] 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*[-/y] mice exhibit aberrant spine morphology[1,11]. We

57   observed spine dysmorphogenesis in *Fmr1*[-/y] mice as evidenced by increased density of

dendritic spines in CA1 hippocampal pyramidal neurons, along with fewer mature stubby and mushroom spines, and an increased number of immature filopodia-like spines (**Fig. 1f,g,h**). Ten-day metformin administration corrected the dendritic abnormalities in *Fmr1*[-/y] mice (**Fig. 1f,g,h**).

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

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

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

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

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83   targets, MAP2 and synapsin, with no effect on eEF2 and PUM2 levels[14] (**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*[-/y] 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[12] or endocrine regulation outside the brain of *Fmr1*[-/y] mice.

88

89   Ten-day metformin administration did not activate Ampk in the prefrontal cortex and

90   hippocampus of *Fmr1*[-/y] 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[15,16]. 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[17,18], 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*[-/y] mice (**Supplementary Fig. 9**)[19].

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[20]. 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[21].

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-/y* 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-/y* mice[13]. 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-/y* mice[1,5], 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[22]. Such peripheral

122   phenotypes are linked to autism, intellectual disability and FXS, and have been shown to

123   affect brain plasticity[23].

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126 **METHODS**

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

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129 **SUPPLEMENTARY INFORMATION**

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

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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.

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146 **COMPETING FINANCIAL INTERESTS**

147 The authors declare no competing financial interest.

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176

FIGURE LEGENDS

178

179 **Figure 1** Chronic metformin treatment corrects social deficit, repetitive behavior, aberrant

180 dendritic spine morphology and exaggerated LTD in *Fmr1$^{-/y}$* 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$^{-/y}$* (n = 10), and metformin-treated

186 WT (n = 9) and *Fmr1$^{-/y}$* (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$^{-/y}$* (n = 10), and

188 metformin-treated WT (n = 8) and *Fmr1$^{-/y}$* (n = 12). (**f**) Golgi-cox staining of CA1 dendritic

189 spines in metformin or vehicle-injected WT and *Fmr1$^{-/y}$* mice. Scale bar: 2 μm. (**g**)

190 Quantification of spine density, measured as the number of spines per 10 μm 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 μm for 10 min) in slices prepared from (**i**) vehicle-treated WT (n = 9) and *Fmr1^{-}*

194 $^{/y}$ (n = 17) mice, and (**j**) metformin-treated WT (n = 9) and *Fmr1$^{-/y}$* (n = 15) mice. (**k**) fEPSP

195 slope during the last 10 minutes of recording. All values are shown as mean ± 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*^{-/y} mice. Vehicle-treated WT (n = 6) and *Fmr1*^{-/y}

202   (n = 6), and metformin-treated WT (n = 6) and *Fmr1*^{-/y} (n = 7). (**b**) Western blots of lysates

203   from hippocampal slices incubated with puromycin to measure basal rates of protein

204   synthesis. β-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*^{-/y} 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 ± 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.

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ONLINE METHODS

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

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

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

Three-chamber sociability and preference for social novelty tests. The apparatus consisted of three Plexiglas chambers: the central chamber (36 cm x 28 cm x 30 cm) was divided from two side chambers (each chamber: 29 cm x 28 cm x 30 cm) by Plexiglas walls, as previously described (Stoelting Co.)[24,25]. Each side was accessible to the mouse from the center through a doorway covered by a removable sliding door. A camera was mounted above the apparatus to record testing. The test consisted of 3 phases: habituation, sociability, and preference for social novelty. In the first part, three-month old male mice were placed in the middle chamber and were allowed to explore all three empty chambers for 10 min. After this period of habituation, mice were gently guided back to the center chamber of the apparatus and the 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*$^{-/y}$ 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

13

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 $(t_{novel} - t_{familiar})/(t_{novel} + t_{familiar})$. 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 β-

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); β-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 μm 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 NaH$_2$PO$_4$, 2 mM

364   MgSO$_4$, 2 mM CaCl$_2$, 26 mM 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*$^{-/y}$ 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_2 and 95% O_2 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 $CsMeSO_3$, 8 CsCl, 0.6 EGTA, 10 diNa-phosphocreatine, 10

389 HEPES, 4 ATP-Mg^{2+}, 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_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$,

392   2 $MgSO_4$, 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 mm 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 μm bin along the apical shaft. Spines were assigned one of

417 the five morphological categories based on published methods[24-26]; 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 $Fmr1^{-/y}$ mice, we measured *de novo* protein synthesis in hippocampal slices

424 using the SUnSET puromycin incorporation assay[24,27]. Transverse hippocampal slices (400

425 μm) were prepared from 5-6 week old mice and allowed to recover for at least 3 h. Puromycin

426 labeling was performed as described[24,27,28]. Briefly, the slices were incubated with puromycin

427 (Sigma, 5 μg/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 β-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

19

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.[26]. 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
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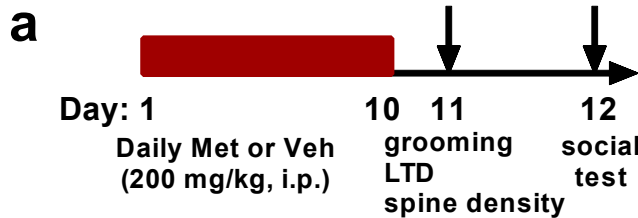
a Day: 1 — 10 Daily Met or Veh (200 mg/kg, i.p.) | 11 grooming LTD spine density | 12 social test

Legend: ☐ WT Veh ■ Fmr1⁻/y Veh ■ WT Met ■ Fmr1⁻/y Met

b Preference for social novelty — Time spent sniffing (s); S1 S2 groups; ***, **, N.S.

c Time in chamber (s); S1 Centre S2; **, *, N.S.

d Time grooming (s); ***, **, N.S.

e Number of grooming bouts; ***, N.S.

f WT Veh | Fmr1⁻/y Veh | WT Met | Fmr1⁻/y Met

g Spine Density (per 10 mm); ***, N.S.

h Fraction of total spines; S/M F groups; ***, *, N.S.

i WT Veh | Fmr1⁻/y Veh; fEPSP slope (% baseline) vs Time (min); DHPG; 1.0 mV, 50 ms

j WT Met | Fmr1⁻/y Met; fEPSP slope (% baseline) vs Time (min); DHPG

k fEPSP slope (% baseline); *, N.S.
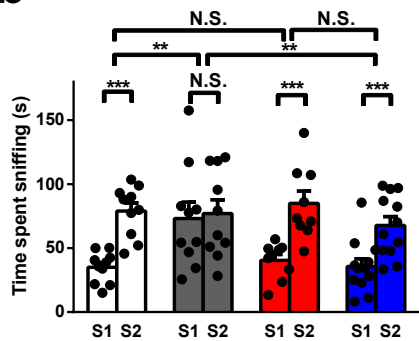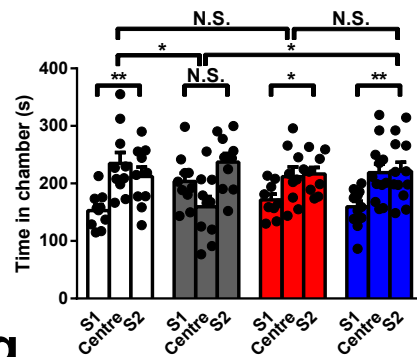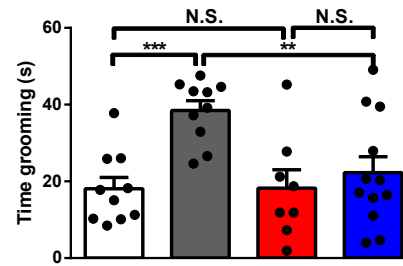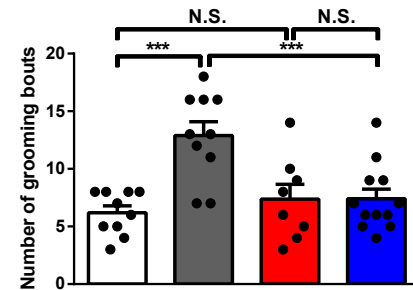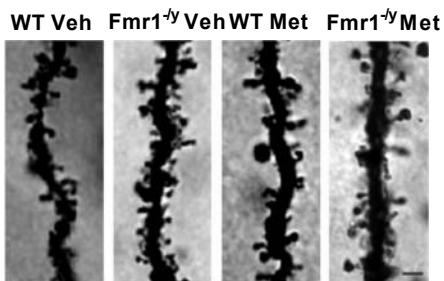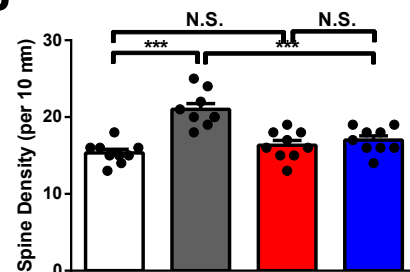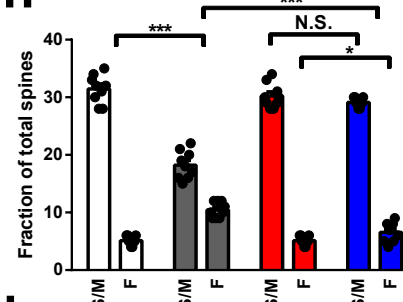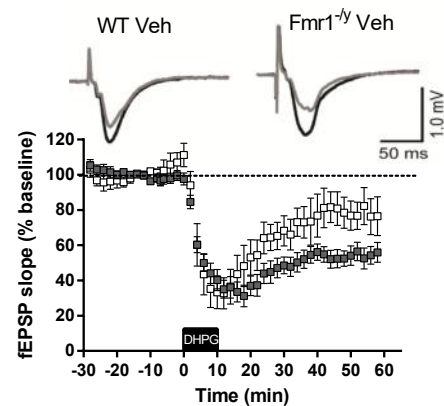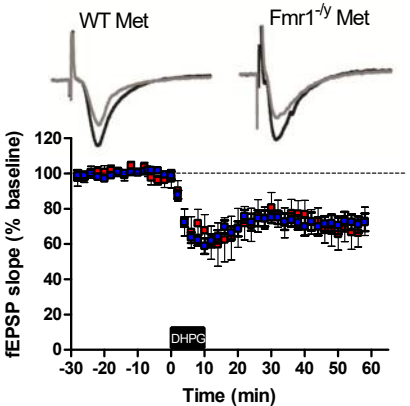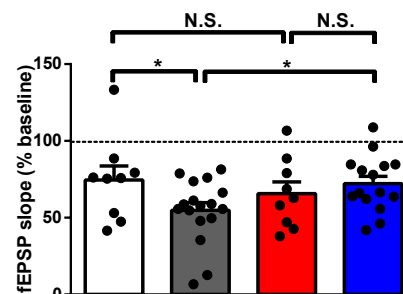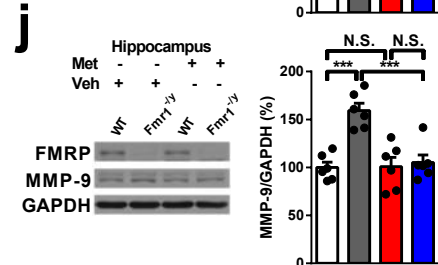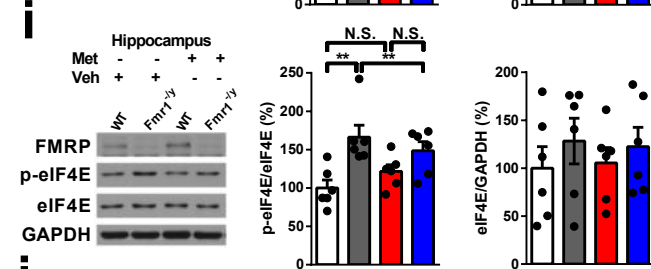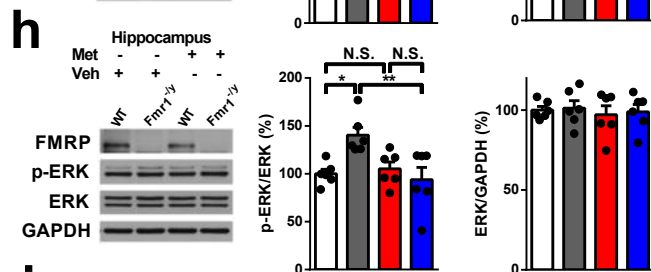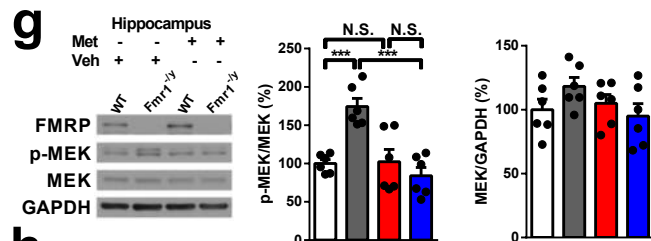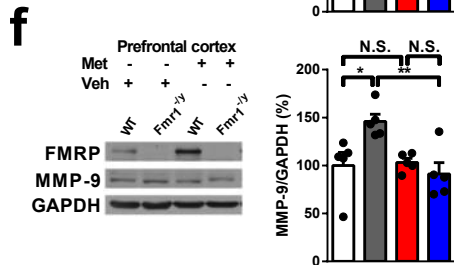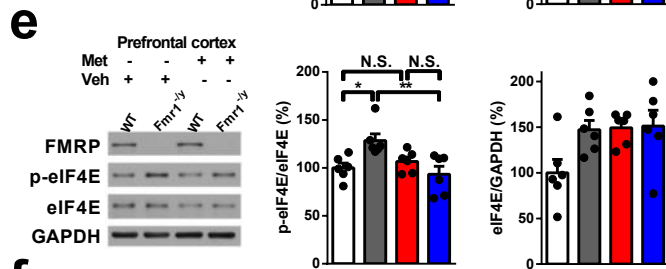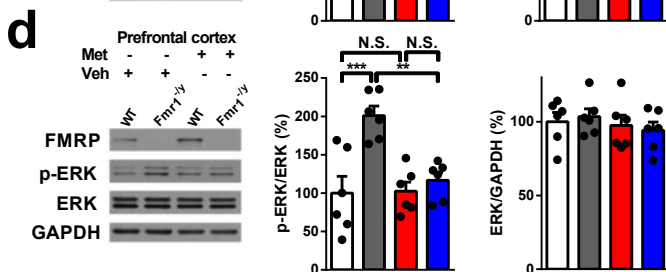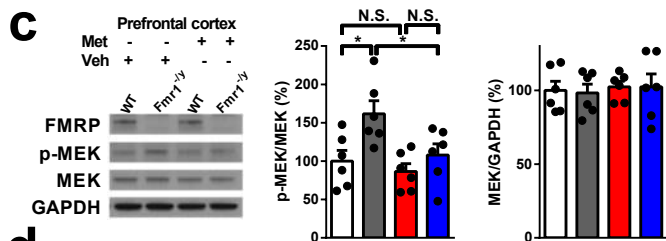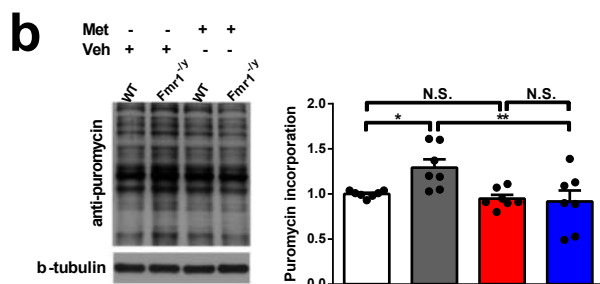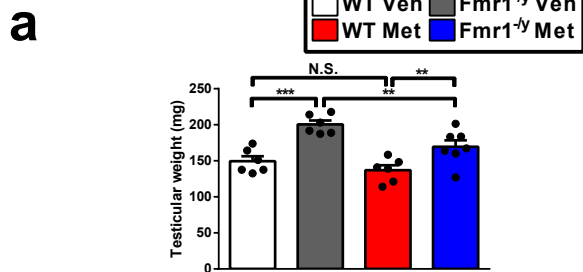
SUPPLEMENTARY FIGURES
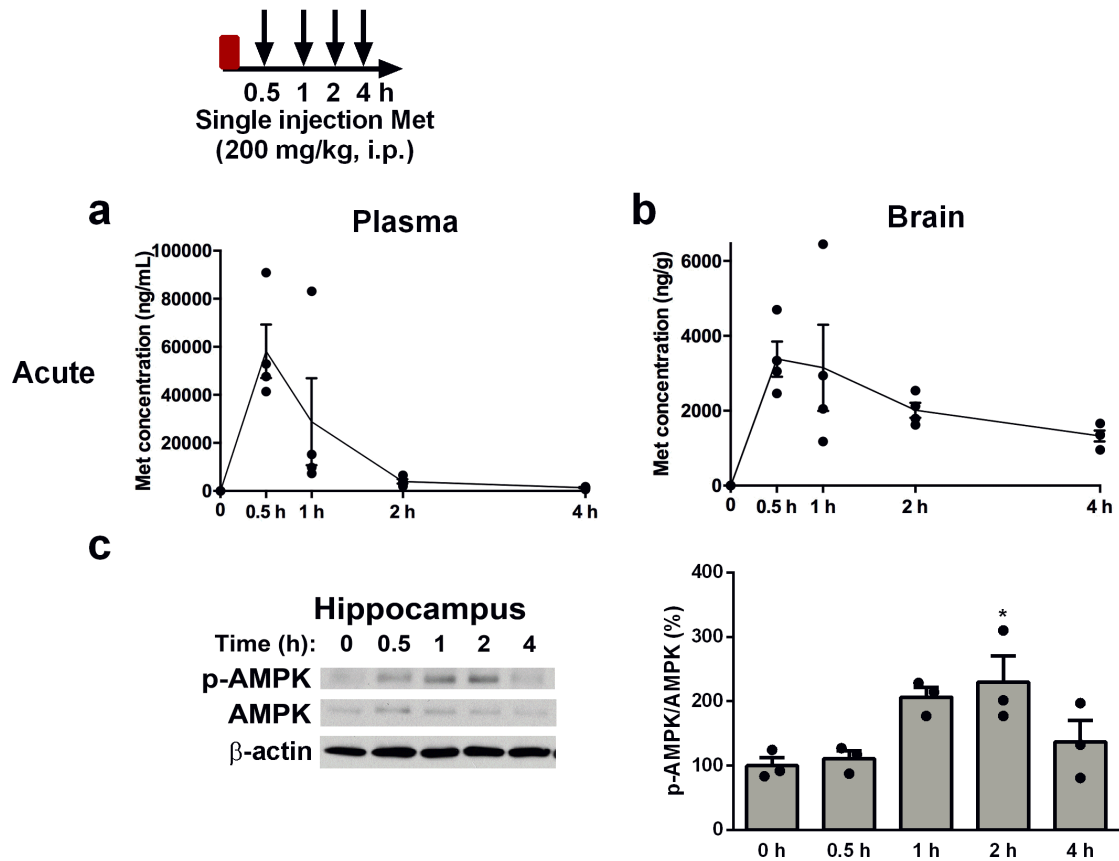
Supplementary Figure 1 Pharmacokinetic study and AMPK activation *in vivo* after acute metformin (200 mg/kg) treatment. The concentrations of metformin were measured by LC-MS/MS in the plasma (**a**), and brain (**b**) at different time points (0, 0.5, 1, 2, and 4 h) after a single metformin injection (200 mg/kg, i.p.) (n = 4 in each group). (**c**) Representative immunoblots of hippocampal lysates from vehicle- and metformin-treated WT and *Fmr1*[-/y] mice probed for total and phosphorylated AMPK (n = 3 in each group). β-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 ± s.e.m. *P < 0.05, versus all other groups; calculated by one-way ANOVA with Tukey's *post hoc* test.
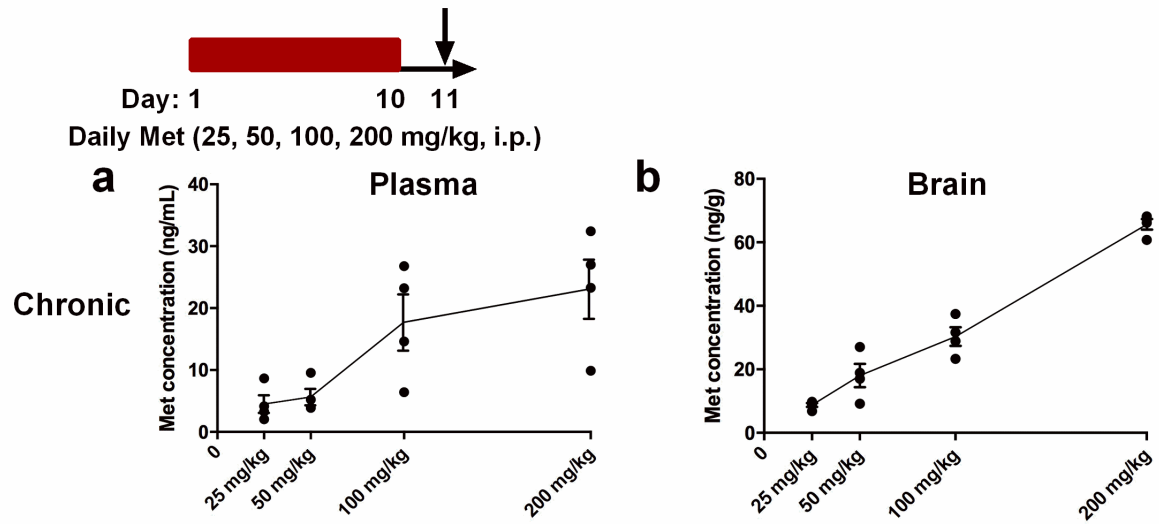
Supplementary Figure 2 Metformin concentrations *in vivo* in plasma and brain after a 10-day chronic metformin treatment. The concentrations of metformin were measured by LC-MS/MS in the plasma (**a**), and brain (**b**) 24 h after last metformin injection (25, 50, 100, and 200 mg/kg, i.p.) (n = 4 in each group).
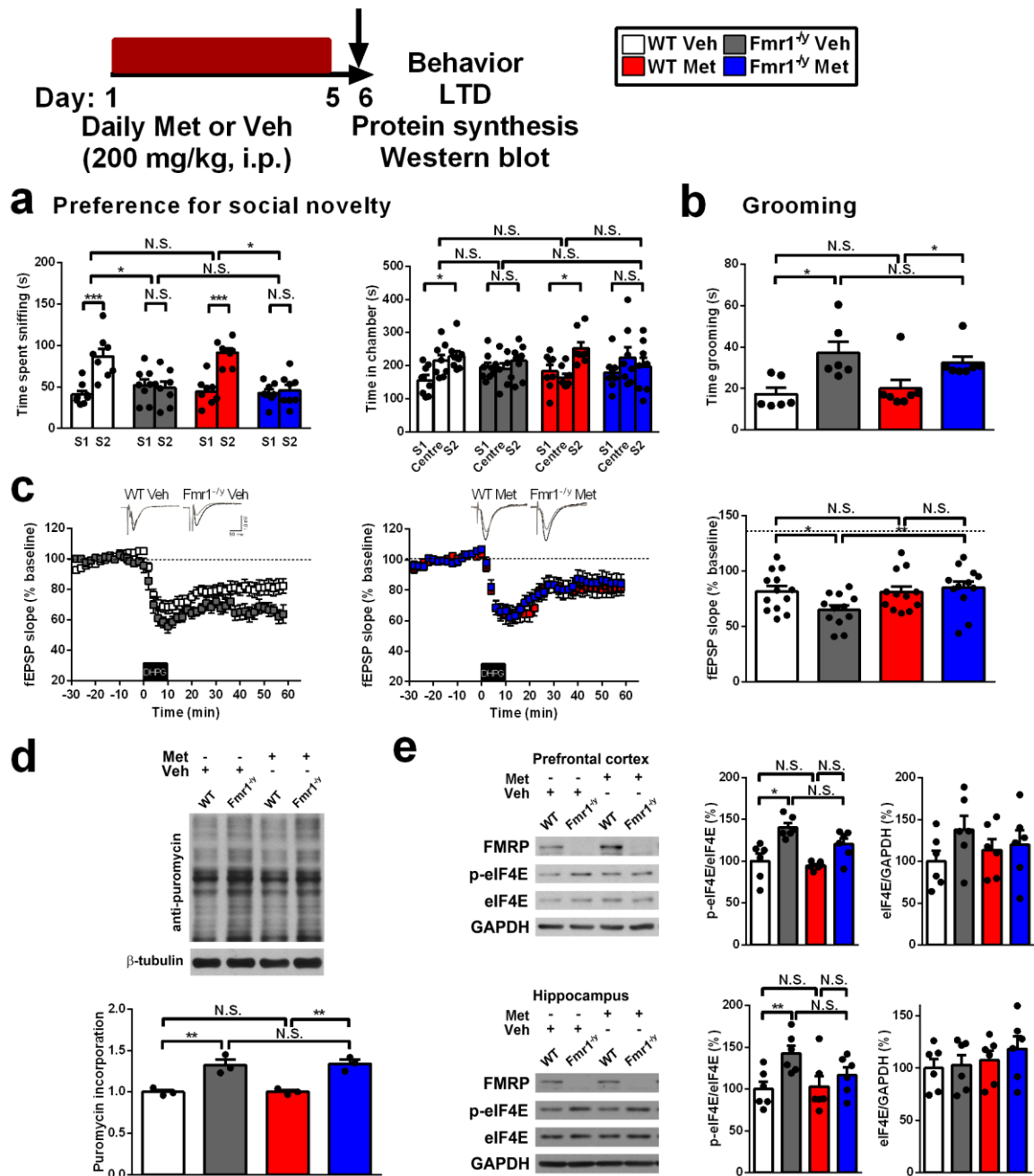
Supplementary Figure 3 Five-day metformin treatment (200 mg/kg/day, i.p.) does not rescue impaired social and grooming behavior, general translation, and phosphorylated eIF4E in *Fmr1⁻/y* mice. (**a**) Preference for social novelty was assessed in the three-chamber social interaction test by measuring 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⁻/y* (n = 6) mice, and metformin-treated WT (n = 7) and *Fmr1⁻/y* (n

= 7) mice. (**c**) Time-plots of mGluR-LTD measured in CA1 in response to DHPG (50 μM for 10 min) in slices prepared from vehicle-treated WT (n = 13) and *Fmr1*$^{-/y}$ (n = 12) mice, and metformin-treated WT (n = 12) and *Fmr1*$^{-/y}$ (n = 12) mice. Quantification (right) of mGluR-LTD during the last 10 min of recording. Exaggerated mGluR-LTD in metformin-treated *Fmr1*$^{-/y}$ mice was rescued. (**d**) Western blots of lysates from hippocampal slices incubated with puromycin to measure basal rates of protein synthesis and β-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*$^{-/y}$ 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 ± 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.
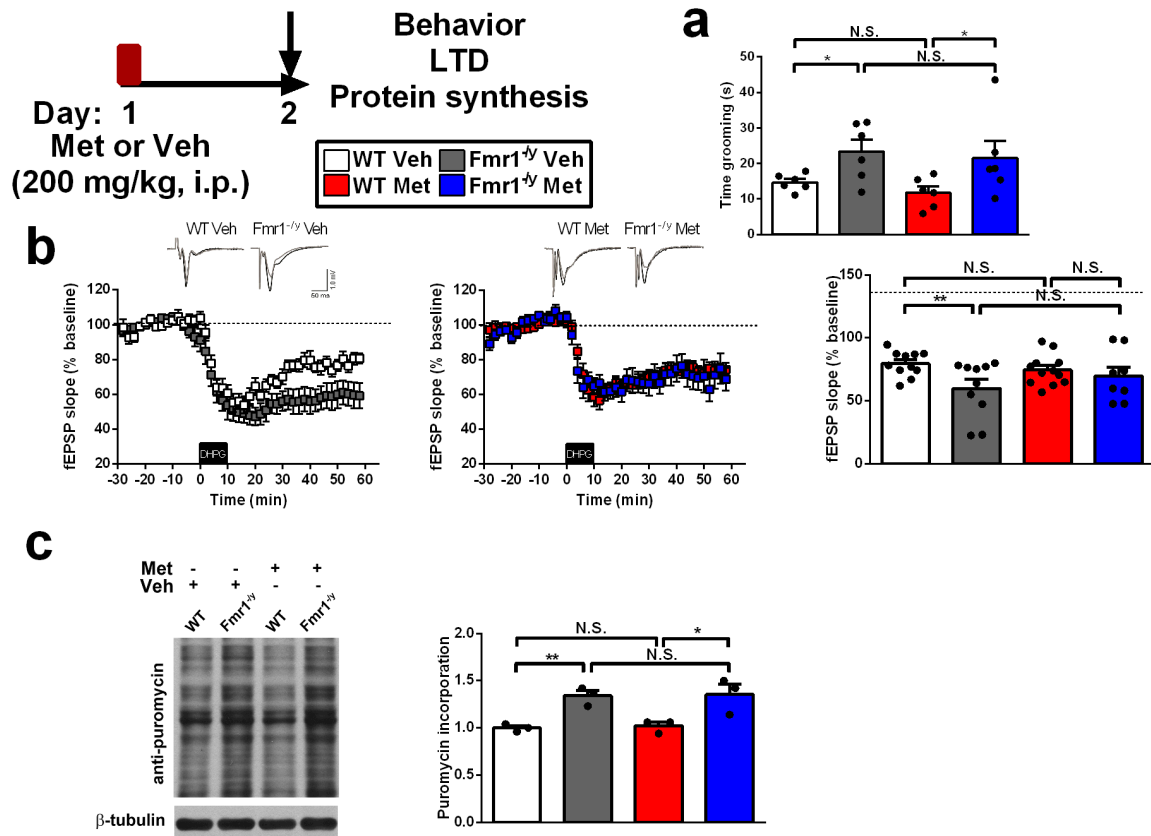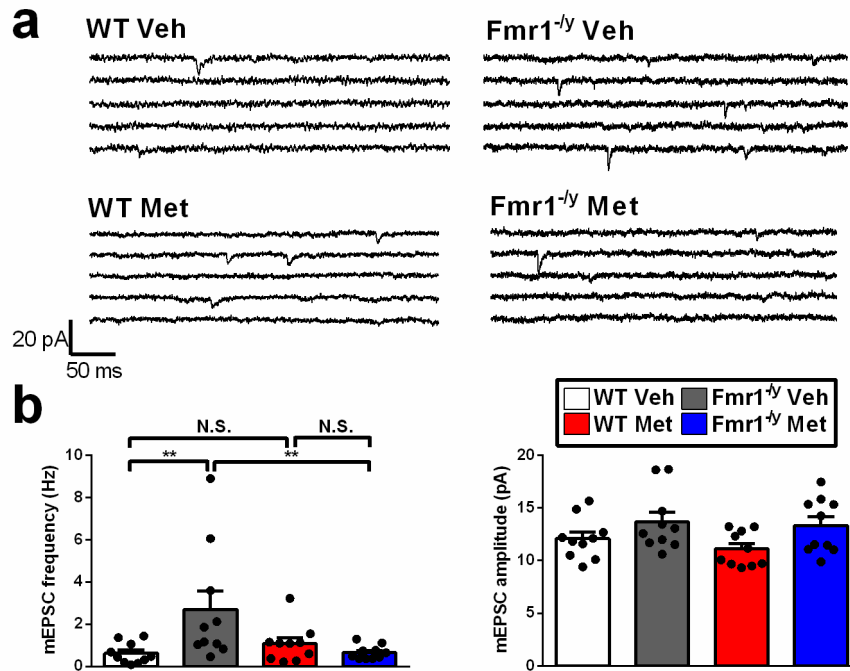
Supplementary Figure 4 One-day metformin treatment (200 mg/kg/day, i.p.) did not rescue increased grooming, exaggerated LTD, and increased general translation in *Fmr1$^{-/y}$* mice. (**a**) Self-grooming test with total time spent grooming; calculated by two-way ANOVA with Tukey's *post hoc* test. n = 6 mice for each group. (**b**) Time-plots of mGluR-LTD measured in CA1 in response to DHPG (50 μM for 10 min) in slices prepared from vehicle-treated WT (n = 11) and *Fmr1$^{-/y}$* (n = 10) mice, and metformin-treated WT (n = 12) and *Fmr1$^{-/y}$* (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 β-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 ± 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.
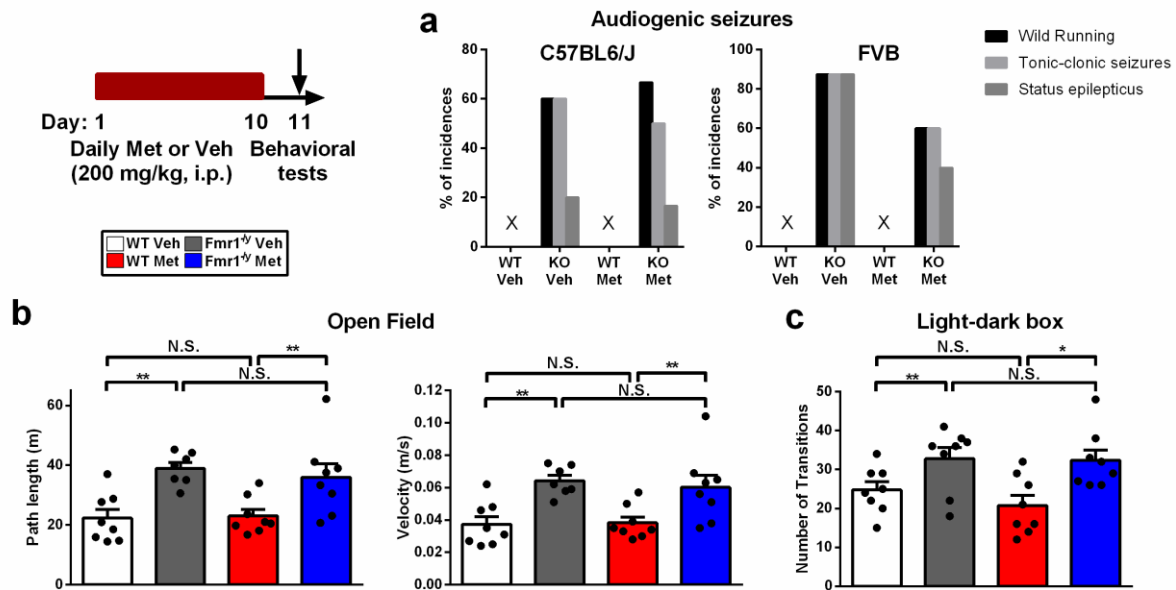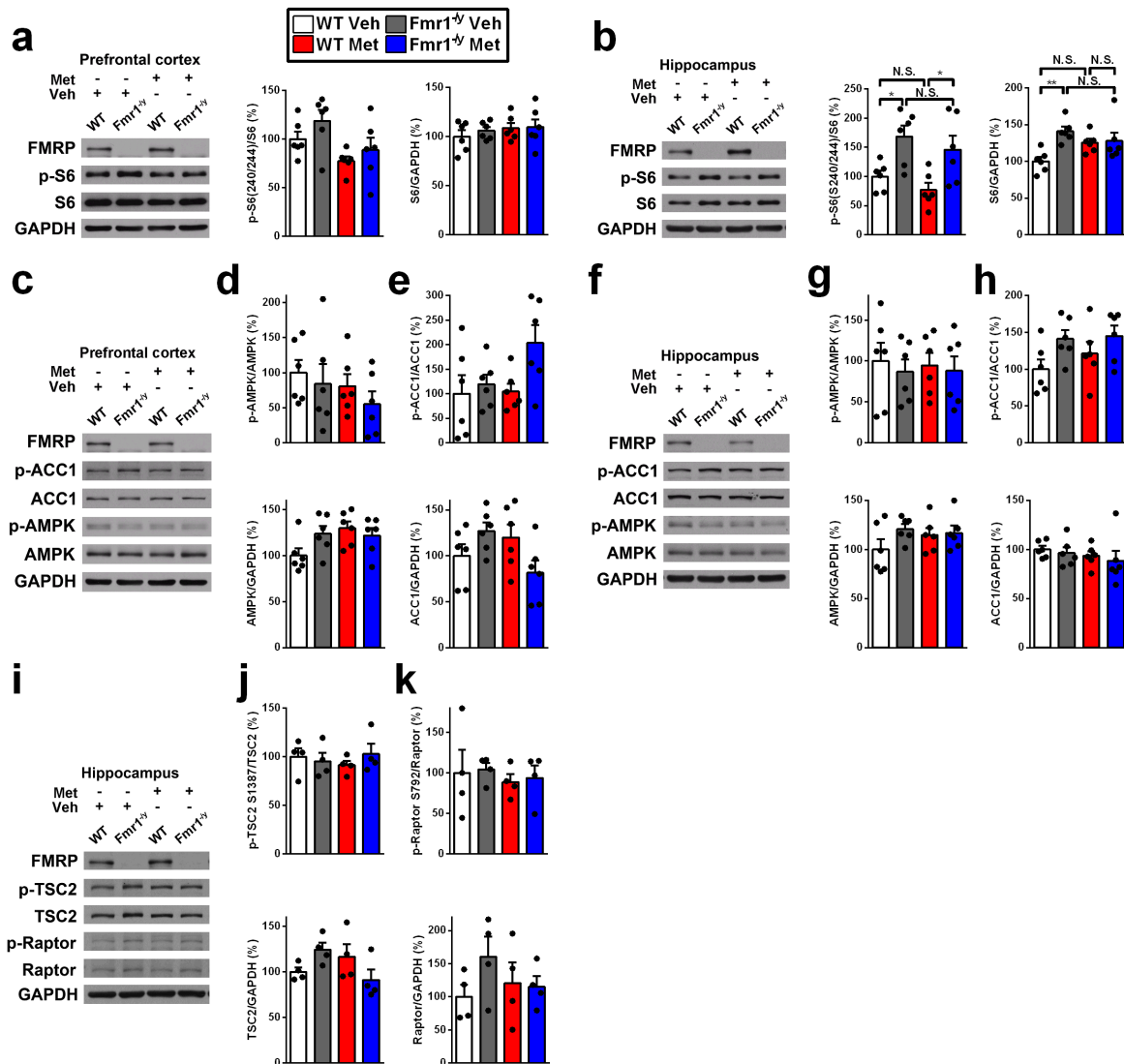
Supplementary Figure 5 Effect of chronic metformin treatment (200 mg/kg/day, 10 days, i.p.) in *Fmr1^{-/y}* and WT mice on audiogenic seizures and hyperactivity. Audiogenic seizures (**a**) were tested on *Fmr1^{-/y}* and WT mice in a C57BL6/J (**a, left panel**) (vehicle-treated WT (n = 2) and *Fmr1^{-/y}* (n = 5) mice, and metformin-treated WT (n = 6) and *Fmr1^{-/y}* (n = 6) mice) and FVB background (**a, right panel**) (vehicle-treated WT (n = 7) and *Fmr1^{-/y}* (n = 8) mice, and metformin-treated WT (n = 7) and *Fmr1^{-/y}* (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^{-/y}* mice showed reduced occurrence of audiogenic seizures. Vehicle-treated *Fmr1^{-/y}* 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^{-/y}* (n = 7) mice, and metformin-treated WT (n = 8) and *Fmr1^{-/y}* (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^{-/y}* 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.
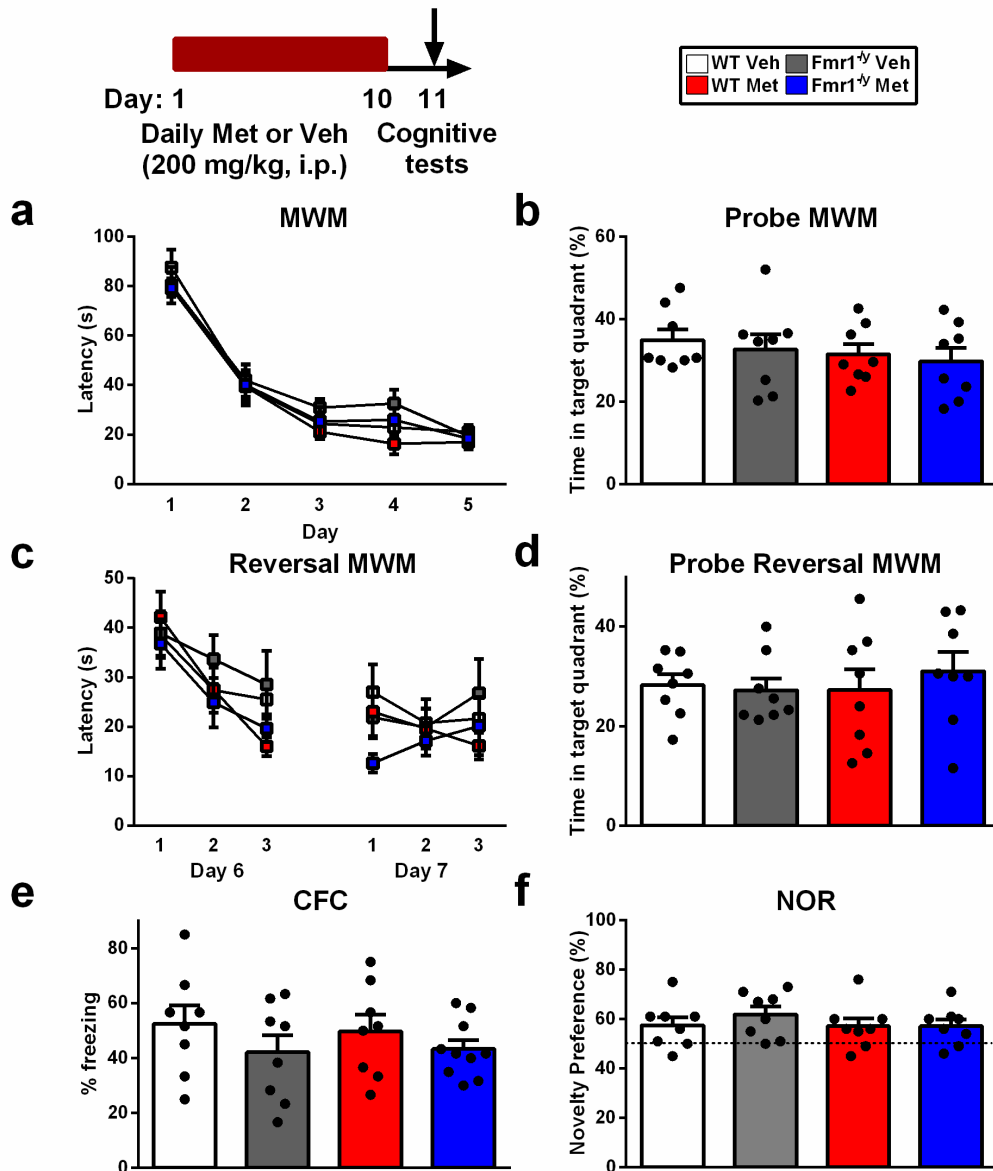
Supplementary Figure 6 Three behavioral tasks to study cognition in 10-day metformin-treated (200 mg/kg, i.p.) *Fmr1⁻/y* and WT mice (C57BL6/J). Vehicle-treated *Fmr1⁻/y* mice did not show a significant cognitive impairment compared to vehicle-treated WT mice in spatial learning during the 5-day acquisition (**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⁻/y* and WT mice. Vehicle-treated WT (n = 8) and *Fmr1⁻/y* (n = 8) mice, and metformin-treated WT (n = 8) and *Fmr1⁻/y* (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⁻/y* mice. (**a**) Representative traces of mEPSCs from pyramidal cells in hippocampal slice cultures from WT and *Fmr1⁻/y* mice treated with vehicle or 50 μM 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⁻/y* neurons (2.71 ± 0.87 Hz) as compared to WT neurons (0.62 ± 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*$^{-/y}$ mice. Representative immunoblots and blot quantification of prefrontal cortex (**a**) and hippocampal (**b**) lysates from vehicle- and metformin-treated WT and *Fmr1*$^{-/y}$ 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*$^{-/y}$ 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*$^{-/y}$ 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 ± 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.
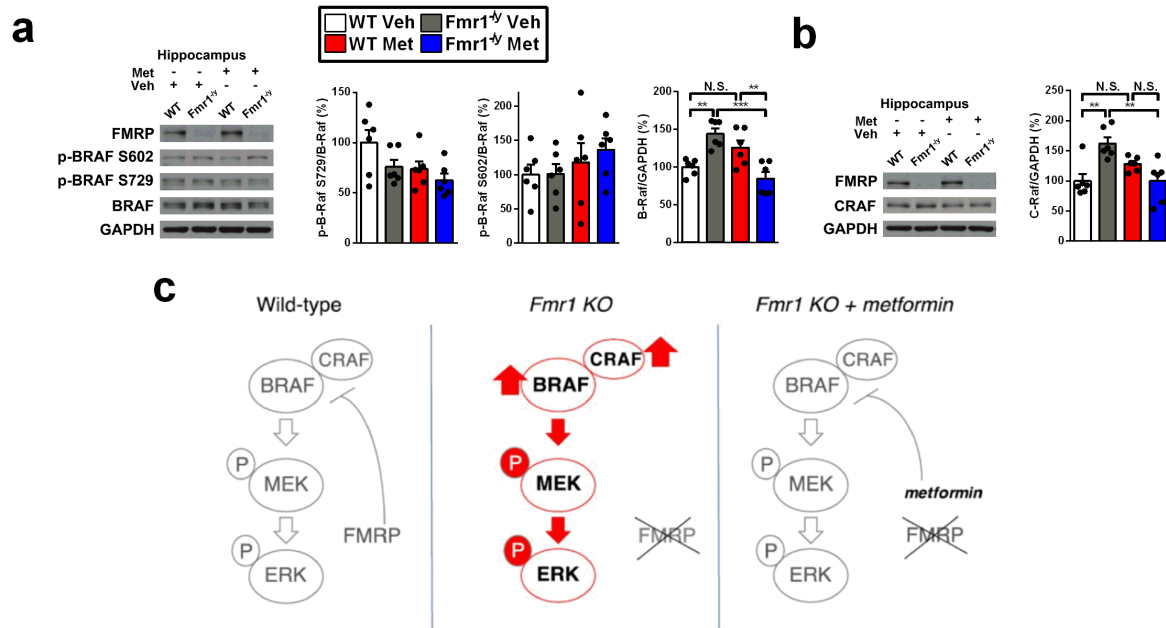
Supplementary Figure 9 Chronic metformin treatment reduced total B-Raf and C-Raf proteins in the hippocampus of *Fmr1$^{-/y}$* mice. Representative immunoblots and blot quantification of total and phosphorylated B-Raf (**a**), and total C-Raf (**b**) from vehicle- and metformin-treated WT and *Fmr1$^{-/y}$* 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 ± 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$^{-/y}$* mouse.
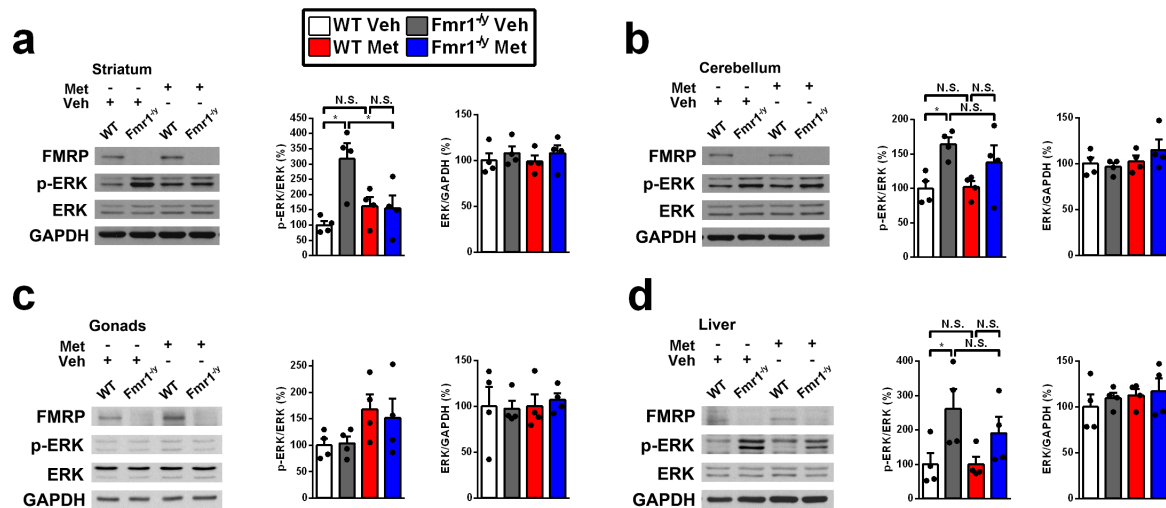
Supplementary Figure 10 Chronic metformin treatment reduced phosphorylated ERK in the striatum, but not in cerebellum, gonads and liver of *Fmr1$^{-/y}$* mice. Representative immunoblots and blot quantification of total and phosphorylated ERK in the striatum (**a**), cerebellum (**b**), gonads (**c**), and liver (**d**) from vehicle- and metformin-treated WT and *Fmr1$^{-/y}$* 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 ± 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.
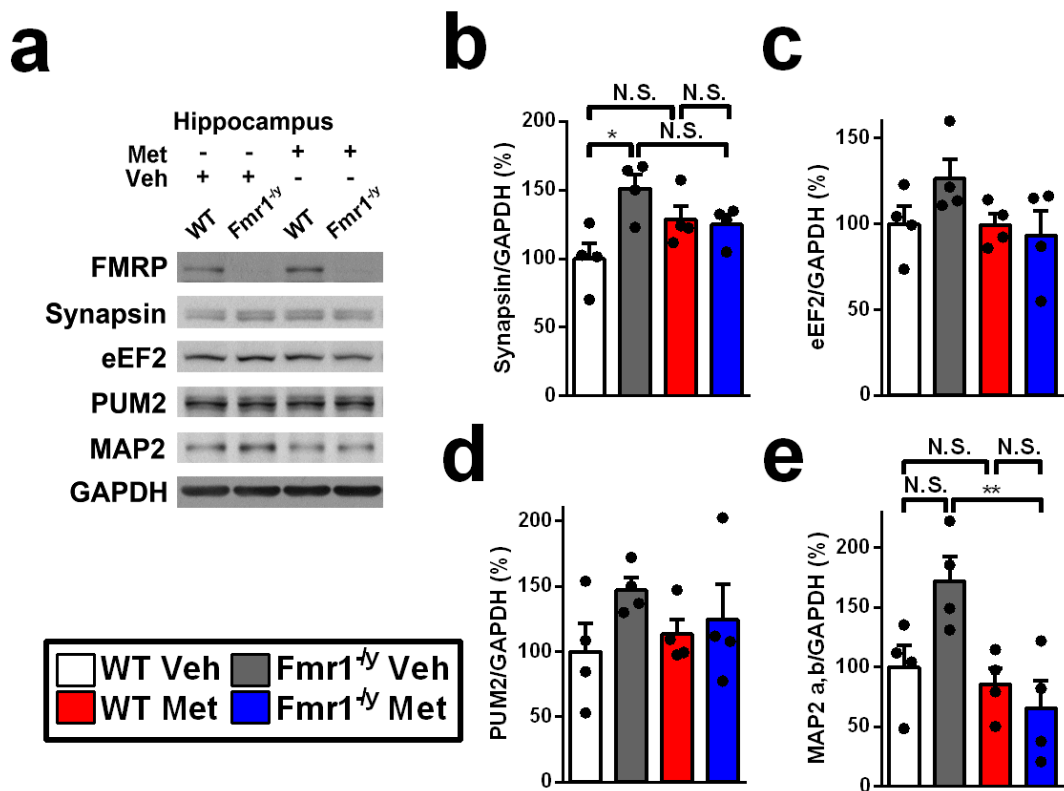
**Supplementary Figure 11** Chronic metformin treatment reduced levels of FMRP targets, synapsin and MAP2 a,b in the hippocampus of *Fmr1$^{-/y}$* mice. Representative immunoblots (**a**) and blot quantification of synapsin (**b**), eEF2 (**c**), PUM2 (**d**) and MAP2 a,b (**e**) in the hippocampus from vehicle- and metformin-treated WT and *Fmr1$^{-/y}$* 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 ± 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.
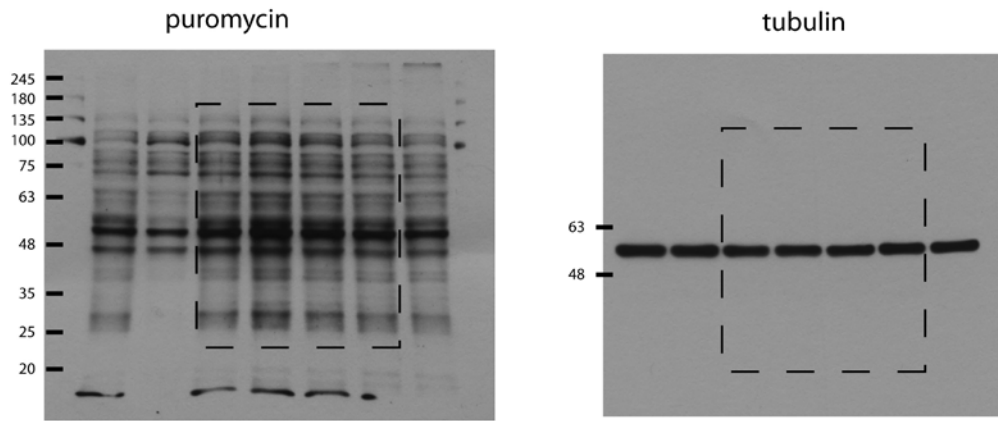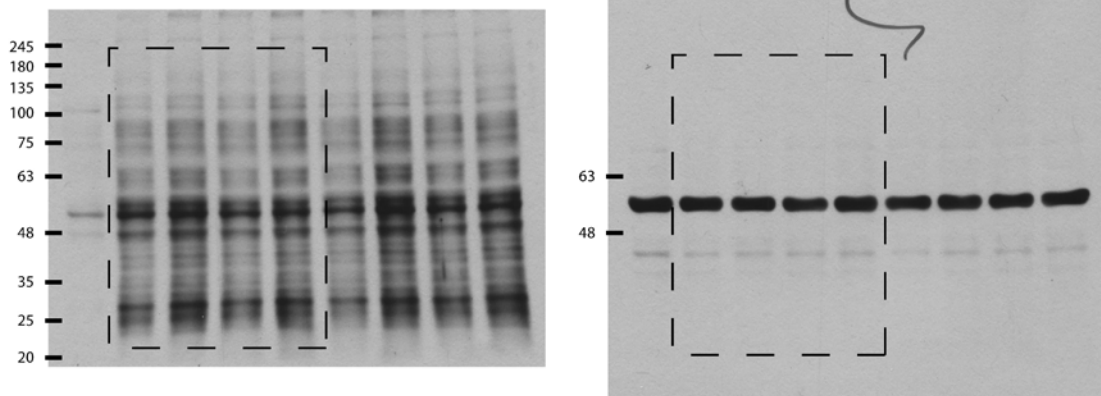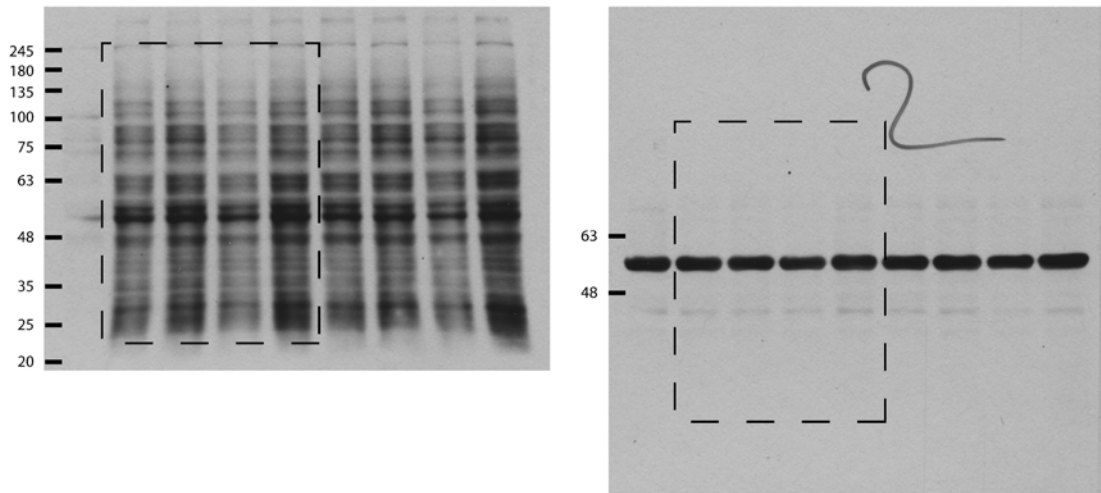
Supplementary Figure 12 Original images of representative western blots of *de novo* protein synthesis
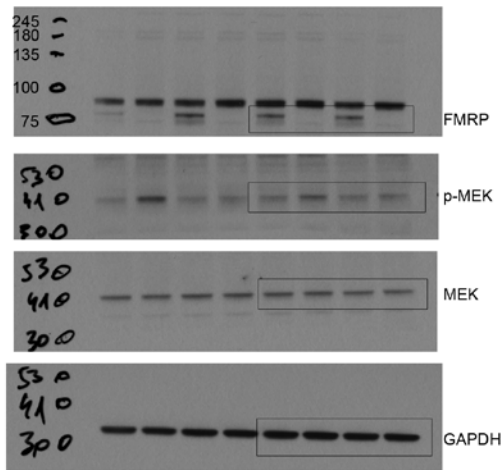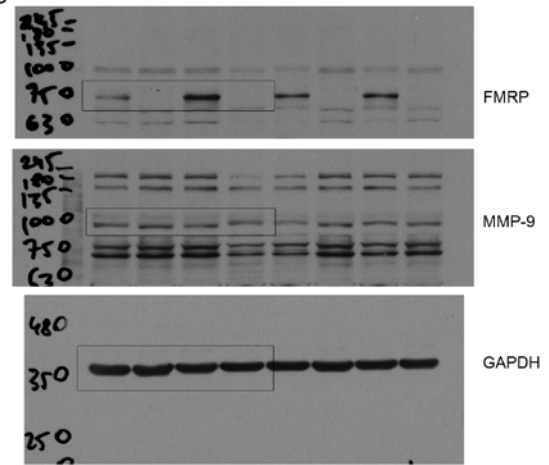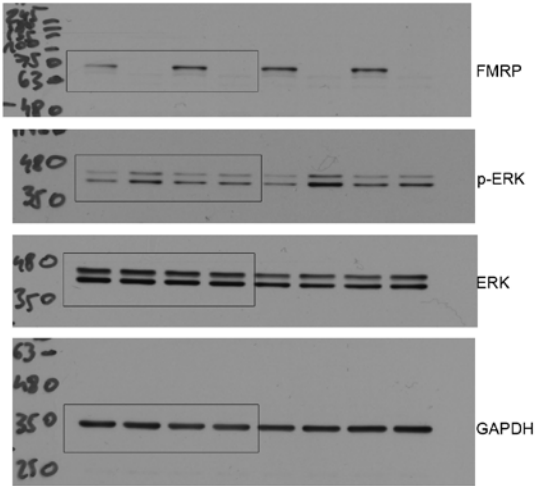
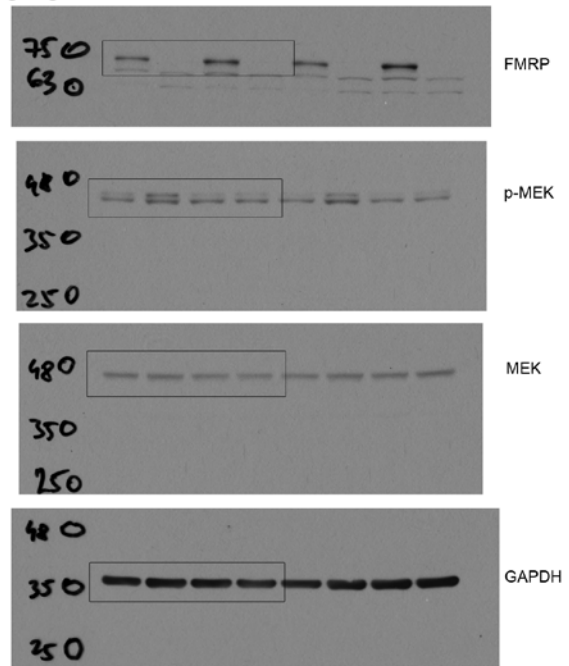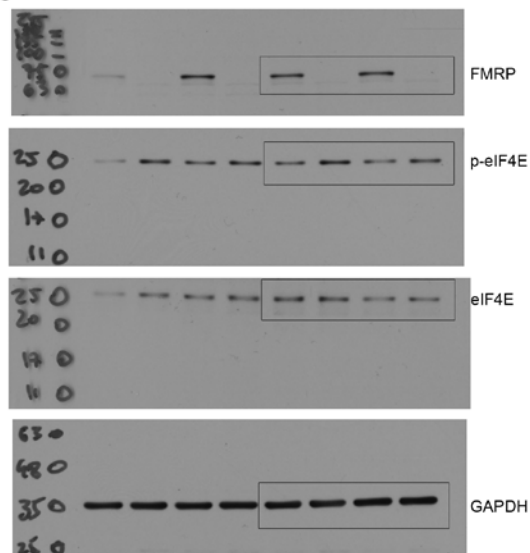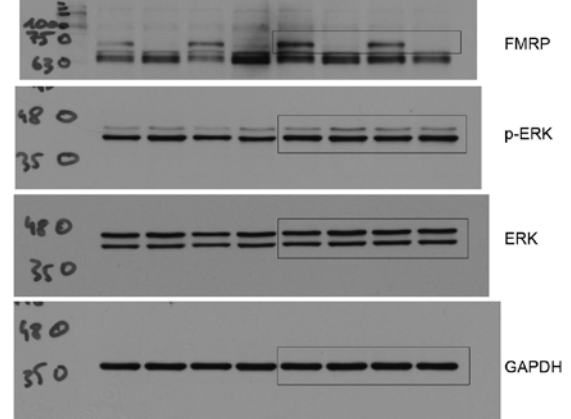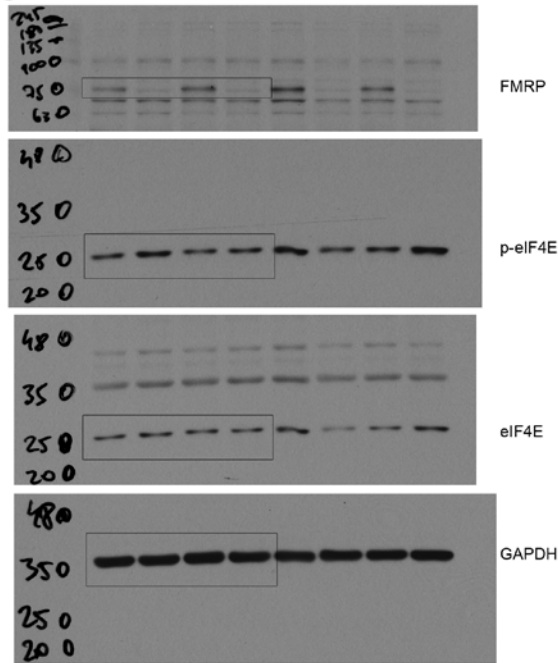**Supplementary Figure 13** Original images of representative western blots in Fig. 2
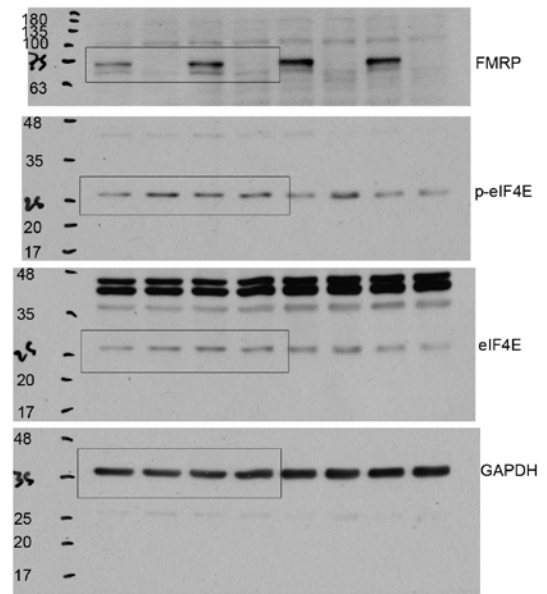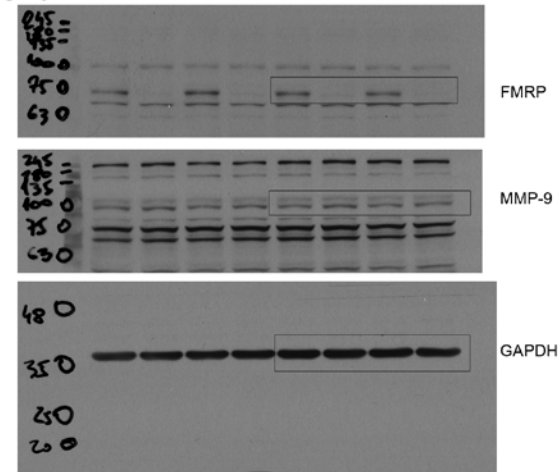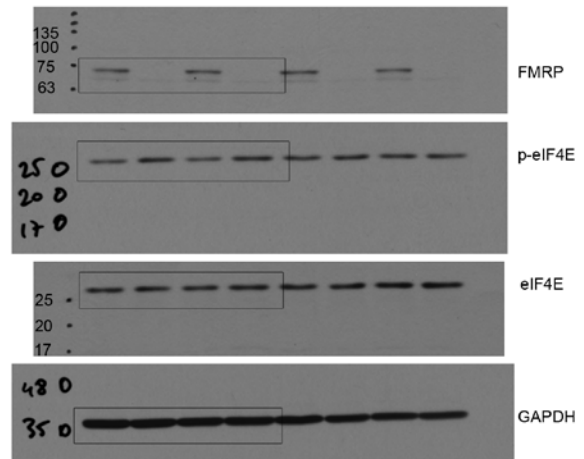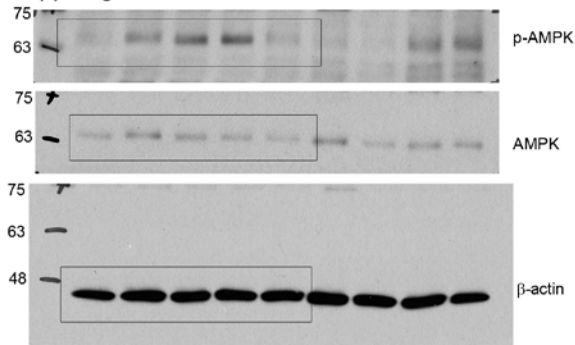
Supplementary Figure 14 Original images of representative western blots in Fig. 2, and Supplementary Figs. 1 and 3
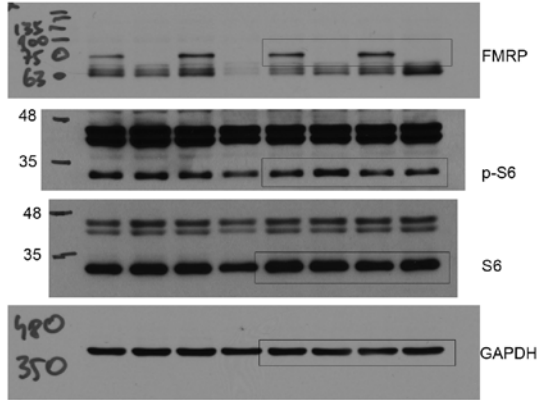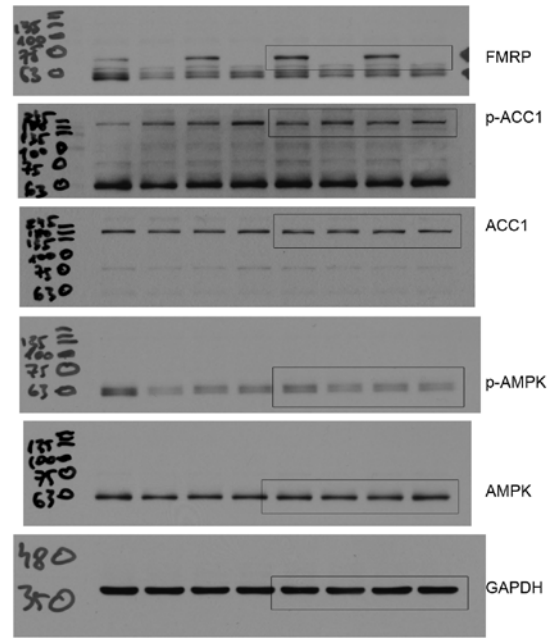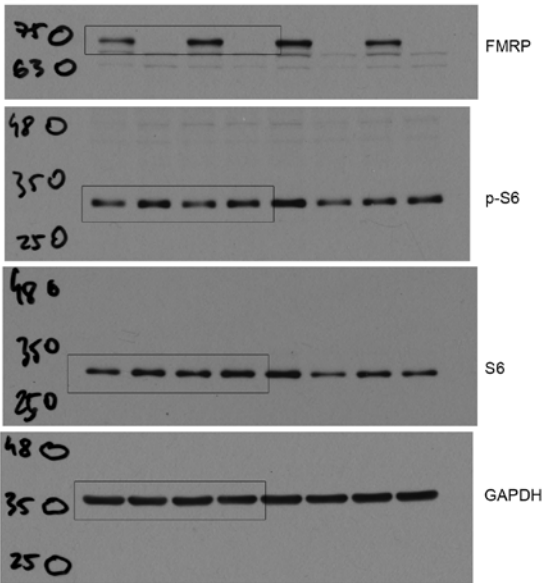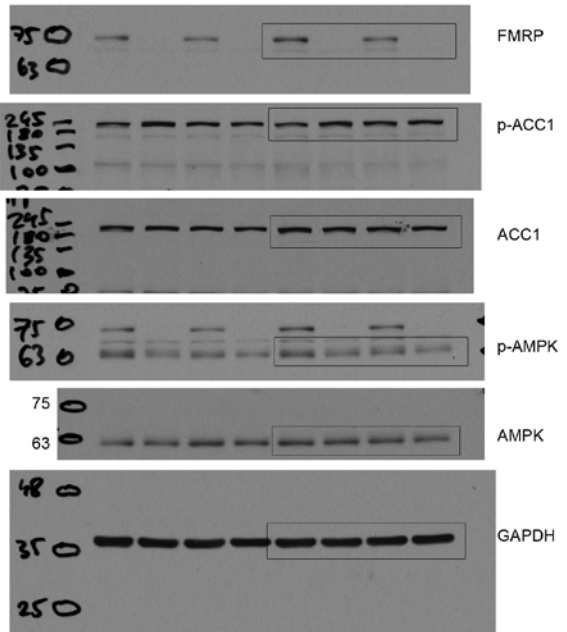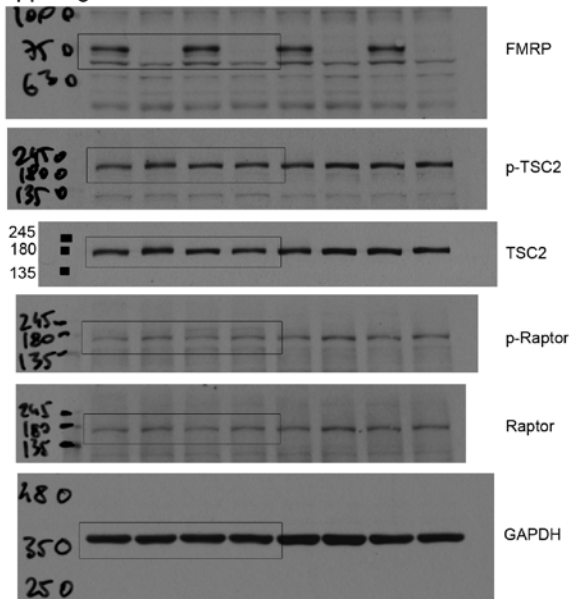
Supplementary Figure 15 Original images of representative western blots in Supplementary Fig. 8

Supp. Fig. 9a

FMRP

p-BRAF
S602

p-BRAF
S729

BRAF

GAPDH

Supp. Fig. 9b

FMRP

CRAF

GAPDH

Supp. Fig10a

FMRP

p-ERK

ERK

GAPDH

Supp. Fig. 10b

FMRP

p-ERK

ERK

GAPDH

Supp. Fig. 10c

FMRP

p-ERK

ERK

GAPDH

Supp. Fig. 10d

FMRP

p-ERK

ERK

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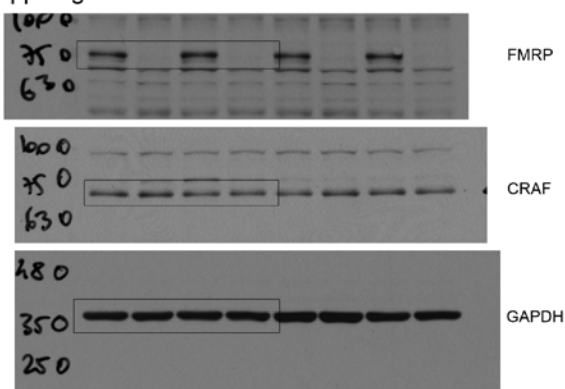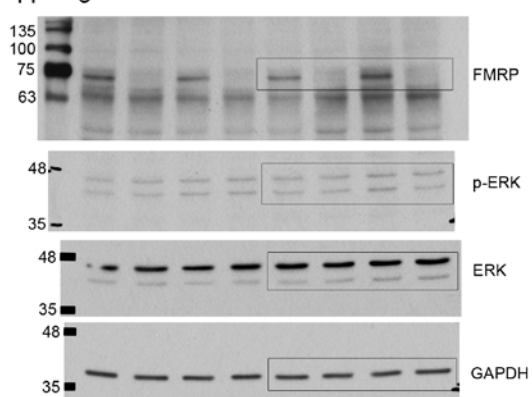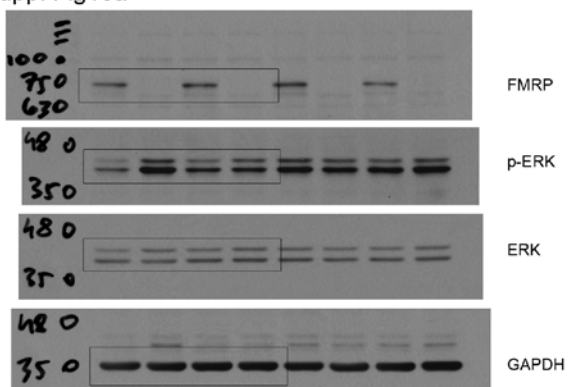
Supplementary Figure 16 Original images of representative western blots in Supplementary Figs. 9 and 10

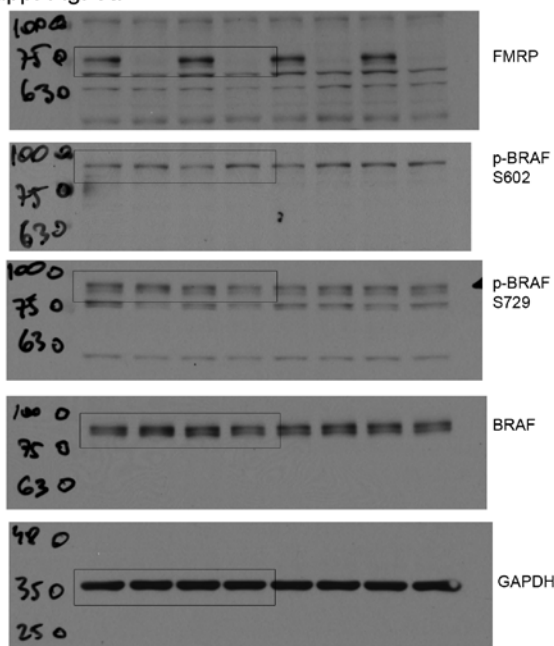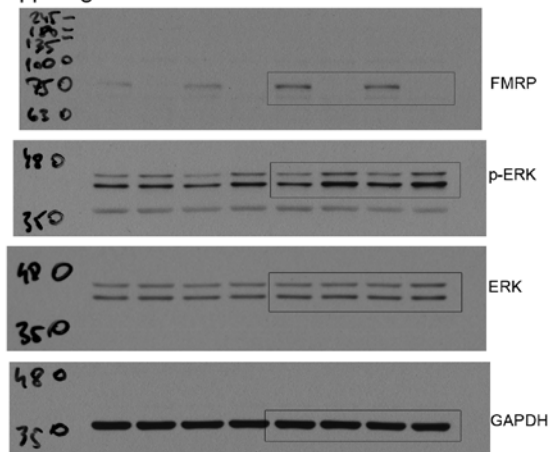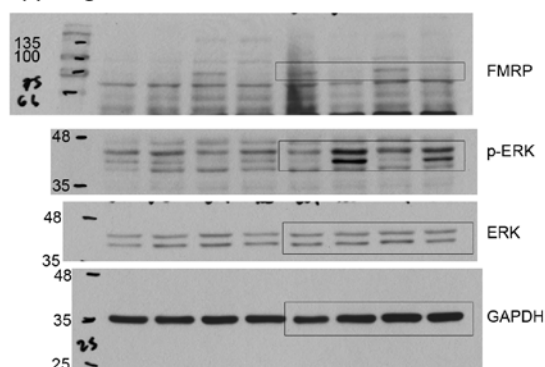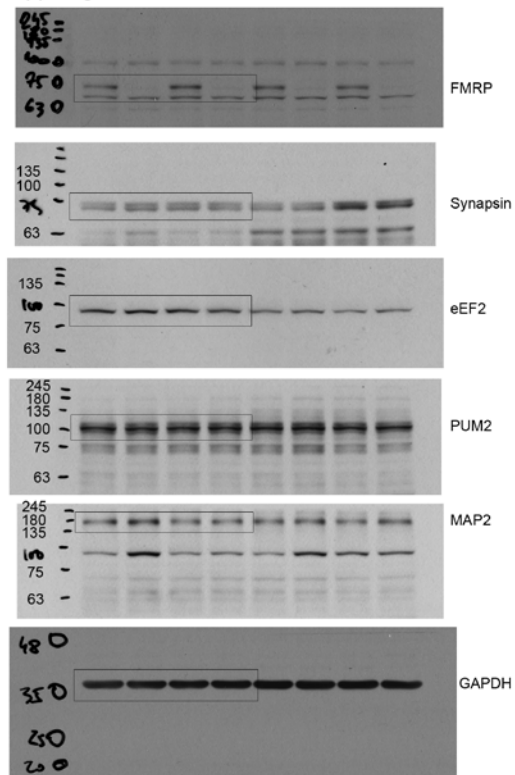**Supplementary Figure 17** Original images of representative western blots in Supplementary Fig. 11

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.
1b, c: Preference for social novelty WT Veh (n = 10) Fmr1$^{-/y}$ Veh (n = 10) WT Met (n = 9) Fmr1$^{-/y}$ Met (n = 12)	Two-way mixed ANOVA *Time sniffing* Chamber: $F_{(1,37)} = 48.173$, $p < 0.001$ Group: $F_{(3,37)} = 2.329$, $p = 0.090$ Chamber x Group: $F_{(3,37)} = 4.392$, $p = 0.010$	*Post hoc* Tukey's test: WT Veh vs KO Veh, $p = 0.006$ WT Met vs KO Met, $p = 0.971$ WT Veh vs WT Met, $p = 0.963$ KO Veh vs KO Met, $p = 0.005$	S1: WT Veh 35 ± 4 KO Veh 73 ± 13 WT Met 40 ± 5 KO Met 36 ± 6 S2: WT Veh 79 ± 6 KO Veh 77 ± 11 WT Met 85 ± 10 KO Met 68 ± 7
	Time in chamber Chamber: $F_{(1,37)} = 25.241$, $p < 0.001$ Group: $F_{(3,37)} = 3.600$, $p = 0.022$ Chamber x Group: $F_{(3,37)} = 0.456$, $p = 0.715$	*Post hoc* Tukey's test: WT Veh vs KO Veh, $p = 0.013$ WT Met vs KO Met, $p = 0.968$ WT Veh vs WT Met, $p = 0.997$ KO Veh vs KO Met, $p = 0.017$	S1: WT Veh 153 ± 10 KO Veh 204 ± 14 WT Met 171 ± 10 KO Met 159 ± 9 Centre: WT Veh 235 ± 19 KO Veh 159 ± 18 WT Met 212 ± 17 KO Met 219 ± 15 S2: WT Veh 212 ± 16 KO Veh 237 ± 15 WT Met 217 ± 11 KO Met 222 ± 15
1d, e: Grooming WT Veh (n = 10) Fmr1$^{-/y}$ Veh (n = 10) WT Met (n = 8) Fmr1$^{-/y}$ Met (n = 12)	Two-way ANOVA *Time* Genotype: $F_{(1,36)} = 10.662$, $p = 0.002$ Treatment: $F_{(1,36)} = 4.613$, $p = 0.039$ Genotype x Treatment: $F_{(1,36)} = 4.782$, $p = 0.035$	*Post hoc* Tukey's test: WT Veh vs KO Veh, $p < 0.001$ WT Met vs KO Met, $p = 0.455$ WT Veh vs WT Met, $p = 0.979$ KO Veh vs KO Met, $p = 0.003$	WT Veh 18.1 ± 3.0 KO Veh 38.5 ± 2.5 WT Met 18.2 ± 4.8 KO Met 22.3 ± 4.1
	Number of bouts Genotype: $F_{(1,36)} = 11.672$, $p = 0.002$ Treatment: $F_{(1,36)} = 4.767$, $p = 0.036$ Genotype x Treatment: $F_{(1,36)} = 11.386$, $p = 0.002$	*Post hoc* Tukey's test: WT Veh vs KO Veh, $p < 0.001$ WT Met vs KO Met, $p = 0.977$ WT Veh vs WT Met, $p = 0.428$	WT Veh 6.2 ± 0.6 KO Veh 12.9 ± 1.2 WT Met 7.4 ± 1.3

		KO Veh vs KO Met, p < 0.001	KO Met 7.3 ± 0.9
1g, h: Spine density

WT Veh (n = 4)
Fmr1$^{-/y}$ Veh (n = 4)
WT Met (n = 4)
Fmr1$^{-/y}$ Met (n = 4) | Two-way ANOVA

Nr of spines
Genotype: F(1,32) = 26.741, p < 0.001
Treatment: F(1,32) = 6.000, p = 0.02
Genotype x Treatment: F(1,32) = 16.667, p < 0.001 | *Post hoc* Tukey's test:
WT Veh vs KO Veh, p < 0.001
WT Met vs KO Met, p = 0.447
WT Veh vs WT Met, p = 0.257
KO Veh vs KO Met, p < 0.001 | WT Veh 15.3 ± 0.5
KO Veh 21.0 ± 0.8
WT Met 16.3 ± 0.6
KO Met 17.0 ± 0.6 |
| | *Mushroom*
Genotype: F(1,32) = 108.587, p < 0.001
Treatment: F(1,32) = 49.390, p < 0.001
Genotype x Treatment: F(1,32) = 77.527, p < 0.001 | *Post hoc* Tukey's test:
WT Veh vs KO Veh, p < 0.001
WT Met vs KO Met, p = 0.262
WT Veh vs WT Met, p = 0.218
KO Veh vs KO Met, p < 0.001 | WT Veh 31.4 ± 0.8
KO Veh 18.2 ± 0.8
WT Met 30.2 ± 0.7
KO Met 29.1 ± 0.3 |
| | *Filopodial*
Genotype: F(1,32) = 71.558, p < 0.001
Treatment: F(1,32) = 23.558, p < 0.001
Genotype x Treatment: F(1,32) = 23.558, p < 0.001 | *Post hoc* Tukey's test:
WT Veh vs KO Veh, p < 0.001
WT Met vs KO Met, p = 0.016
WT Veh vs WT Met, p = 1.000
KO Veh vs KO Met, p < 0.001 | WT Veh 5.1 ± 0.3
KO Veh 10.4 ± 0.4
WT Met 5.1 ± 0.3
KO Met 6.6 ± 0.6 |
| **1k**: LTD

WT Veh (n = 9)
Fmr1$^{-/y}$ Veh (n = 17)
WT Met (n = 9)
Fmr1$^{-/y}$ Met (n = 15) | Two-way ANOVA

Genotype: F(1,46) = 1.075, p = 0.305
Treatment: F(1,46) = 0.457, p = 0.503
Genotype x Treatment: F(1,46) = 4.222, p < 0.046 | *Post hoc* Tukey's test:
WT Veh vs KO Veh, p = 0.032
WT Met vs KO Met, p = 0.480
WT Veh vs WT Met, p = 0.393
KO Veh vs KO Met, p = 0.028 | WT Veh 75 ± 9
KO Veh 55 ± 5
WT Met 66 ± 8
KO Met 72 ± 5 |
| **2a**: Testicle weight

WT Veh (n = 6)
Fmr1$^{-/y}$ Veh (n = 6)
WT Met (n = 6)
Fmr1$^{-/y}$ Met (n = 7) | Two-way ANOVA

Genotype: F(1,21) = 32.605, p < 0.001
Treatment: F(1,21) = 8.846, p = 0.007
Genotype x Treatment: F(1,21) = 1.596, p = 0.220 | *Post hoc* Tukey's test:
WT Veh vs KO Veh, p < 0.001
WT Met vs KO Met, p = 0.004
WT Veh vs WT Met, p = 0.248
KO Veh vs KO Met, p = 0.006 | WT Veh 149 ± 6
KO Veh 200 ± 5
WT Met 137 ± 6
KO Met 170 ± 8 |
| **2b**: *De novo* protein synthesis

WT Veh (n = 7)
Fmr1$^{-/y}$ Veh (n = 7)
WT Met (n = 7)
Fmr1$^{-/y}$ Met (n = 7) | Two-way ANOVA

Genotype: F(1,24) = 2.454, p = 0.130
Treatment: F(1,24) = 6.198, p = 0.02
Genotype x Treatment: F(1,24) = 3.782, p = 0.064 | *Post hoc* Tukey's test:
WT Veh vs KO Veh, p = 0.021
WT Met vs KO Met, p = 0.792
WT Veh vs WT Met, p = 0.704
KO Veh vs KO Met, p = 0.005 | WT Veh 1.00 ± 0.01
KO Veh 1.29 ± 0.09
WT Met 0.95 ± 0.04
KO Met 0.92 ± 0.12 |
| **2c-j**: Western blot
For all data except for MMP-9 in prefrontal cortex:

WT Veh (n = 6) | Two-way ANOVA
Prefrontal cortex

p-MEK/MEK
Genotype: F(1,20) = 8.693, p = 0.008 | *Post hoc* Tukey's test:

WT Veh vs KO Veh, p = 0.027
WT Met vs KO Met, p = 0.705 | WT Veh 100 ± 14
KO Veh 162 ± 17 |

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

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

2 h (n = 3) 4 h (n = 3)			
Supplementary 2a, 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 *Plasma* Time: $F(3,12) = 7.017$, $p = 0.006$ *Brain* Time: $F(3,12) = 98.789$, $p < 0.001$	*Post hoc* Tukey's test: 25 vs 50, $p = 0.996$ 25 vs 100, $p = 0.078$ 25 vs 200, $p = 0.012$ *Post hoc* Tukey's test: 25 vs 50, $p = 0.091$ 25 vs 100, $p < 0.001$ 25 vs 200, $p < 0.001$	
Supplementary 3a: Preference for social novelty WT Veh (n = 8) Fmr1$^{-/y}$ Veh (n = 8) WT Met (n = 8) Fmr1$^{-/y}$ Met (n = 8)	Two-way mixed ANOVA *Time sniffing* Chamber: $F(1,28) = 30.489$, $p < 0.001$ Group: $F(3,28) = 5.016$, $p = 0.007$ Chamber x Group: $F(3,28) = 10.210$, $p < 0.001$ *Time in chamber* Chamber: $F(1,28) = 8.850$, $p = 0.006$ Group: $F(3,28) = 1.735$, $p = 0.183$ Chamber x Group: $F(3,28) = 0.964$, $p = 0.424$	*Post hoc* 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, $p = 0.821$ *Post hoc* 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, $p = 0.694$	
Supplementary 3b 5-day metformin grooming WT Veh (n = 6) Fmr1$^{-/y}$ Veh (n = 6) WT Met (n = 7) Fmr1$^{-/y}$ Met (n = 7)	Two-way ANOVA *Time grooming* Genotype: $F(1,20) = 14.904$, $p < 0.001$ Treatment: $F(1,20) = 0.004$, $p = 0.947$ Genotype x Treatment: $F(1,20) = 1.020$, $p = 0.325$	*Post hoc* 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, $p = 0.427$	
Supplementary 3c 5-day metformin LTD WT Veh (n = 13) Fmr1$^{-/y}$ Veh (n = 12) WT Met (n = 12) Fmr1$^{-/y}$ 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: $F(1,45) = 4.232$, $p = 0.045$	*Post hoc* 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, $p = 0.008$	
Supplementary 3d 5-day metformin *De novo* protein synthesis WT Veh (n = 3) Fmr1$^{-/y}$ 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: $F(1,8) = 0.023$, $p = 0.883$	*Post hoc* 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$^{-/y}$ Met (n = 3)			
Supplementary 3e 5-day metformin Western blot WT Veh (n = 6) Fmr1$^{-/y}$ Veh (n = 6) WT Met (n = 6) Fmr1$^{-/y}$ Met (n = 6)	Two-way ANOVA *Prefrontal cortex* *p-eIF4E/eIF4E* 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 *Hippocampus* *p-eIF4E/eIF4E* 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	*Post hoc* 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 *Post hoc* 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	
Supplementary 4a 1-day metformin grooming WT Veh (n = 6) Fmr1$^{-/y}$ Veh (n = 6) WT Met (n = 6) Fmr1$^{-/y}$ Met (n = 6)	Two-way ANOVA *Time grooming* 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	*Post hoc* 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	
Supplementary 4b 1-day metformin LTD WT Veh (n = 11) Fmr1$^{-/y}$ Veh (n = 10) WT Met (n = 12) Fmr1$^{-/y}$ Met (n = 8)	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	*Post hoc* 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	
Supplementary 4c 1-day metformin *De novo* protein synthesis WT Veh (n = 3) Fmr1$^{-/y}$ Veh (n = 3) WT Met (n = 3) Fmr1$^{-/y}$ Met (n = 3)	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	*Post hoc* 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	
Supplementary 5b Open Field WT Veh (n = 8) Fmr1$^{-/y}$ Veh (n = 7) WT Met (n = 8) Fmr1$^{-/y}$ Met (n = 8)	Two-way ANOVA *Path length* 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 *Velocity*	*Post hoc* 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 *Post hoc* Tukey's test:	

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

Supplementary 6f NOR WT Veh (n = 8) Fmr1$^{-/y}$ Veh (n = 8) WT Met (n = 8) Fmr1$^{-/y}$ Met (n = 8)	Two-way ANOVA Genotype: $F_{(1,28)} = 0.488$, $p = 0.491$ Treatment: $F_{(1,28)} = 0.669$, $p = 0.402$ Genotype x Treatment: $F_{(1,28)} = 0.488$, $p = 0.491$		
Supplementary 7b mEPSC WT Veh (n = 10) Fmr1$^{-/y}$ Veh (n = 10) WT Met (n = 10) Fmr1$^{-/y}$ Met (n = 10)	Two-way ANOVA *Frequency* Genotype: $F_{(1,36)} = 3.247$, $p = 0.080$ Treatment: $F_{(1,36)} = 2.952$, $p = 0.094$ Genotype x Treatment: $F_{(1,36)} = 7.204$, $p = 0.011$ *Amplitude* Genotype: $F_{(1,36)} = 6.766$, $p = 0.013$ Treatment: $F_{(1,36)} = 0.864$, $p = 0.359$ Genotype x Treatment: $F_{(1,36)} = 0.185$, $p = 0.670$	*Post hoc* Tukey's test: WT Veh vs KO Veh, $p = 0.003$ WT Met vs KO Met, $p = 0.537$ WT Veh vs WT Met, $p = 0.499$ KO Veh vs KO Met, $p = 0.004$ *Post hoc* Tukey's test: WT Veh vs KO Veh, $p = 0.134$ WT Met vs KO Met, $p = 0.039$ WT Veh vs WT Met, $p = 0.343$ KO Veh vs KO Met, $p = 0.726$	
Supplementary 8a-k: Western blot For all data except for p-TSC2 and p-Raptor in hippocampus: WT Veh (n = 6) Fmr1$^{-/y}$ Veh (n = 6) WT Met (n = 6) Fmr1$^{-/y}$ Met (n = 6)	Two-way ANOVA *Prefrontal cortex* *p-S6/S6* Genotype: $F_{(1,20)} = 2.594$, $p = 0.123$ Treatment: $F_{(1,20)} = 7.681$, $p = 0.012$ Genotype x Treatment: $F_{(1,20)} = 0.152$, $p = 0.701$ *p-AMPK/AMPK* Genotype: $F_{(1,20)} = 0.999$, $p = 0.329$ Treatment: $F_{(1,20)} = 1.353$, $p = 0.258$ Genotype x Treatment: $F_{(1,20)} = 0.060$, $p = 0.809$ *p-ACC1/ACC1* Genotype: $F_{(1,20)} = 4.121$, $p = 0.056$ Treatment: $F_{(1,20)} = 2.326$, $p = 0.143$ Genotype x Treatment: $F_{(1,20)} = 1.851$, $p = 0.189$ *Hippocampus* *p-S6/S6* Genotype: $F_{(1,20)} = 16.293$, $p < 0.001$ Treatment: $F_{(1,20)} = 1.782$, $p = 0.197$ Genotype x Treatment: $F_{(1,20)} = 0.001$, $p = 0.975$	*Post hoc* Tukey's test: WT Veh vs KO Veh, $p = 0.505$ WT Met vs KO Met, $p = 0.824$ WT Veh vs WT Met, $p = 0.358$ KO Veh vs KO Met, $p = 0.148$ *Post hoc* Tukey's test: WT Veh vs KO Veh, $p = 0.047$ WT Met vs KO Met, $p = 0.043$ WT Veh vs WT Met, $p = 0.770$ KO Veh vs KO Met, $p = 0.794$	

		Post hoc Tukey's test:	
	S6/GAPDH	WT Veh vs KO Veh, p = 0.006	
	Genotype: $F_{(1,20)} = 7.909$, p = 0.011	WT Met vs KO Met, p = 0.995	
	Treatment: $F_{(1,20)} = 0.584$, p = 0.454	WT Veh vs WT Met, p = 0.133	
	Genotype x Treatment: $F_{(1,20)} = 6.149$, p = 0.022	KO Veh vs KO Met, p = 0.626	
	p-AMPK/AMPK		
	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		
		Post hoc Tukey's test:	
	p-ACC1/ACC1	WT Veh vs KO Veh, p = 0.180	
	Genotype: $F_{(1,20)} = 5.626$, p = 0.028	WT Met vs KO Met, p = 0.615	
	Treatment: $F_{(1,20)} = 0.835$, p = 0.371	WT Veh vs WT Met, p = 0.698	
	Genotype x Treatment: $F_{(1,20)} = 0.397$, p = 0.536	KO Veh vs KO Met, p = 0.997	
WT Veh (n = 4)			
$Fmr1^{-/y}$ Veh (n = 4)	*p-TSC2/TSC2*		
WT Met (n = 4)	Genotype: $F_{(1,12)} = 0.184$, p = 0.676		
$Fmr1^{-/y}$ Met (n = 4)	Treatment: $F_{(1,12)} = 0.005$, p = 0.947		
	Genotype x Treatment: $F_{(1,12)} = 0.915$, p = 0.358		
WT Veh (n = 4)			
$Fmr1^{-/y}$ Veh (n = 4)	*p-Raptor/Raptor*		
WT Met (n = 4)	Genotype: $F_{(1,12)} = 0.076$, p = 0.788		
$Fmr1^{-/y}$ Met (n = 4)	Treatment: $F_{(1,12)} = 0.384$, p = 0.547		
	Genotype x Treatment: $F_{(1,12)} = 0.000$, p = 0.983		
Supplementary 9a, b:	Two-way ANOVA		
Western blot	*Hippocampus*	*Post hoc* Tukey's test:	
	b-Raf/GAPDH	WT Veh vs KO Veh, p = 0.004	
WT Veh (n = 6)	Genotype: $F_{(1,20)} = 0.044$, p = 0.836	WT Met vs KO Met, p = 0.007	
$Fmr1^{-/y}$ Veh (n = 6)	Treatment: $F_{(1,20)} = 4.555$, p = 0.045	WT Veh vs WT Met, p = 0.124	
WT Met (n = 6)	Genotype x Treatment: $F_{(1,20)} = 29.514$, $p < 0.001$	KO Veh vs KO Met, $p < 0.001$	
$Fmr1^{-/y}$ Met (n = 6)			
	p-b-Raf S729/ b-Raf		
	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-b-Raf S602/ b-Raf		
	Genotype: $F_{(1,20)} = 0.253$, p = 0.620		

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

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