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

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

mTORC1 and ERK pathways, we reasoned that metformin could have beneficial therapeutic effects in *Fmr1*$^{-/y}$ mice[9].

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

Neurons from FXS patients and *Fmr1*$^{-/y}$ mice exhibit aberrant spine morphology[1,11]. We 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

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

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

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

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

METHODS

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

SUPPLEMENTARY INFORMATION

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

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AUTHOR CONTRIBUTIONS

I.G., A.K. and J.P. designed the experiments, performed data analysis and wrote the 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. and C.G.G. designed and carried out experiments. A.S., V.T.T., I.A.G. and K.G. assisted with experiments. K.N. supervised the project. J-C.L., C.G.G. and N.S. supervised the project, designed experiments and edited the manuscript. All authors revised the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1 Chronic metformin treatment corrects social deficit, repetitive behavior, aberrant dendritic spine morphology and exaggerated LTD in *Fmr1*$^{-/y}$ mice. (**a**) Metformin or vehicle was injected i.p. over 10 days (200 mg/kg/day) followed by analysis of social behavior, grooming, dendritic spine morphology, and LTD. 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) (**b**); and time spent in each chamber (**c**). Vehicle-treated WT (n = 10) and *Fmr1*$^{-/y}$ (n = 10), and metformin-treated WT (n = 9) and *Fmr1*$^{-/y}$ (n = 12). (**d**) Self-grooming test with total time spent grooming and (**e**) total number of grooming bouts. Vehicle-treated WT (n = 10) and *Fmr1*$^{-/y}$ (n = 10), and metformin-treated WT (n = 8) and *Fmr1*$^{-/y}$ (n = 12). (**f**) Golgi-cox staining of CA1 dendritic spines in metformin or vehicle-injected WT and *Fmr1*$^{-/y}$ mice. Scale bar: 2 μm. (**g**) Quantification of spine density, measured as the number of spines per 10 μm and (**h**) spine subtype analysis (S/M = spiny/mushroom; F = filopodial) presented as a fraction of total spines for each subtype (n = 4 in each group). mGluR-LTD was measured in CA1 in response to DHPG (50 μm for 10 min) in slices prepared from (**i**) vehicle-treated WT (n = 9) and *Fmr1*$^{-/y}$ (n = 17) mice, and (**j**) metformin-treated WT (n = 9) and *Fmr1*$^{-/y}$ (n = 15) mice. (**k**) fEPSP slope during the last 10 minutes of recording. All values are shown as mean ± s.e.m. ***P < 0.001, **P < 0.01, *P < 0.05 versus all other groups; N.S., not significant; calculated by two-way ANOVA with Tukey's *post hoc* test.

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

vehicle- and metformin-treated WT and *Fmr1*$^{-/y}$ mice. Vehicle-treated WT (n = 6) and *Fmr1*$^{-/y}$ (n = 6), and metformin-treated WT (n = 6) and *Fmr1*$^{-/y}$ (n = 7). (**b**) Western blots of lysates from hippocampal slices incubated with puromycin to measure basal rates of protein synthesis. β-tubulin was used as a loading control. Puromycin incorporation is presented as percentage change relative to vehicle-treated WT slices (n = 7 in each group). Representative western blots of lysates from vehicle- and metformin-treated WT and *Fmr1*$^{-/y}$ mice and quantification of phosphorylation and total levels of (**c**) MEK, (**d**) ERK, (**e**) eIF4E and (**f**) MMP-9 in prefrontal cortex and (**g**) MEK, (**h**) ERK, (**i**) eIF4E and (**j**) MMP-9 in hippocampus. 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 (n = 6 in each group, n = 5 for MMP-9 in prefrontal cortex). All values (**a-h**) are shown as mean ± s.e.m. ***P < 0.001, **P < 0.01, *P < 0.05 versus all other groups; N.S., not significant; calculated by two-way ANOVA with Tukey's *post hoc* test.

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

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

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

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

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

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

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

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

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

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

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

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

LTD recordings. For analysis of hippocampal LTD, male 31- to 34-day-old wildtype or $Fmr1^{-/y}$, treated with either saline or metformin (as described above) were used. After obtaining hippocampal slices (400 μm thickness), CA1 and CA3 hippocampal regions were isolated by a surgical excision and incubated for 2 h at 32°C in oxygenated artificial cerebral spinal fluid for recovery (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose). Later, slices were placed in a

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

Miniature EPSC recordings. Organotypic hippocampal slices were prepared from WT and *Fmr1*$^{-/y}$ mice (postnatal day 4-6). The brain was removed and dissected in Hanks' balanced salt solution (Invitrogen)-based medium. Corticohippocampal slices (400 μm thick) were obtained with a McIlwain tissue chopper (Campden Instruments). Slices were placed on Millicell culture plate inserts (Millipore) and incubated in OptiMem (Invitrogen)-based medium in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C.

Experiments were performed after 14-20 days in culture. Cultures were treated with metformin (50 μM) or vehicle (Optimem media) for 4-5 days before electrophysiology experiments which were performed blinded to treatment. Whole-cell recordings were obtained from CA1 pyramidal neurons using borosilicate pipettes (3–6 MΩ) filled with intracellular solution containing (in mM) 132 $CsMeSO_3$, 8 CsCl, 0.6 EGTA, 10 diNa-phosphocreatine, 10 HEPES, 4 ATP-Mg^{2+}, 0.4 GTP-Na (pH 7.25-7.30 with CsOH, 275-280 mOsmol).

Spontaneous miniature EPSCs (mEPSCs) were recorded in the presence of TTX (5 nM; Abcam) in ACSF containing (in mM) 124 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgSO_4$, 10 D-Glucose, (pH 7.37-7.41 with NaCl, 295-305 mOsm). Recordings were obtained using a Multiclamp 700 A amplifier and a 1440 A Digidata acquisition board (Molecular Devices). Signals were low-pass-filtered at 2 kHz, digitized at 20 kHz and stored on a PC. mEPSCs were recorded in whole cell voltage-clamp at a holding potential of -70 mV and identification of mEPSCs was confirmed by application of CNQX (10 μM). Access resistance was routinely monitored and recordings were only included if <30 MΩ and with variation <25% over the recording period. For analysis, mEPSC traces were filtered at 2.8 kHz (Bessel filter) using pClamp10 software (Molecular Devices) and miniature events were analyzed using MiniAnalysis (Synaptosoft). Two-way ANOVA with Tukey's *post hoc* test was used to assess statistical significance.

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

ANOVA with Tukey's *post hoc* test. For each neuron, the spine morphology was determined by the first 10 spines in every 25 μm bin along the apical shaft. Spines were assigned one of the five morphological categories based on published methods[24-26]; A:Thin, B:Stubby, C:Mushroom, D:Filopodia, E:Branched. Chi-Square analysis was used to compare the distribution of spines in these categories between genotypes. For statistical analysis, we used two-way ANOVA with Tukey's *post hoc* test.

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

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

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

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

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

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

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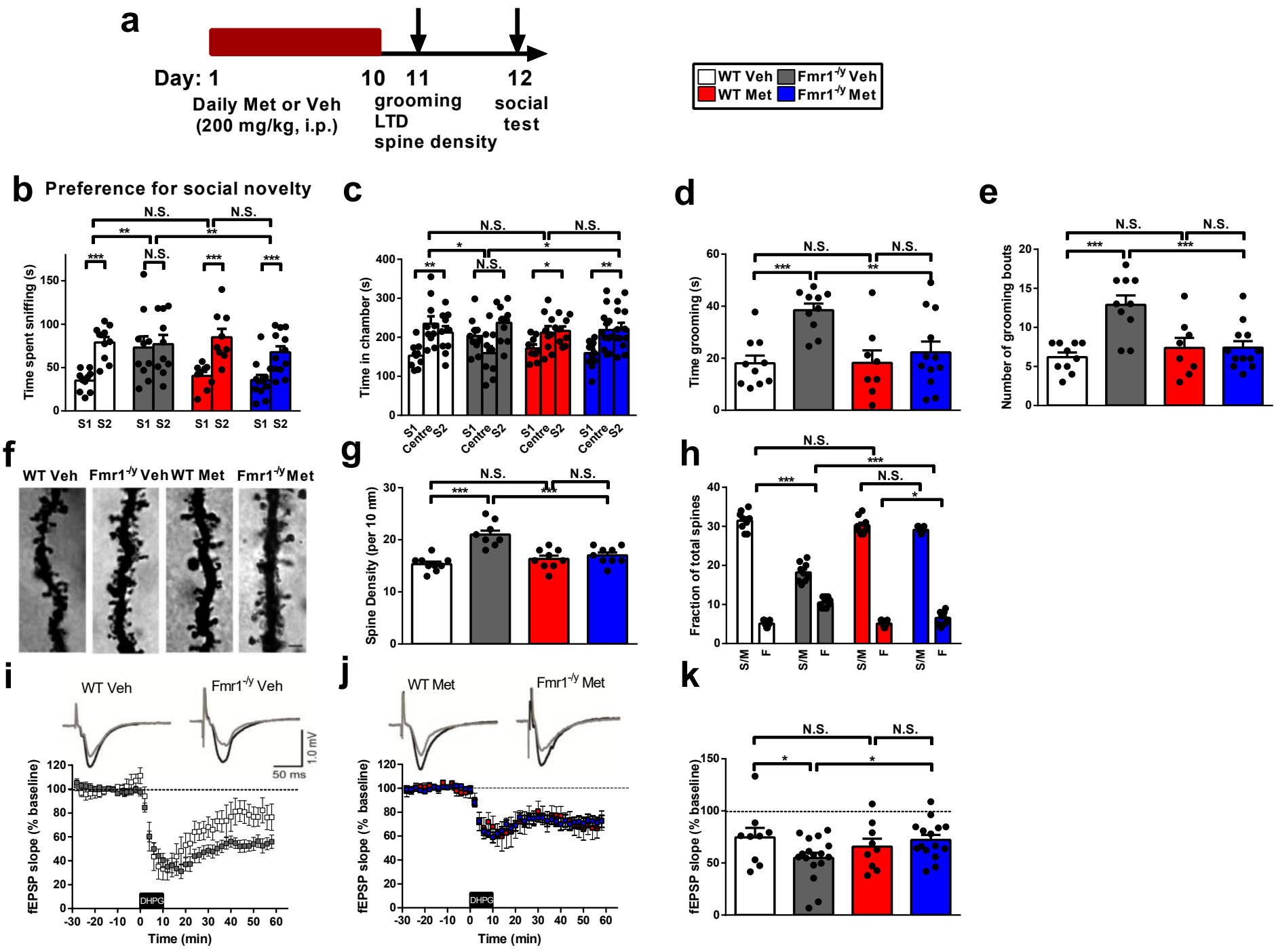
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Day: 1
Daily Met or Veh
(200 mg/kg, i.p.)
10 11
grooming
LTD
spine density
12
social
test
WT Veh
Fmr1-/y Veh
WT Met
Fmr1-/y Met
b Preference for social novelty
Time spent sniffing (s)
S1 S2
c
Time in chamber (s)
S1 Centre S2
d
Time grooming (s)
e
Number of grooming bouts
f
WT Veh
Fmr1-/y Veh
WT Met
Fmr1-/y Met
g
Spine Density (per 10 mm)
h
Fraction of total spines
S/M
F
i
WT Veh
Fmr1-/y Veh
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50 ms
fEPSP slope (% baseline)
DHPG
Time (min)
j
WT Met
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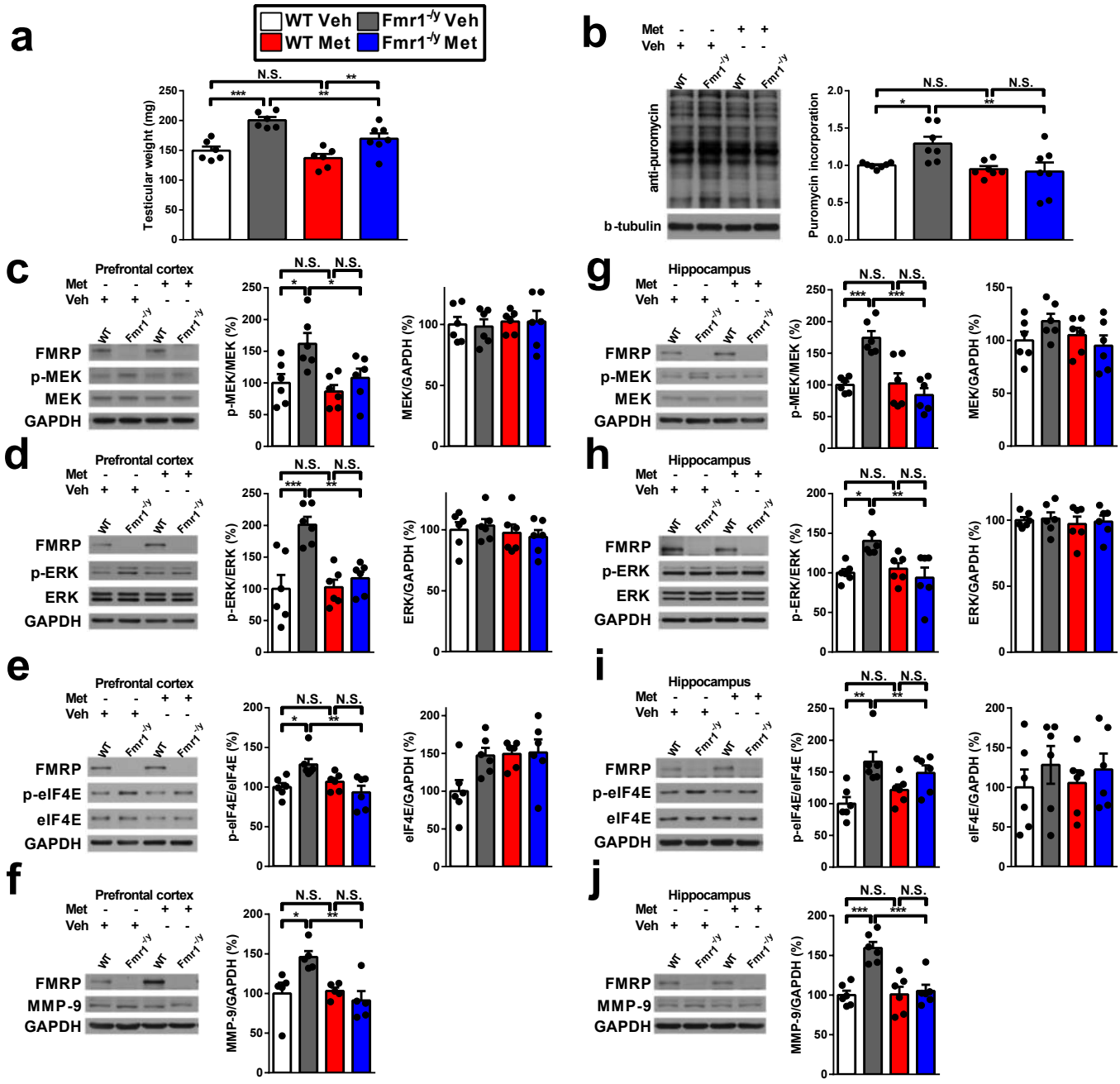
k
N.S.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WT Veh
Fmr1-/y Veh
WT Met
Fmr1-/y Met
Testicular weight (mg)
N.S.
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**
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b
Met
Veh
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Fmr1-/y
anti-puromycin
b-tubulin
Puromycin incorporation
N.S.
N.S.
*
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c
Prefrontal cortex
FMRP
p-MEK
MEK
GAPDH
p-MEK/MEK (%)
MEK/GAPDH (%)
d
Prefrontal cortex
FMRP
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ERK
GAPDH
p-ERK/ERK (%)
ERK/GAPDH (%)
e
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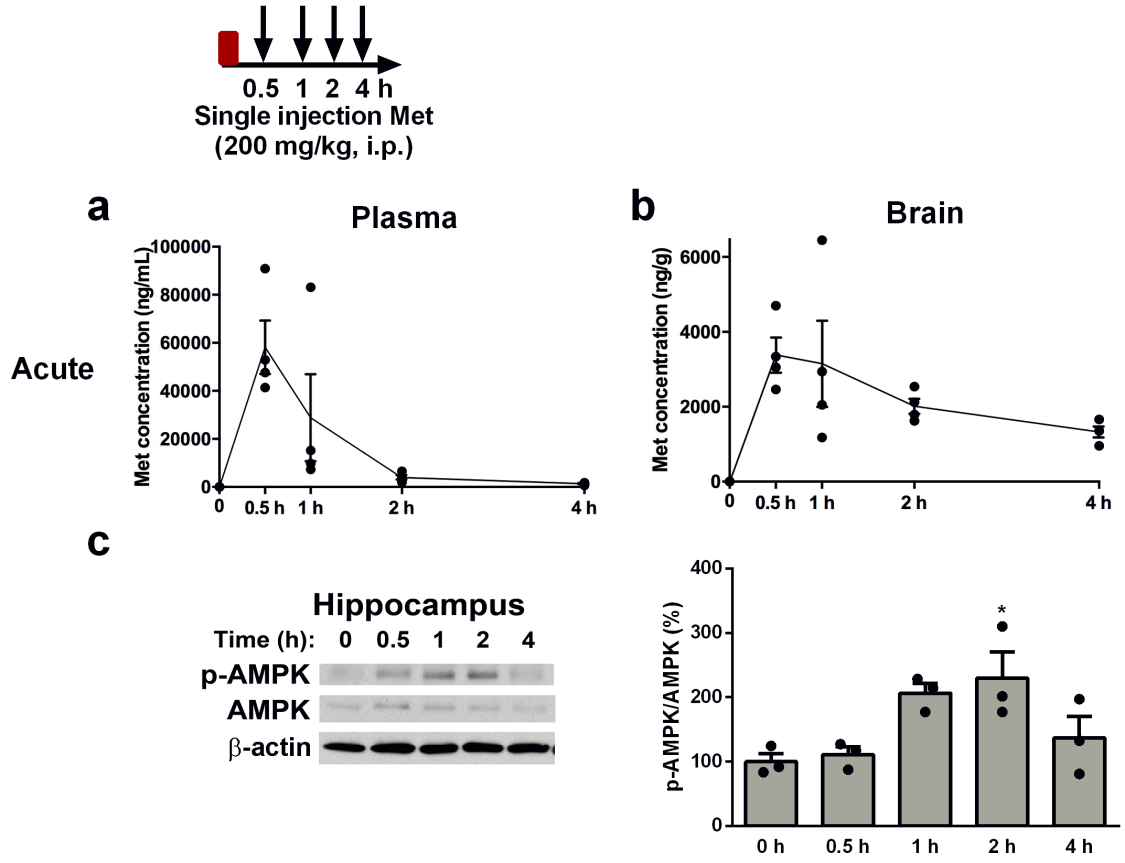
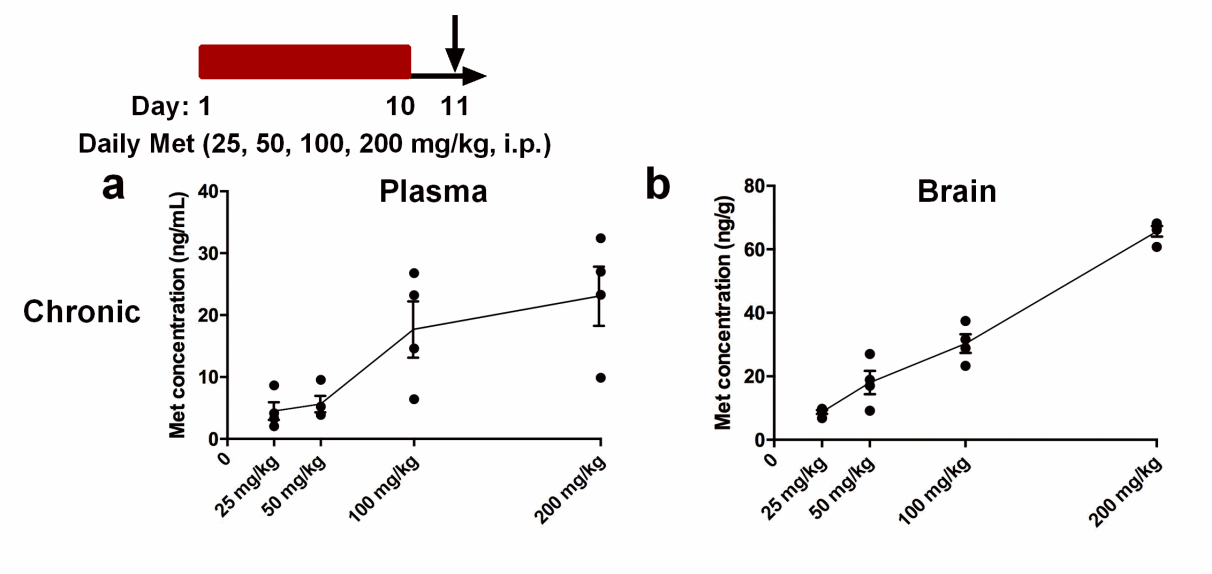
FMRP
p-eIF4E
eIF4E
GAPDH
p-eIF4E/eIF4E (%)
eIF4E/GAPDH (%)
f
Prefrontal cortex
FMRP
MMP-9
GAPDH
MMP-9/GAPDH (%)
g
Hippocampus
FMRP
p-MEK
MEK
GAPDH
p-MEK/MEK (%)
MEK/GAPDH (%)
h
Hippocampus
FMRP
p-ERK
ERK
GAPDH
p-ERK/ERK (%)
ERK/GAPDH (%)
i
Hippocampus
FMRP
p-eIF4E
eIF4E
GAPDH
p-eIF4E/eIF4E (%)
eIF4E/GAPDH (%)
j
Hippocampus
FMRP
MMP-9
GAPDH
MMP-9/GAPDH (%)

SUPPLEMENTARY INFORMATION

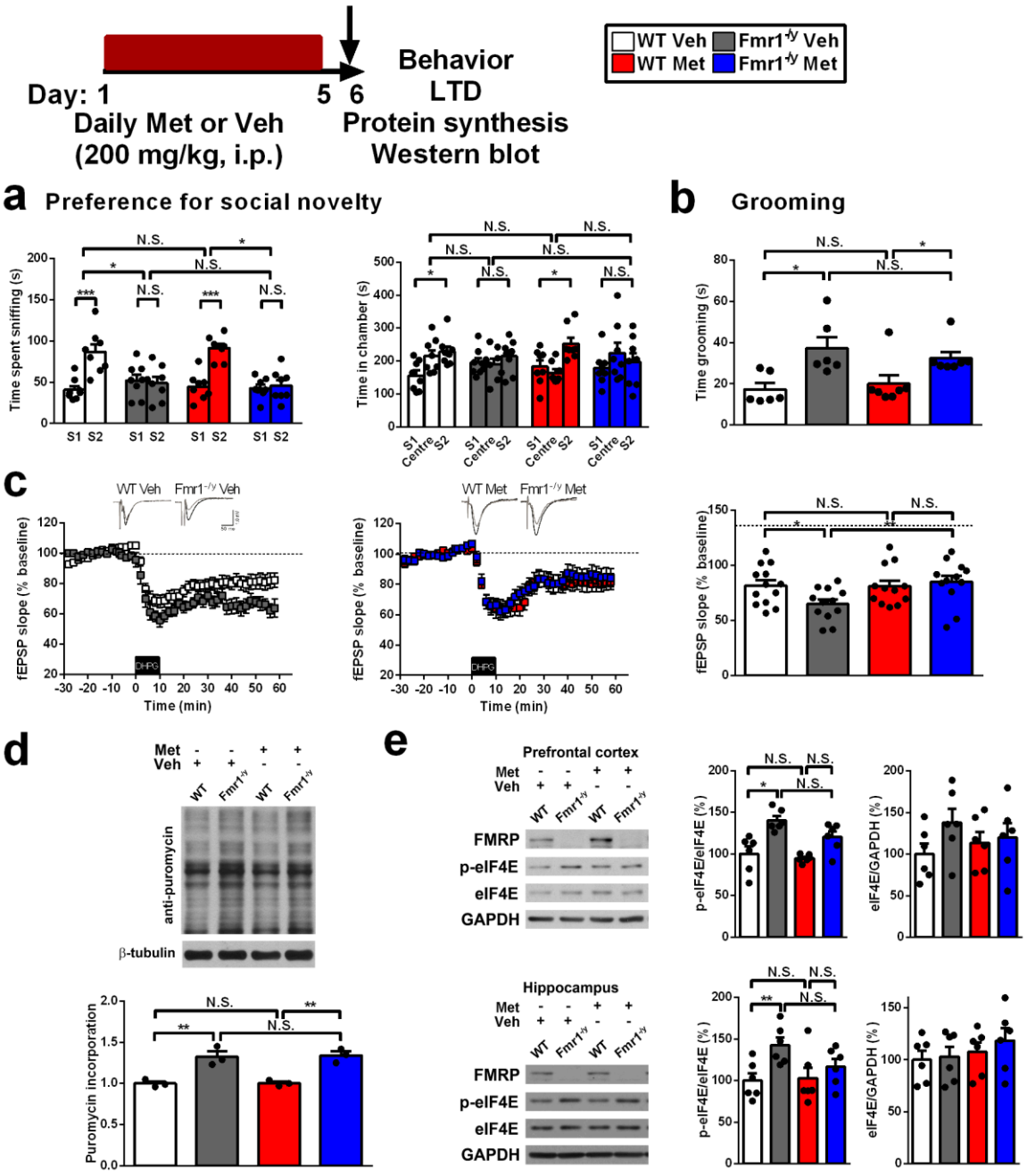
SUPPLEMENTARY FIGURES



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*$^{-/y}$ 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.

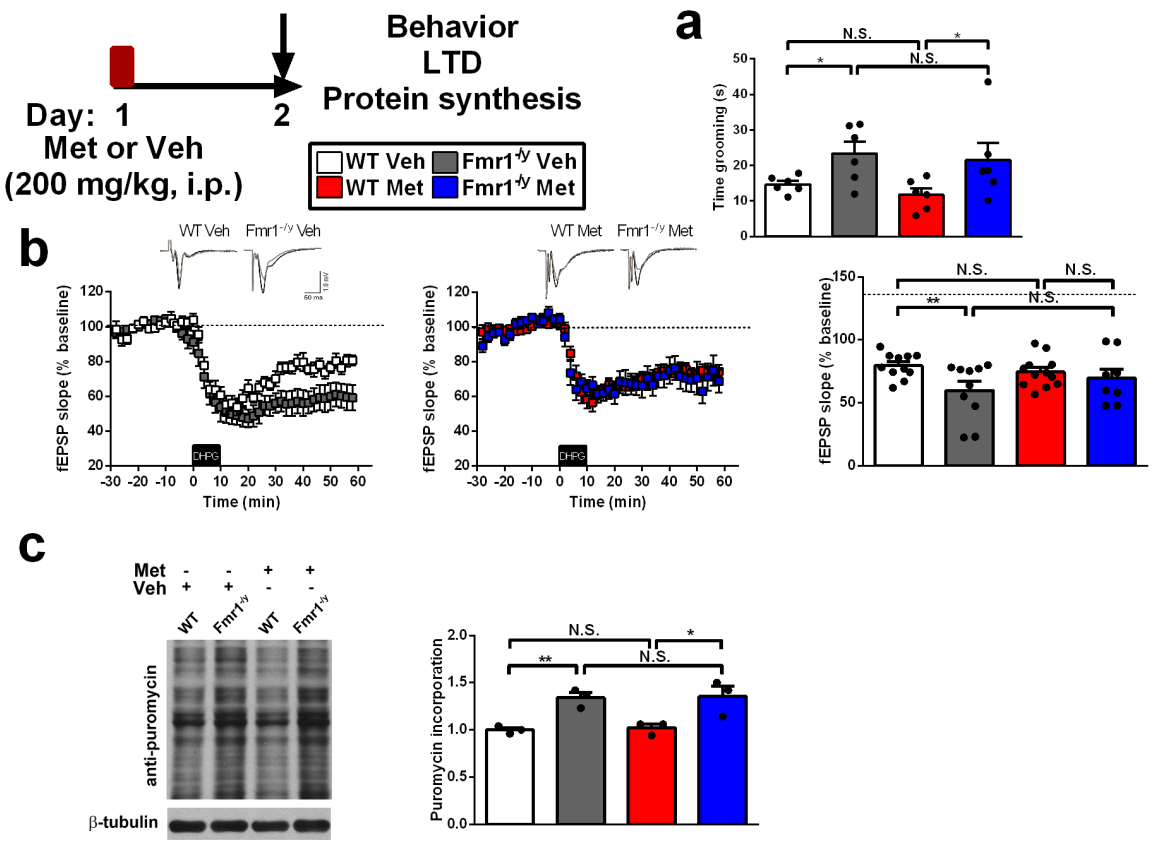


Supplementary Figure 2 Metformin concentrations *in vivo* in plasma and brain after a 10-day chronic metformin treatment. The concentrations of metformin were measured by LC-MS/MS in the plasma (**a**), and brain (**b**) 24 h after last metformin injection (25, 50, 100, and 200 mg/kg, i.p.) (n = 4 in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

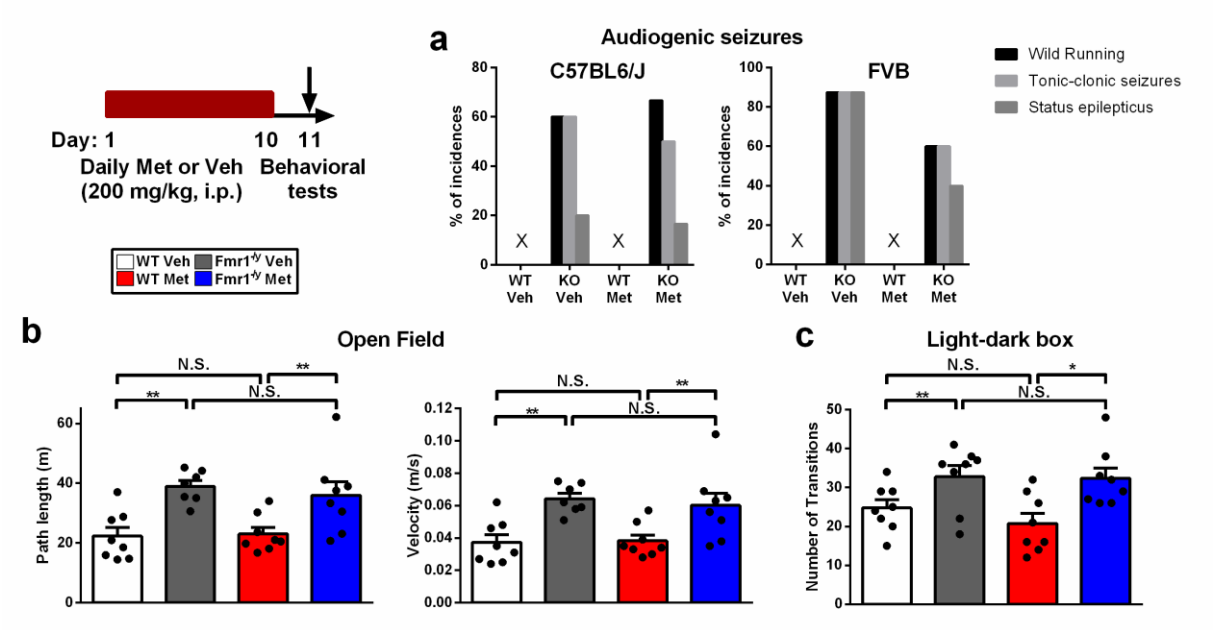


Supplementary Figure 3 Five-day metformin treatment (200 mg/kg/day, i.p.) does not rescue impaired social and grooming behavior, general translation, and phosphorylated eIF4E in *Fmr1*$^{-/y}$ mice. (**a**) Preference for social novelty was assessed in the three-chamber social interaction test by measuring 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

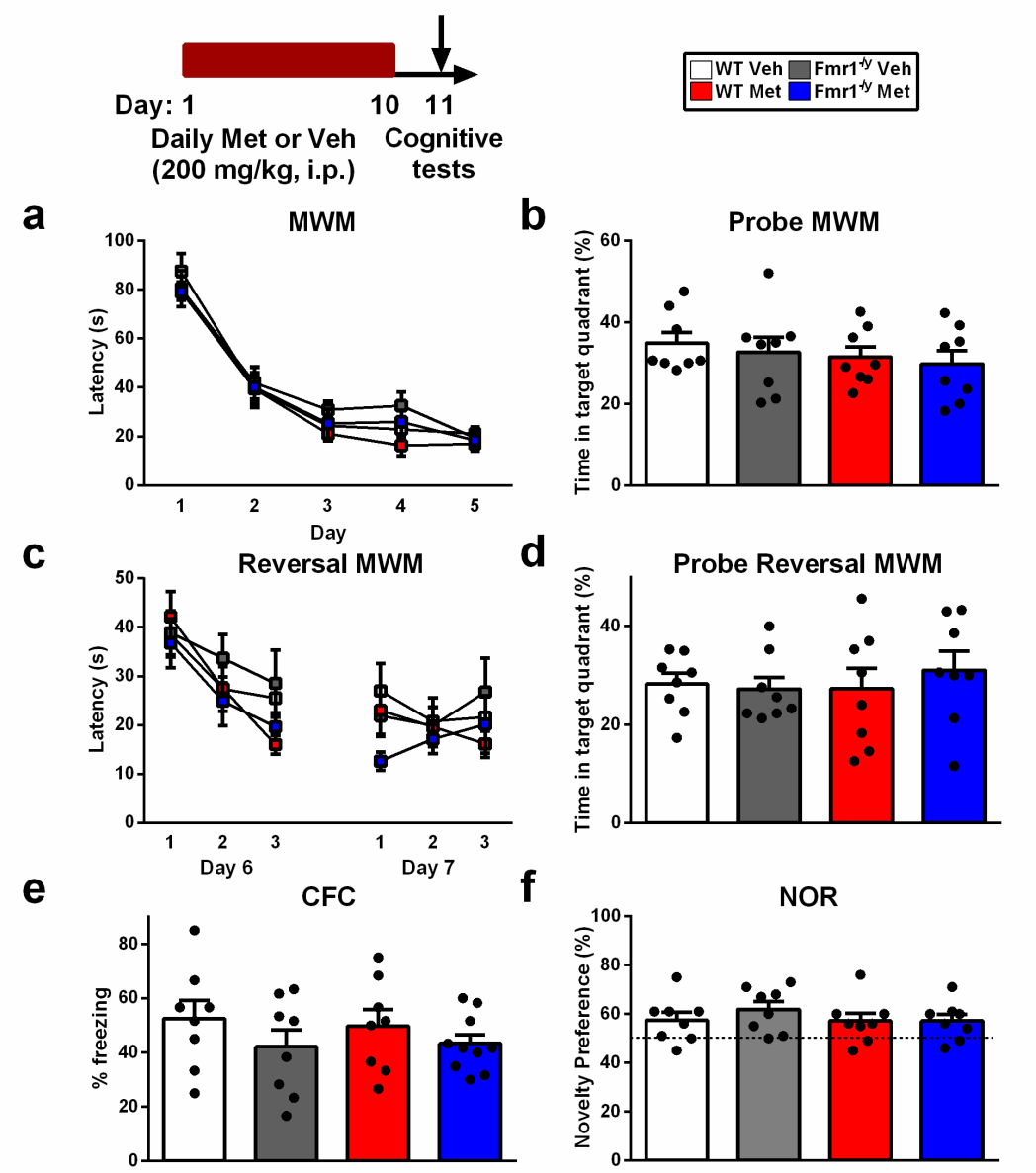
= 7) mice. (**c**) Time-plots of mGluR-LTD measured in CA1 in response to DHPG (50 μM for 10 min) in slices prepared from vehicle-treated WT (n = 13) and *Fmr1*$^{-/y}$ (n = 12) mice, and metformin-treated WT (n = 12) and *Fmr1*$^{-/y}$ (n = 12) mice. Quantification (right) of mGluR-LTD during the last 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.



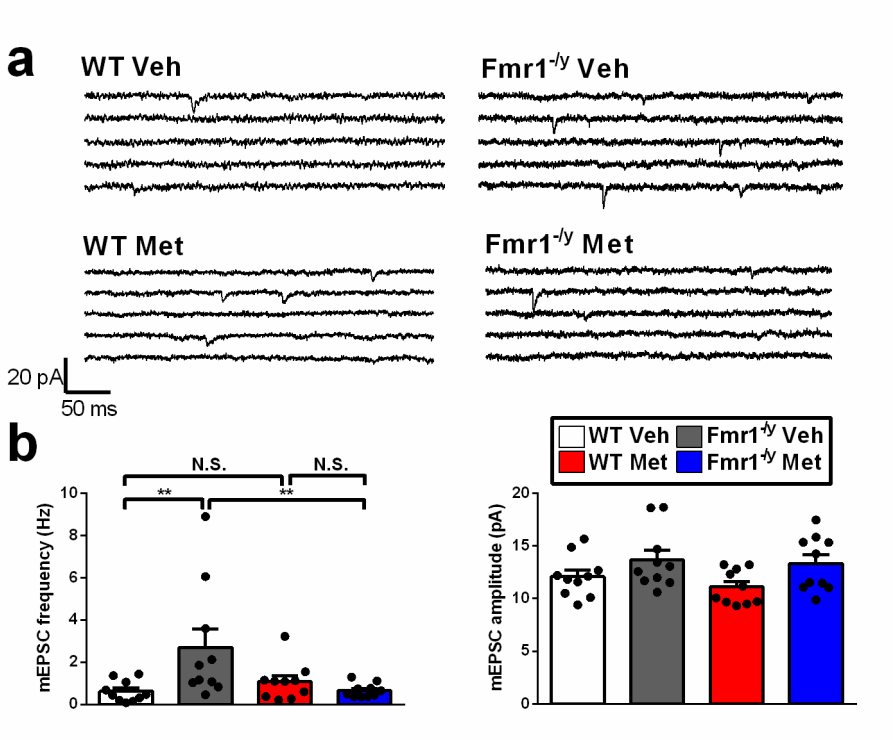
Supplementary Figure 4 One-day metformin treatment (200 mg/kg/day, i.p.) did not rescue increased grooming, exaggerated LTD, and increased general translation in *Fmr1*$^{-/y}$ mice. (**a**) Self-grooming test with total time spent grooming; calculated by two-way ANOVA with Tukey's *post hoc* test. n = 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.



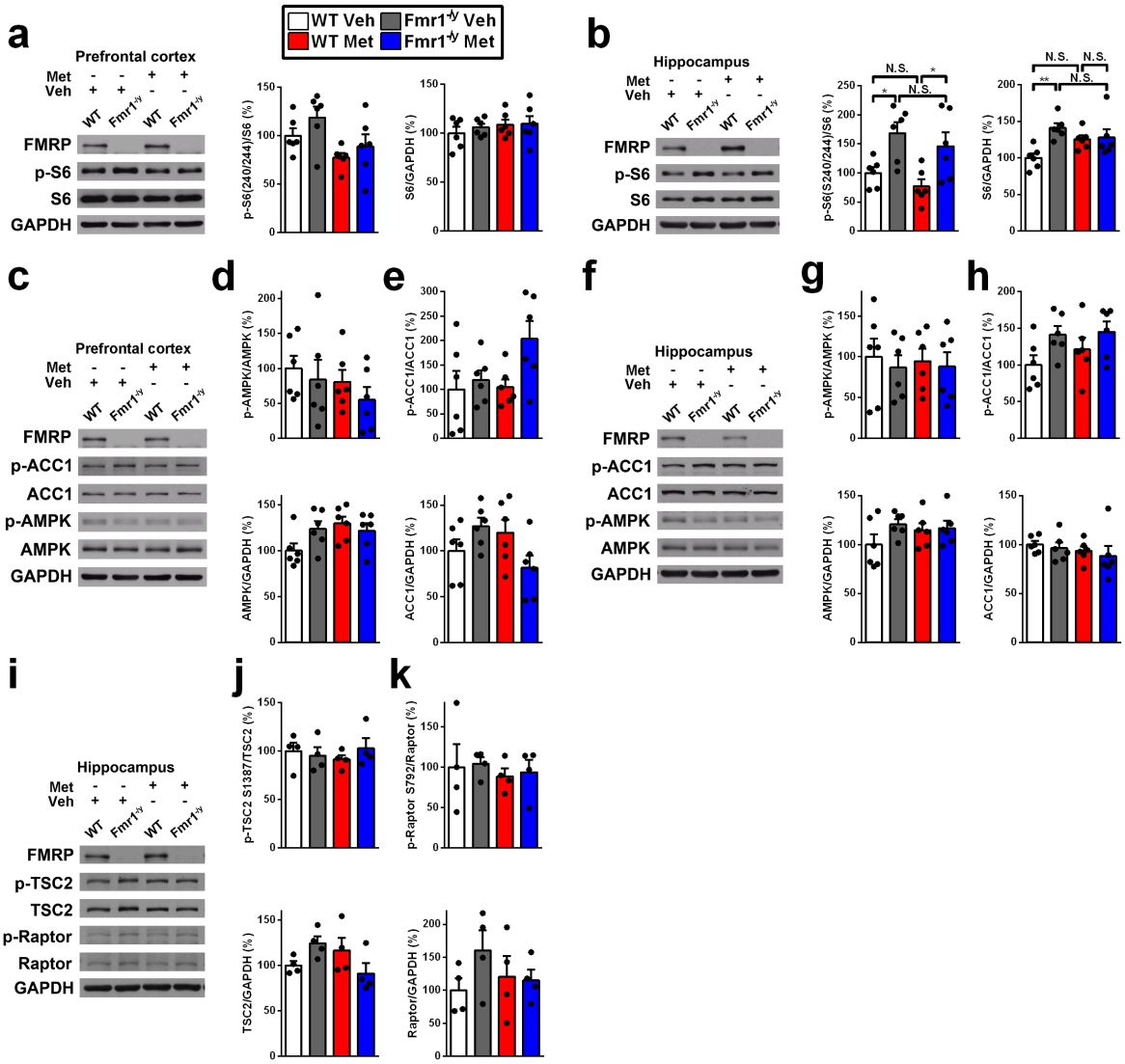
Supplementary Figure 5 Effect of chronic metformin treatment (200 mg/kg/day, 10 days, i.p.) in *Fmr1*$^{-/y}$ and WT mice on audiogenic seizures and hyperactivity. Audiogenic seizures (**a**) were tested on *Fmr1*$^{-/y}$ and WT mice in a C57BL6/J (**a, left panel**) (vehicle-treated WT (n = 2) and *Fmr1*$^{-/y}$ (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).

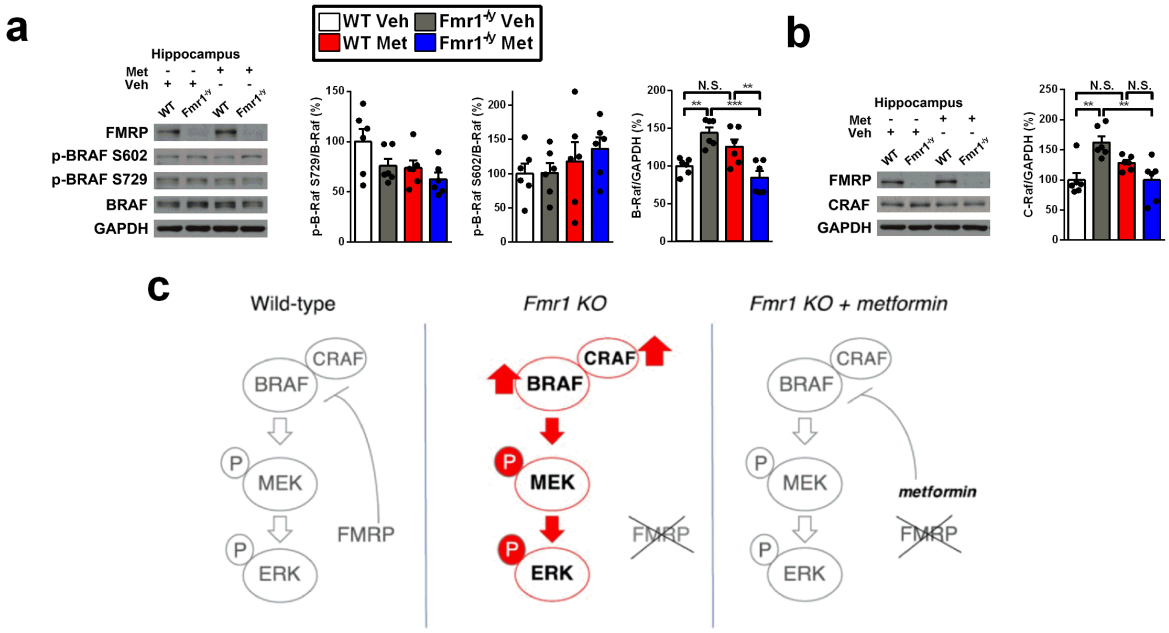


Supplementary Figure 7 Metformin restores excitatory synaptic activity in *Fmr1*$^{-/y}$ mice. (**a**) Representative traces of mEPSCs from pyramidal cells in hippocampal slice cultures from WT and *Fmr1*$^{-/y}$ mice treated with vehicle or 50 μM metformin for 4-5 days prior to recording. (**b**) Bar 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 ± s.e.m.

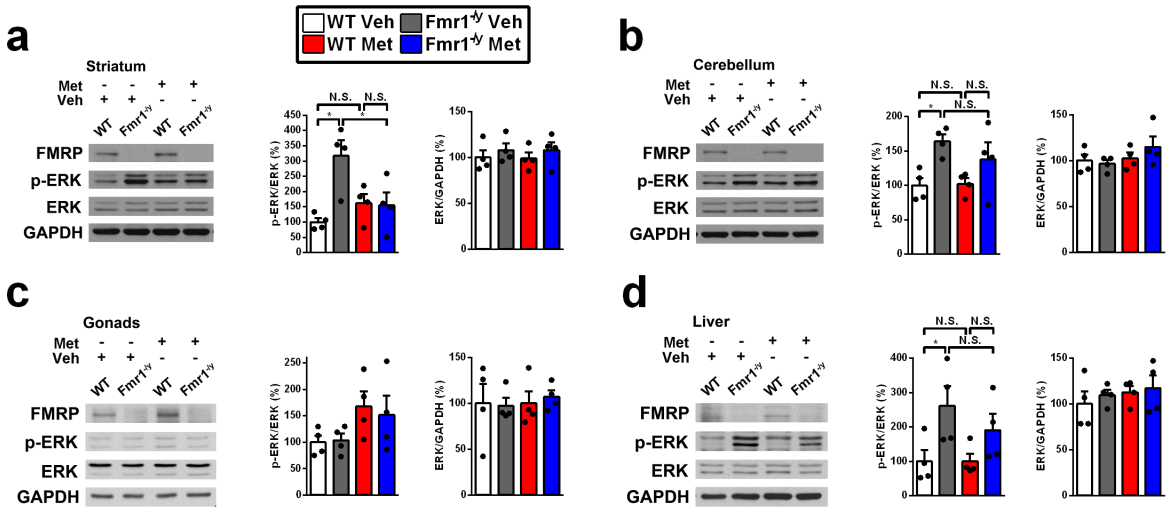


Supplementary Figure 8 Chronic metformin treatment failed to reduce phosphorylation of S6 (S240/244), AMPK, ACC1, TSC2 and Raptor in *Fmr1*$^{-/y}$ mice. Representative immunoblots and blot quantification of prefrontal cortex (**a**) and hippocampal (**b**) lysates from vehicle- and metformin-treated 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

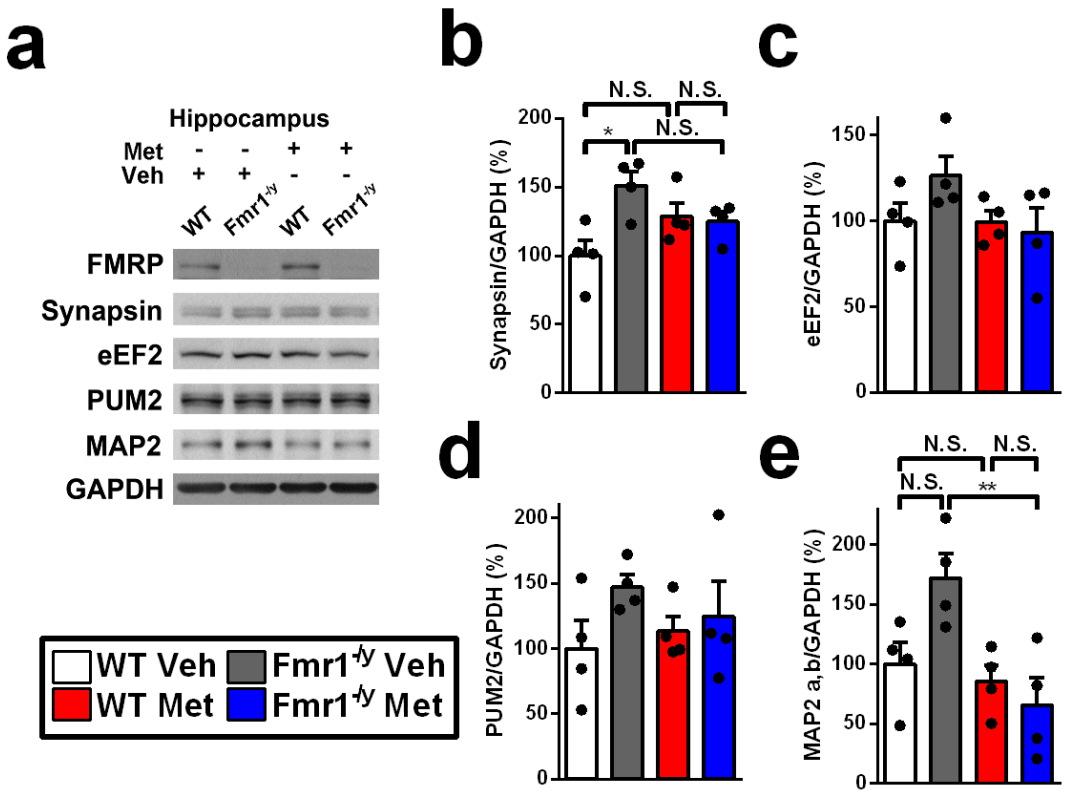
vehicle-treated WT. Values are shown as mean ± s.e.m. **P < 0.01, *P < 0.05, N.S. not significant, versus all other groups; calculated by two-way repeated measures ANOVA with Tukey's *post hoc* test.



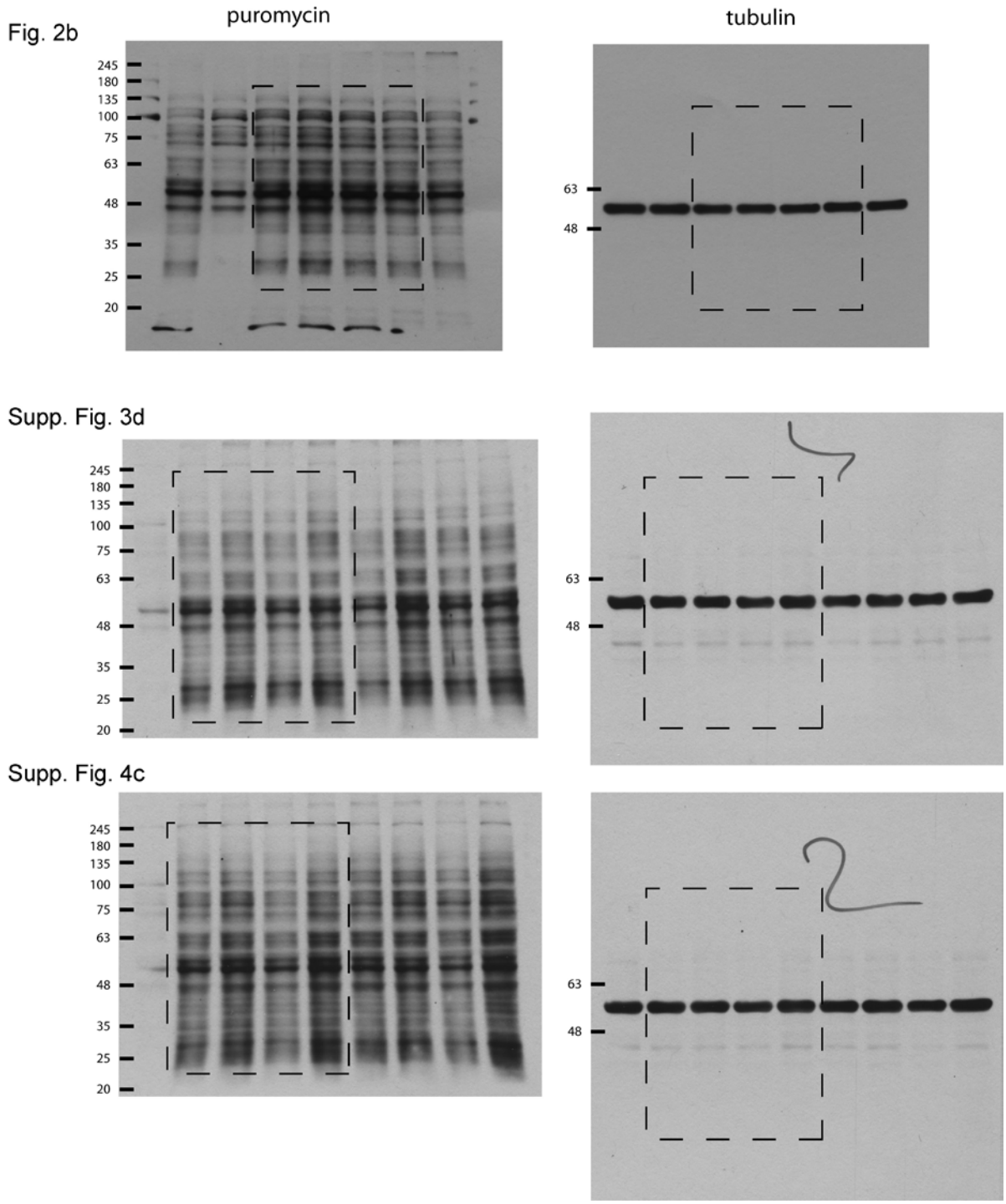
Supplementary Figure 9 Chronic metformin treatment reduced total B-Raf and C-Raf proteins in the hippocampus of *Fmr1*$^{-/y}$ mice. Representative immunoblots and blot quantification of total and phosphorylated B-Raf (**a**), and total C-Raf (**b**) from vehicle- and metformin-treated WT and *Fmr1*$^{-/y}$ 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.



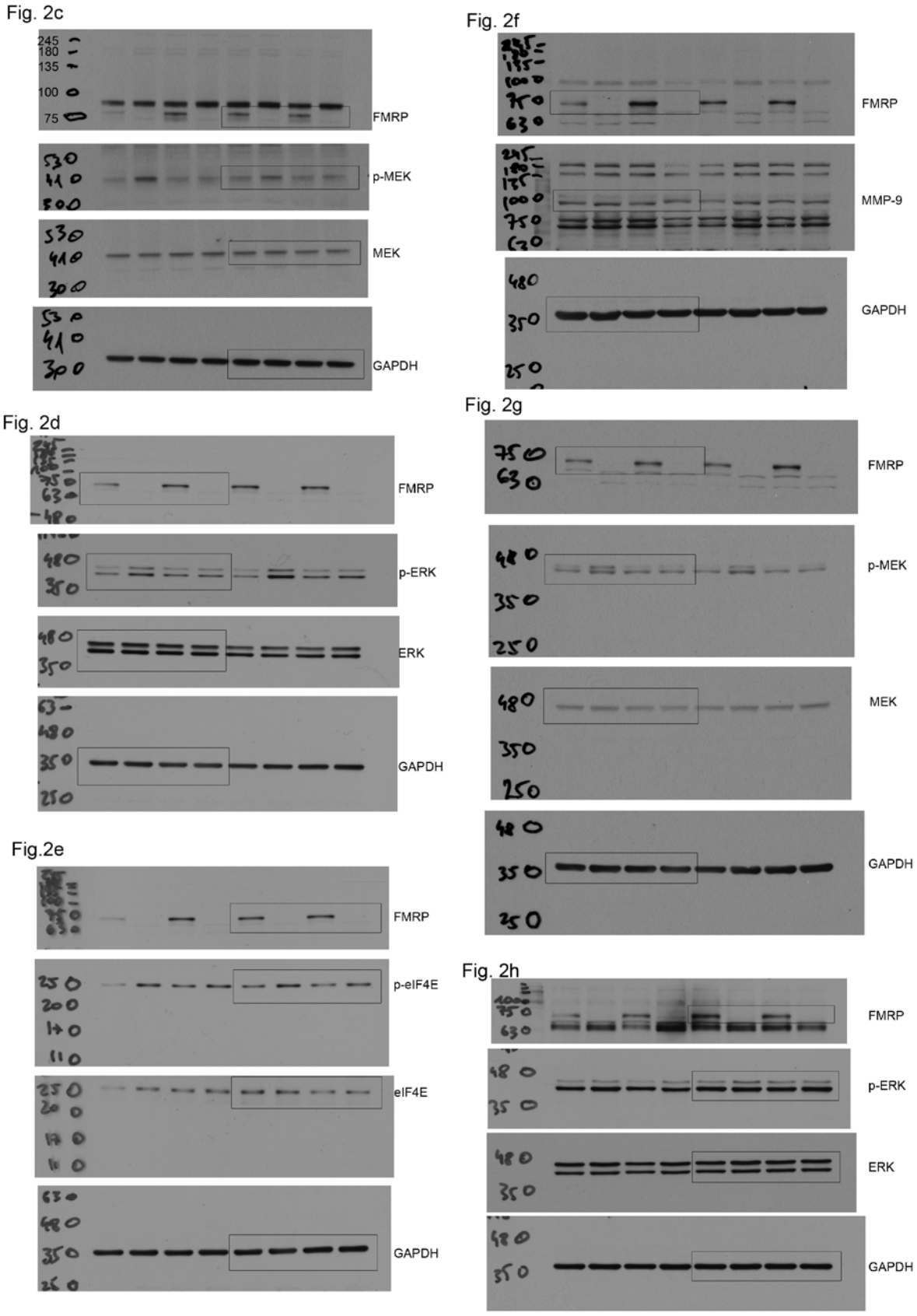
Supplementary Figure 10 Chronic metformin treatment reduced phosphorylated ERK in the striatum, but not in cerebellum, gonads and liver of *Fmr1*$^{-/y}$ mice. Representative immunoblots and blot quantification of total and phosphorylated ERK in the striatum (**a**), cerebellum (**b**), gonads (**c**), 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.



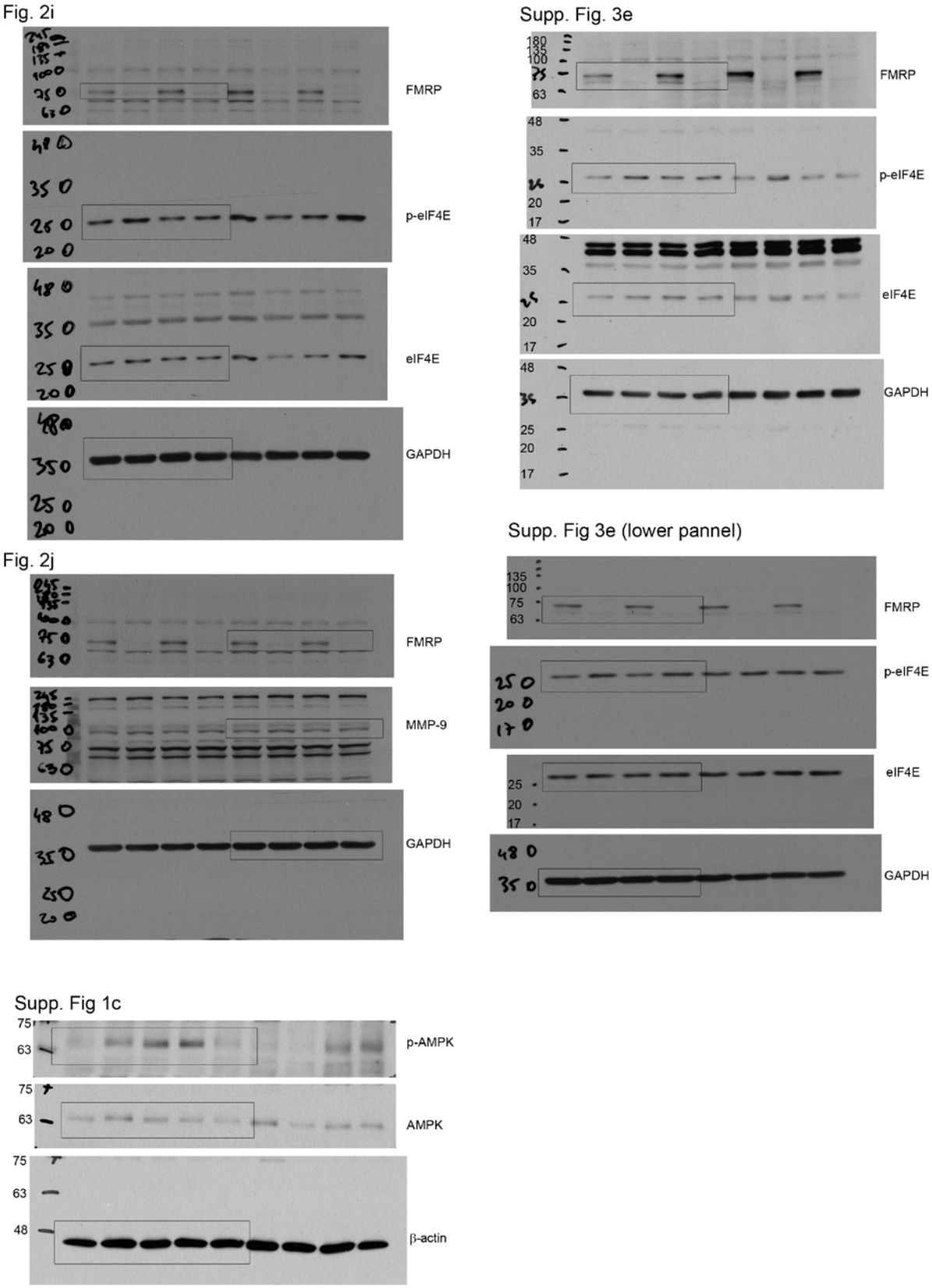
Supplementary Figure 11 Chronic metformin treatment reduced levels of FMRP targets, synapsin and MAP2 a,b in the hippocampus of *Fmr1*$^{-/y}$ mice. Representative immunoblots (**a**) and blot quantification of synapsin (**b**), eEF2 (**c**), PUM2 (**d**) and MAP2 a,b (**e**) in the hippocampus from 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.



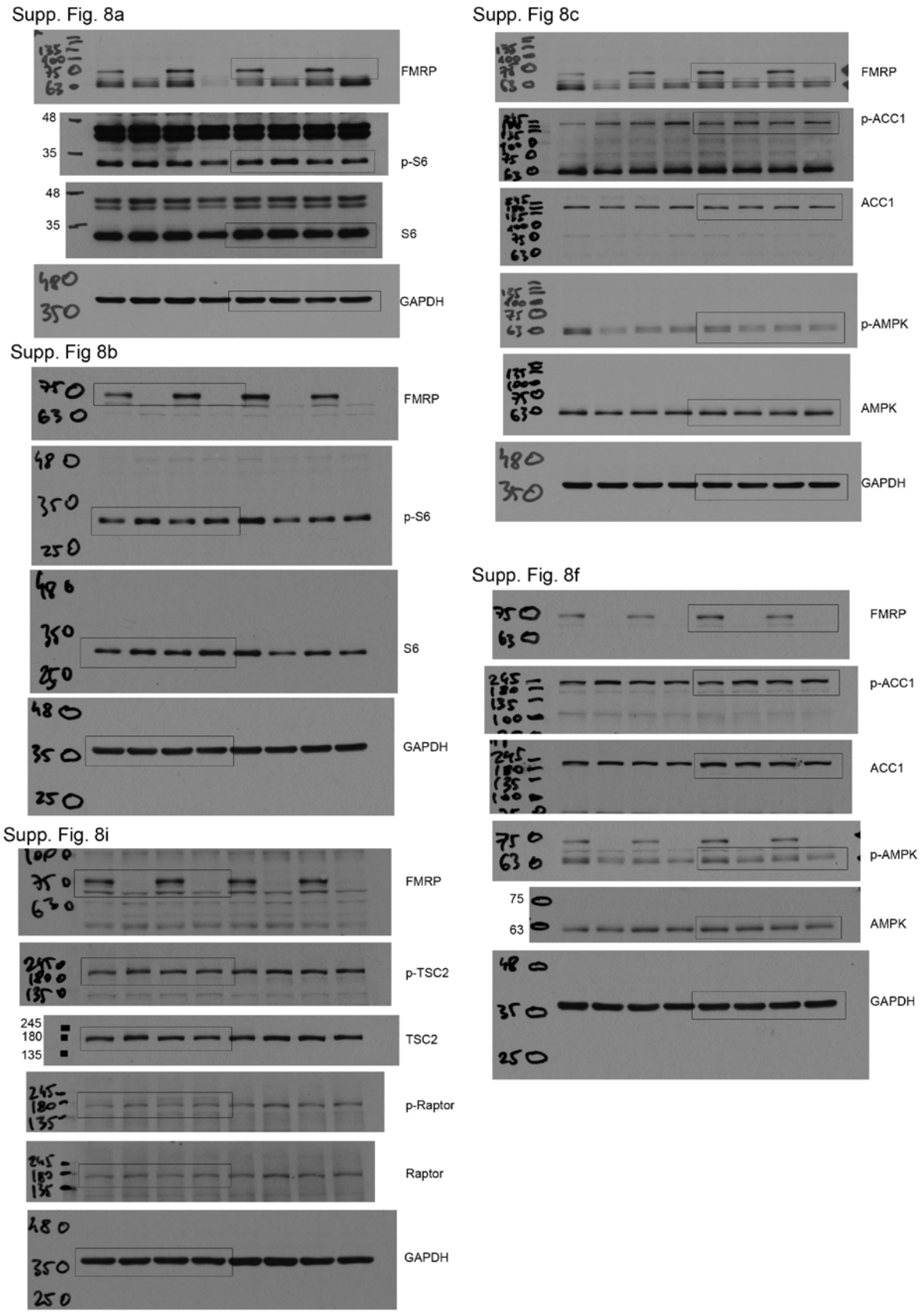
Supplementary Figure 12 Original images of representative western blots of *de novo* protein synthesis



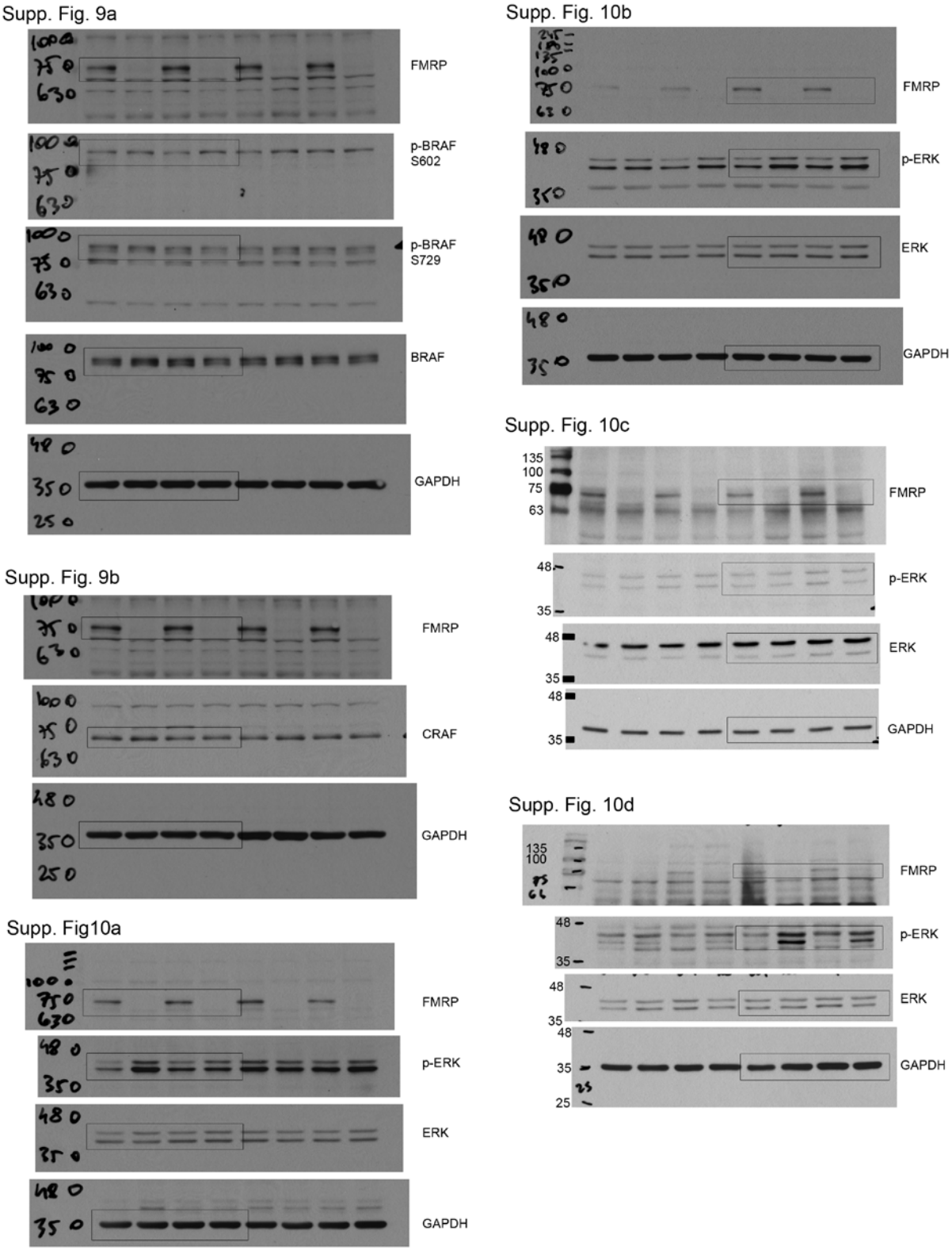
Supplementary Figure 13 Original images of representative western blots in Fig. 2



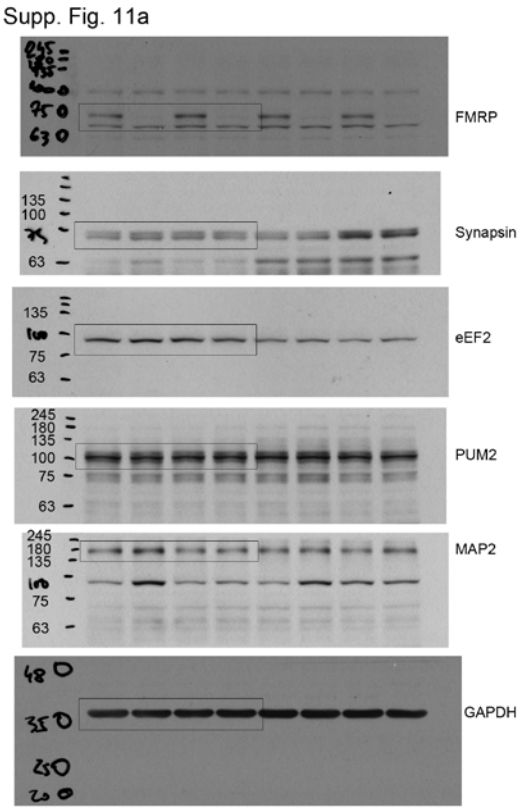
Supplementary Figure 14 Original images of representative western blots in Fig. 2, and Supplementary Figs. 1 and 3



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



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	Two-way mixed ANOVA		
	Time sniffing	*Post hoc* Tukey's test:	S1:
WT Veh (n = 10)	Chamber: F(1,37) = 48.173, p < 0.001	WT Veh vs KO Veh, p = 0.006	WT Veh 35 ± 4
Fmr1$^{-/y}$ Veh (n = 10)	Group: F(3,37) = 2.329, p = 0.090	WT Met vs KO Met, p = 0.971	KO Veh 73 ± 13
WT Met (n = 9)	Chamber x Group: F(3,37) = 4.392, p = 0.010	WT Veh vs WT Met, p = 0.963	WT Met 40 ± 5
Fmr1$^{-/y}$ Met (n = 12)		KO Veh vs KO Met, p = 0.005	KO Met 36 ± 6
			S2:
			WT Veh 79 ± 6
			KO Veh 77 ± 11
			WT Met 85 ± 10
			KO Met 68 ± 7
	Time in chamber	*Post hoc* Tukey's test:	S1:
	Chamber: F(1,37) = 25.241, p < 0.001	WT Veh vs KO Veh, p = 0.013	WT Veh 153 ± 10
	Group: F(3,37) = 3.600, p = 0.022	WT Met vs KO Met, p = 0.968	KO Veh 204 ± 14
	Chamber x Group: F(3,37) = 0.456, p = 0.715	WT Veh vs WT Met, p = 0.997	WT Met 171 ± 10
		KO Veh vs KO Met, p = 0.017	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	Two-way ANOVA		
WT Veh (n = 10)	*Time*	*Post hoc* Tukey's test:	
Fmr1$^{-/y}$ Veh (n = 10)	Genotype: F(1,36) = 10.662, p = 0.002	WT Veh vs KO Veh, p < 0.001	WT Veh 18.1 ± 3.0
WT Met (n = 8)	Treatment: F(1,36) = 4.613, p = 0.039	WT Met vs KO Met, p = 0.455	KO Veh 38.5 ± 2.5
Fmr1$^{-/y}$ Met (n = 12)	Genotype x Treatment: F(1,36) = 4.782, p = 0.035	WT Veh vs WT Met, p = 0.979	WT Met 18.2 ± 4.8
		KO Veh vs KO Met, p = 0.003	KO Met 22.3 ± 4.1
	Number of bouts	*Post hoc* Tukey's test:	
	Genotype: F(1,36) = 11.672, p = 0.002	WT Veh vs KO Veh, p < 0.001	WT Veh 6.2 ± 0.6
	Treatment: F(1,36) = 4.767, p = 0.036	WT Met vs KO Met, p = 0.977	KO Veh 12.9 ± 1.2
	Genotype x Treatment: F(1,36) = 11.386, p = 0.002	WT Veh vs WT Met, p = 0.428	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 *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 *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 = 0.447 WT Veh vs WT Met, p = 0.257 KO Veh vs KO Met, 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 *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 15.3 ± 0.5 KO Veh 21.0 ± 0.8 WT Met 16.3 ± 0.6 KO Met 17.0 ± 0.6 WT Veh 31.4 ± 0.8 KO Veh 18.2 ± 0.8 WT Met 30.2 ± 0.7 KO Met 29.1 ± 0.3 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)	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$^{-/y}$ Met (n = 6)			
	MEK/GAPDH		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
	p-ERK/ERK	*Post hoc* 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
	ERK/GAPDH		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
	p-eIF4E/eIF4E	*Post hoc* 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
	eIF4E/GAPDH		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)	*MMP-9/GAPDH*	*Post hoc* Tukey's test:	
Fmr1$^{-/y}$ 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$^{-/y}$ 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
	Hippocampus	*Post hoc* Tukey's test:	
	p-MEK/MEK	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
	MEK/GAPDH		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-ERK/ERK Genotype: F(1,20) = 2.749, p = 0.113 Treatment: F(1,20) = 5.618, p = 0.028 Genotype x Treatment: F(1,20) = 8.715, p = 0.008 *ERK/GAPDH* Genotype: F(1,20) = 0.109, p = 0.744 Treatment: F(1,20) = 0.350, p = 0.561 Genotype x Treatment: F(1,20) = 0.004, p = 0.950 *p-eIF4E/eIF4E* Genotype: F(1,20) = 16.194, p < 0.001 Treatment: F(1,20) = 18.973, p < 0.001 Genotype x Treatment: F(1,20) = 3.989, p = 0.059 *eIF4E/GAPDH* Genotype: F(1,20) = 0.739, p = 0.400 Treatment: F(1,20) = 0.769, p = 0.391 Genotype x Treatment: F(1,20) = 3.866, p = 0.063 *MMP-9/GAPDH* Genotype: F(1,20) = 16.803, p < 0.001 Treatment: F(1,20) = 11.770, p = 0.003 Genotype x Treatment: F(1,20) = 12.526, p = 0.002	*Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.019 WT Met vs KO Met, p = 0.797 WT Veh vs WT Met, p = 0.976 KO Veh vs KO Met, p = 0.006 *Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.002 WT Met vs KO Met, p = 0.494 WT Veh vs WT Met, p = 0.366 KO Veh vs KO Met, p = 0.001 *Post hoc* Tukey's test: WT Veh vs KO Veh, p < 0.001 WT Met vs KO Met, p = 0.978 WT Veh vs WT Met, p = 0.999 KO Veh vs KO Met, p < 0.001	WT Veh 100 ± 5 KO Veh 140 ± 8 WT Met 105 ± 7 KO Met 94 ± 13 WT Veh 100 ± 2 KO Veh 101 ± 5 WT Met 97 ± 6 KO Met 99 ± 5 WT Veh 100 ± 5 KO Veh 166 ± 16 WT Met 122 ± 9 KO Met 149 ± 12 WT Veh 100 ± 22 KO Veh 128 ± 24 WT Met 105 ± 16 KO Met 122 ± 20 WT Veh 100 ± 6 KO Veh 159 ± 8 WT Met 101 ± 10 KO Met 105 ± 8
Supplementary 1a, b: Pharmacokinetics 0 h (n = 4) 0.5 h (n = 4) 1 h (n = 4) 2 h (n = 4) 4 h (n = 4)	One-way ANOVA *Plasma* Time: F(4,15) = 6.964, p = 0.002 *Brain* Time: F(4,15) = 5.936, p = 0.005	 *Post hoc* Tukey's test: 0 vs 0.5, p = 0.005 0.5 vs 1, p = 0.241 0.5 vs 2, p = 0.008 0.5 vs 4, p = 0.006 *Post hoc* Tukey's test: 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: Western blot 0 h (n = 3) 0.5 h (n = 3) 1 h (n = 3)	One-way ANOVA *Hippocampus* p-AMPK/AMPK Time: F(4,10) = 5.072, p = 0.017	*Post hoc* Tukey's test: 0 vs 0.5, p = 0.998 0 vs 1, p = 0.089 0 vs 2, p = 0.033 0 vs 4, p = 0.845	

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) WT Met (n = 4) Fmr1$^{-/y}$ Met (n = 4)	*p-TSC2/TSC2* 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		
WT Veh (n = 4) Fmr1$^{-/y}$ Veh (n = 4) WT Met (n = 4) Fmr1$^{-/y}$ Met (n = 4)	*p-Raptor/Raptor* 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		
Supplementary 9a, b: Western blot WT Veh (n = 6) Fmr1$^{-/y}$ Veh (n = 6) WT Met (n = 6) Fmr1$^{-/y}$ Met (n = 6)	Two-way ANOVA *Hippocampus* *b-Raf/GAPDH* 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 < 0.001	*Post hoc* 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 < 0.001	
	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* Genotype: F(1,20) = 2.498, p = 0.130 Treatment: F(1,20) = 2.616, p = 0.121 Genotype x Treatment: F(1,20) = 17.981, p < 0.001	 *Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.003 WT Met vs KO Met, p = 0.267 WT Veh vs WT Met, p = 0.278 KO Veh vs KO Met, p = 0.003	
Supplementary 10a-d: Western blot WT Veh (n = 4) Fmr1$^{-/y}$ Veh (n = 4) WT Met (n = 4) Fmr1$^{-/y}$ Met (n = 4)	Two-way ANOVA *Striatum* *p-ERK/ERK* Genotype: F(1,12) = 6.057, p = 0.030 Treatment: F(1,12) = 3.793, p = 0.075 Genotype x Treatment: F(1,12) = 6.325, p = 0.027 *Cerebellum* *p-ERK/ERK* Genotype: F(1,12) = 11.142, p = 0.006 Treatment: F(1,12) = 0.644, p = 0.438 Genotype x Treatment: F(1,12) = 0.923, p = 0.356 *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* *p-ERK/ERK* Genotype: F(1,12) = 11.427, p = 0.005 Treatment: F(1,12) = 0.245, p = 0.629 Genotype x Treatment: F(1,12) = 0.729, p = 0.410	 *Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.019 WT Met vs KO Met, p = 1.000 WT Veh vs WT Met, p = 0.977 KO Veh vs KO Met, p = 0.036 *Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.044 WT Met vs KO Met, p = 0.374 WT Veh vs WT Met, p = 0.999 KO Veh vs KO Met, p = 0.611 *Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.048 WT Met vs KO Met, p = 0.326 WT Veh vs WT Met, p = 0.994 KO Veh vs KO Met, p = 0.777	
Supplementary 11b-e Western blot WT Veh (n = 4) Fmr1$^{-/y}$ Veh (n = 4) WT Met (n = 4) Fmr1$^{-/y}$ Met (n = 4)	Two-way ANOVA *Hippocampus* *Synapsin/GAPDH* Genotype: F(1,12) = 5.891, p = 0.032 Treatment: F(1,12) = 0.019, p = 0.891 Genotype x Treatment: F(1,12) = 7.966, p = 0.015 *eEF2/GAPDH*	 *Post hoc* Tukey's test: WT Veh vs KO Veh, p = 0.014 WT Met vs KO Met, p = 0.992 WT Veh vs WT Met, p = 0.210 KO Veh vs KO Met, p = 0.280	

	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	