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

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Fragile X syndrome (FXS) is the leading monogenic cause of autism spectrum disorders (ASD). Trinucleotide repeat expansions in FMR1 abolish FMRP expression, leading to hyperactivation of ERK and mTOR signaling upstream of mRNA translation. Here we show that metformin, the most widely used drug for type 2 diabetes, rescues core phenotypes in Fmr1−/y mice and selectively normalizes ERK signaling, eIF4E phosphorylation and the expression of MMP-9. Thus, metformin is a potential FXS therapeutic.

Dysregulated mRNA translation is linked to core pathologies diagnosed in FXS, such as social and behavioral problems, developmental delays and learning disabilities1,2. In the brains of individuals with FXS and knockout mice (Fmr1−/y; X-linked Fmr1 deletion in male mice), loss of the 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 pathways1,2. Consistent with increased ERK activity, eukaryotic initiation factor 4E (eIF4E) phosphorylation is elevated in the brains of individuals with FXS and Fmr1−/y mice, thereby promoting translation of the mRNA encoding matrix metalloproteinase 9 (MMP-9), which is also elevated in the brains of both individuals with FXS and Fmr1−/y mice1,5. In accordance with these findings, knockout of Mmp9 rescues the majority of known cellular and behavioral phenotypes in Fmr1−/y mice. MMP-9 degrades components of the extracellular matrix, including proteins that are important for synaptic function and maturation, which have been implicated in FXS and 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 prognosis6. Metformin inhibits mitochondrial respiratory chain complex 1, leading to a decrease in the 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 reported7,8. As metformin suppresses translation by inhibiting the mTORC1 and ERK pathways, we reasoned that metformin could have beneficial therapeutic effects in Fmr1−/y mice9.

Adult (aged 8–12 weeks) wild-type (WT) and Fmr1−/y mice were injected intraperitoneally (i.p.) with metformin (200 mg per kg bodyweight per day, a dose previously used in preclinical studies3) or vehicle for 10 d (Fig. 1a). Metformin, as previously reported10, crossed the blood–brain barrier (BBB), with lower concentrations found in the brain than in 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 their preference for social novelty, showing no preference for interaction with the novel stimulus (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 individuals with FXS that is recapitulated in Fmr1−/y mice as increased self-grooming1,11. Metformin reversed the increased grooming in Fmr1−/y mice (Fig. 1d) and decreased the number of grooming bouts (Fig. 1e) measured 24 h after the last injection. Prolonged exposure to metformin was required to rescue behavioral deficits, as metformin treatment of Fmr1−/y mice for 1 or 5 d 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 after metformin treatment and observed no cognitive impairment. Ten-day treatment with metformin reduced the incidence of seizures but did not have an effect on hyperactivity (Supplementary Figs. 5 and 6).

Neurons from individuals with FXS and Fmr1−/y mice exhibit aberrant spine morphology1,11. We observed spine dysmorphogenesis in Fmr1−/y mice, as evidenced by increased density of dendritic spines for CA1 hippocampal pyramidal neurons, along with fewer mature spines with a stubby or mushroom morphology and an increased number of immature, filopodia-like spines (Fig. 1f–h). Metformin administration for 10 d corrected the dendritic abnormalities in Fmr1−/y mice (Fig. 1f–h).

Fmr1−/y mice also display exaggerated group 1 metabotropic glutamate receptor (mGlur)-dependent long-term depression (LTD) of synaptic transmission1,12. Metformin treatment for 10 d rescued

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Received 5 June 2016; accepted 7 April 2017; published online 15 May 2017; doi:10.1038/nm.4335
A hallmark of post-adolescent male individuals with FXS and Fmr1<sup>−/−</sup> mice is macroorchidism<sup>11,12</sup>. Metformin administration for 10 d restored the levels of protein synthesis were exaggerated LTD (<sub>50</sub>mg/kg, i.p.) in Fmr1<sup>−/−</sup> mice<sup>1,2,12,13</sup>. Metformin treatment for 10 d reduced the levels of phosphorylated mitogen-activated protein kinase kinase (MEK), ERK and EIF4E and the total levels of MMP-9 in the prefrontal cortex and hippocampus of Fmr1<sup>−/−</sup> mice (Fig. 2c–j), whereas the levels of phosphorylated S6 remained elevated in the hippocampus (Supplementary Figs. 8a,b and 9). Similarly, metformin treatment for 10 d rescued increased phosphorylated ERK levels in the striatum, but not in the cerebellum (Supplementary Fig. 10a,b), of Fmr1<sup>−/−</sup> mice and affected the known specific synaptic FMRP targets MAP2 and synapsin, with no effect on eEF2 and PUM2 levels<sup>14</sup> (Supplementary Fig. 11). Apart from the brain, the level of ERK phosphorylation was increased in the liver but not in the gonads of Fmr1<sup>−/−</sup> mice in the increased phosphorylation.

Metformin treatment for 10 d did not rescue the increased ERK phosphorylation in the liver (Supplementary Fig. 10d), implicating other pathways<sup>12</sup> or endocrine regulation outside the brain of Fmr1<sup>−/−</sup> mice in the increased phosphorylation.
by the lack of increased levels of phosphorylated AMPK and its downstream substrates ACC1, TSC2, raptor and B-Raf (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 AMPK phosphorylation in the brain\textsuperscript{15,16}. It is not immediately clear why metformin

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**Figure 2** Chronic metformin treatment corrects macroorchidism, decreases 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 mice (n = 6), vehicle-treated Fmr1–/y mice (n = 6), metformin-treated WT mice (n = 6), metformin-treated Fmr1–/y mice (n = 7). (b) Immunoblots and quantification 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). (c–j) Representative immunoblots of lysates from vehicle- and metformin-treated WT and Fmr1–/y mice and quantification of phosphorylation and total levels of MEK (c), ERK (d), eIF4E (e) and MMP-9 (f) in prefrontal cortex and MEK (g), ERK (h), eIF4E (i) and MMP-9 (j) in the hippocampus. GAPDH was used as a loading control. For quantification, the phospho-protein signal was normalized first against total protein signal and is presented relative to the signal for vehicle-treated WT mice (n = 6 mice in each group). Full-length immunoblots are shown in Supplementary Figures 12–14.

Each point represents data from an individual mouse, and all values in a–j are shown as mean ± s.e.m. ***P < 0.001, **P < 0.01, *P < 0.05 versus all other groups; calculated by two-way ANOVA with Tukey’s post hoc test.
administration for 10 d does not have this effect; however, in accordance with previous studies, a single injection of 200 mg per kg bodyweight i.p. of metformin induced a transient increase in the levels of phosphorylated AMPK (Supplementary Fig. 1c). A plausible explanation is that the change in ERK signaling following chronic metformin treatment is due to the rescue of elevated expression of B-Raf and c-Raf in Fmr1−/y mice (Supplementary Fig. 9).9

Presently, there is no cure for FXS or ASD, and recently completed clinical trials in teenagers or adults with FXS were not promising.20 Our data show that metformin, the most widely used US Food & Drug Administration (FDA)-approved anti-diabetic for patients aged 10 years and older, corrects most phenotypic deficits in the adult FXS mouse model. Thus, metformin, which for long-term safety and tolerability have been 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 melanogaster fragile X model.21 We present a potential molecular mechanism to explain how metformin ameliorates FXS phenotypes by showing that chronic metformin treatment corrects enhanced Raf–MEK–ERK signaling and MMP-9 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-dependent LTD and decreased general protein synthesis in Fmr1−/y mice.15 Metformin, however, corrects a broader range of phenotypes than lovastatin. In combination, these findings bolster the idea that aberrant ERK activity has a critical role in engendering FXS-like phenotypes in FXS. Because Mmp9 mRNA translation is stimulated by eIF4E phosphorylation and knockout of Mmp9 reversed abnormal phenotypes in Fmr1−/y mice,15 it is highly likely that the rescue by metformin is selectively mediated via ERK- and eIF4E-dependent normalization of MMP-9 expression in the brain, providing a very strong mechanistic explanation for the action of metformin; however, we cannot exclude the possibility of an unidentified, peripherally mediated rescue mechanism, given that metformin inhibits gluconeogenesis and alters 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, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
This work is supported by the FRAXA Research Foundation, Brain Canada/FNC, a CHF foundation grant (FDN-148423) and Brain & Behavior Research Foundation grants (24365) to N.S.; a Wellcome Trust/Royal Society Sir Henry Dale grant (107687/Z/15/Z) to C.G.G.; the Canada Research Chair Program (950-231066) to J.-C.L.; and a Brain Canada/NeuroDevNet Postdoctoral Training Award to J.P.

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 interests.

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ONLINE METHODS

Knockout mice and metformin administration. Generation of Fmr1<sup>−/−</sup> mice (the Fmr1 gene is on the mouse X chromosome; thus, male animals have a −/− genotype, where y corresponds to the mouse Y chromosome) on a C57BL/6 background (Jackson Laboratories, 003025) has been previously described<sup>3</sup>. Food and water were provided ad libitum, and mice were kept on a 12-h light/12-h dark cycle (7:00–19:00, light period). After weaning at postnatal day 21, mice were group housed (maximum of five mice per cage) by sex. Cages were maintained in ventilated racks in rooms with controlled temperature (20–21 °C) and humidity (~55%). Standard corncob bedding was used for housing (Harlan Laboratories, Inc.). All animals received a 10-d chronic treatment with metformin (200 mg per kg bodyweight per day, i.p.) or vehicle (saline), except when indicated otherwise. Injection groups were randomized over all cages. All procedures were in compliance with 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 × 28 cm × 30 cm) was divided from two side chambers (each chamber: 29 cm × 28 cm × 30 cm) by Plexiglas walls (Stoelting Co.) as previously described<sup>1,24</sup>. 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 three phases: habituation, sociability and preference for social novelty. In the first part, male mice aged 3 months 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, an unfamiliar mouse (stranger 1), which was enclosed in a wire cage to ensure that only the test mouse could initiate social interaction, was placed into one of the two remaining side chambers. An empty wire cage that was identical to the wire cage housing stranger 1 was placed on the corresponding spot in the other side chamber. The side doors were then opened simultaneously to allow the test mouse to explore the three-chamber apparatus for 10 min to assess sociability. At the end of the 10-min period, the test mouse was gently guided to the central chamber and the sliding doors were closed. In the final part of the test, a new, unfamiliar mouse (stranger 2) was placed in the previously empty wire cage, and the test mouse could explore the three chambers for an additional 10 min to assess preference for social novelty. Stranger mice consisted of age- and sex-matched C57BL/6 mice that were group housed (four mice per cage) and were used in a counterbalanced way. The empty wire cages were alternated between side chambers for different test mice. Stranger 1 and stranger 2 mice always came from different home cages. Mice were tested in the morning during the light phase. Time spent directly sniffing, defined as the time the test mouse spent in direct nose contact with wire cages, time spent in each chamber and the number of transitions into the chambers were scored manually. Data were scored in a manner that was blinded to mouse genotype—and, if possible, by a third party—using a stopwatch. Statistical analysis included mixed ANOVA with a Tukey’s post hoc test for multiple comparisons.

Self-grooming test. The setup consisted of a new Plexiglas cage equal in size to the home cage, containing approximately 1 cm of bedding material but no nesting material. A camera was placed vertically in front of the cage for recording. Fmr1<sup>−/−</sup> and WT mice (males aged 3 months) were placed in the new Plexiglas cage and allowed to explore for 20 min. The first 10 min of the experiment was considered to be the habituation phase; the final 10 min was used to acquire self-grooming data. The total time spent grooming and the total number of grooming bouts were used to analyze grooming behavior. Data were manually scored in a manner blinded to mouse genotype—and, if possible, by a third party—using a stopwatch. All measures were analyzed with two-way ANOVA with Tukey’s post hoc test.

Audiogenic seizures. Mice (male, postnatal day (P) 21–24) were chronically injected for 10 d with metformin (200 mg per kg bodyweight) or vehicle before experimentation. Mice were individually habituated in an isolated, sound-insulated behavioral chamber made of transparent plastic (outside dimensions: 28 cm × 17 cm × 16 cm) for 2 min and were subjected to a 130-dB acoustic stimulus using a personal alarm (Vigilant) for 2 min; during this time, the incidences of wild running, tonic-clonic seizures and status epilepticus were recorded.

Open-field exploration. Animals (male, aged 8–12 weeks) were first habituated to the dimly lit experimental room (~15 lx) for 30 min and were then individually placed in an illuminated clear Plexiglas chamber (40 cm × 40 cm × 40 cm, −1.200 lx) with a white floor. Animals were allowed to explore freely for 10 min following an initial 1-min habituation phase. Total path length and velocity were calculated using ANY-maze (Stoelting Co.) as a measure of hyperactive behavior.

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 cm × 40 cm × 40 cm) and a chamber with three clear Plexiglas walls with an open top. Mice (male, aged 8–12 weeks) were placed into the ‘light’ side (~390 lx) and allowed to explore freely for 10 min. An entry was defined as the mouse placing all four feet into each zone.

Morris water maze and reversal learning. Chronic daily metformin (200 mg per kg bodyweight) or vehicle (saline) administration started 5 d before training and lasted throughout the whole course of testing, for a total of 10 d. 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, aged 8–12 weeks) were handled daily for 3 d before the start of the experiment. During the experiment, mice were trained three times per day with an intertrial interval of 30 min over five consecutive days (days 1–5). Each trial lasted 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 (on day 6), the platform was removed and each mouse was allowed to swim for 30 s. For the test of the reversal learning paradigm, in which the hidden platform was relocated to the opposite quadrant (days 6 and 7), mice received the same training procedure as described above. The platform was removed for the probe test of reversal learning (day 8) to assess spatial retention. The experiment was recorded with a video tracking system (HVS Image), whereby the latency to reaching the platform during acquisition and the time spent in the target quadrant during the probe trials were determined.

Contextual fear conditioning. During acquisition (5 min), two foot shocks of 0.7 mA for 1 s each that were separated by 60 s were administered after an initial 2-min period of context exposure. Twenty-four hours after training, mice (male, aged 8–12 weeks) 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, in which animals were assigned 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 1, mice (male, aged 8–12 weeks) were first habituated for 15 min in a square testing arena (40 cm × 40 cm) followed by 15 min in an opaque box, before being returned to their home cages. On days 2 and 3, mice were put back in the arena for 15 min and presented with two identical objects (familiar) within specific areas (counterbalanced for location of objects). Mice were first allowed to freely explore the arena and objects, followed by a 15-min interval in which the mice were in an opaque box; after that, mice were returned to their home cages. On day 4, one of the objects (used for days 2 and 3) 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 spent interacting with both objects. To assess preferential attention to an object, a discrimination index was calculated as:

\[
\text{Index} = \frac{(t_{\text{novel}} - t_{\text{familiar}}) + (t_{\text{novel}} + t_{\text{familiar}})}{2}
\]

A positive index represents a preference for the novel object.
Immunoblotting and antibodies. Brain tissue (from male mice aged 3 months) 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, 0.1 mM EGTA pH 8.0, 10 mM NaF, 1 mM β-glycerophosphate and 1 mM sodium orthovanadate) containing protease inhibitors (Roche). Protein extracts were denatured with heat and resolved by SDS–PAGE or gradient gels (Thermo Fisher Scientific). Following electrophoresis, proteins were transferred to nitrocellulose membranes and immunoblotting was performed. Membranes were stripped in 25 mM glycine-HCl pH 2.0 and 1% SDS for 30 min at room temperature, followed by washing in TBST before reprobing. Immunoreactivity was detected by enhanced chemiluminescence (plus-ECL, PerkinElmer, Inc.) after exposure to X-ray film (Denville Science, 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); GAPDH (sc-32233, Santa Cruz); β-actin (A5441, Sigma); second-tubulin (T8328, Sigma) and presented as percentage change relative to control. For statistical analysis of immunoblot results, we used two-way ANOVA with Tukey’s post hoc test.

Analysis of neuronal morphology by Golgi–Cox staining. Four male mice per genotype and treatment (aged 3 months) were used for morphological analysis.

LTD recordings. For analysis of hippocampal LTD, male WT or Fmr1−/− mice aged 31–34 d, treated with either saline or metformin (as described above), were used. After obtaining hippocampal slices (400 µm thick), the CA1 and CA3 hippocampal regions were isolated by 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 NaH2PO4, 2 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3 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. Glass electrodes (2–3 MΩ) were filled with ACSF and gently placed on the CA1 stratum radiatum to record iEPSPs 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 iEPSPs with 60% maximal amplitude. mGlur-LTD was induced by perfusion with the group I mGluR agonist DHPG (50 µM; Tocris Bioscience) for 10 min in ACSF. iEPSPs were recorded for a total of 60 min after the onset of induction. The iEPSP slope was measured on digitized analog recordings using the Clampfit analyze function and by using the cursor to define quantifiable epochs between 10% and 90% of the maximal iEPSP amplitude. This setting excluded fiber volley and population spikes. Data were then analyzed using two-way ANOVA with Tukey’s post hoc test.

Measurement of de novo protein synthesis. To assess whether metformin corrected increased translation in Fmr1−/− mice, we measured de novo protein synthesis in hippocampal slices using the SUnSET puromycin incorporation assay1,12. Transverse hippocampal slices (400 µm thick) were prepared from the brains of mice aged 5–6 weeks and were allowed to recover for at least 3 h. Puromycin labeling was then performed as described12,18. Briefly, the slices were incubated with puromycin (P7255, Sigma, 5 µg/ml in ACSF) for 45 min and then processed for immunoblotting, as described above, using an anti-puromycin antibody (EQ0001, KeraFast). Slices processed in parallel but not incubated with puromycin served as an unlabeled control. The quantity of protein synthesis was determined by measuring total lane signal from 15–250 kDa and subtracting signal from the unlabeled protein control. Signals were quantified using ImageJ, normalized to β-tubulin (T8328, Sigma) and presented as percentage change relative to control. For statistical analysis of immunoblot results, we used two-way ANOVA with Tukey’s post hoc test.

Metformin bioanalysis and LC–MS/MS. WT mice on a C57BL/6J background (Charles River Laboratories; males aged 8–10 weeks) were used for the study. Food and water were provided ad libitum, and mice were kept on a 12-h light/12-h dark cycle (light period, 7:00–19:00). For pharmacokinetic analysis, the mice received a single dose of metformin (200 mg per kg bodyweight, i.p.) and plasma and brain tissues were collected at 0, 0.5, 1, 2 and 4 h after drug administration. For the dose-response study, mice were treated for 10 d with 25, 50, 100 or 200 mg of metformin per kg bodyweight per day (i.p.) and plasma and brain tissues were collected 24 h after the last injection. Brain tissue homogenate and plasma concentrations of metformin were 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 the salt factor as applicable. Calibration spiking solutions were prepared at 10.0, 20.0, 50.0, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000 and 100,000 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 three parts distilled water per gram of tissue for a final processing dilution factor of fourfold. The resultant blank tissues were used for matrix calibration standards, which were prepared the same day as analysis on ice at 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100, 250, 500, 1,000, 2,500 and 5,000 ng/ml by spiking blank brain tissue homogenate and plasma matrices at 1:20 with the appropriate metformin spiking solution. Subsequently, aliquots of the matrix samples, matrix calibration standards and matrix blanks were taken and protein was precipitated by the addition of 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 1,150 g 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 an LC–MS/MS system: AB Sciex QTRAP 6500 with a Shimadzu Nexera UPLC system using a ZIC-HILIC 2.1 mm × 50 mm analytical column (EMD Millipore) with a 3.5-µm pore size. An injection volume of 1.5 µl was used 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 were generated with positive electrospray ionization (ESI+) using multiple-reaction monitoring (MRM) of the following transitions: metformin, 130.324/60.100 Da; 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 genotype and treatment during testing and scoring. To determine 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 ref. 27. All experimental n values represent individual animals unless otherwise stated—technical replicates of some immunoblots 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.** The data supporting the findings of this study are available from the corresponding author upon reasonable request.