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Supplementary Materials for

mGlu$_7$ potentiation rescues cognitive, social, and respiratory phenotypes in a mouse model of Rett syndrome

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Supplementary Materials and Methods

Reporter Gene Construction and Luciferase Assay

The restriction sites used to clone the *GRM7* promoter into the pGL4 expression vector were SacI and HindIII, and the restriction sites used to clone *MECP2* into the pIRESpuro3 vector were NotI and BamHI. HEK293 cells were plated in 24-well plates at a density of 5 x 10^4 cells per well and transfected with the pGL4 vector with and without *MECP2* using Fugene 6 (Promega) at a 3:1 ratio of transfection reagent to DNA. The growth medium was aspirated and 100 µl of 5X passive lysis buffer (Promega) was added to the cells. The cells were freeze-thawed twice and then 10 µl of each sample was transferred to a 96-well plate. Luciferase was detected using the Dual-Glo Luciferase Assay System (Promega). After luciferase was measured, samples were treated with 10 µl of the Dual-Glo Stop & Glo reagent (Promega) to neutralize luciferase luminescence and allow for the detection of *Renilla* luminescence.

Drugs

The full names of compounds used are as follows: VU0422288 (*N*-[3-Chloro-4-[(5-chloro-2-pyridinyl)oxy]phenyl]-2-pyridinecarboxamide), VU0155094 (methyl 4-[3-[2-(4-acetamidophenyl)sulfanylacetyl]-2,5-dimethylpyrrol-1-yl]benzoate), LSP4-2022 ((2*S*)-2-amino-4-(([4-(carboxymethoxy)phenyl](hydroxy)methyl)(hydroxy)phosphoryl)butanoic acid), ADX88178 (5-Methyl-*N*-(4-methylpyrimidin-2-yl)-4-(1*H*-pyrazol-4-yl)thiazol-2-amine) and ADX71743 (6-(2,4-dimethylphenyl)-2-ethyl-4,5,6,7-tetrahydro-1,3-benzoxazol-4-one). All drugs used for electrophysiology experiments were diluted in artificial cerebrospinal fluid (ACSF,in mM: 126 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$ and 1 $MgSO_4$), and drugs used for behavioral experiments were diluted in 10% Tween 80.

Animals

All of the animals used in the present study were group housed with food and water given *ad libitum* and maintained on a 12 hour light/dark cycle. Animals were cared for in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All studies were approved by the Institutional Animal Care and Use Committee for Vanderbilt University and took place during the light phase. P39-P50 B6.129P2(C)-*Mecp2*$^{tm1.1Bird}$/J on a C57BL6/J congenic background, Jackson Labs strain #003890 males (*Mecp2*$^{-/y}$), 18-20 week old *Mecp2*$^{+/-}$ females, or wild-type littermate controls (*Mecp2*$^{+/y}$ or *Mecp2*$^{+/+}$) were used in all studies. The *Grm7* knockout mice used in antibody validation experiments were cryorecovered from the Mutant Mouse Regional Resource Center and maintained under

identical conditions. Wild type male Sprague-Dawley rats, aged 8-10 weeks and weighing ~300g, were purchased from Harlan Laboratories for in vivo pharmacokinetic experiments.

Quantitative Real-time PCR (QRT-PCR)

Cortex, hippocampus and striatum were microdissected from P39-55 *Mecp2*$^{-/y}$ and *Mecp2*$^{+/y}$ mice, and human samples were obtained from the University of Maryland and the Harvard Brain Bank. All samples were homogenized with a mortar and pestle and total RNA was prepared using TRIzol Reagent (Invitrogen) in accordance with manufacturer's instructions. RNA-quality was verified using an Agilent Bioanalyzer High Sensitivity Chip. Total RNA from each brain region was DNase-treated with Roche Turbo DNase kit, and cDNA from 2 μg total RNA was synthesized using the VILO kit (Invitrogen). RT-qPCR on cDNA from 25 ng of initial RNA template was then run in triplicate using Taqman Fast Reagent Mix (Life Technologies). Values exceeding two times the standard deviation were classified as outliers. Each value was compared to the average delta-cycle threshold (Ct) value acquired for *Mecp2*$^{+/y}$ control mice and calculated as percent-relative to the average control Δ-Ct.

Total and Synaptosomal Protein Preparation

Tissue samples were homogenized using a hand-held motorized mortar and pestle in radioimmunoprecipitation assay buffer (RIPA) containing 10 mM Tris-HCL, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1% Triton™ X-100, and 1% Deoxycholate. After homogenization, samples were centrifuged for 20 minutes at 15,000g at 4 ºC. The supernatant was then transferred to new tubes and protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce). For synaptosome preparations, the cortex and hippocampus were microdissected from *Mecp2*$^{-/y}$, *Mecp2*$^{+/-}$ and age-matched control mice. The tissue was homogenized in 9 ml of ice-cold sucrose/HEPES (0.32 M sucrose, 4.2 mM HEPES, pH 7.4) using a Teflon-glass homogenizer (Wheaton Science Products). The homogenate was centrifuged at 1,000g for 5 minutes at 4 °C and the resultant supernatant was centrifuged at 12,000g for 15 minutes at 4° C. The final pellets containing synaptosomes were re-suspended in Krebs-Ringer-HEPES buffer (KRH, 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.2 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM HEPES pH 7.4). Protein concentrations were determined using a BCA protein assay (Pierce).

Extracellular Field Potential Recordings

P45-P50 *Mecp2*$^{-/y}$ and 20 week old *Mecp2*$^{+/-}$ mice, as well as age matched *Mecp2*$^{+/y}$ and *Mecp2*$^{+/+}$ controls, were anesthetized with isoflurane and then decapitated. Brains were rapidly removed and submerged in ice-cold sucrose cutting

buffer containing 230 mM sucrose, 2.5 mM KCl, 8 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 10 mM glucose, and 26 mM $NaHCO_3$ saturated with 95%/5% O_2/CO_2. A block of tissue containing hippocampus was trimmed, embedded in agarose, and coronal slices 400 μm thick were cut using a Compresstome VF-200 (Precisionary Instruments). Slices were transferred to a holding chamber containing N-methyl-D-glucamine (NMDG)-HEPES recovery solution (in mM, 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$, pH 7.3, 305 mOsm) for 15 minutes at 32 ^{0}C. Slices were then transferred to a room temperature modified artificial cerebral spinal fluid (ACSF) containing (in mM) 126 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$ and 1 $MgSO_4$, and 600 μM sodium ascorbate for at least 1 hour. Subsequently, slices were transferred to a submersion recording chamber and continuously perfused (2 mL/min) with ACSF containing (in mM) 126 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$ heated to 32 °C. All solutions were continuously bubbled with 95%/5% O_2/CO_2. Borosilicate glass electrodes were pulled using a Flaming/Brown micropipette puller (Sutter Instruments) and had a resistance of 3-5 MΩ when filled with ACSF. Paired-pulse field excitatory postsynaptic potentials (fEPSPs) were evoked (100 μs duration, every 20 sec spaced 20 ms apart) at the CA3-CA1 border by placing a glass recording electrode in the stratum radiatum of CA1. Input-output curves were generated for each slice and the stimulation intensity was adjusted to 50% of the maximum response for subsequent experiments. Paired-pulse ratios (PPR) were calculated as the ratio between the slope of the second fEPSP divided by the slope of the first fEPSP. PPRs were calculated at several inter-stimulus intervals (ISI) ranging from 10-500 ms.

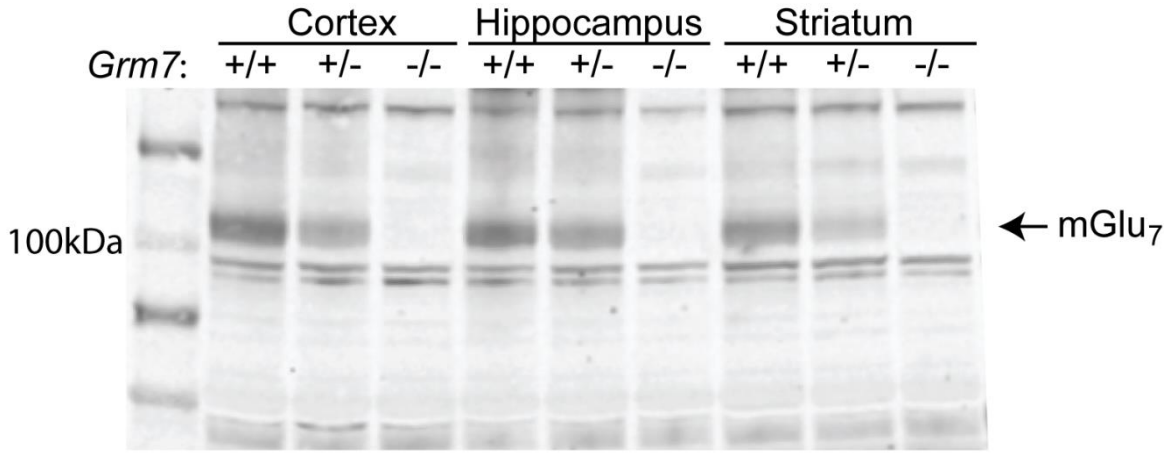
In Vivo Pharmacokinetic Analysis

VU0422288 was formulated in 10% Tween 80 in sterile water (4 mg/mL) at a concentration of 10 mg/kg and administered intraperitoneally (i.p.) to adult male Sprague-Dawley rats (Harlan Laboratories). One hour following VU0422288 administration, animals were euthanized via decapitation under isoflurane to obtain blood and brain samples. Plasma was isolated from the blood samples by centrifugation and stored at -80 ºC until analysis. Brain samples were quickly washed with ice-cold phosphate-buffered saline (PBS) and frozen on dry ice. Analysis of plasma and brain VU0422288 concentrations was performed as previously described (18, 23). Briefly, plasma and brain samples were protein-precipitated with three volumes of ice-cold acetonitrile containing internal standard, centrifuged (4000 g, 4 ºC, 5 min), and the supernatants transferred and diluted 1:1 (v/v) for LC-MS/MS analysis. Samples were analyzed via electrospray ionization (ESI) LC-MS/MS on an AB Sciex API-4000 triple-quadrupole instrument that was coupled with

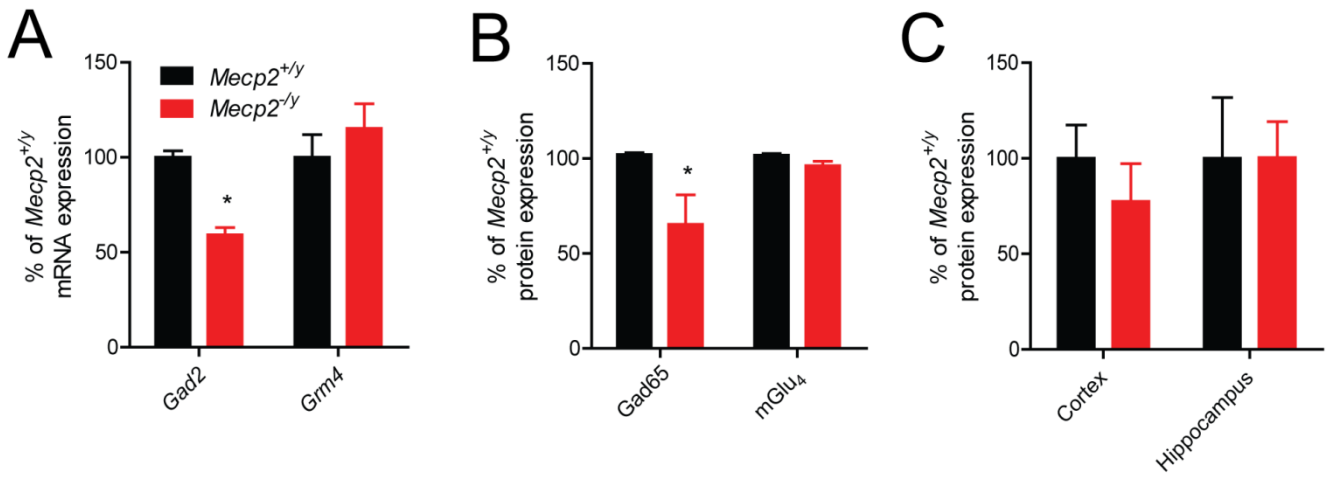
Shimadzu LC-10AD pumps) and a Leap Technologies CTC PAL auto-sampler and concentrations (ng/mL or ng/g) of VU0422288 were determined using a matrix-matched 10-point standard curve.

Brain Homogenate and Plasma Binding

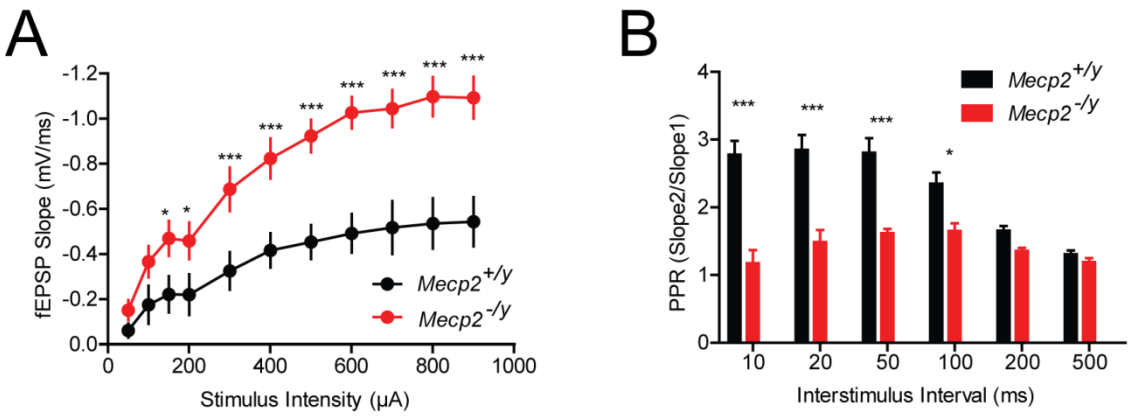
The binding of VU0422288 was determined in rat plasma or brain homogenates via equilibrium dialysis using RED plates (Thermo Fisher Scientific). A mixture of sample homogenate and VU0422288 at a final concentration of 5 μM was added to the *cis* side (red) of a RED plate while an equal volume of PBS (25 mM, pH 7.4) was added to the *trans* side. The plate was then sealed and incubated at 37 ºC for 4 hours. At the completion of the incubation, an aliquot from each chamber was removed and diluted 1:1 with either plasma (*cis*) or PBS (*trans*) and transferred to a new 96-well plate. The proteins were precipitated using ice-cold acetonitrile and the samples were then centrifuged at 3000 rcf for 10 minutes to isolate the supernatant. After centrifugation, the supernatant was removed and the concentrations of VU0422288 from each sample were determined via liquid chromatography-tandem mass spectrometry as previously described (26, 30).



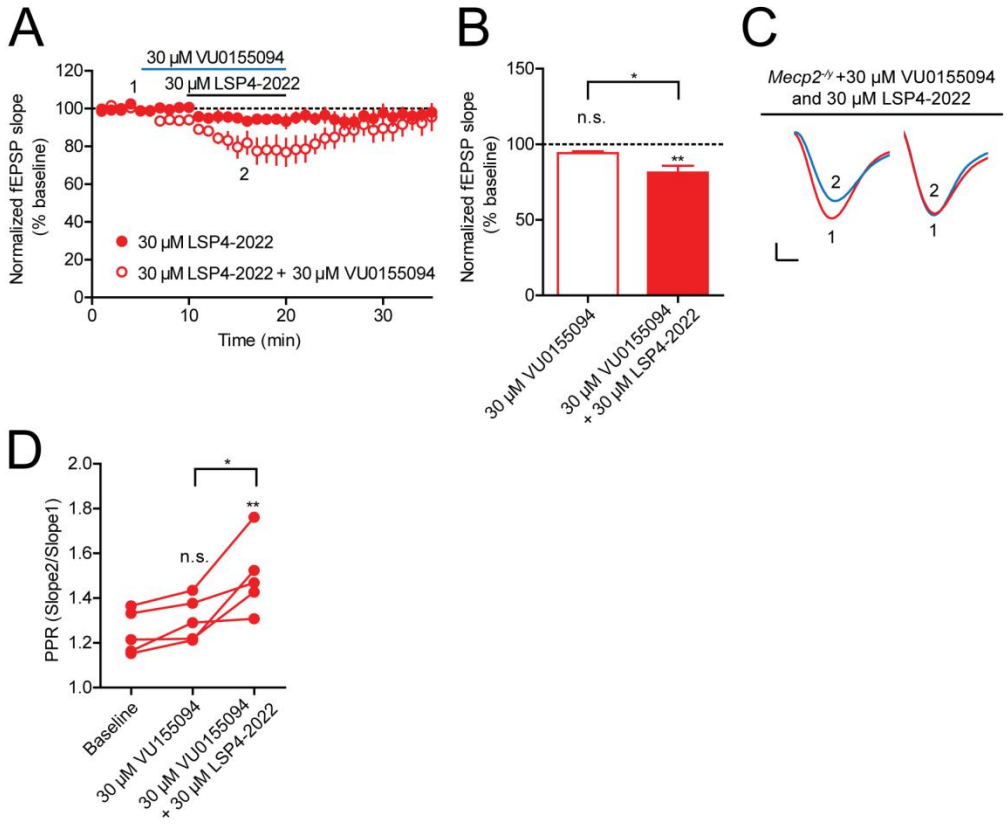
Supplementary Figure 1. Antibody #07-239 is selective for $mGlu_7$. Odyssey fluorescent Western blot. Cortical, hippocampal and striatal protein isolates from *Grm7*$^{+/+}$, *Grm7*$^{+/-}$ and *Grm7*$^{-/-}$ mice were probed with anti-$mGlu_7$ (Millipore, #07-239) to confirm the specificity of the antibody used for these studies. The predicted size of $mGlu_7$ is 107 kDa.



Supplementary Figure 2. Gad65, but not mGlu$_4$, expression is reduced in *Mecp2*$^{-/y}$ mice. (**A**) *Gad2* and *Grm4* mRNA expression in the cortex of *Mecp2*$^{+/y}$ and *Mecp2*$^{-/y}$ mice % of *Mecp2*$^{+/y}$, *Gad2*: 59.2 ± 3.9 (*p < 0.05), *Grm4*: 115.2 ± 13.0, p = 0.0173, Two-way ANOVA, genotype x gene with Bonferonni's post hoc, n = 3-5, df = 1, 12. (**B**) Gad65 (*Gad2*) total protein expression in the cortex of *Mecp2*$^{+/y}$ and *Mecp2*$^{-/y}$ mice.% of *Mecp2*$^{+/y}$, Gad65: 65.3 ± 15.4 (*p < 0.05), mGlu$_4$: 96.2 ± 2.4, p = 0.0452, Two-way ANOVA, genotype x gene with Bonferonni's post hoc, n = 3-4, df = 1, 10. **(C)** Quantification of Figure 2D, vGlut2 synaptosome blots. % of *Mecp2*$^{+/y}$: 77.4 ± 8.9, p =0.09 and hippocampus 100.4 ± 8.4, p =0.99. n = 5 / region / genotype; Student's t-test.

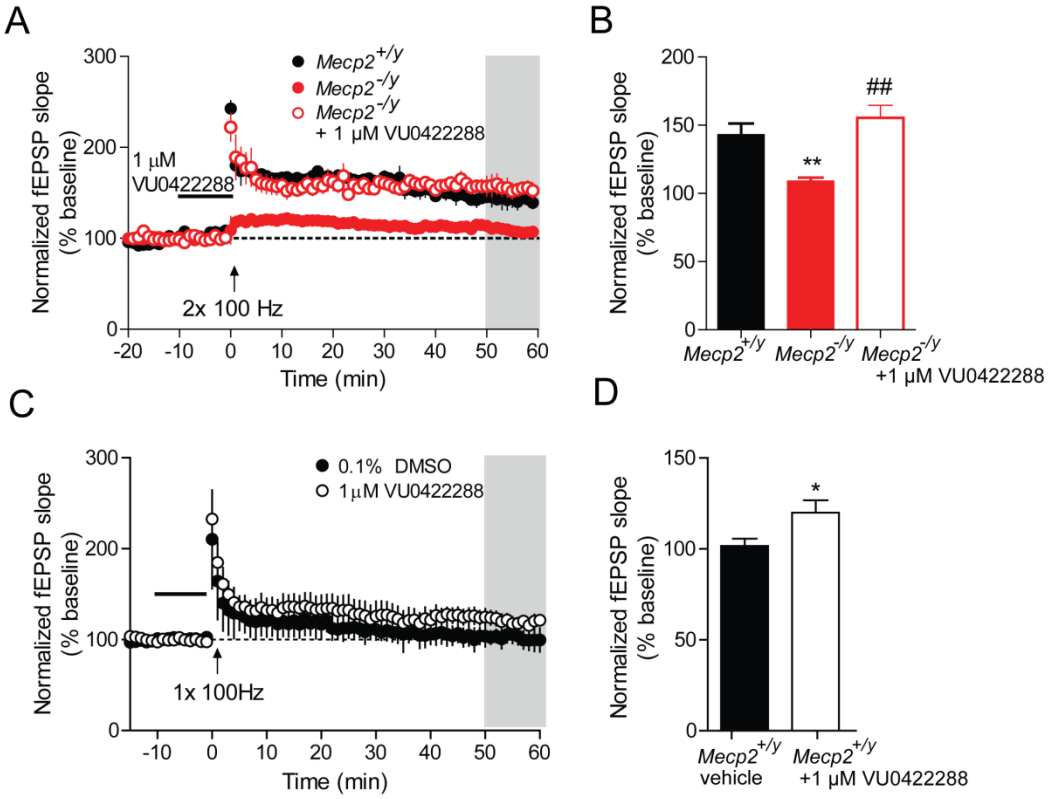


Supplementary Figure 3. *Mecp2*$^{-/y}$ hippocampal slices display enhanced excitatory transmission at the SC-CA1 synapse. fEPSPs were generated by stimulating axon fibers from CA3 with a bipolar electrode. (**A**) fEPSPs evoked by increasing stimulation intensities 50-900 μA in slices from *Mecp2*$^{-/y}$ and *Mecp2*$^{+/y}$ mice (overall p = 0.0002, Two-way ANOVA with repeated measures, genotype x stimulation intensity with Bonferonni's post hoc, n = 7, 7 (slices, mice), df = 1, 120, *p < 0.05, ***p < 0.001). (**B**) PPR at interstimulus intervals (ISIs) between 10 - 100 ms in slices from *Mecp2*$^{-/y}$ and *Mecp2*$^{+/y}$ mice (overall p = 0.0071, Two-way ANOVA, genotype x ISI with Bonferonni's post hoc, n = 3-4, 4 (slices, mice), df = 1, 25, *p < 0.05, ***p < 0.001).

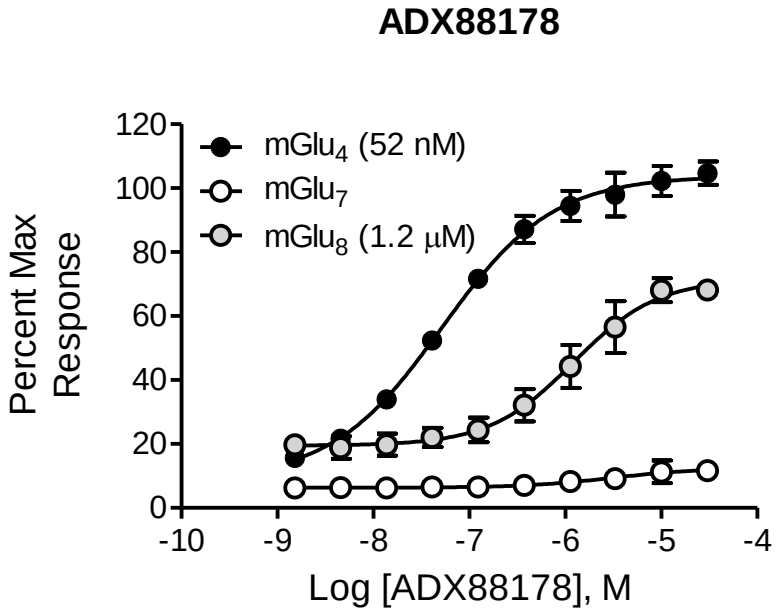


Supplementary Figure 4. VU0155094, a PAM that is structurally distinct from VU0422288, also rescues the efficacy of LSP4-2022 on fEPSPs at SC-CA1 in *Mecp2*$^{-/y}$ slices. Paired fEPSPs were generated by stimulating the axon fibers from CA3 with a bipolar electrode. (**A**) Bath application of 30 µM VU0155094 for 5 minutes alone and then for 10 minutes in combination with 30 µM LSP4-2022 (empty symbols) compared to 30 µM LSP4-2022 alone (filled symbols). Data are normalized to the average baseline fEPSP slope. VU0155094 application is denoted by a solid blue line and LSP4-2022 addition is denoted by a solid black line. (**B**) Quantification of normalized fEPSP slopes during VU0155094 addition alone (white bar) or in combination with LSP4-2022 (red bar). n.s. indicates not significantly different from baseline (% of baseline, VU0155094 alone: 94.2 ± 1.3, VU0155094 + LSP4-2022: 81.3 ± 4.6, **p = 0.0064, One-way repeated measures ANOVA with Bonferonni's post hoc, n = 5, 5 (slices, mice), df = 4, 8, *p < 0.05). (**C**) Paired-pulse traces from an individual, representative experiment during application of 30 µM VU0155094 alone (1, red trace) and 30 µM VU0155094 plus 30 µM LSP4-2022 (2, blue trace). Scale bars represent 0.2 mV by 2 ms. (**D**) Quantification of PPR at baseline, following 30 µM VU0155094 application alone, and in response to coadminstration of 30 µM VU0155094 and 30 µM LSP4-2022. Values for individual experiments are represented as points connected by a line. n.s. indicates not significantly different from baseline (1.2 ± 0.04 at baseline vs. 1.3 ± 0.04 during VU0155094 alone vs. 1.5 ±0.07 during

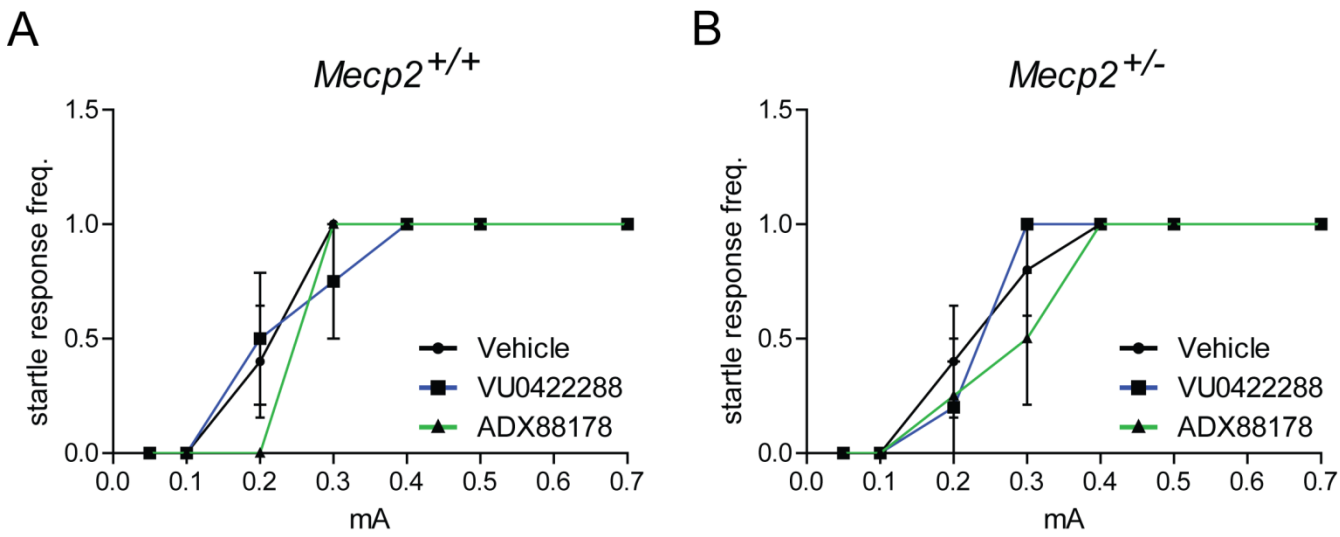
VU0155094 + LSP4-2022, $p = 0.0021$, One-way repeated measures ANOVA with Bonferonni's post hoc, $n = 5$, $df = 4, 8$, $*p < 0.05$, $**p < 0.001$).



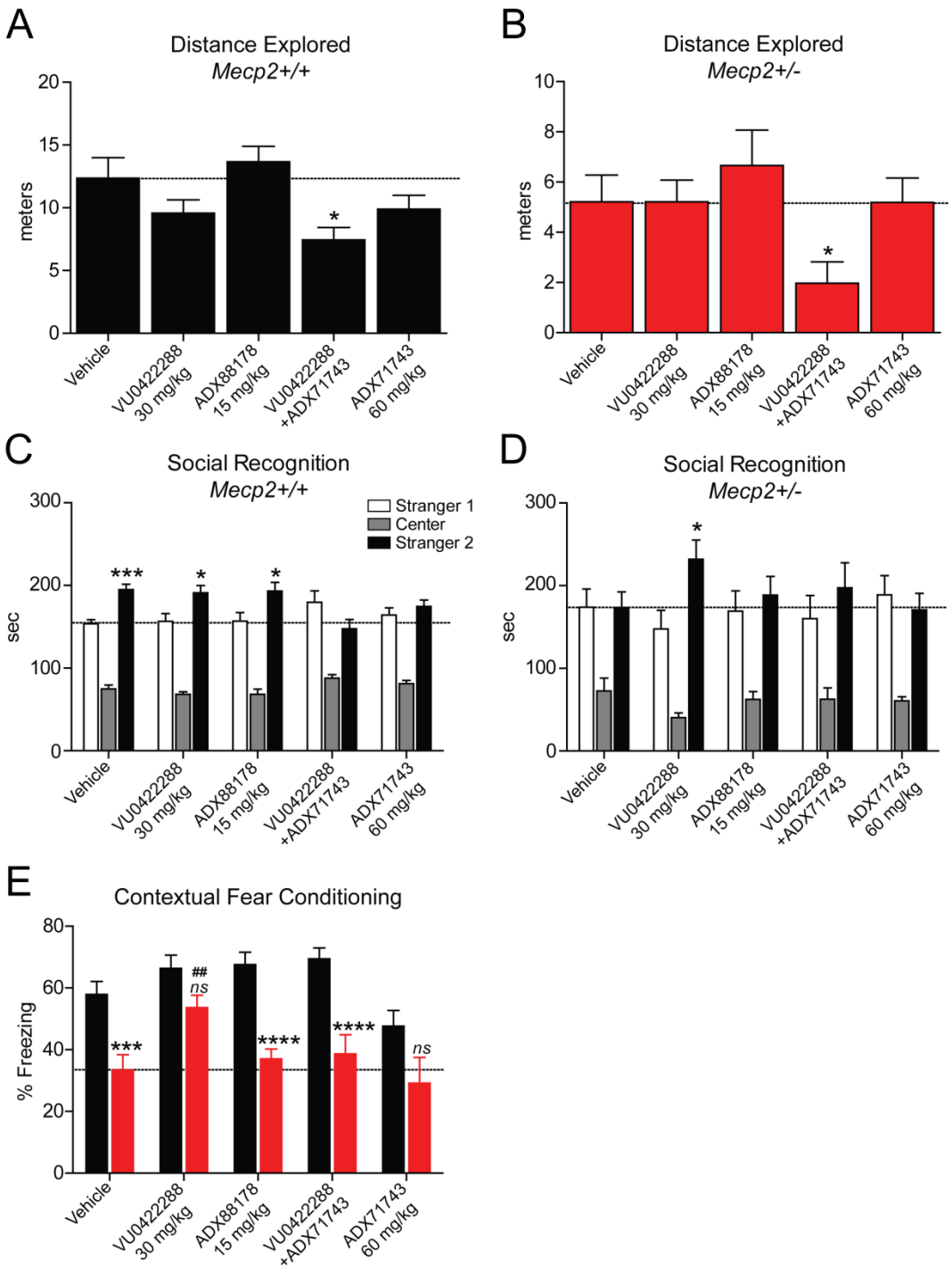
Supplementary Figure 5. VU0422288 rescues attenuated LTP at the SC-CA1 synapse in *Mecp2*$^{-/y}$ mice. (**A**) LTP was induced by applying two trains of 100 Hz stimulation lasting 1 second with a 20 second inter-stimulus interval. LTP in *Mecp2*$^{-/y}$ slices treated with vehicle (filled red circles) and 1 μM VU0422288 (empty red circles), compared to LTP in *Mecp2*$^{+/y}$ slices treated with vehicle (filled black circles). (**B**) Quantification of LTP was determined by averaging the values during the last 10 minutes of recording (shaded grey area in **A**). (% of baseline, *Mecp2*$^{+/y}$: 143.1 ± 8.1, *Mecp2*$^{-/y}$: 109.0 ± 2.6, overall p = 0.006, *Mecp2*$^{-/y}$ + VU0422288: 155.4 ± 9.2% of baseline, One-way ANOVA with Bonferonni's post hoc, **p < 0.01, n = 3-4, 3-4 (slices, mice),. # denotes a comparison with vehicle-treated mice of the same genotype. (**C**) Threshold LTP in *Mecp2*$^{+/y}$ slices treated with vehicle (filled symbols) and 1 μM VU0422288 (empty symbols). (**D**) Bar graph depicting LTP quantified by averaging the values during the last 10 minutes of recording in (**C**). (Shaded grey area in **C**). % of baseline, vehicle: 101.6 ± 4.0, VU0422288: 119.9 ± 6.7, One-way ANOVA with Bonferonni's post hoc, p = 0.041 n = 6, 6 (slices, mice).



Supplementary Figure 6. ADX88178 is active at $mGlu_4$ and $mGlu_8$, but not at $mGlu_7$. Increasing concentrations of ADX88178 were applied 2 minutes prior to the addition of an EC_{20} concentration of either glutamate (for $mGlu_4$ and $mGlu_8$) or L-AP4 (for $mGlu_7$) in calcium mobilization assays as described in Sakrikar et al. Responses were normalized to the % maximal response induced by the control PAM, VU0422288, for each cell line; this compound induces a maximal level of potentiation in each cell line. For each receptor, n=3 independent assays run in triplicate.

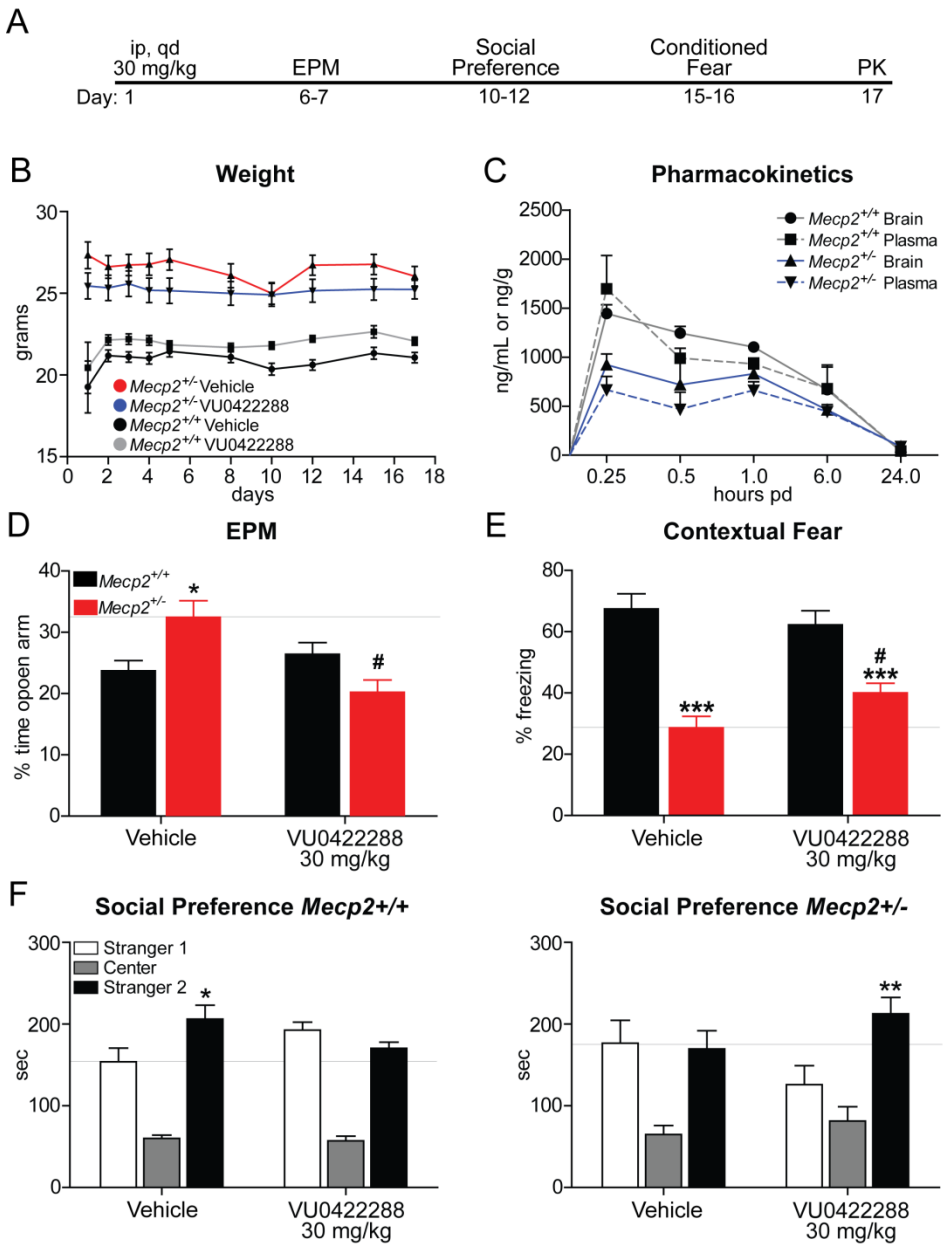


Supplementary Figure 7. Startle response threshold is not affected by VU0422288 and ADX88178 treatment in *Mecp2*$^{+/+}$ or *Mecp2*$^{+/-}$ mice. (**A**) In control *Mecp2*$^{+/+}$ mice, startle response was 0.3 ± 0.1 mA regardless of treatment. (**B**) Similar to what was observed in *Mecp2*$^{+/-}$ mice, startle response was 0.3 ± 0.1 mA independent of compound type. n = 5 / genotype / treatment. Two-way ANOVA with Bonferoni post hoc analysis, no significant difference observed between treatment groups.



Supplementary Figure 8. Coadministration of VU0422288 and ADX71743 confirm mGlu$_7$'s role in cognition but evokes sedative effects. (**A**) Distance traveled in the habituation phase of the social recognition assay for *Mecp2*$^{+/+}$ control mice, as assessed at T_{max} of each compound (T_{max} defined by in-house pharmacokinetic analysis). (*Mecp2*$^{+/+}$: Vehicle (n = 9): 12.4 ± 1.63 m, VU0422288 (n = 8, 30 mg/kg): 9.6 ± 1.06 m, ADX88178 (n = 10, 15 mg/kg): 13.7 ± 1.24 m, VU0422288 + ADX71743: 7.4 ± 1.00 m (*p < 0.05), ADX71743 (60 mg/kg): 9.9 ± 1.11 m). (**B**) Distance traveled in the habituation phase of the social recognition assay for *Mecp2*$^{+/-}$ mice. (*Mecp2*$^{+/-}$: vehicle: 5.2 ± 1.07 m, VU0422288: 5.2 ± 0.87 m, ADX88178: 6.7 ± 1.41, VU0422288 + ADX71743: 1.97 ± 0.85 m (*p < 0.05), ADX71743: 5.2 ± 0.98 m.)

(C-E) Extension of Figure 4. ADX71743 and ADX71743 + VU0422288 were excluded from primary figure due to sedative effects. (**C**) Three chamber social preference in *Mecp2*$^{+/+}$ mice comparing time spent with a familiar mouse (stranger 1, white) and a novel mouse (stranger 2, black) (vehicle: 153.7 ± 5.0s stranger 1 vs. 194.9 ± 6.4s stranger 2, ***p < 0.001; VU0422288: 156.7 ± 9.2s stranger 1 vs. 191.1 ± 8.7s stranger 2, *p < 0.05; ADX88178: 156.7 ± 10.1s stranger 1 vs. 193.1 ± 10.5s stranger 2, *p < 0.05); however, social recognition is lost in *Mecp2*$^{+/+}$ control mice treated with the VU0422288 + the $mGlu_7$ NAM ADX71743, or ADX71743 alone (VU0422288 + ADX71743: 179.7 ± 13.5s stranger 1 vs 147.7 ± 11.2s stranger 2, ADX71743: 164.2 ± 8.7s stranger 1 vs 274.6 ± 7.7s stranger 2. (**D**) Three chamber social preference in *Mecp2*$^{+/-}$ mice comparing time spent with a familiar mouse (stranger 1, white) and a novel mouse (stranger 2, black) (vehicle: 174.0 ± 21.8 stranger 1 vs. 173.3 ± 19.1 stranger 2: VU0422288: 147.7 ± 22.3 stranger 1 vs. 231.8 ± 23.4 stranger 2, *p < 0.05; ADX88178: 169.1 ± 24.7 stranger 1 vs. 188.6 ± 22.6 stranger 2, (VU0422288 + ADX71743: 160.0 ± 28.13s stranger 1 vs 197.4 ± 30.20s stranger 2, ADX71743: 188.77 ± 23.28s stranger 1 vs 170.7 ± 20.04s stranger 2). Student's t-test, stranger 1 vs stranger 2. (**E**) Contextual Fear Conditioning in *Mecp2*$^{+/+}$ (black) and *Mecp2*$^{+/-}$ mice (red) treated with vehicle (*Mecp2*$^{+/+}$: 57.9 ± 4% (n = 22), *Mecp2*$^{+/-}$: 33.4 ± 4.9% (n = 16), **p < 0.001), 30 mg/kg VU0422288 (*Mecp2*$^{+/+}$: 66.3 ± 4.3% (n = 17), *Mecp2*$^{+/-}$: 53.6 ± 4.1% (n = 15), ##p < 0.01, *Mecp2*$^{+/-}$ + VU0422288 is not significantly different from *Mecp2*$^{+/+}$ + vehicle), 15 mg/kg of the $mGlu_{4,8}$ PAM ADX88178 (*Mecp2*$^{+/+}$: 67.6 ± 3.9% (n = 10), *Mecp2*$^{+/-}$: 37.0 ± 3.2% (n = 15), ****p < 0.0001), coadministration of VU0422288 and the $mGlu_7$ NAM ADX71743 (*Mecp2*$^{+/+}$: 69.4 ± 3.5% (n = 10), *Mecp2*$^{+/-}$: 38.7 ± 6.2% (n = 17), ****p < 0.0001), and treatment ADX71743 alone (*Mecp2*$^{+/+}$: 47.6 ± 5.1% (n = 17), *Mecp2*$^{+/-}$: 29.2 ± 8.3% (n = 11). Two-way ANOVA, genotype x drug, df = 4, 148 with two-tailed Student's t tests used for post hoc analysis. # denotes a comparison with vehicle-treated mice of the same genotype.



Supplementary Figure 9. Efficacy is conserved with repeated VU0422288 administration. (**A**) 30 mg/ kg VU0422288 was delivered via intraperitoneal (ip) injection, once daily and elevated plus maze (EPM), 3-chamber social preference and conditioned fear analysis was performed at T_{max} on the days noted above. n=15 / genotype / group. (**B**) Daily weights of *Mecp2*$^{+/+}$ and *Mecp2*$^{+/-}$ mice during 17 day subchronic administration of vehicle and VU0422288 ($p < 0.01$ at all time-points comparing *Mecp2*$^{+/+}$ relative to *Mecp2*$^{+/-}$ in both treatment groups, two-way ANOVA with Bonferoni posttest). (**C**) Brain and plasma were isolated from n = 3 mice at 0.25, 0.5, 1, 6 and 24 hrs after dose #17. VU0422288 exhibits brain to plasma ratios of 1.04 and 1.10 in *Mecp2*$^{+/+}$ and *Mecp2*$^{+/-}$ mice, respectively. T_{max} was 0.25 hrs for both genotypes and brain C_{max} was 1445 ng/g in *Mecp2*$^{+/+}$ mice and 923 ng/g in *Mecp2*$^{+/-}$ mice. (**D**) Elevated plus maze in vehicle-treated *Mecp2*$^{+/+}$ (black) and *Mecp2*$^{+/-}$ (red) mice (% time open arm: 23.7 ± 1.7 s vs 32.5 ± 2.7 s, *p >

0.05), and those treated subchronically (6-7 days) with VU0422288 (20.2 ± 2.0 s, $^{\#}p < 0.05$). (**E**) Contextual fear conditioning in vehicle-treated *Mecp2*$^{+/+}$ and *Mecp2*$^{+/-}$ mice (*Mecp2*$^{+/+}$: 67.4 ± 5%, *Mecp2*$^{+/-}$: 28.6 ± 3.7%, ***$p < 0.001$) and in those treated subchronically (15-16 days) with 30 mg/kg VU0422288 (*Mecp2*$^{+/+}$: 62.2 ± 4.6%, *Mecp2*$^{+/-}$: 40.0 ± 3.2%, #$p < 0.05$. Student's t tests used for post hoc analysis). (**F**) Three chamber social preference comparing time spent with the familiar mouse (stranger 1, white) and preference for stranger 2 (black) in in *Mecp2*$^{+/+}$ and *Mecp2*$^{+/-}$ mice treated with vehicle (*Mecp2*$^{+/+}$: 153.9 ± 16.8 s stranger 1 vs. 206.0 ± 17.0 s stranger 2, *$p < 0.05$; *Mecp2*$^{+/-}$: 176.6 ± 28.0 s stranger 1 vs. 169.7 ± 22.3 s stranger 2), and in *Mecp2*$^{+/+}$ and *Mecp2*$^{+/-}$ mice following repeat administration (10-12 days) of VU0422288 (*Mecp2*$^{+/+}$: 192.7 ± 9.6 s stranger 1 vs. 170.3 ± 7.7 s stranger 2; *Mecp2*$^{+/-}$: 125.9 ± 23.3 s stranger 1 vs. 212.7 ± 20.0 s stranger 2, **$p < 0.01$).

Code	AN Number	Age	Sex	PMI (hr)	Mutation	%XCI	Region	Sub -reg	Cause of Death
Rett-1	AN19242	10	F	NA	R255X	63.9	Cortex	BA4	
Rett-2	AN05852	24	F	14	R168X		Cortex	BA4	
Rett-3	AN15579	11	F	9.6	R255X	38.9	Cortex	BA4	
Rett-4	AN04573	12	F	5	R270X	49.1	Cortex	BA4	
Rett-5	AN08016	8	F	2.9	R255X		Cortex	BA4	
Rett-6	AN02091	24	F	15.78	R255X		Cortex	BA4	
Rett-7	AN04121	10	F	23.5	R270X		Cortex	BA4	
Rett-8	AN01193	23	F	10.5	Unknown		Cortex	BA4	
Rett-9	AN17129	22	F	2.5	Unknown		Cortex	BA4	

	Age	PMI
AVG	16.0	10.4
SEM	2.3	2.5

Code	Maryland BB	Age	Sex	PMI (hr)	Mutation	%XCI	Region	Sub -reg	Cause of Death
CTL-1	#1038	24	F	7	None (WT)		Cortex	BA4	Accidental Head Injury
CTL-2	#1708	8	F	20	None (WT)		Cortex	BA4	Accidental Head Injury
CTL-3	#5161	10	F	22	None (WT)		Cortex	BA4	Accidental Hanging
CTL-4	#5554	13	F	15	None (WT)		Cortex	BA4	Suicidal Hanging
CTL-5	#5669	24	F	29	None (WT)		Cortex	BA4	Hypertensive CV Disease
CTL-6	#5844	42	F	12	None (WT)		Cortex	BA4	Cardiac Arythmia
CTL-7	#5566	15	F	23	None (WT)		Cortex	BA4	Hypertrophic Cardiomyopathy
CTL-8	#5309	14	F	8	None (WT)		Cortex	BA4	Streptococcal Toxic Shock

	Age	PMI
AVG	18.7	17.0
SEM	3.9	2.7

Supplementary Table 1. Human motor cortex sample data. PMI = post mortem interval, XCI = X chromosome inactivation *cause of death in RTT samples not specified.

IP Administration of 10 mg / kg VU0422288 in Rat					
Project	mGlu7 PAM				
Study	IP Delivery				
Animals	Rat, SD (N=2)				
Vehicle	IP: 10% Tween 80 in water (4 mg/mL)				
Dose	10 mg/kg				
Time points	IP: 1 hr				
Matrix	EDTA plasma, brain				
Analyte	VU0422288				
MW	360.2 (free base)				
LLOQ	0.5 ng/mL				
VU0422288-2					
Concentration (ng/mL or g)					
Dose (mg/kg)	Time (hr)	Plasma Systemic	HPV	Brain	Brain : Plasma Ratio
10	1	778	514	1264	1.62
	1	3680	2580	6340	1.72

Supplementary Table 2. Pharmacokinetic analysis of VU0422288. IP = intraperitoneal, SD = sprague dawley, HPV=hepatic portal vein.