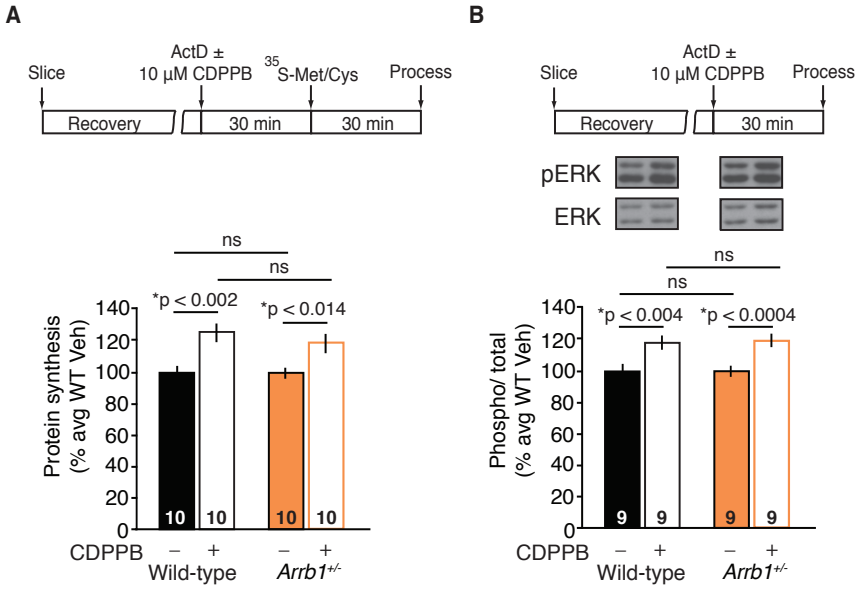

Figure S1. Related to Figure 1. β-arrestin1 is not necessary for protein synthesis-dependent ERK1/2 activation. **(A)** Schematic illustrates experimental timeline. Protein synthesis was similarly elevated in WT slices and *Arrb1*$^{+/-}$ slices stimulated with CDPPB compared to vehicle-treated slices of both genotypes. WT: two-tailed *t* test, t = 3.9158, *p = 0.001, n = 10 animals; *Arrb1*$^{+/-}$ slices: two-tailed *t* test, t = 2.7268, *p = 0.0138, n = 10 animals. Two-way ANOVA, genotype vs. treatment, F = 0.297, p = 0.589). **(B)** Schematic illustrates experimental timeline. Representative immunoblots of ERK1/2 phosphorylation and total ERK protein from hippocampal slices ± 10µM CDPPB stimulation from WT and *Arrb1*$^{+/-}$ mice. WT and *Arrb1*$^{+/-}$ slices stimulated with CDPPB show elevated ERK1/2 phosphorylation compared with vehicle. WT: two-tailed *t* test, t = 3.4122, *p = 0.0036, n = 9 animals; *Arrb1*$^{+/-}$ slices: two-tailed *t* test, t = 4.4550, *p = 0.0004, n = 9 animals. Two-way ANOVA, genotype vs. treatment, F = 0.0752, p = 0.786.

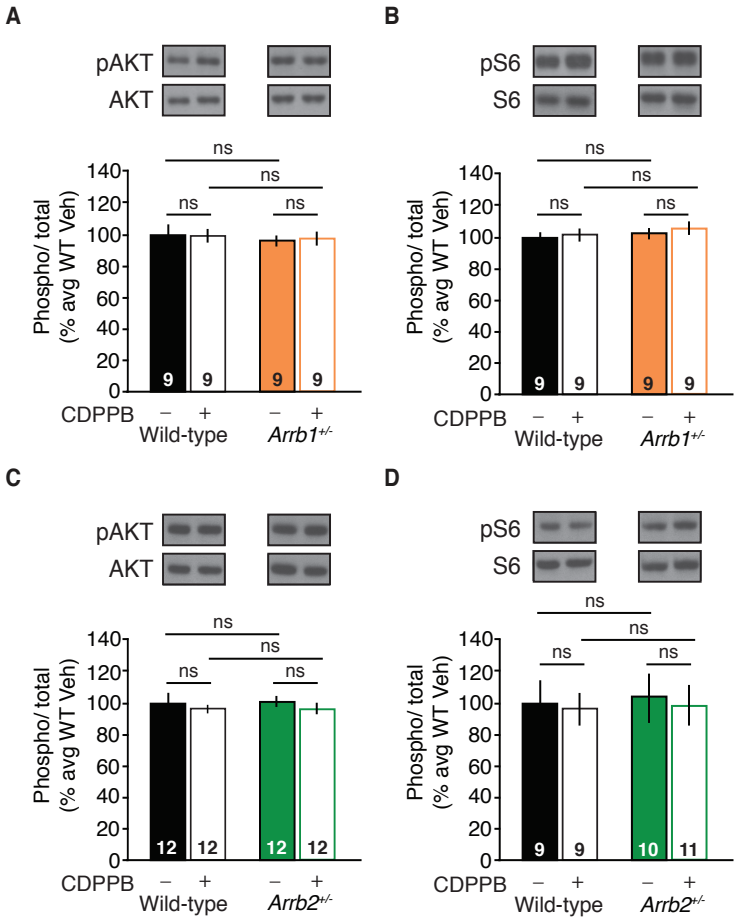

Figure S2. Related to Figure 1. The AKT-mTOR pathway is not activated by CDPPB in wild-type, *Arrb1*$^{+/-}$ or *Arrb2*$^{+/-}$ mice. (**A**) Representative immunoblots of AKT phosphorylation and total AKT protein from hippocampal slices ± 10μM CDPPB stimulation from WT and *Arrb1*$^{+/-}$ mice. Neither WT nor *Arrb1*$^{+/-}$ slices stimulated with CDPPB show elevated AKT phosphorylation compared with vehicle. WT: two-tailed *t* test, t = 0.2531, p = 0.8034, n = 9 animals; *Arrb1*$^{+/-}$ slices: two-tailed *t* test, t = 0.2596, p = 0.7985, n = 9 animals. Two-way ANOVA, genotype vs. treatment, F = 0.124, p = 0.727. (**B**) Representative immunoblots of ribosomal protein S6 phosphorylation and total S6 protein from hippocampal slices ± 10μM CDPPB stimulation from WT and *Arrb1*$^{+/-}$ mice. Neither WT nor *Arrb1*$^{+/-}$ slices stimulated with CDPPB show elevated S6 phosphorylation compared with vehicle. WT: two-tailed *t* test, t = 0.5532, p = 0.5878, n = 9 animals; *Arrb1*$^{+/-}$ slices: two-tailed *t* test, t = 0.9099, p = 0.3764, n = 9 animals. Two-way ANOVA, genotype vs. treatment, F = 0.00479, p = 0.945. (**C**) Representative immunoblots of AKT phosphorylation and total AKT protein from hippocampal slices ± 10μM CDPPB stimulation from WT and *Arrb2*$^{+/-}$ mice. Neither WT nor *Arrb2*$^{+/-}$ slices stimulated with CDPPB show elevated AKT phosphorylation compared with vehicle. WT: two-tailed *t* test, t = 0.6968, p = 0.4932, n = 12 animals; *Arrb2*$^{+/-}$ slices: two-tailed *t* test, t = 1.1195, p = 0.2750, n = 12 animals. Two-way ANOVA, genotype vs. treatment, F = 0.0180, p = 0.894. (**D**) Representative immunoblots of ribosomal protein S6 phosphorylation and total S6 protein from hippocampal slices ± 10μM CDPPB stimulation from WT and *Arrb2*$^{+/-}$ mice. Neither WT nor *Arrb2*$^{+/-}$ slices stimulated with CDPPB show elevated S6 phosphorylation compared with vehicle. WT: two-tailed *t* test, t = 3.4122, p = 0.9297, n = 9, 10 animals; *Arrb1*$^{+/-}$ slices: two-tailed *t* test, t = 0.0563, p = 0.9557, n = 9, 11 animals. Two-way ANOVA, genotype vs. treatment, F = 0.0104, p = 0.920.

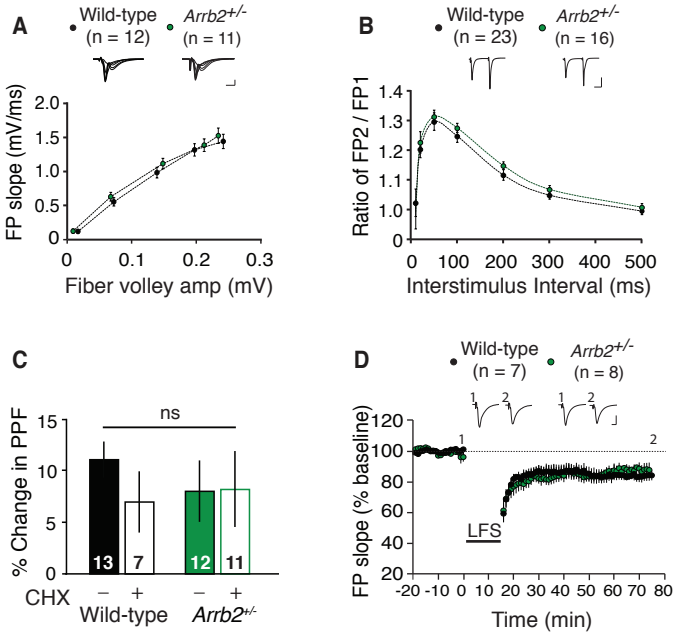

Figure S3. Related to Figure 1. *Arrb2*$^{+/-}$ mice have normal basal synaptic function and NMDAR-dependent LTD. (**A**) Basal synaptic transmission (plotted as fEPSP amplitude against presynaptic fiber volley amplitude) does not differ between genotypes. Scale bars equal 0.5 mV, 20 ms for representative field potential traces. (**B**) Paired pulse facilitation is normal across several inter-stimulus intervals (20, 30, 50, 100, 200, 300, 500 ms) in *Arrb2*$^{+/-}$slices. Scale bars equal 0.5 mV, 20 ms.(**C**) There is no significant difference in paired pulse facilitation between WT and *Arrb2*$^{+/-}$ mice with or without the presence of cycloheximide. (**D**) The magnitude of NMDA receptor-dependent LTD evoked by low frequency stimulation (LFS, 900 pulses at 1 Hz) does not differ between genotypes ($p = 0.610$). Representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals. Scales bars equal 0.5 mV, 5 ms.

Figure 1B

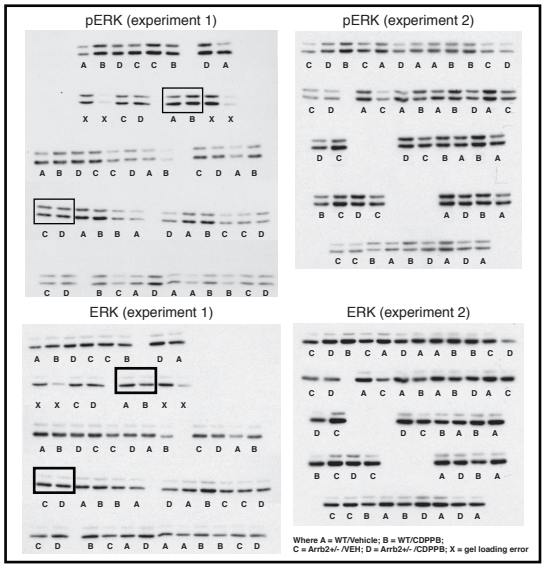

Figure S1B

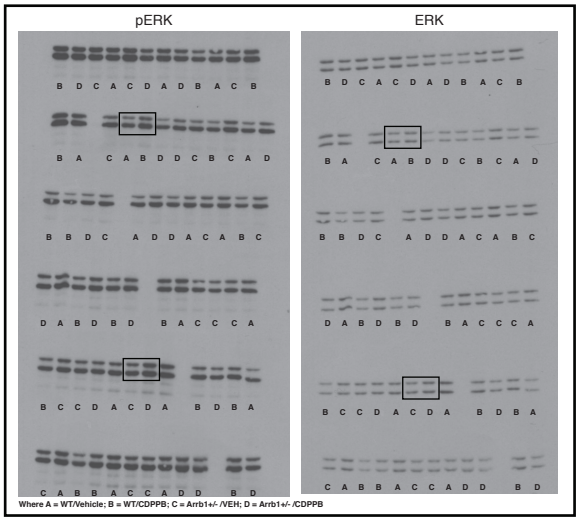

Figure S2A

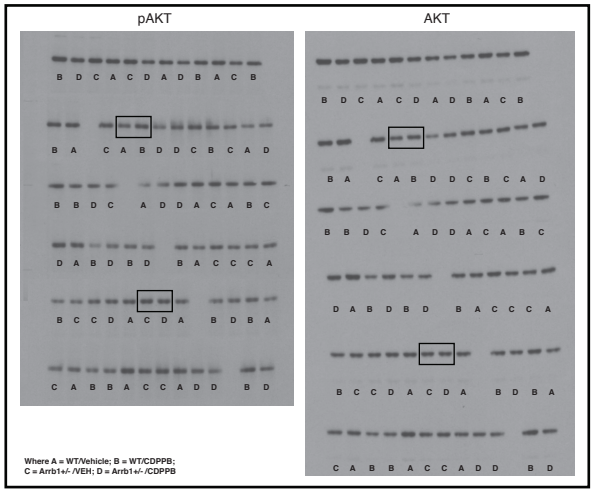

Figure S2B

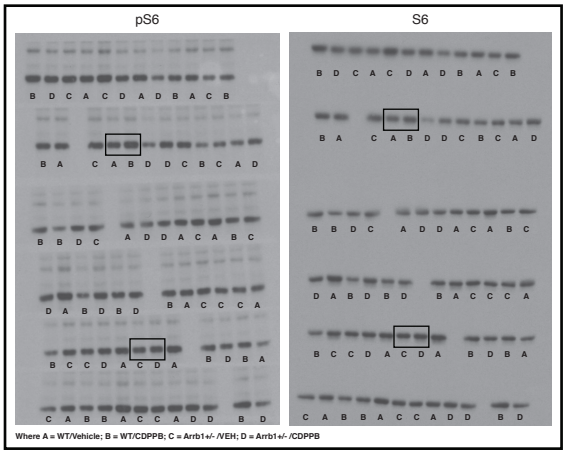

Figure S2C

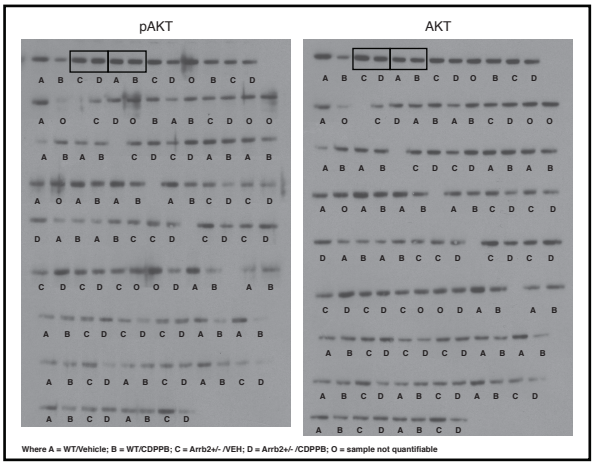

Figure S2D

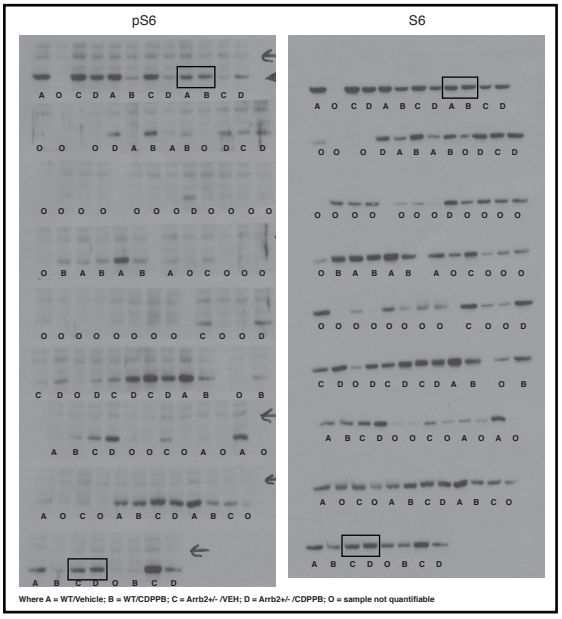

Figure S4. Related to Figure 1, Figure S1 and Figure S2. Uncropped western blots. Boxes indicate representative immunoblots from corresponding figures.

Supplemental Experimental Procedures

Animals

Arrb2$^{+/-}$ male and female mutant mice on the C57Bl/6J clonal background were bred together to produce the WT and *Arrb2*$^{+/-}$ offspring used in this study. *Fmr1*$^{-/+}$ female mice (Jackson Labs) were crossed with *Arrb2*$^{+/-}$ mice to generate double mutant animals. All experimental animals were age-matched male littermates, and were studied with the experimenter blind to genotype and treatment condition. Animals were group housed and maintained on a 12:12 hour, light: dark cycle. The Institutional Animal Care and Use Committee at MIT approved all experimental techniques and all animals were treated in accordance with NIH and MIT guidelines.

Electrophysiology

Slices were prepared as described previously (Dolen et al., 2007). Acute hippocampal slices (350 μm) were prepared from P28-35 animals in ice-cold dissection buffer containing (in mM): NaCl 87, Sucrose 75, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 0.5, $MgSO_4$ 7, Ascorbic acid 1.3, and D-glucose 10 (saturated with 95% O_2 / 5% CO_2). Immediately following slicing the CA3 region was removed. Slices were recovered in artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1, and D-glucose 10 (saturated with 95% O_2/5% CO_2) at 32.5°C for ≥ 3 hours prior to recording. Field recordings were performed in a submersion chamber, perfused with aCSF (2-3 ml/min) at 30ºC. Field EPSPs (fEPSPs) were recorded in CA1 stratum radiatum with extracellular glass electrodes (1-2 MΩ) filled with aCSF. Baseline responses were evoked by stimulation of the Schaffer collaterals at 0.033 Hz with a 2-contact cluster electrode (FHC) using a 0.2 ms stimulus yielding 40-60% of the maximal response. fEPSP recordings were filtered at 0.1 Hz - 1 kHz, digitized at 10 kHz, and analyzed using pClamp9 (Axon Instruments). The initial slope of the response was used to assess changes in synaptic strength. Data were normalized to the baseline response and are presented as group means ± SEM. The input output function was examined by stimulating slices with incrementally increasing current and recording the fEPSP response. Paired pulse facilitation was induced by applying two pulses at different interstimulus intervals. Facilitation was measured by the ratio of the fEPSP slope of stimulus 2 to stimulus 1. NMDAR-dependent LTD was induced by delivering 900 test pulses at 1 Hz. mGlu-LTD was induced by (S)-3,5-Dihydroxyphenylglycine (DHPG, 25 μM) for 5 minutes. In order to determine the protein synthesis dependency of mGlu-LTD, slices were incubated with the protein synthesis inhibitor cycloheximide (60 μM) for at least 10 minutes prior to recording and throughout the entire experiment. The magnitude of LTD was measured by comparing the average response 55-60 minutes post DHPG/LFS application to the average of the last 5 minutes of baseline. Statistical significance for input-output function, paired-pulse facilitation, and mGlu- or NMDAR-dependent plasticity was determined by two-way ANOVA and post-hoc Student's t-tests.

Fluorescence-based calcium imaging in brain slices

Hippocampal slices were prepared as described for electrophysiology experiments and recovered in aCSF for 2 hours at 32°C. Slices were then moved to a small recovery chamber and incubated in oxygenated aCSF containing 20 μM fluo-4-acetoxymethyl ester (fluo-4 AM; ThermoFisher) and 0.1% Pluronic F-127 (ThermoFisher) for 1 hour in the dark. After 1 hour, slices were then washed with oxygenated aCSF containing 1 μM tetrodotoxin (Abcam) and 50 μM D-AP5

(Tocris) for 20 minutes in a recording chamber. A low-powered (4X) objective was used to identify the brain region of interest. CA1 pyramidal neurons were visualized using a 40X water immersion objective and a Nikon Eclipse E600FN microscope. Changes in Ca^{2+} fluorescence were monitored in the presence of 1 μM tetrodotoxin and 50 μM D-AP5 using a Hamamatsu ORCA-100 camera, HCImage software (Hamamatsu), and a mercury arc-lamp and power supply (Nikon). After a 10 second baseline, 25 μM (S)-3,5-DHPG was bath applied. For a subset of experiments, slices were pre-treated with 10 μM U73122 during the wash period to block phospholipase C activity. Data were analyzed using Image J software. Increases in Ca^{2+} mobilization in hippocampal neurons were reported as changes in relative fluorescence divided by the baseline fluorescence ($\Delta F/F$).

Metabolic Labeling

Metabolic labeling of new protein synthesis was performed as previously described (Osterweil et al., 2010). Male P28-P32 littermate mice were anesthetized with isoflurane and the hippocampus was rapidly dissected into ice-cold aCSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$, saturated with 95% O_2 and 5% CO_2). Hippocampal slices (500 μm) were prepared using a Stoelting Tissue Slicer and transferred into 32.5°C aCSF (saturated with 95% O_2 and 5% CO_2) within 5 min. Slices were incubated in aCSF undisturbed for 3.5-4 h to allow for recovery of basal protein synthesis. Actinomycin D (25 μM) and vehicle or CDPPB (10 μM) was then added to the recovery chamber for 30 min to inhibit transcription and stimulate mGlu receptors, respectively after which slices were transferred to fresh aCSF containing ~10 mCi/ml [^{35}S] Met/Cys (Perkin Elmer) for an additional 30 min. Slices were then homogenized, and labeled proteins isolated by TCA precipitation. Samples were read with a scintillation counter and subjected to a protein concentration assay (Bio-Rad). Data was analyzed as counts per minute per microgram of protein, normalized to the [^{35}S] Met/Cys aCSF used for incubation and the average incorporation of all samples analyzed and then normalized to percent wild-type for each experiment. Statistical significance was determined using unpaired t-tests.

Immunoblotting

Hippocampal slices were prepared and recovered as described in metabolic labeling experiments. Sets of slices were stimulated with CDPPB (10 μM) for 30 minutes and then flash frozen in liquid nitrogen immediately after stimulation, prior to processing. Yoked unstimulated slices were also processed to assess basal signaling levels. Immunoblotting was performed according to established methods using primary antibodies to p-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), p-Akt (Ser473) (Cell Signaling Technology), Akt (Cell Signaling Technology), pS6 240/44 (Cell Signaling Technology) and S6 (Cell Signaling Technology). Protein levels were measured by densitometry (Quantity One), and quantified as the densitometric signal of phospho-protein divided by the total protein signal in the same lane.

Inhibitory Avoidance Extinction

Inhibitory avoidance (IA) experiments were performed as previously described (*26*). On the day of testing, P56-P76 animals were placed into the dark compartment of an IA training box (a two-chambered Perspex box consisting of a lighted safe side and a dark shock side separated by a trap door) for 30 seconds followed by 90 seconds in the light compartment for habituation.

Following the habituation period, the door separating the two compartments was opened and animals were allowed to enter the dark compartment. Latency to enter following door opening was recorded ("baseline", time 0, 8-9am); animals with baseline entrance latencies of greater than 120 seconds were excluded. After each animal stepped completely into the dark compartment with all four paws, the sliding door was closed and the animal received a single scrambled foot-shock (0.5mA, 2.0 sec) via electrified steel rods in the floor of the box. This intensity and duration of shock consistently caused animals to vocalize and jump. Animals remained in the dark compartment for 15 sec following the shock and were then returned to their home cages. Six to seven hours following IA training, mice received a retention test ("post-acquisition", time 6 hours, 2p.m.-3p.m.). During post-acquisition retention testing, each animal was placed in the lit compartment as in training; after a 90 second delay, the door opened, and the latency to enter the dark compartment was recorded (cut-off time 537 sec). For inhibitory avoidance extinction (IAE) training, animals were allowed to explore the dark compartment of the box for 200 seconds in the absence of foot-shock (animals remaining in the lit compartment after the cutoff were gently guided, using an index card, into the dark compartment); following IAE training, animals were returned to their home cages. Twenty-four hours following initial IA training, mice received a second retention test ("post-extinction 1", time 24 hours, 8a.m-9a.m.). Animals were tested in the same way as at the six hour time point, followed by a second 200 second extinction trial in the dark side of the box; following training animals were again returned to their home cages. Forty-eight hours following avoidance training, mice received a third and final retention test ("post-extinction 2", time 48 hours, 8a.m.- 9a.m.).

Audiogenic Seizures

AGS experiments were performed as previously described (*26*). Animals at P19-25 (immediately following weaning) were habituated to the behavioral chamber (28x17.5x12 cm transparent plastic box) for 1 minute prior to stimulus onset. AGS stimulus was a 125 dB at 0.25 m siren (modified personal alarm, Radioshack model 49-1010, powered from a DC converter). Seizures were scored for incidence during a 2-minute stimulus presentation or until animal reached AGS endpoint (wild running, status epilepticus, respiratory arrest or death were all scored as seizure activity).

Object Recognition

Object Recognition Task was adapted from experiments previously described. Animals at P56-70 were habituated to a 40 cm x 40 cm x 40 cm box during 2 x 15 minute sessions, spaced 1-2 hours apart. Animals were returned to their home cage in between sessions. 24 hours post habituation animals were exposed to two identical objects for 2 x 10 minute exploration sessions in the same box, spaced 1-2 hours apart. Animals were required to explore each object for at least 10 seconds (for a total of at least 20 seconds) in the first session to be included in the subsequent sessions. 24 hours post object exploration, one object was replaced with a novel object and the animals were allowed to explore the objects for 10 minutes. Time spent sniffing was recording during this exploration period and was characterized by sniffing within 2 cm of each object or directly touching the objects. Time spent climbing or on top of the objects was not included. Familiar and novel object and side placement was randomly assigned, by animal. Discrimination index was calculated as [(time spent exploring novel object) / (time spent exploring novel object + time spent exploring familiar object)].

MK801-induced hyperlocomotion

To determine the effects of genotype on MK801-induced hyperlocomotion, mice were habituated in the open field (40 cm x 40 cm x 40 cm box) for 60 min, followed by the administration of vehicle or MK801 and locomotor activity was recorded for another 60 min. To determine the effects of MTEP by genotype on MK801-induced hyperlocomotion, mice were habituated in the open field for 30 min, followed by the intraperitoneal (i.p.) administration of MTEP (10 mg/kg at a dosing volume of 10 ml/kg). After an additional 30 min, MK801 (0.3 mg/kg at a dosing volume of 10 ml/kg) was administered i.p. and locomotor activity was recorded for another 60 min. The time course of drug-induced changes in ambulation was expressed as cm traveled/5 min over the 120-min session. Sessions were recorded using Plexon's*CinePlex*® Studio and analyzed using Plexon's*CinePlex*® Editor and code written in MATLAB. MK801-induced locomotor activity was scored and analyzed using the average of the final 5 minutes (minute 115-120) per cohort.

Reagents

(S)-3,5-dihydroxyphenylglycine (S-DHPG) was purchased from Tocris. Fresh bottles of DHPG were prepared as a 100x stock in H_2O, divided into aliquots, and stored at -80°C. Fresh stocks were made once a week. Cycloheximide (Sigma) was prepared daily at 100x stock in H_2O. Actinomycin D (Tocris) was prepared as a stock solution of 1mg/mL in 0.01% DMSO and aCSF and stored at -20°C. CDPPB (Tocris) was prepared daily at 75 mM stock in DMSO. U73122 (Tocris) was prepared as a 5 mM stock in DMSO. MK801 (Sigma) was prepared in H_2O daily and .3 mg/kg was injected i.p. at a dosing volume of 10 ml/kg. MTEP (Tocris) was prepared in H_2O daily and 10 mg/kg was injected i.p. at a dosing volume of 10 ml/kg.

Statistics

All data shown represent mean ± SEM. N values represent individual animals and are denoted as values in bar graphs or in parentheses below figures and also noted in the figure legends. For LTD experiments testing for differences between genotypes, ordinary one-way ANOVAs were used. For protein synthesis, western blotting, behavioral, and LTD experiments, post-hoc analysis was performed using either two-tailed *t* tests or Bonferroni's multiple comparisons. For protein synthesis, western blotting, behavioral, or LTD experiments where differences between genotypes and drug treatments were tested, two-way ANOVAs were used with post-hoc analysis performed using two-tailed *t* tests or Bonferroni's test for multiple comparisons. For inhibitory avoidance experiments, repeated measures two-way ANOVA was used. Calcium imaging data was analyzed on a per-animal basis for changes in peak fluorescence using a Mann-Whitney test, and a per-cell basis using a Kolmogorov-Smirnov test. Differences in audiogenic seizure incidence were determined using a two-tailed Fisher's exact test. All experiments were performed blind to genotype and included interleaved controls for genotype and treatment. Results of statistical tests are reported in the figure legends.