Cell Reports

Supplemental Information

Coordination between Translation and Degradation Regulates Inducibility of mGluR-LTD

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Supplemental Methods

Electrophysiology and Western blotting were carried out as described previously (Klein et al., 2013; Younts et al., 2013). Methods from these references are copied below.

Hippocampal slice preparation

Animal procedures were approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines. Male and female Wistar rats (Charles River) aged postnatal day (P20-P25) or C57BL/6 mice (P20–P25) were deeply anesthetized with isoflurane and then killed by decapitation. Sam68 KO mice were bred into a C57BL/6 background (Richard et al., 2005). The brain was removed and quickly placed in ice-cold cutting solution containing the following (in mM): 215 sucrose, 20 glucose, 26 $NaHCO_3$, 4 $MgCl_2$, 4 $MgSO_4$, 1.6 NaH_2PO_4, 1 $CaCl_2$, and 2.5 KCl. Hippocampi were mounted on an agar block, and transverse slices 400 µm thick were prepared with a DTK-2000 microslicer (Dosaka EM). Slices were placed in a hot water bath at 30 °C, in a holding chamber containing 50% cutting solution and 50% artificial CSF (ACSF) recording solution containing the following (in mM): 124 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, and 1.3 $MgSO_4$. After 30 min, the 1:1 solution was switched to ACSF at 30 °C and the holding chamber was removed from the water bath. Slices recovered in ACSF at room temperature for at least 1 h, and then were transferred to a submersion-type, temperature-controlled recording chamber (TC-344B, Warner Instruments) and perfused with ACSF at 2 ml/min using a peristaltic pump (Dynamax RP-1, Rainin). Experiments were performed at 30°C. All solutions were equilibrated for at least 30 min with 95% O_2 and 5% CO_2, pH 7.4.

Electrophysiology

Conventional single whole-cell voltage-clamp recordings were performed using a MultiClamp 700B amplifier (Molecular Devices). For single-cell voltage-clamp experiments (holding potential = −60 mV), CA1 pyramidal cells located ~150 µm deep in the slice were blind patched with a micropipette (2.3–3.4 MΩ) fabricated on a two-step micropipette puller (PP-830 or PC-10, Narishige) and filled with an intracellular recording solution containing the following (in mM): 135 K-gluconate, 3 KCl, 0.04 $CaCl_2$, 0.1 EGTA, 10 NaCl, and 10 HEPES, pH 7.2 (280–290 mOsm). Sucrose was used to adjust osmolarity. To assess cell stability, series and input resistances were monitored with a 5 mV, 80 ms hyperpolarizing test pulse, and cells with >15% change in series resistance were excluded from analysis. To elicit synaptic responses, paired, monopolar square-wave voltage or current pulses (100–200 µs pulse width) were delivered through a stimulus isolator (Isoflex, AMPI) connected to a broken tip (~10–20 µm) stimulating patch-type micropipette filled with ACSF. Typically, stimulating pipettes were placed in the middle of CA1 stratum radiatum. Stimulus intensity was adjusted to give comparable magnitude synaptic responses across experiments (~200–500 pA for whole-cell recordings and ~0.6 mV for extracellular field recordings). Baseline stimulation was delivered at 0.1 Hz, before and after the induction of long-term plasticity.

Oligodeoxynucleotides

Chimeric ODNs containing phosphorothioate linkages (indicated by *) between the three bases on the 5′ and 3′ ends and phosphodiester internal linkages were synthesized, HPLC purified,

ultrafiltrated, and sterilized (Fisher Scientific, Eurofins) (Messaoudi et al., 2007). Oligos were diluted in internal solution and used at a final concentration of 150 µM. Arc antisense oligo #1 (A*G*T* GTA GTC GTA GCC ATC A*G*C*; targets bp 700-720 of the Arc gene) or control mismatch oligos for #1 (A*G*G* GTA TTC GAA GCT ATC C*G*C*) (Waung et al., 2008). A half hour baseline was recorded to allow oligos to infuse into the neuron. The ARC AS oligo was capable of blocking mGluR-LTD, but LTD could be rescued by inhibition of the proteasome with 5 µM MG132 for one hour prior to recording and during the baseline and induction (open red circles). EPSCs were recorded using a K-gluconate internal solution. The recording configuration was switched to current clamp, with no current injection, during DHPG application and 5 minutes after, and then switched back to voltage clamp for the remainder of the recording.

Data acquisition and statistical analysis

Stimulation and acquisition were controlled with custom software written in IgorPro (Wavemetrics). Output signals from whole-cell recordings were acquired at 5 kHz, filtered at 2.4 kHz, and stored online. Statistical analyses were performed using OriginPro 9.0 (OriginLab). ANOVAs were performed on experiments with multiple groups followed by post-hoc pairwise analyses with Bonferroni correction. An asterisk denotes a P value of <0.05, and “n.s” indicates a P value >0.05. All values are expressed as mean ± SEM. Student's paired and unpaired *t*-tests were used to assess within-group and between-group differences, respectively. Electrophysiological experiments with Sam68 animals were conducted blind to genotype for data acquisition and analysis (Fig 1D, Supp Fig 1A, Supp Fig 1B, Supp Fig 1G). Initial, exploratory experiments for Fig 1C were not performed blind to genotype. These experiments were later repeated blind to genotype for data acquisition and analysis. The two data sets revealed similar results and were combined to generate the graph in Fig 1C.

Reagents

Reagents were bath applied following dilution into ACSF from stock solutions stored at −20°C prepared in water or DMSO, depending on the manufacturer's recommendation. The final DMSO concentration was <0.01% total volume. Reagents 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), (R,*S*)-3,5-dihydroxyphenylglycine (DHPG) were purchased from Abcam Biochemicals. Z-Leu-Leu-Leu-CHO ((S)-MG132), lactacystin, and rapamycin were purchased from Cayman. Picrotoxin, cycloheximide, and salts for making ACSF and internal solutions were purchased from Sigma-Aldrich. DL-APV, CPCCOEt, and (R,S)-3,5-DHPG were obtained from the Chemical Synthesis and Drug Supply Program of the National Institute of Mental Health

Drug Treatment and Western Blots

After recovery acute hippocampal slices were transferred from recovery chamber to a six-well dish for drug treatment in a 30 °C water bath. The appropriate number of slices was placed in each well with ACSF and bubbled. After a brief acclimation period drugs were added to the wells as indicated in the text. Antibodies used were APP (C6.1; 1 µg/ml; gift from Paul Matthews): OPHN1 (H-100; 1 µg/ml; Santa Cruz Biotechnology): FMRP (ab191411; 1 µg/ml; abcam), (ab27455; 1 µg/ml; abcam): PSD95 (K28/43; 1:2,000; UC Davis/National Institutes of Health NeuroMabFacility): ARC (C7; 2 µg/ml; Santa Cruz Biotechnology). Western

blots/membranes were incubated overnight at 4°C with primary antibodies used at the concentrations indicated in 1% BSA/PBS and processed according to standard conditions.

Immunopreciptation and Ubiuitination Western Blots

After recovery acute hippocampal slices were transferred from recovery chamber to a six well dish for drug treatment in a 30 °C water bath. The appropriate number of slices was placed in each well with ACSF and bubbled. After a brief acclimation period drugs were added to the wells. In both the vehicle and DHPG conditions the proteasome was blocked by addition of 5 μM MG132 for 30 minutes. After this pre-incubation period slices were treated with either DHPG (DHPG (100 μM, 6 min), or a vehicle. Slices were then transferred to fresh ASCF and chased for a 30 minutes wash-out period. Immediately following wash-out, slices were lysed in Ub-buffer (50 mM Tris pH 7.4, 2% SDS, 10 mM n-ethylmaleimide, and 2 mM EDTA). IPs, using standard conditions, were performed on at least 500 μgs of protein with 1 μg of Ab. Before addition of the Ab, lysate was diluted with buffer (50 mM Tris pH 7.4, 10% glycerol, and 1% Tx-100) to reduce SDS to < 0.2%. In the case of ARC it was necessary to IP at least 2 mg of protein with 2.5 μg of Ab. Ubiquitination was detected by anti-Ub Ab (PD41, Santa Cruz), or (FK2, Enzo Life Sciences).

Subcellular Fractionation for Synaptosomes

Brain tissue was Dounce homogenized in a 0.32 M sucrose solution and centrifuged at low speed to remove heavy cellular components. The supernatants then were centrifuged at 30,000 × g to collect the light membrane fraction. After resuspension, the pellets were layered on top of a 0.85 M, 1 M, and 1.2 M sucrose step gradient and centrifuged at 82,500 × g for 2 h. The synaptosomal fraction was collected at the 1 M/1.2 M sucrose interface and pelleted down at 45,000 × g for 30 min.

Supplemental References

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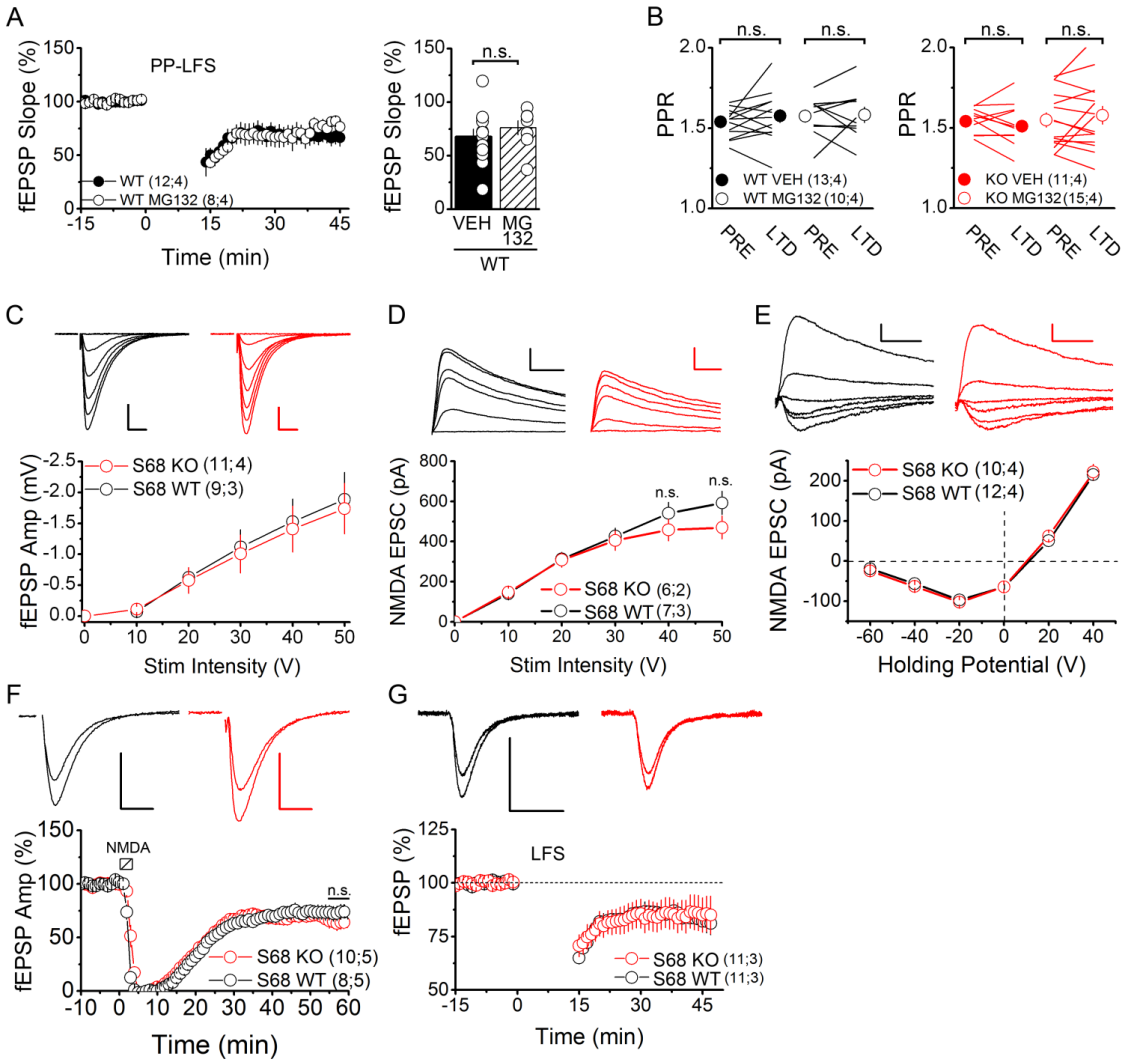

Figure S1. **Effects of proteasome inhibition on synaptic plasticity and synaptic characterization of Sam68 KO mice. Related to Figure 1.**

A: Data reproduced from Fig 1D. Field recordings in acute hippocampal slices at Schaffer collateral synapses of mGluR-LTD induced by PP-LFS for 15 min (2 pulses at 50 ms ISI, 1Hz). Additional experiments measuring mGluR-LTD in WT slices treated with MG132 have been added. For PP-LFS experiments recordings were performed at 30° C, in the presence of 50 µM picrotoxin and 50 µM D-APV. The extracellular concentration of KCl was also increased from 2.5 mM to 5 mM. 5 µM MG132 for 1 hour before and during PP-LFS had no effect on the magnitude of LTD in WT slices (black open circles) compared to vehicle-treated WT controls (black filled circles). Right, average fEPSP slope during the last five minutes of recording. (WT, 67.7 ± 7.3; WT MG132, 76.2 ± 6.5).

B: Two pulses with a 100 ms ISI were used to measure the paired pulse ratios at baseline (PRE) and after (LTD) induction of mGluR-LTD during the experiments summarized in Fig 1C. No significant difference in PPR was observed in any of our experimental conditions (WT, 1.5 ± .02; WT MG132, 1.5 ± .04; KO, 1.5 ± .02, KO MG132, 1.5 ± .03).

C: Field EPSP input/output curve reveals no significant difference in baseline transmission between Sam68 WT and KO mice. Scale bars are 0.5 mV / 25 ms.

D: Whole-cell EPSC NMDA input/output curve reveals no difference in baseline NMDA transmission recorded at +40 mV between Sam68 WT and KO mice. Scale bars are 150 pA / 25 ms.

E: Whole-cell EPSC NMDA input/voltage curve reveals no difference in baseline NMDA transmission between Sam68 WT and KO Mice. Scale bars are 50 pA/ 25 ms.

F: Field recordings in acute hippocampal slices from Sam68 KO and WT littermates of chemically induced NMDA-LTD at Schaffer collateral synapses induced by 40 µM NMDA for 3.5 min reveal no difference in the magnitude of LTD

G: Field recordings in acute hippocampal slices from Sam68 KO and WT littermates of synaptically induced NMDA-LTD at Schaffer collateral synapses induced by LFS (1 Hz for 15 min) reveal no difference in the magnitude of LTD.

Summary data consist of mean ± s.e.m.

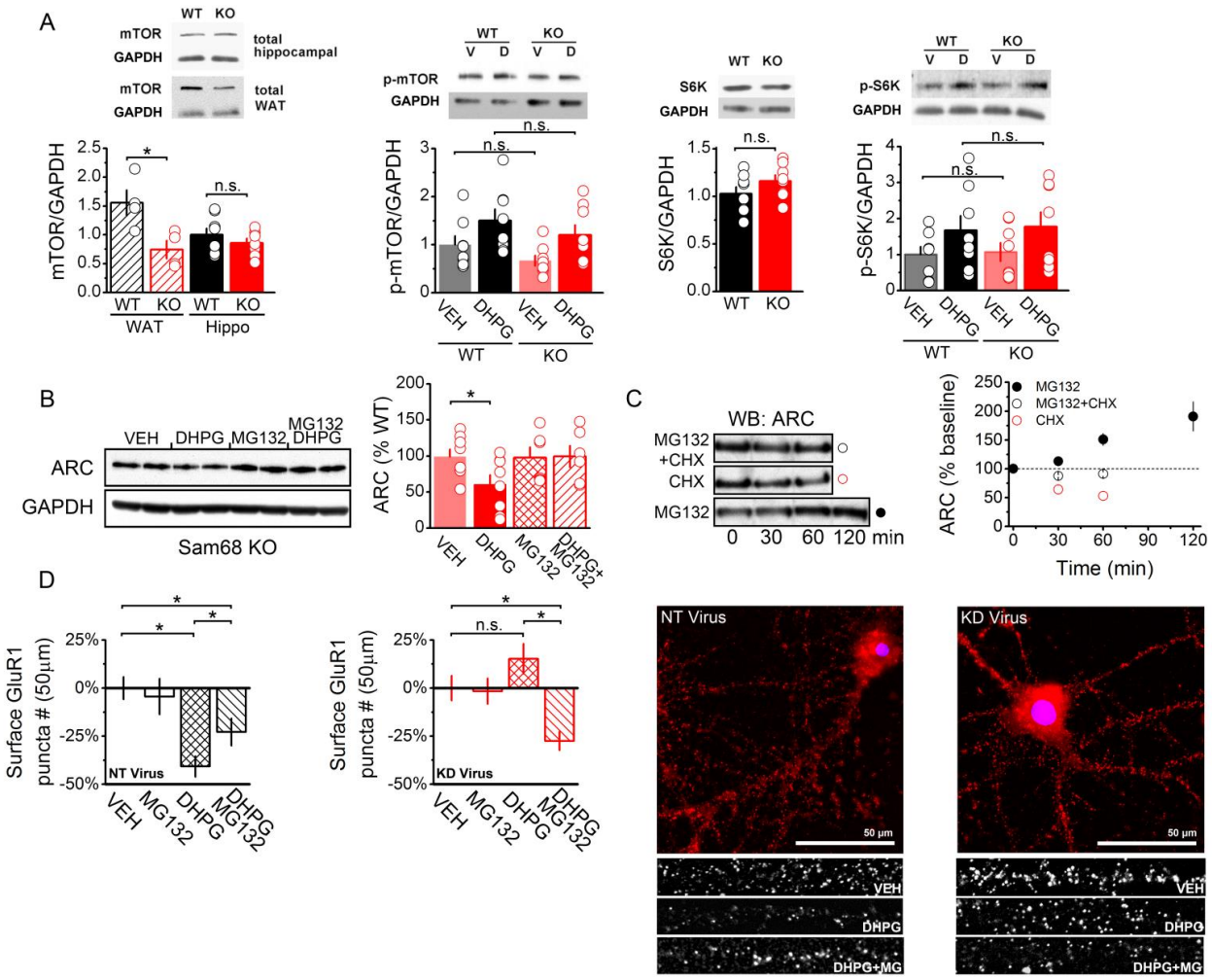

Figure S2. Control experiments showing normal mTOR signaling and AMPAR internalization in Sam68-deficient neurons. Related to Figures 1 and 3.

A: Quantification from Westerns from total hippocampal (Hippo) and white adipose tissue (WAT) of mTOR and S6Kinase for WT or Sam68 KO animals. For WAT experiments dots represent a separate sample of WAT taken from perirenal and perigonadal regions (4 samples, 2 mice each WT and KO). For hippocampal experiments dots represent pooled lysate from 3 hippocampal slices. The average for each condition was generated from 8 separate experiments using slices from 4 mice each of KO and WT. Slices were treated either with vehicle (V) or 50 µM DHPG for 5 min (D) and lysates were prepared immediately following to quantify levels of phospho-mTOR or p-S6K. Asterisks indicate a p value < 0.05 from a paired student's t-test.

B: Data reproduced from Fig 1A. Additional experiments measuring ARC protein levels in KO slices treated with MG132 for 1 hr have been added. Western blot of ARC levels from hippocampal slices. Slices from Sam68 KO or WT animals were treated with vehicle (VEH) or 50 µM (R,S)-3,5-DHPG for 5 min. 5 µM (S)-MG132 was added for 1 hour before and during DHPG treatment. Right, densitometric quantification normalized to GAPDH. Each dot represents a separate experiment consisting of pooled lysate from 3 slices. The average for each condition was generated from 8 separate experiments using slices from 4 KO and WT mice. Black asterisks denote a significant difference from WT (VEH) levels. [**(VEH)** KO, 100 ± 6.8; KO MG, 98.0 ± 14.3 **(DHPG)** KO, 60.5 ± 13.1; KO (MG132), 99.3 ± 14.9].

C: Western blot time course plot of ARC protein levels during three separate experimental conditions. Left, western blots of ARC expression during treatment with either MG132+CHX, CHX, or MG132, drugs were applied at zero time point. Right, densitometric quantification of ARC levels at indicated time points. Data is averaged from three independent experiments.

D: GluR1 surface staining in dissociated hippocampal cultures (21 DIV). Cultures were infected at 14 DIV with lentivirus expressing either a non-targeting shRNA (NT) or a previously validated shRNA against Sam68 (KD) (Klein et al, 2013). The number of GluR1 reactive, surface punta were quantified by immunocytochemistry. In both NT and KD cultures four conditions were examined; vehicle-treated, MG132-treated (10 µM, 30 min), DHPG-treated (100 µM, 5 min), or MG132- (10 µM, 30 min) then DHPG-treated (100 µM, 5 min). In NT cultures both DHPG-, or MG132/DHPG-treatment resulted in a significant decrease in surface GluR1 staining. In contrast, DHPG-treatment did not result in a reduction in GluR1 staining in KD cultures. However, co-treatment with MG132 and DHPG rescued the decrease in surface staining in KD cultures. Values in bar graph represent percent change from VEH condition. In all conditions at least 3 coverslips, and 20 dendrites were imaged. [**(NT)** VEH, 0 ± 5.8; MG132, -4.4 ± 9.3; DHPG, -39.4 ± 5.4; MG132/DHPG, -22.8 ± 7.1: **(KD)** VEH, 0 ± 6.5; MG132, -1.7 ± 6.6; DHPG, 15.3 ± 7.8; MG132/DHPG, -27.5 ± 4.9]

Summary data consist of mean ± s.e.m.

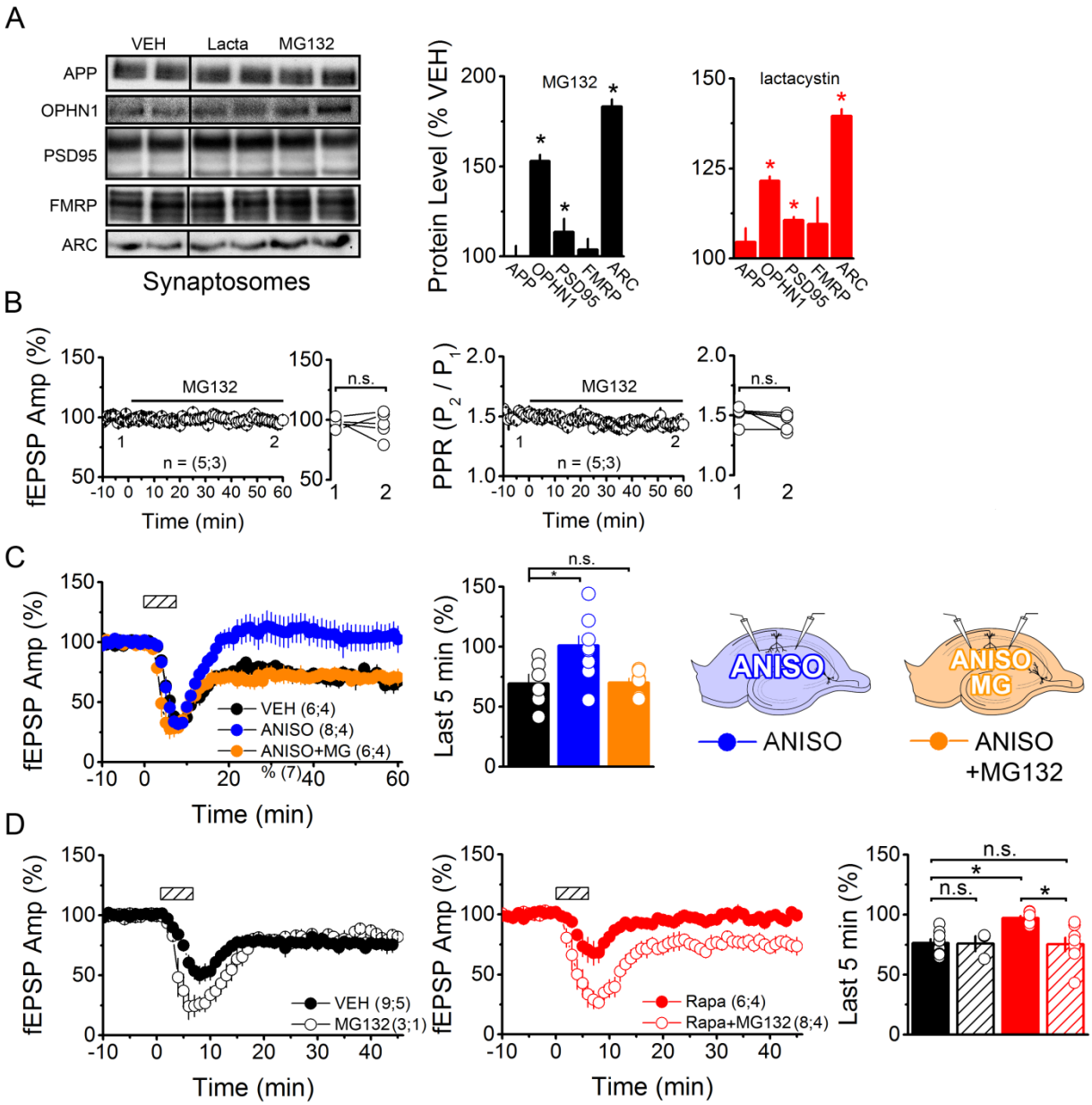

Figure S3. Control experiments showing how protein translation and degradation affect PRP abundance and synaptic function. Related to Figures 2 and 3.

A: Western blots of PRP levels from hippocampal synaptosomal fractions indicate blocking the proteasome with either 5 μM MG132 or 1 μM lactacystin (Lacta) for one hour similarly results in elevated levels of selected PRPs. Right, densitometric quantification, the average for each condition was generated from 4 separate experiments using slices from 4 rats. Each separate experiment consists of pooled lysate from 8 slices which was used to generate synaptosomal fractions. Asterisks denote PRP levels significantly different than vehicle conditions.

B: Left, field recordings at Schaffer collateral synapses from rat hippocampal slices treated with MG132 for 1 hr do not show a change in the amplitude of fEPSPs. Scatter plot displays no significant difference in the average fEPSPs of the first and last 5 minutes for each experiment (student's paired t-test). Right, field recordings show no change in the paired pulse ratio (PPR) after 1 hr application of 5 μM MG132. Scatter plot displays no significant difference in the average PPRs of the first and last 5 minutes for each experiment (student's paired t-test).

C: Inhibition of the proteasome relieves the block of mGluR-LTD by the translational inhibitor anisomycin. Field recordings from acute hippocampal slices (mouse) treated with either 20 μM anisomycin (ANISO, blue) or 20 μM anisomycin and 5 μM MG132 (ANISO/MG, orange) for 1 hr. Dashed boxes show time of DHPG application (50 μM, 5 min). Right, average fEPSP slope during the last five minutes of recording indicates inhibiting the proteosome rescues mGluR-LTD blocked by translational inhibition [VEH, 71.5 ± 5.8; ANISO, 97.8 ± 8.5; ANISO+MG132, 70.5 ± 4.9]. "n" values are (pathways;animals).

D: Inhibition of the proteasome relieves the block of mGluR-LTD by the mTOR inhibitor rapamycin. Field recordings from acute hippocampal slices (mouse, 6 weeks old) treated with either 20 nM rapamycin (RAPA, filled red circles) or 20 nM rapa and 5 μM MG132 (RAPA+MG, open red circles) for 1 hr. Control experiments were vehicle treated (closed black circles), or MG132 treated (open black circles). Dashed boxes show time of DHPG application (50 μM, 5 min). Right, average fEPSP slope during the last five minutes of recording indicates inhibiting the proteosome rescues mGluR-LTD blocked by mTOR inhibition [VEH, 75.4 ± 3.7; MG132, 78.4 ± 4.1; RAPA, 97.1 ± 2.0; RAPA+MG132, 75.3 ± 5.6].

Summary data consist of mean ± s.e.m.

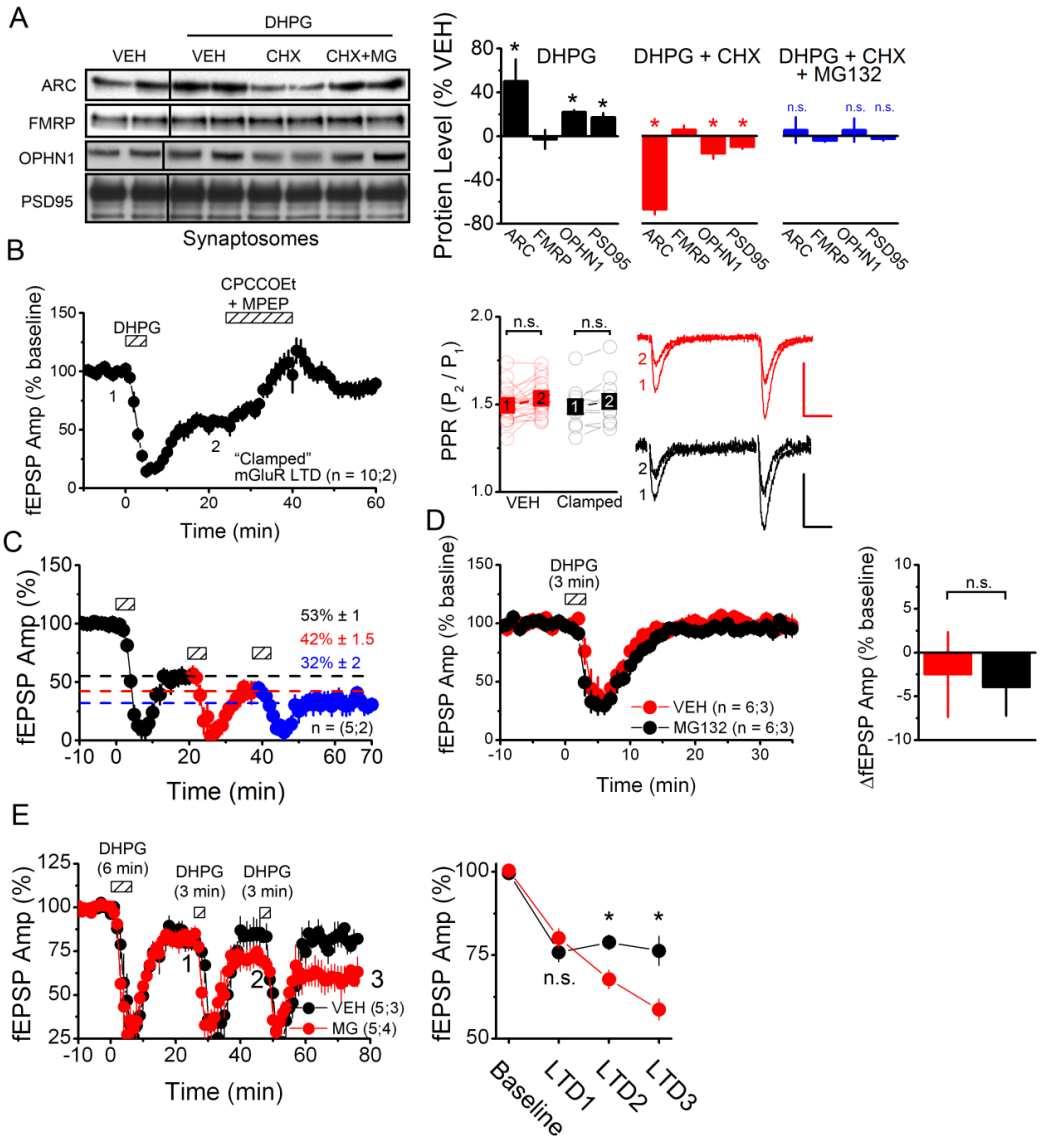

Figure S4. Clamping PRP levels affects inducibility but not reversibility of mGluR-LTD. Related to Figures 3 and 4.

A: Western blots of PRP levels from hippocampal synaptosomal fractions prepared from acute slices preincubated for 1 hour in either vehicle (VEH), 60 μM cycloheximide (CHX), or 60 μM CHX and 5 μM MG132 (CHX+MG132). After 1 hour, slices from all three conditions were treated with DHPG (100 μM, 6 min) and immediately processed into synaptosomal fractions. Right, densitometric analyses reveal increased synaptic levels of select PRPs in response to DHPG treatment. In contrast, CHX pretreatment results in a reduction in synaptic PRP levels following DHPG application. Importantly, synaptic PRP levels are "clamped" following DHPG treatment by co-incubation in CHX and MG132 (asterisks denote PRP levels significantly different than baseline levels prior to DHPG application). The average for each condition was generated from 4 separate experiments using slices from 4 rats. Each separate experiment consists of pooled lysate from 8 slices which was used to generate synaptosomal fractions.

B: Left, field recordings from rat hippocampal slices reveal that mGluR-LTD induced in "clamped" conditions (CHX+MG132) does not manifest with a change in PPR, and depends on sustained mGluR signalling. Right, "clamped' mGluR-LTD does not alter PPRs similar to mGluR-LTD induced under normal conditions.

C: Field recordings at Schaffer collateral synapses from rat hippocampal slices demonstrate mGluR-LTD becomes saturated only after multiple applications of DHPG. Each color change represents a separate application of 100 μM DHPG for 6 min. Colored numbers represent average fEPSP amplitude of last 5 minutes for each DHPG application.

D: Left, field recordings from acute hippocampal slices (rat) treated with a vehicle (VEH, red) or 5 μM MG132 (MG132, black) for 1 hr. A subthreshold stimulus of DHPG (100 μM, 3 min) resulted in a transient depression, but failed to induce LTD in either condition. Right, average fEPSP amplitude during the last five minutes show similar failure to induce mGluR-LTD.

E: Left, field recordings from acute hippocampal slices (rat) treated with a vehicle (VEH, black) or 5 μM MG132 (MG132, red) for 1 hr. 5 μM MG132 continued to be in the bath until the second LTD induction. An initial round of LTD was induced (100 μM DHPG, 6 min), followed by two subthreshold stimului (100 μM DHPG, 3 min). Dashed boxes show time of DHPG application. Right, scatter plot displaying average amount of LTD at each induction in both condtions. Under vehicle conditions a subthreshold stimulus did not, on average, produce a second or third round of LTD. However, inhibition of the proteasome allows for multiple subsequent rounds of LTD to be induced.

Summary data consist of mean ± s.e.m.