Supplemental Data

A Cdh1-APC/FMRP Ubiquitin Signaling Link

Drives mGluR-Dependent Synaptic Plasticity in the Mammalian Brain

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Supplemental Figures:

Figures S1 and S2, Related to Figure 1

Figure S3, Related to Figure 2

Figure S4, Related to Figure 3

Figure S5, Related to Figure 5

Figure S6, Related to Figure 6

Figure S1

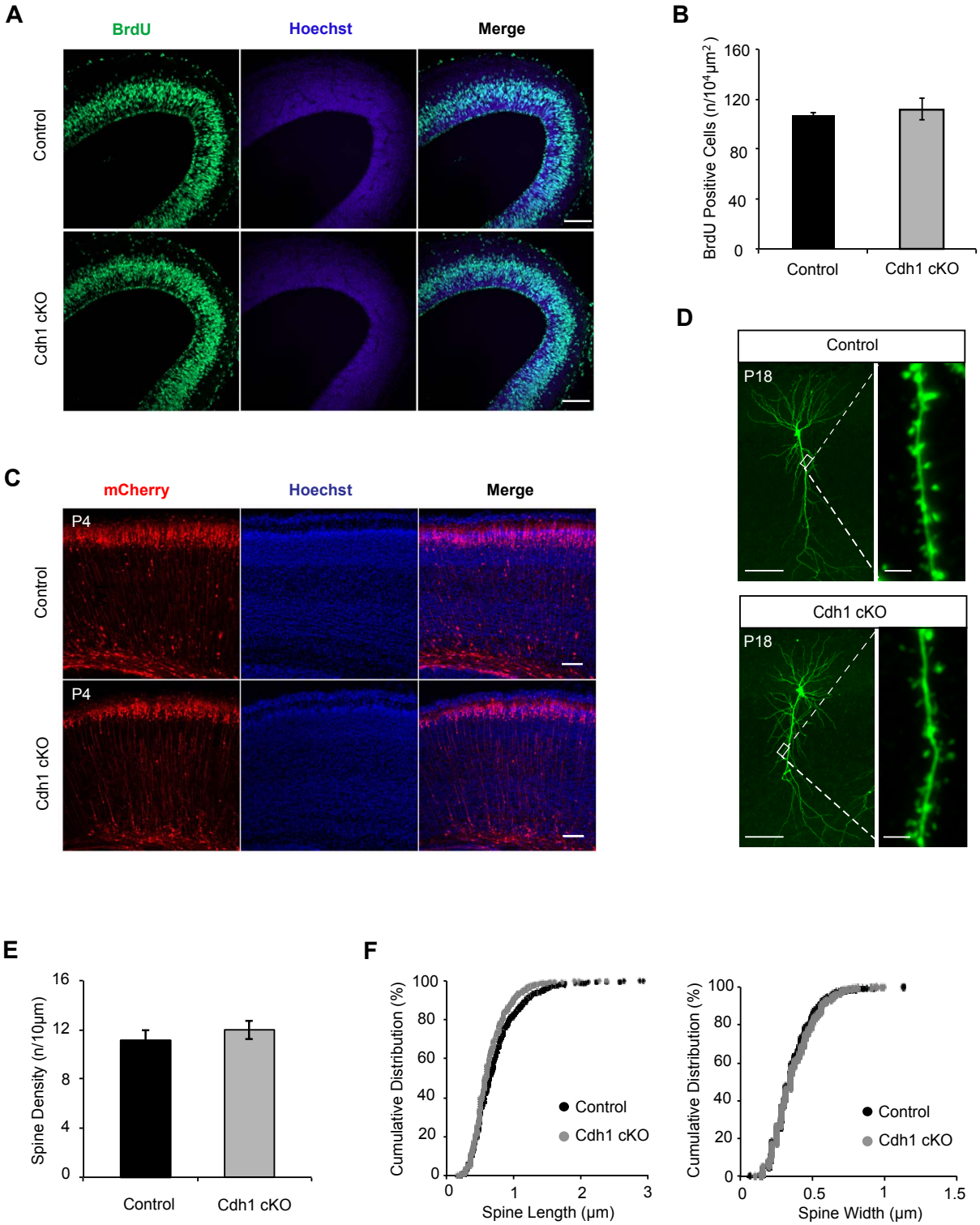
A
BrdU
Hoechst
Merge
Control
Cdh1 cKO
B
BrdU Positive Cells (n/10⁴μm²)
160
120
80
40
0
Control
Cdh1 cKO
C
mCherry
Hoechst
Merge
P4
Control
Cdh1 cKO
D
Control
P18
Cdh1 cKO
P18
E
Spine Density (n/10μm)
16
12
8
4
0
Control
Cdh1 cKO
F
Cumulative Distribution (%)
100
80
60
40
20
0
Control
Cdh1 cKO
0
1
2
3
Spine Length (μm)
Cumulative Distribution (%)
Control
Cdh1 cKO
0
0.5
1
1.5
Spine Width (μm)

Figure S1: Conditional Cdh1 knockout does not appear to alter the structure of the forebrain or dendritic spine density and morphology of CA1 hippocampal neurons.

(A) Timed pregnant (E15) female mice were intraperitoneally injected with BrdU (200 mg/kg body weight) for 2.5 hours. Coronal sections of E15 embryonic brains from control and conditional Cdh1 knockout mice were subjected to BrdU staining with the BrdU antibody (Developmental Studies Hybridoma Bank, G3G4). Scale bars represent 100 μm.

(B) Quantification of results shown in (A). The number of BrdU-positive cells in conditional Cdh1 knockout mice did not show significant difference as compared to control mice (P=0.61; Control: n=5 animals; Cdh1 cKO: n=5 animals).

(C) E15 control and conditional Cdh1 knockout mouse embryos were electroporated *in utero* with pCAG-mCherry and analyzed at P4. Conditional knockout of Cdh1 did not appear to alter the positioning of neurons in the cerebral cortex (Control: n=5 animals; Cdh1 cKO: n=7 animals). Scale bars represent 100 μm.

(D, E, F) CA1 neurons in acute hippocampal slices from P18 control or conditional Cdh1 knockout mice were injected with Biocytin followed by *post-hoc* Biocytin staining with Alexa Fluor 488-Avidin. Scale bars in (D) represent 100 μm in left panels and 2 μm in right panels. Knockout of Cdh1 had little or no effect on the dendritic spine density (E, P=0.66), spine length (F, P=0.13) and spine width (F, P=0.99) in CA1 hippocampal neurons as compared to control mice (Control: n=990 spines from 4 neurons; Cdh1 cKO: n=950 spines from 4 neurons).

Figure S2

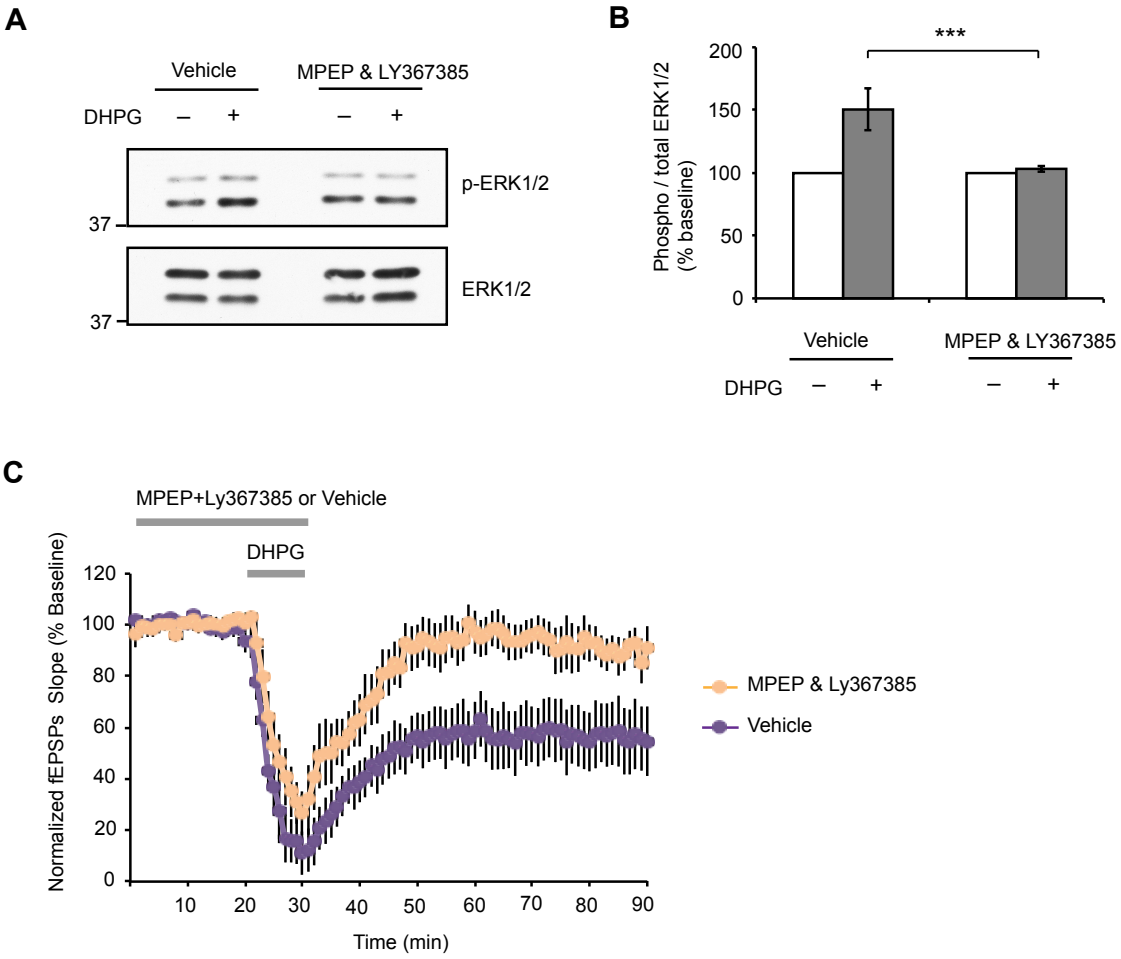
A
Vehicle
MPEP & LY367385
DHPG
–
+
–
+
p-ERK1/2
ERK1/2
37
37
B
***
Phospho / total ERK1/2
(% baseline)
200
150
100
50
0
Vehicle
MPEP & LY367385
DHPG
–
+
–
+
C
MPEP+Ly367385 or Vehicle
DHPG
Normalized fEPSPs Slope (% Baseline)
120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
Time (min)
MPEP & Ly367385
Vehicle

Figure S2: Group 1 mGluR antagonists MPEP and LY367385 block DHPG-induced ERK1/2 phosphorylation and mGluR-LTD in the hippocampus.

(A) Lysates of acute hippocampal slices pretreated with the mGluR5-specific antagonist MPEP (10 μM) and the mGluR1-specific antagonist LY367385 (100 μM) or vehicle for 1 hour and treated with or without DHPG (50 μM for 10 min). Lysates were immunoblotted with the ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) antibodies.

(B) Quantification of phospho- *vs* total- ERK1/2 level shown in (A). Application of MPEP and LY367385 blocked DHPG-induced ERK1/2 phosphorylation ($P<0.005$; Vehicle: 150.7±16.7% of baseline, n=4 animals; MPEP+LY367385: 103.3±2.5% of baseline, n=4 animals).

(C) Application of MPEP and LY367385 blocked DHPG-induced mGluR-LTD in hippocampus as compared with the vehicle ($P<0.05$; Vehicle: 56.6±12.8% of baseline, n=7 slices from 3 animals; MPEP+LY367385: 90.0±6.7% of baseline, n=6 slices from 3 animals).

Figure S3

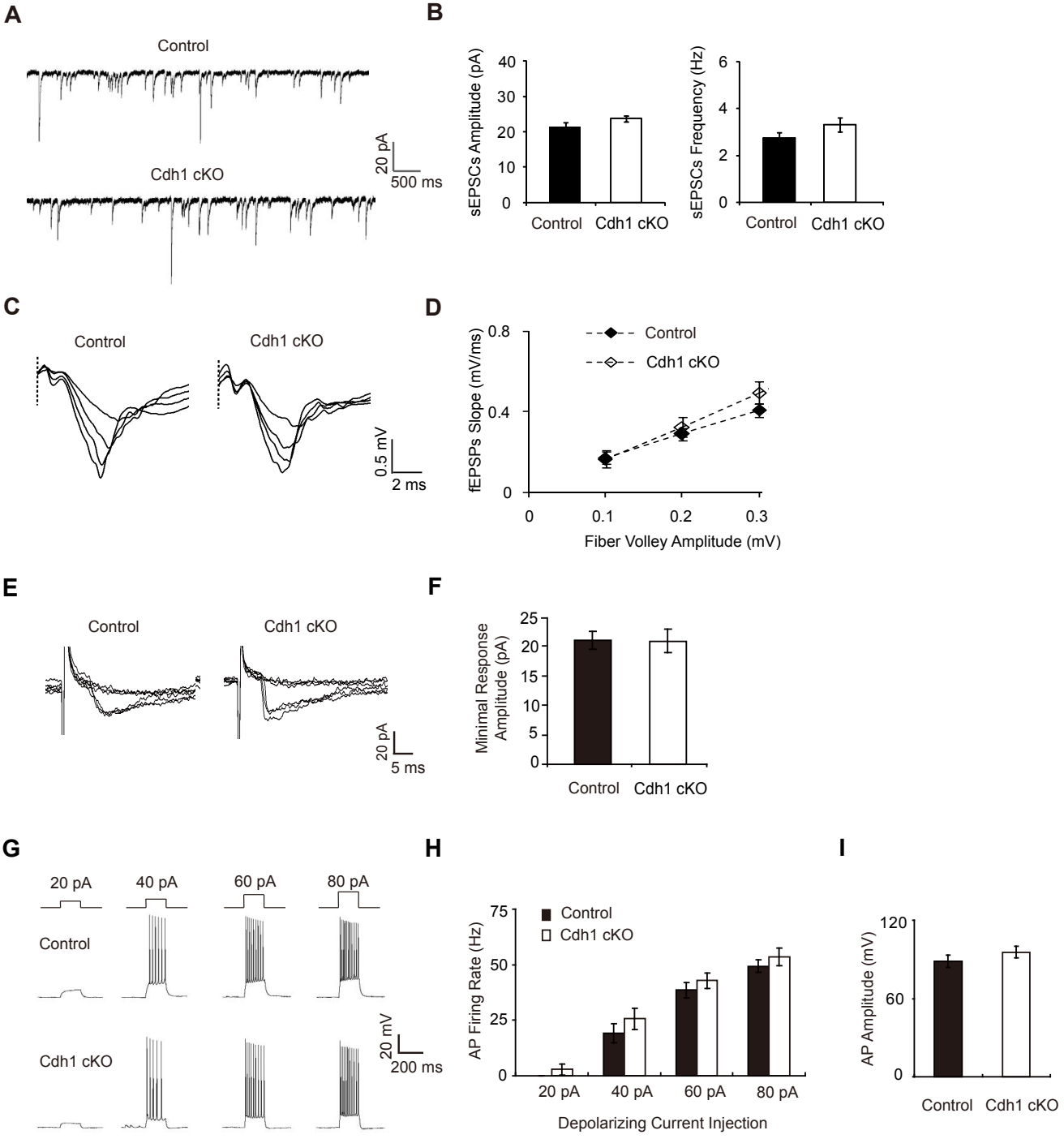
A
Control
Cdh1 cKO
20 pA
500 ms
B
sEPSCs Amplitude (pA)
40
30
20
10
0
Control
Cdh1 cKO
sEPSCs Frequency (Hz)
6
4
2
0
Control
Cdh1 cKO
C
Control
Cdh1 cKO
0.5 mV
2 ms
D
Control
Cdh1 cKO
fEPSPs Slope (mV/ms)
0.8
0.4
0
0
0.1
0.2
0.3
Fiber Volley Amplitude (mV)
E
Control
Cdh1 cKO
20 pA
5 ms
F
Minimal Response Amplitude (pA)
25
20
15
10
5
0
Control
Cdh1 cKO
G
20 pA
40 pA
60 pA
80 pA
Control
Cdh1 cKO
20 mV
200 ms
H
Control
Cdh1 cKO
AP Firing Rate (Hz)
75
50
25
0
20 pA
40 pA
60 pA
80 pA
Depolarizing Current Injection
I
AP Amplitude (mV)
120
60
0
Control
Cdh1 cKO

Figure S3: Conditional Cdh1 knockout does not alter the basal synaptic transmission and intrinsic neuronal excitability in CA1 neurons.

(A, B) Spontaneous EPSCs were recorded in CA1 neurons from control or conditional Cdh1 knockout mice. Knockout of Cdh1 had little or no effect on the sEPSC amplitude (B; *P*=0.13) and sEPSC frequency (B; *P*=0.16) (Control: n=27 neurons; Cdh1 cKO: n=25 neurons).

(C, D) Evoked field EPSPs in response to increasing intensity of electrical stimulation at Schaffer collaterals were recorded in the hippocampal CA1 area from control or conditional Cdh1 knockout mice. Slopes of fEPSPs are plotted against with the peak amplitude of fiber volleys. No significant difference of evoked field EPSPs were observed between control and conditional Cdh1 knockout mice (D; *P*=0.91, 0.58, 0.24 for electrical stimulations inducing 0.1 mV-, 0.2 mV-, 0.3 mV-fiber volley, respectively; Control: n=8 slices; Cdh1 cKO: n=10 slices).

(E, F) Minimum electrical stimulation induced synaptic responses in hippocampal CA1 neurons from control or conditional Cdh1 knockout mice. Minimum stimulation was determined with stimulation intensity that evoked synaptic responses at ~50% success rate. No significant difference of the minimum stimulation induced responses was observed between control and conditional Cdh1 knockout mice (F; *P*=0.97; Control: n=6 neurons; Cdh1 cKO: n=7 neurons).

(G, H, I) Hippocampal CA1 neurons were recorded under current clamp configuration. Action potentials (APs) were evoked by injection of increasing steps of depolarizing current. No significant difference of AP firing rates (H; *P*= 0.24, 0.38, 0.56, 0.57 for 20 mA-, 40 mA-, 60 mA-, 80 mA-current injection, respectively) and AP amplitude (I; *P*=0.17) were observed between control and conditional Cdh1 knockout mice (Control: n=7 neurons; Cdh1 cKO: n=8 neurons).

Figure S4

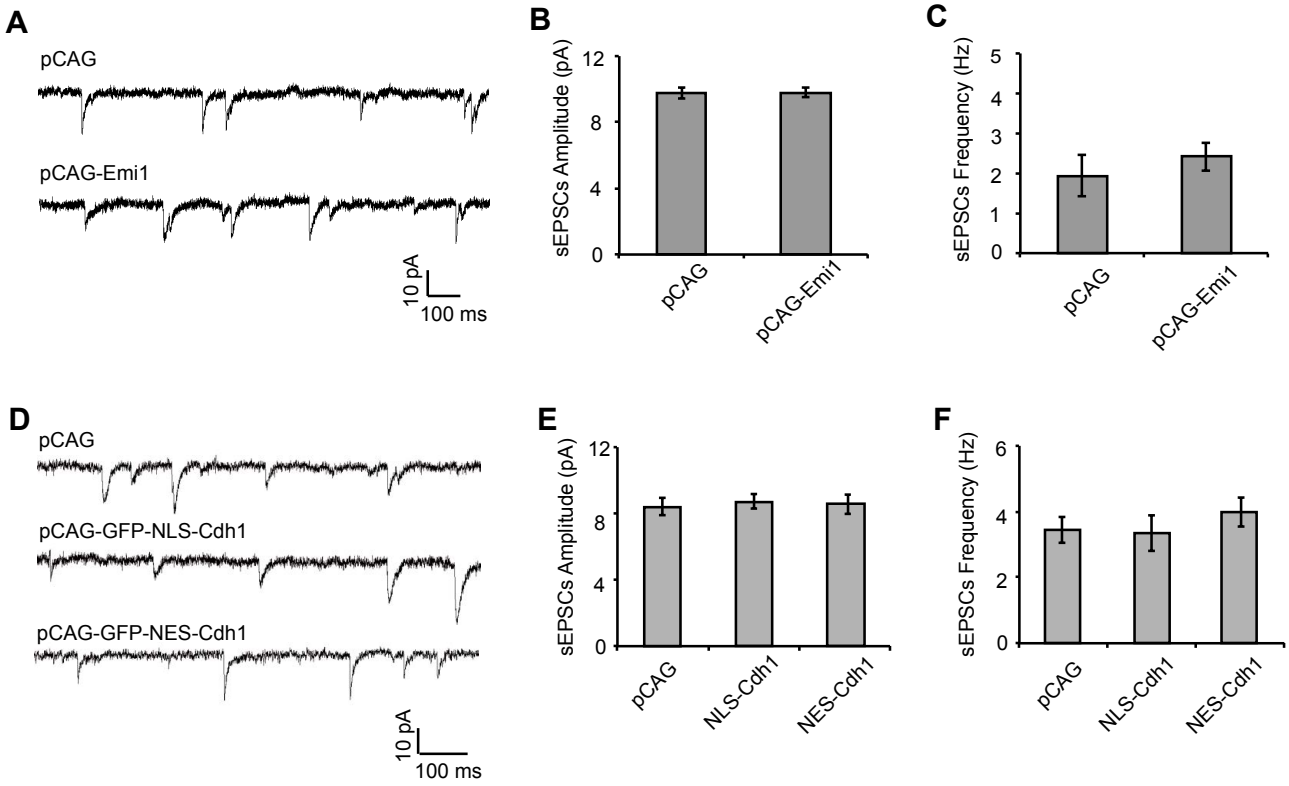
A
pCAG
pCAG-Emi1
10 pA
100 ms
B
sEPSCs Amplitude (pA)
12
8
4
0
pCAG
pCAG-Emi1
C
sEPSCs Frequency (Hz)
5
4
3
2
1
0
pCAG
pCAG-Emi1
D
pCAG
pCAG-GFP-NLS-Cdh1
pCAG-GFP-NES-Cdh1
10 pA
100 ms
E
sEPSCs Amplitude (pA)
12
8
4
0
pCAG
NLS-Cdh1
NES-Cdh1
F
sEPSCs Frequency (Hz)
6
4
2
0
pCAG
NLS-Cdh1
NES-Cdh1

Figure S4: Expression of Emi1, NES-Cdh1, or NLS-Cdh1 does not alter the basal synaptic transmission in CA1 neurons.

(A, B, C) Sponetaneous EPSCs were recorded in hippocampal CA1 neurons expressing pCAG-Emi1 or pCAG empty vector in wild type mice. Expression of Emi1 had little or no effect on sEPSCs amplitude (B; *P*=0.931) and sEPSCs frequency (C; *P*=0.445) (pCAG-Emi1: n=11 neurons; pCAG empty vector: n=10 neurons).

(D, E, F) Sponetaneous EPSCs were recorded in hippocampal CA1 neurons expressing cytoplasmic Cdh1 (pCAG-GFP-NES-Cdh1), nuclear Cdh1 (pCAG-GFP-NLS-Cdh1) or pCAG empty vector under the background of Cdh1 knockout mice. Expression of NES-Cdh1 or NLS-Cdh1 had little or no effect on sEPSCs amplitude (E; *P*=0.980) and sEPSCs frequency (F; *P*=0.923) (pCAG-GFP-NES-Cdh1: n=13 neurons; pCAG-GFP-NLS-Cdh1: n=14 neurons; pCAG empty vector: n=14 neurons).

Figure S5

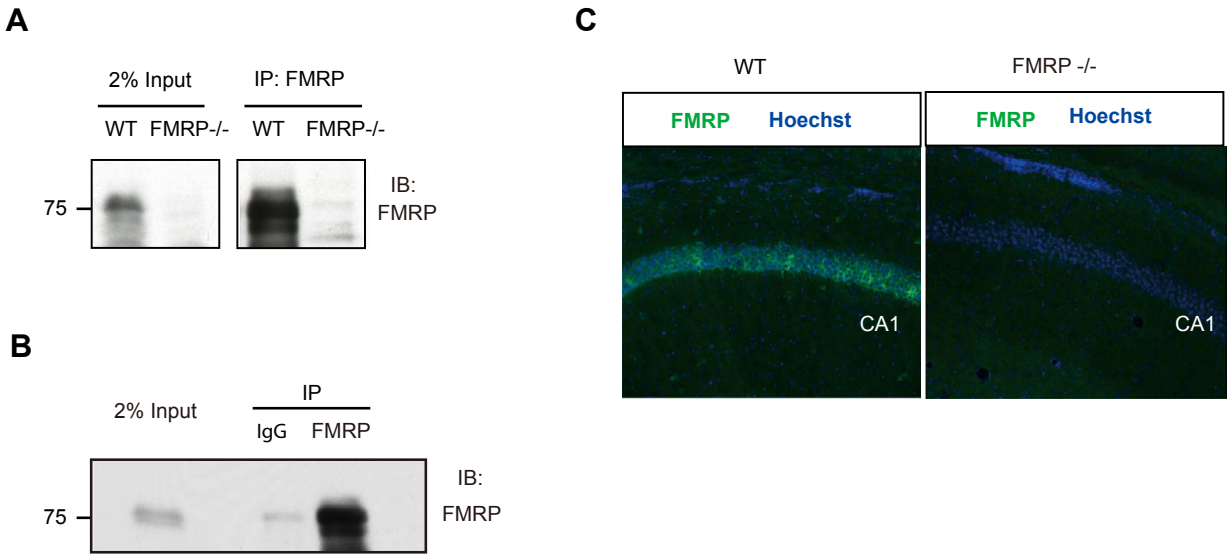
A
2% Input
IP: FMRP
WT FMRP-/- WT FMRP-/-
75
IB:
FMRP
B
2% Input
IP
IgG FMRP
75
IB:
FMRP
C
WT
FMRP -/-
FMRP Hoechst
FMRP Hoechst
CA1
CA1

Figure S5: Validation of the FMRP antibody in immunoprecipitation and immunohistochemistry analyses.

(A) Lysates of the hippocampus from wild type or FMRP knockout mice were immunoprecipitated with the FMRP antibody (Ab17722) followed by immunoblotting with the FMRP antibody (1C3).

(B) Lysates of the hippocampus from wild type mice were immunoprecipitated with the FMRP antibody (Ab17722) or IgG control followed by immunoblotting with the FMRP antibody (1C3).

(C) Sections of the hippocampus from wild type or FMRP knockout mice were subjected to immnunohistochemistry with the FMRP antibody (Ab17722) and counterstained with the DNA dye bisbenzimide (Hoechst).

Figure S6

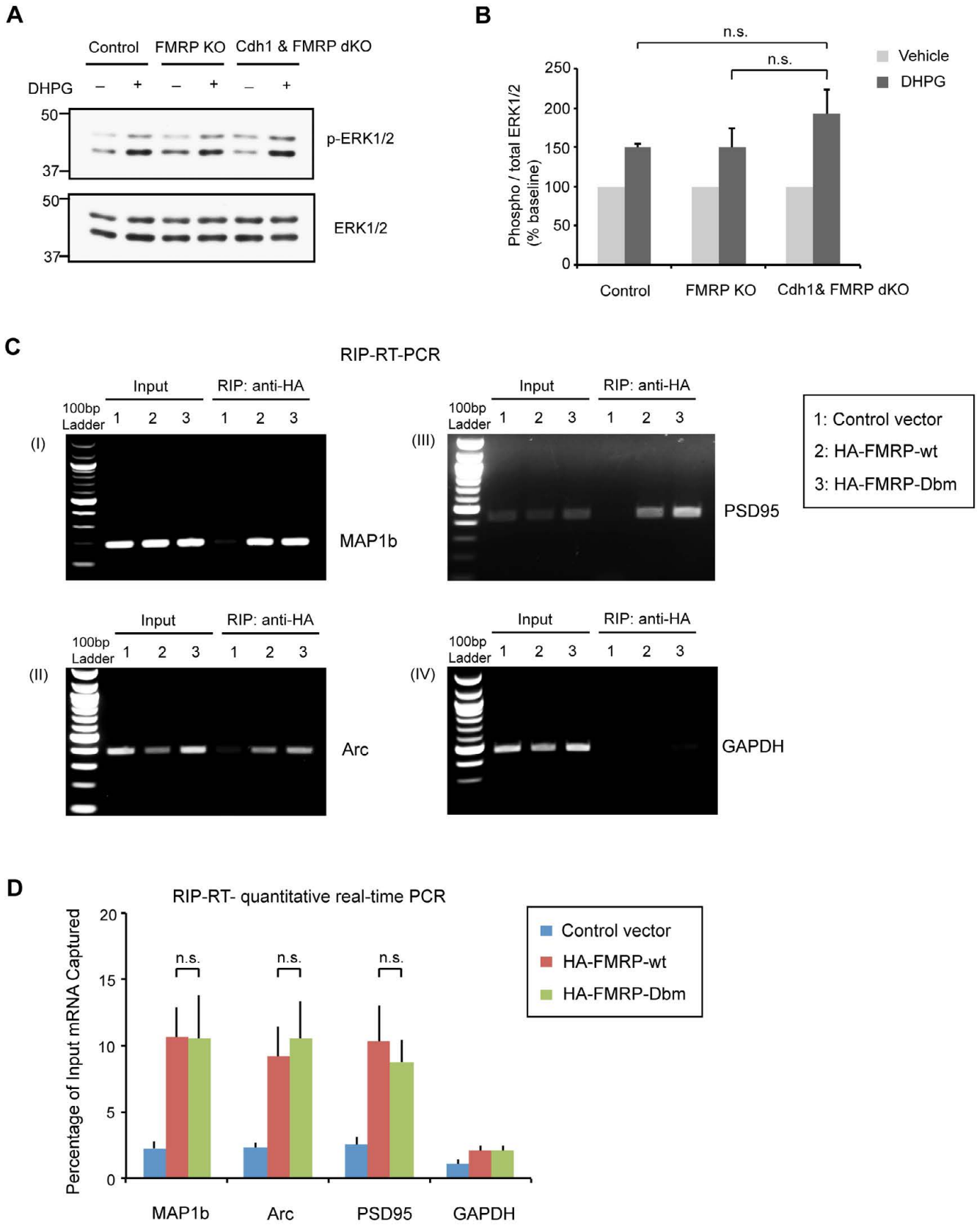
A
Control
FMRP KO
Cdh1 & FMRP dKO
DHPG
–
+
–
+
–
+
50
37
p-ERK1/2
50
37
ERK1/2
B
n.s.
n.s.
Vehicle
DHPG
Phospho / total ERK1/2
(% baseline)
250
200
150
100
50
0
Control
FMRP KO
Cdh1& FMRP dKO
C
RIP-RT-PCR
Input
RIP: anti-HA
100bp
Ladder
1
2
3
1
2
3
(I)
MAP1b
(III)
PSD95
(II)
Arc
(IV)
GAPDH
1: Control vector
2: HA-FMRP-wt
3: HA-FMRP-Dbm
D
RIP-RT- quantitative real-time PCR
Percentage of Input mRNA Captured
20
15
10
5
0
n.s.
n.s.
n.s.
Control vector
HA-FMRP-wt
HA-FMRP-Dbm
MAP1b
Arc
PSD95
GAPDH

Figure S6: FMRP & Cdh1 double knockout does not alter general mGluR function and the D-box mutation does not affect mRNA binding properties of FMRP.

(A, B) Lysates of acute hippocampal slices from control, FMRP knockout, FMRP and Cdh1 double knockout mice treated with or without DHPG (50 μM for 10 min) immunoblotted with the ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) antibodies. Quantification of phospho- *vs* total- ERK1/2 level shown in (B). Knockout of FMRP or double knockout of Cdh1 and FMRP had little or no effect on DHPG-induced ERK1/2 phosphorylation (B; *P*=0.29; *P*=0.34) (Control: 150.0±3.9% of baseline, n=2 animals; FMRP knockout: 150.7±22.7% of baseline, n=2 animals; Cdh1 and FMRP double knockout: 192.1±30.7% of baseline, n=3 animals).

(C) Mouse N2A cells transfected with wild type FMRP (HA-FMRP-wt), the D-box mutant FMRP (HA-FMRP-Dbm), or empty vector were subjected to UV crosslinking followed by RNA-immunoprecipitation (RIP) with the anti-HA agarose beads. RT-PCR analyses were conducted using specific primers for FMRP target mRNAs (MAP1b, Arc and PSD95) and control mRNA (GAPDH). The D-box mutation had little or no effect on the ability of FMRP to associate with target mRNAs as compared to wild type FMRP (n=5 independent experiments). Input represents 5% of lysate. Expected size of PCR products: MAP1b 185bp; PSD95 432bp; Arc 386bp; GAPDH 474bp.

(D) RNA-immunoprecipitation (RIP) as shown in (C), followed by RT and quantitative real-time PCR using specific primers for MAP1b, Arc, PSD95 and GAPDH. The D-box mutation of FMRP had little or no effect on the ability of FMRP to associate with the mRNA targets (MAP1b, Arc, and PSD95) as compared to wild type FMRP (n=8; *P*= 0.97 for MAP1b; *P*=0.72 for Arc; *P*=0.70 for PSD95).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials

The antibodies purchased include mouse anti-Cdh1 (Thermo Scientific, DH01), rabbit anti-FMRP (Abcam, Ab17722), mouse anti-FMRP (Millipore, 1C3), mouse anti-Cdc27 (Santa Cruz, AF3.1), anti-GluR2 extracellular (Millipore, clone 6C4), mouse anti-Flag M2 affinity gel (Sigma Aldrich, A2220), rabbit anti-Flag (Sigma Aldrich, F7425), rabbit anti-PARP (Cell signaling), mouse anti-ubiquitin (Millipore, FK2), rabbit anti-ERK1/2 (Cell signaling), rabbit anti-p-ERK1/2 T202/Y204 (Cell signaling), mouse anti-actin (Santa Cruz, C2), mouse anti-α-tubulin (Santa Cruz, B7), mouse anti-HA agarose conjugated (HA-7, Sigma Aldrich, A2095), rat anti-HA (Roche, 3F10), mouse anti-HA (Covance, 101P), chicken anti-GFP (Abcam, Ab13970) and rabbit anti-DsRed (Clonetech, 632496).

S-DHPG, MPEP, Ly367385, DL-AP5, MG132, Anisomycin and N-ethylmaleimide were purchased from Sigma Aldrich.

Plasmids

The expression plasmids for Flag-Cdh1, GFP-NES-Cdh1, GFP-NLS-Cdh1 have been described (Stegmüller et al., 2006). GFP-NES-Cdh1 and GFP-NLS-Cdh1 were subcloned into pCAG vector (Matsuda and Cepko, 2007). *FMR1* cDNA was amplified from mouse brain cDNA by PCR with primers (For: 5'aaagaattcgaggagctggtggtggaagtg3'; Re: 5'aaactcgagttagggtactccatt caccag3'), and then cloned into pcDNA3-HA vector to generate pcDNA3-HA-FMRP. The D-box mutation of FMRP was generated by site-directed mutagenesis with the primers (For: 5'gatgcagtgaaaaaggctgctagctttgctgaatttgctgaagat3'; Re: 5'agccttttttcactgcatcttgatcctctccat3'). HA-FMRP-wt and HA-FMRP-Dbm were subcloned into pCAG vector. Dephosphorylated mimic FMRP S499A and phosphorylated mimic FMRP S499D plasmid were generated by site-directed mutagenesis with the primers (S499A: For: 5'tctgaagcatcaaatgctgctgaaacagaatctgac3'; Re:

5’gtcagattctgtttcagcagcatttgatgcttcaga3’. S499D: For: 5’tctgaagcatcaaatgctgacgaaacagaatctgac3’; Re: 5’gtcagattctgtttcgtcagcatttgatgcttcaga3’). The Flag-Emi1 expression plasmid has been described (Konishi et al., 2004). Flag-Emi1 was subcloned into pCAG vector.

Subcellular fractionation

Nuclear and cytoplasmic fractions were prepared from P15 mouse hippocampus. Tissues were lysed in hypotonic buffer containing 10 mM Hepes-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, and protease inhibitors. The homogenate was centrifuged at 800 g for 5 min at 4°C to isolate nuclei. The pelleted nuclei were washed twice in hypotonic buffer and resuspended in nuclear lysis buffer containing 20mM Hepes-KOH pH 7.9, 400 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors. After 30 min incubation at 4°C, the nuclear fraction was centrifuged at 15000 rpm for 10 min and the supernatant was collected as the nuclear fraction. The homogenate that remained after isolating the nuclei was centrifuged at 800g for 10 minutes to eliminate the contaminated nuclei and the supernatant was then collected as the cytoplasmic fraction. Equal volume of cytosolic and nuclear fractionation was loaded in western blot.

Immunohistochemistry

For immunofluorescence analyses of cell surface AMPA receptor, GluR2, primary hippocampal neurons 14 days after plating from P0 mice were treated with or without DHPG (50μM) for 10min. 20min after DHPG washout, neurons were fixed with 4% PFA, blocked with blocking buffer without detergent (10% goat serum and 3% BSA in PBS), and subjected to immunofluorescence analyses with mouse anti-GluR2 extracellular (Millipore, clone 6C4, 1:150 dilution) for cell surface GluR2 staining.

For FMRP degradation assays, acute hippocampal slices were pretreated with the protein

synthesis inhibitor Anisomycin (25μM) for 1 hour, and then treated with or without DHPG (50uM) for 10min. 20 minutes after DHPG washout, acute hippocampal slices were fixed with 4% PFA. Sections of 20μm were cut from 400μm-thick slices and subjected to immunofluorescence analyses with rabbit anti-FMRP antibody (Abcam Ab17722, 1:400 dilution). In other experiments, the following primary antibodies were used: chicken anti-GFP (Abcam, 1:500 dilution), and rabbit anti-DsRed (Clontech, 1:500 dilution). Alexa Fluor 488- or Cy3-conjugated goat anti-rabbit, anti-mouse or anti-chicken IgG was used as secondary antibodies. The DNA dye bisbenzimide (Hoechst 33258) was used to label cell nuclei. Confocal images were acquired with ZEISS LSM 510 Pascal system with identical scanning configurations for all samples in the same experiment.

Calcium Imaging

Primary hippocampal neurons 14 days after plating from P0 mice were loaded with 7.5μM Oregon Green 488 BAPATA-1, AM (Life Technologies) and Pluronic F127 (1:1000; Life Technologies) in Neurobasal medium at 37°C for 30min, and washed 3 times with Neurobasal medium. During calcium imaging, neurons were incubated in buffer containing 125mM NaCl, 5mM KCl, 20mM HEPES, 1.5mM $CaCl_2$, 1.5mM $MgCl_2$, 10mM glucose and pH 7.4. After baseline calcium activities were obtained, DHPG (50μM final concentration) was applied to the bath solution and images were recorded for an additional 5min. Images were obtained using an Olympus BX 50WI confocal microscope with a LUMPlanFl/lR 40x/0.80w objective and processed with MetaMorph software.

I*n utero* electroporation

Timed pregnant E15 mice were used for hippocampal *in utero* electroporation. Briefly, expression plasmids under the CAG promoter (2 μg/μl) together with pCAG-mCherry (1 μg/μl)

were injected into the lateral ventricle of E15 mouse embryos within the uterus. Electric pulses (35 V for 50 ms, with 950 ms inter-pulse intervals, 5 pulses) were applied to the brain to electroporate the hippocampus with a 5 mm-diameter platinum tweezertrode (BTX, 45-0489) and a square wave pulse electroporator (BTX, ECM830) as described (Mejia et al., 2013; Pacary et al., 2012; Zhang et al., 2013).

BrdU assay

Timed pregnant female mice (E15) were intraperitoneally injected with BrdU (200 mg/kg body weight). 2.5 hours after BrdU injection, brains of E15 embryos were dissected and fixed in 4% PFA overnight at 4°C followed by cryoprotection with 30% sucrose. Brain sections were subjected to antigen retrieval in 10 mM citric acid (pH 6) at 95-100°C for 15min followed by immunohistochemical analyses with a BrdU antibody (Developmental Studies Hybridoma Bank, G3G4, 1:200).

Biocytin injection and *post-hoc* staining

Biocytin labeling was performed as described (Huang et al., 2010). Intracelluar recording solution containing 1% Biocytin was loaded into CA1 neurons from P18 animals through recording electrode (3-4 MΩ) for around 20 minutes. Slices were fixed in 4% PFA for 2 hours and then dehydrated in 30% sucrose at 4°C. Slices were subjected to histochemical analyses with Alexa Fluor 488-conjugated-Avidin (1:500).

Nissl staining

Brain sections were delipidized by 70%, 95%, 100% ethanol for 4 min each, and then Xylene for 10 min, followed by rehydration in 100%, 95%, 70%, 50% ethanol for 4 min each. Sections were stained with 0.04% Cresyl violet for 5 min. After two dips in dH_2O, sections were

dehydrated with 50%, 70%, 95%, 100% ethanol for 1 min each, and then placed into Xylene for 10 min and mounted.

RNA immunoprecipitation, RT-PCR and RT-Real time PCR

RNA immunoprecipitation (RIP) was performed as described (Keene et al., 2006). Mouse N2A neuroblastoma cells transfected with wild type FMRP (HA-FMRP-wt), the D-box mutant FMRP (HA-FMRP-Dbm), or empty vector were subjected to UV crosslinking for 400mJ/cm^2 in Stratalinker. Cells were lysed in polysome lysis buffer containing 0.5% NP40, 100mM KCl, 5mM MgCl2, 10mM HEPES, 1mM DTT and 100U/ml RNaseOUT (Invitrogen) and 600U/ml SUPERase.In RNase Inhibitor (Invitrogen) and 1:100 proteinase inhibitor cocktail (Sigma). Monoclonal anti-HA−Agarose beads (Sigma) were used for protein-RNA immunoprecipitation for 2.5 hours at 4°C. After immunoprecipitation, beads were washed 4 times in NT2 buffers containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM MgCl2, 0.1% NP40. Proteins were digested by proteinase K (300μg/ml, NEB) in NT2 buffer for 30min at 50°C. RNA from immunoprecipitation complex and input lysate were extracted using Trizol LS (Invitrogen). Reverse transcription was performed using SuperScript® III Reverse Transcriptase kit (Invirogen). Quantitative real-time PCR was performed using the LightCycler® 480 System with LightCycler® 480 SYBR Green I Master (Roche). Gene specific primers for PCR and quantitative real-time PCR are as follows.

	PCR primers	Real-time PCR primers
MAP1b-F	gaccgtggtggtggaagc	gaccgtggtggtggaagc
MAP1b-R	cggatccccagctcgatgtt	cggatccccagctcgatgtt
Arc-F	aagctggagaacaacttggacgg	atccagaaccacatgaatgggcc
Arc-R	cccccaagactgatattgctgag	catacagtgtctggtacaggtcc
PSD95-F	ggcttcattcccagcaaacg	ggcttcattcccagcaaacg
PSD95-R	catcaaggatgcagtgcttc	catcaaggatgcagtgcttc
GAPDH-F	tgctggtgctgagtatgtcg	tgctggtgctgagtatgtcg
GAPDH-R	gcatgtcagatccacaacgg	gcatgtcagatccacaacgg

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