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(54) Title: TREATMENT OF FRAGILE X SYNDROME BY INHIBITION OF CDH1-APC

(57) Abstract: Described herein are methods for the treatment of fragile X syndrome and other cognitive disorders related to impairment of mGluR-dependent LTD and/or FMRP gene dysfunction by administration of an agent that inhibits the Cdh1-APC complex.



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TREATMENT OF FRAGILE X SYNDROME BY INHIBITION OF CDH1-APC**RELATED APPLICATIONS**

This application claims the benefit of priority to United States Provisional Patent
5 Application serial number 62/007,692, filed June 4, 2014; the contents of which are hereby
incorporated by reference.

GOVERNMENT INTEREST

This invention was made with Government support under National Institutes of
Health Grant NS051255. The Government has certain rights in the invention.

10 BACKGROUND

Synaptic plasticity plays a fundamental role in the adaptive responses of the nervous
system to experience. Two forms of synaptic plasticity, long-term potentiation (LTP) and
long-term depression (LTD), have been characterized at several synapses in the mammalian
brain and may represent physiological correlates of learning and memory. Many
15 neurological diseases, including developmental disorders of cognition, are characterized by
defects in synaptic plasticity.

Alteration of LTD is a prominent feature of fragile X syndrome (also known as
Martin-Bell syndrome or Escalante's syndrome), the most common known monogenic
cause of intellectual disability and autism spectrum disorder. Fragile X syndrome is
20 associated with reduced expression or activity of fragile X syndrome protein FMRP (the
protein product of the FMR1 gene). Reduced expression or activity of FMRP triggers an
increase in a form of LTD that is induced by the activation of metabotropic glutamate
receptors (mGluR-LTD). Fragile X syndrome is most often associated with reduced
expression of FMRP due to expansion of a CGG trinucleotide repeat in the FMR1 gene,
25 which results in gene methylation and transcriptional silencing. However, in some cases of
fragile X syndrome, the activity of FMRP is reduced as the result of mutations in the FMR1
gene. In addition to fragile X syndrome, defects in FMRP is also associated with mental
retardation, premature ovarian failure, autism, Parkinson's disease, developmental delays
and other cognitive defects. There is currently no drug treatment for fragile X syndrome.

30 Thus, there is a need for new compositions and methods for the treatment of
diseases and disorders associated with impairment of mGluR-dependent LTD and/or
reduced expression or function of FMRP, such as cognitive disorders and including fragile
X syndrome.

SUMMARY

In certain aspects, provided herein are methods for treating or preventing a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced
5 expression or activity of FMRP (*e.g.*, fragile X syndrome, fragile X permutation, mental retardation, premature ovarian failure, autism, Parkinson's disease, cognitive impairment).

In some embodiments, the method includes administering to a subject an agent that inhibits Cdh1-APC. In some embodiments, the agent inhibits the expression of Cdh1. In some embodiments, the agent inhibits the formation of a complex between Cdh1 and APC.
10 In some embodiments, the agent inhibits the activity of Cdh1-APC (*e.g.*, the ubiquitin ligase activity of Cdh1-APC).

In some embodiments, the agent is a small molecule. In certain embodiments the agent is tosyl-L-arginine methyl ester (TAME) or a TAME prodrug. In some embodiments the agent is a TAME prodrug comprising a TAME derivative in which a guanidine group is
15 protected by a carbamate group. In some embodiments, the agent is a TAME prodrug comprising an esterase-activatable N,N'-bis(acyloxymethyl carbamate) derivative of TAME. In some embodiments, the agent is proTAME.

In some embodiments, the agent is an interfering nucleic acid molecule specific for Cdh1. In some embodiments, the agent is an antisense molecule, an siRNA molecule, an
20 shRNA molecule or a miRNA molecule.

In some aspects, provided herein is a method of determining whether a test agent (*e.g.*, a small molecule) is a candidate therapeutic agent for the treatment of a disease or disorder associated with impairment of mGluR-dependent LTD and/or dysfunction of in the expression or activity of FMRP (*e.g.*, fragile X syndrome, fragile X permutation, mental
25 retardation, premature ovarian failure, autism, Parkinson's disease, cognitive impairment). In some embodiments, the method includes the step of forming a test reaction mixture comprising: a Cdh1 polypeptide or fragment thereof; an APC polypeptide or fragment thereof; and a test agent. In some embodiments, the method includes the step of incubating the test reaction mixture under conditions conducive for the formation of a complex
30 between the Cdh1 polypeptide or fragment thereof and the APC polypeptide or fragment thereof. In some embodiments, the method includes the step of determining the amount of the complex in the test reaction mixture. In some embodiments, a test agent that reduces the amount of the complex in the test reaction mixture compared to the amount of the complex

in a control reaction mixture is a candidate therapeutic agent for the treatment of a cognitive disorder. In some embodiments, the test agent is a member of a library of test agents.

In some embodiments, the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture does not comprise a test agent.

- 5 In some embodiments, the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture comprises a placebo agent instead of a test agent.

In some embodiments, the test reaction mixture is formed by adding the test agent to a mixture comprising the Cdh1 polypeptide or fragment thereof and the APC polypeptide or
10 fragment thereof. In some embodiments, the test reaction mixture is formed by adding the APC polypeptide or fragment thereof to a mixture comprising the test agent and the Cdh1 polypeptide or fragment thereof. In some embodiments, the test reaction mixture is formed by adding the Cdh1 polypeptide or fragment thereof to a mixture comprising the test agent and the APC polypeptide or fragment thereof.

- 15 In some embodiments, the APC polypeptide or fragment thereof is anchored to a solid support in the test reaction mixture. In some embodiments, the test reaction mixture is incubated under conditions conducive to the binding of the Cdh1 polypeptide or fragment thereof to the anchored APC polypeptide or fragment thereof. In some embodiments, the method further comprises the step of isolating Cdh1 polypeptide or fragment thereof bound
20 to the APC polypeptide or fragment thereof from the Cdh1 polypeptide or fragment thereof not bound to the APC polypeptide or fragment thereof. In some embodiments, the amount of complex in the test reaction mixture is determined by detecting the amount of APC polypeptide or fragment thereof bound to the Cdh1 polypeptide or fragment thereof. In some embodiments, the Cdh1 polypeptide or fragment thereof is linked to a detectable
25 moiety.

- In some embodiments, the Cdh1 polypeptide or fragment thereof is anchored to a solid support in the test reaction mixture. In some embodiments, the test reaction mixture is incubated under conditions conducive to the binding of the APC polypeptide or fragment thereof to the anchored Cdh1 polypeptide or fragment thereof. In some embodiments, the
30 method further comprises the step of isolating APC polypeptide or fragment thereof bound to the Cdh1 polypeptide or fragment thereof from the APC polypeptide or fragment thereof not bound to the Cdh1 polypeptide or fragment thereof. In some embodiments, the amount of complex in the test reaction mixture is determined by detecting the amount of APC

polypeptide or fragment thereof bound to the Cdh1 polypeptide or fragment thereof. In some embodiments, the APC polypeptide or fragment thereof is linked to a detectable moiety.

BRIEF DESCRIPTION OF THE DRAWINGS

5 **Figure 1** shows that hippocampal mGluR-LTD is impaired in conditional Cdh1 knockout mice. **(A)** Lysates of hippocampus from *Emx-Cre;Cdh1^{loxP/loxP}* (Cdh1 cKO) mice and control *Cdh1^{loxP/loxP}* mice were immunoblotted with the Cdh1 and actin antibodies, the latter to serve as loading control. **(B)** Coronal brain sections from P22 *Emx-Cre;Cdh1^{loxP/loxP}* (Cdh1 cKO) mice and control *Cdh1^{loxP/loxP}* mice were subjected to Nissl

10 staining. Scale bars represent 500 μ m. **(C)** High frequency stimulation (100 pulses at 100 Hz, 1 s) induced LTP at hippocampal CA1 synapses in both control *Cdh1^{loxP/loxP}* mice and *Emx-Cre;Cdh1^{loxP/loxP}* (Cdh1 cKO) mice ($P=0.94$; Control: $160.4 \pm 9.3\%$ of baseline, $n=8$ slices from 4 animals; Cdh1 cKO: $161.3 \pm 9.6\%$ of baseline, $n=7$ slices from 4 animals). In this and all subsequent electrophysiology figures, evoked synaptic responses over the time

15 course of the experiment were normalized to the average of baseline responses. Statistical analyses of long-term plasticity were performed with data from 50-60 minutes post-stimulus. Representative recording traces from conditional Cdh1 knockout and control mice were taken at times indicated by the numbers 1 and 2. Trace 1 represents baseline responses and trace 2 represents responses 1 hour after LTD or LTP induction. **(D)** Low frequency

20 stimulation (900 pulses at 1 Hz, 15 min) induced NMDAR-LTD at hippocampal CA1 synapses in both control *Cdh1^{loxP/loxP}* mice and *Emx-Cre;Cdh1^{loxP/loxP}* (Cdh1 cKO) mice ($P=0.98$; Control: $65.4 \pm 8.0\%$ of baseline, $n=8$ slices from 5 animals; Cdh1 cKO: $65.6 \pm 6.9\%$ of baseline, $n=10$ slices from 6 animals). **(E)** DHPG (50 μ M, 10 min) induced significantly reduced mGluR-LTD at hippocampal CA1 synapses in *Emx-Cre;Cdh1^{loxP/loxP}*

25 (Cdh1 cKO) mice as compared to the control *Cdh1^{loxP/loxP}* mice ($P<0.01$) or control *Emx-Cre* mice ($P<0.001$) (Cdh1 cKO: $92.0 \pm 2.4\%$ of baseline, $n=13$ slices from 7 animals; Control-*Cdh1^{loxP/loxP}*: $74.5 \pm 4.5\%$ of baseline, $n=8$ slices from 5 animals; Control-*Emx-Cre*: $71.6 \pm 3.5\%$ of baseline, $n=10$ slices from 5 animals). **(F)** Paired-pulse low frequency stimulation (900 paired-pulses at 1 Hz with 50 ms inter-pulse interval, 15 min), in the

30 presence of NMDAR antagonist DL-AP5 (100 μ M), induced significantly reduced mGluR-LTD at hippocampal CA1 synapses in *Emx-Cre;Cdh1^{loxP/loxP}* (Cdh1 cKO) mice as compared with control *Cdh1^{loxP/loxP}* mice ($P<0.01$; Cdh1 cKO: $99.5 \pm 10.0\%$ of baseline, $n=9$ slices from 5 animals; Control: $65.2 \pm 9.2\%$ of baseline, $n=10$ slices from 6 animals).

Figure 2 shows that conditional knockout of *Cdh1* does not alter the structure of the forebrain or dendritic spine density and morphology of CA1 hippocampal neurons. **(A)** Timed pregnant female mice were intraperitoneally injected with BrdU (200 mg/kg body weight) for 2.5 hours. Coronal sections from E15 embryonic brains were subjected to BrdU staining with a BrdU antibody (Developmental Studies Hybridoma Bank, G3G4). BrdU positive cells indicate the proliferation of neural precursor cells in the ventricular zone in both conditional *Cdh1* knockout mice and control *Cdh1^{loxP/loxP}* mice. Scale bars represent 100 μ m. **(B)** Quantification of results presented in **(A)**. The number of BrdU-positive cells in conditional *Cdh1* knockout mice was not different from control *Cdh1^{loxP/loxP}* mice ($P=0.61$; Control: $n=5$ animals; *Cdh1* cKO: $n=5$ animals). **(C)** E15 conditional *Cdh1* knockout and control mouse embryos were electroporated in utero with pCAG-mCherry and analyzed at P4. Conditional *Cdh1* knockout 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 μ m. **(D, E, F)** CA1 neurons in acute hippocampal slices from P18 control and conditional *Cdh1* knockout mice were labeled with biocytin followed by staining with Alexa Fluor 488-Avidin. Scale bars in left panels represent 100 μ m; right panels: 2 μ m. **(D)**. Conditional *Cdh1* knockout had no effect on the CA1 dendritic spine density (**E**, $P=0.66$), spine length (**F**, $P=0.13$) and spine width (**F**, $P=0.99$) in CA1 hippocampal neurons (Control: $n=990$ spines from 4 neurons; *Cdh1* cKO: $n=950$ spines from 4 neurons).

Figure 3 shows that conditional *Cdh1* knockout does not alter the intrinsic excitability and basal synaptic transmission of CA1 neurons. **(A, B)** Spontaneous EPSCs were recorded in conditional *Cdh1* knockout and control mouse CA1 neurons. Conditional *Cdh1* knockout had little or no effect on the sEPSC amplitude (**E**; $P=0.13$) and sEPSC frequency (**E**; $P=0.16$) (Control: $n=27$ neurons; *Cdh1* cKO: $n=25$ neurons). **(C, D)** Evoked field EPSPs were recorded in conditional *Cdh1* knockout and control mouse hippocampal CA1 area in response to increasing intensity of electrical stimulation at Schaffer Collaterals. Slopes of fEPSPs are plotted against with the peak amplitude of fiber volleys. No significant difference of evoked field EPSCs were observed between conditional *Cdh1* knockout and control mice (**G**; $P=0.91, 0.58, 0.24, 0.12$ for electrical stimulations inducing 0.1 mV-, 0.2 mV-, 0.3 mV-, 0.4 mV fiber volley, respectively; Control: $n=8$ slices; *Cdh1* cKO: $n=10$ slices). **(E, F)** Minimum electrical stimulation induced synaptic responses in conditional *Cdh1* knockout and control hippocampal CA1 neurons. 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 conditional Cdh1 knockout and control mice (I; $P=0.97$; 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 (A). No significant difference of AP firing rates (B; $P=0.24$, 0.38, 0.56, 0.57 for 20 mA-, 40 mA-, 60 mA-, 80 mA-current injection, respectively) and AP amplitude (C; $P=0.17$) were observed between conditional Cdh1 knockout and control CA1 neurons (Control: $n=7$ neurons; Cdh1 cKO: $n=8$ neurons).

Figure 4 shows that Cdh1-APC operates in the cytoplasm rather than the nucleus to regulate mGluR-LTD. **(A)** Nuclear (N) and cytosolic (C) fractionations were prepared from P18 hippocampus and cortex and immunoblotted with the Cdh1, SnoN and α -tubulin antibodies. SnoN, and α -Tubulin served as the nuclear and cytoplasmic marker, respectively. **(B)** Schematic diagram of in utero electroporation to hippocampus at E15 embryos and whole cell patch clamp recording of mCherry-positive transfected CA1 neurons in 3-week old conditional Cdh1 knockout mice. **(C)** E15 mice electroporated with a plasmid expressing GFP-NES-Cdh1 (a-d) or GFP-NLS-Cdh1 (e-h) together with a mCherry expressing plasmid were allowed to develop until P20. Brain sections were subjected to immunohistochemical analyses with the GFP and DsRed antibodies and the DNA dye bisbenzimidazole (Hoechst 33258). GFP-NES-Cdh1 and GFP-NLS-Cdh1 appeared to be predominantly in the cytoplasm and nucleus, respectively. Areas inside the white box (a-h) are enlarged in a'-h'. Scale bars represent 100 μ m. Notably, although GFP-NLS-Cdh1 displayed modest expression in the soma in addition to robust expression in the nucleus, GFP-NES-Cdh1 was restricted to the cytoplasm and excluded from the nucleus in CA1 neurons. **(D)** The conditional Cdh1 knockout-induced mGluR-LTD deficit was effectively reversed by expression of cytoplasmic Cdh1 (GFP-NES-Cdh1) ($P<0.01$), but not nuclear Cdh1 (GFP-NLSCdh1) ($P>0.05$), compared with expression of pCAG empty vector in conditional Cdh1 knockout mice. mGluR-LTD in CA1 hippocampal neurons expressing GFP-NES-Cdh1 in the background of Cdh1 knockout is comparable in magnitude to control *Cdh1*^{loxP/loxP} neurons. (Control neurons: $65.0 \pm 6.8\%$ of baseline, $n=12$ from 7 animals; Cdh1 CKO neurons: $91.6 \pm 7.0\%$ of baseline, $n=12$ from 6 animals; GFP-NES-Cdh1-expressing neurons in Cdh1 knockout background: $64.9 \pm 4.5\%$ of baseline, $n=11$ from 7 animals; GFP-NLS-Cdh1-expressing neurons in Cdh1 knockout background: $96.3 \pm$

4.4% of baseline, n=12 from 6 animals; Neurons transfected with pCAG empty vector in Cdh1 knockout background: $99.8 \pm 6.1\%$ of baseline, n=12 from 5 animals).

Figure 5 shows that FMRP is a novel substrate of the ubiquitin ligase Cdh1-APC.

(A) Lysates of 293T cells expressing Flag-Cdh1, HA-FMRP-wt and HA-FMRP-Dbm were immunoprecipitated with the Flag antibody followed by immunoblotting with the HA and Flag antibodies. Input was also immunoblotted with the HA and Flag antibodies. Wild type FMRP, but not D-box mutant of FMRP, formed a complex with Cdh1. (B) Lysates of 293T cells expressing Flag-Cdh1, HA-FMRP-wt and HA-FMRP-Dbm were immunoprecipitated with the HA antibody followed by immunoblotting with the HA and Flag antibodies. Input was also immunoblotted with the HA and Flag antibodies. Cdh1 formed a complex with wild type FMRP but not D-box mutant of FMRP. (C) Lysates of 293T cells expressing Myc-Cdc20 and HA-FMRP-wt were immunoprecipitated with the Myc antibody followed by immunoblotting with the HA and Myc antibodies. Input was also immunoblotted with the antibodies. Cdc20 failed to form a complex with FMRP. (D) Conserved sequences of FMRP D-box motif from mouse, rat and human are listed. The Dbox mutation (Dbm) in FMRP is illustrated. (E) Lysates of acute hippocampal slices pretreated with MG132 (20 μ M for 1.5 h) and then treated with DHPG (50 μ M for 10 min) were immunoprecipitated with the FMRP antibody (Ab17722) followed by immunoblotting with the ubiquitin (FK2), Cdh1 (DH01) and FMRP (1C3) antibodies. Endogenous level of ubiquitinated FMRP was substantially reduced in hippocampal slices from conditional Cdh1 knockout mice as compared to control Cdh1loxP/loxP mice. (F) Lysates of acute hippocampal slices from control Cdh1loxP/loxP and conditional Cdh1 knockout mice, pretreated with anisomycin (25 μ M for 1 h), incubated with or without DHPG (50 μ M for 10 min), and collected immediately after or 20 min after DHPG washout, were immunoblotted with FMRP (1C3) and Actin antibodies, the latter to serve as loading control. DHPG10'/0' represents 0 min after DHPG (10 min) treatment; DHPG10'/20' represents 20 min after DHPG (10 min) treatment. Quantification of FMRP level from immunoblotting assays is shown in graph. FMRP level was normalized to Actin level in each sample. Endogenous FMRP was significantly reduced 20 min after DHPG treatment in the control *Cdh1*^{loxP/loxP} slices as compared to baseline level ($P < 0.05$; Control: $71.7 \pm 8.1\%$ of baseline, n=3 animals), but not in the conditional Cdh1 knockout slices ($P > 0.05$; Cdh1 cKO: $126.5 \pm 19.2\%$ of baseline, n=3 animals). (G) Acute hippocampal slices from control Cdh1loxP/loxP and conditional Cdh1 knockout mice, pretreated with anisomycin (25 μ M for 1 h), were incubated with or

without DHPG (50 μ M for 10 min) and collected 20 min after drug washout. Sections from acute hippocampal slices were subjected to immunohistochemistry with the FMRP (Ab17722) antibody and the DNA dye bisbenzimidazole (Hoechst 33258). Quantification of FMRP fluorescent intensities at CA1 areas in immunohistochemistry assays is shown in graph. Endogenous FMRP was significantly decreased in response to DHPG treatment in control *Cdh1^{loxP/loxP}* slices as compared to baseline level ($P < 0.05$; Control: 61.7 ± 8.4 % of baseline, $n = 3$ animals), but not in the conditional *Cdh1* knockout slices ($P > 0.05$; *Cdh1* cKO: 112.2 ± 13.0 % of baseline, $n = 3$ animals). Scale bars represent 100 μ m.

Figure 6 shows that FMRP knockout suppresses the conditional *Cdh1* knockout-induced impaired mGluR-LTD phenotype. (A) Field recording of DHPG (50 μ M, 10 min) induced mGluR-LTD at hippocampal CA1 synapses in *Cdh1* and FMRP double knockout (*FMRI^{-/-};Emx-Cre;Cdh1^{loxP/loxP}*), conditional *Cdh1* knockout (*Emx-Cre;Cdh1^{loxP/loxP}*), FMRP knockout (*FMRI^{-/-};Cdh1^{loxP/loxP}*) and control (*Cdh1^{loxP/loxP}*) mice, respectively. DHPG-induced mGluR-LTD in *Cdh1* and FMRP double knockout mice was significantly increased compared to conditional *Cdh1* knockout mice ($P < 0.001$), but indistinguishable from mGluR-LTD in FMRP knockout slices. (*Cdh1* and FMRP dKO: 68.5 ± 2.9 % of baseline, $n = 17$ slices from 8 animals; *Cdh1* cKO: 90.9 ± 1.6 % of baseline, $n = 13$ slices from 7 animals; FMRP KO: 68.0 ± 4.2 % of baseline, $n = 12$ slices from 7 animals; Control: 76.0 ± 3.4 % of baseline, $n = 12$ slices from 6 animals). (B) mGluR-LTD in (A) was quantified as percent reduction in fEPSPs 50-60 minutes post-DHPG treatment compared to baseline fEPSPs. mGluR-LTD in *Cdh1* and FMRP double knockout mice was exaggerated and indistinguishable from FMRP knockout mice (*Cdh1* and FMRP dKO: 31.5 ± 2.9 % of LTD, $n = 17$ slices from 8 animals; *Cdh1* cKO: 9.1 ± 1.6 % of LTD, $n = 13$ slices from 7 animals; FMRP KO: 32.0 ± 4.2 % of LTD, $n = 12$ slices from 7 animals; Control: 24.0 ± 3.4 % of LTD, $n = 12$ slices from 6 animals).

DETAILED DESCRIPTION

General

Provided herein are methods and compositions for treating and/or preventing a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced expression or activity of FMRP through the inhibition *Cdh1*-APC. For example, in some embodiments, provided herein are methods of treating and/or preventing fragile X syndrome, fragile X permutation, mental retardation, premature ovarian failure, autism, Parkinson's disease and/or cognitive impairment in a subject by administering to the subject

an agent that inhibits Cdh1-APC. In some embodiments, provided herein are methods of identifying agents that inhibit Cdh1-APC for the treatment or prevention fragile X syndrome, fragile X permutation, mental retardation, premature ovarian failure, autism, Parkinson's disease and/or cognitive impairment.

5 Among E3 ubiquitin ligases, the anaphase-promoting complex (APC) has emerged as a critical and pleiotropic regulator of neuronal morphogenesis and synaptic connectivity in the nervous system. Cdh1 and the Cdh1-related protein Cdc20 represent the key regulatory and coactivating subunits of the APC. Cdh1-APC and Cdc20-APC control the morphogenesis of axons and dendrites, respectively, in the rodent cerebellar cortex.

10 Whereas Cdh1-APC acts in the nucleus to limit axon growth, Cdc20-APC acts at the centrosome to drive the elaboration of dendrite arbors. These findings indicate that spatial control of the APC plays a critical role in determining its pleiotropic functions. Cdh1-APC also controls synaptic plasticity including EphA4-dependent homeostatic plasticity in forebrain neurons and LTP in the amygdala. However, the role of Cdh1-APC in LTD has
15 previously been unexplored.

 Disclosed herein is a signaling link between the major ubiquitin ligase Cdh1-APC and the fragile X syndrome protein FMRP that governs mGluR-dependent LTD in the mammalian brain. As described herein, FMRP is a novel substrate of Cdh1-APC in the regulation of mGluRLTD. Cdh1 forms a complex with FMRP, and mutation of a conserved
20 Cdh1 recognition motif, the D-box, within FMRP disrupts the interaction of FMRP with Cdh1. Knockout of Cdh1 impairs mGluR activation-induced ubiquitination and degradation of FMRP in the hippocampus. Knockout of FMRP completely suppresses the Cdh1 conditional knockout-induced phenotype of impaired mGluR-LTD. Inhibition of Cdh1-APC therefore reduces FMRP degradation and increases the level and activity of FMRP.

25 In several cognitive disorders, including most cases of fragile X syndrome, FMRP expression and/or activity is reduced but not absent. Inhibitors of Cdh1-APC are therefore useful for the treatment of such disorders.

 Thus, in certain aspects, provided herein are methods for treating or preventing a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced
30 expression or activity of FMRP (*e.g.*, fragile X syndrome, fragile X permutation, mental retardation, premature ovarian failure, autism, Parkinson's disease, cognitive impairment) by administering to a subject an agent that inhibits Cdh1-APC. In some embodiments, the agent inhibits the expression or activity of Cdh1. In some embodiments, the agent inhibits

the formation of a complex between Cdh1 and APC. In some embodiments, the agent inhibits the activity of Cdh1-APC (*e.g.*, the ubiquitin ligase activity of Cdh1-APC).

Definitions

For convenience, certain terms employed in the specification, examples, and
5 appended claims are collected here.

The articles “*a*” and “*an*” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

As used herein, the term “*administering*” means providing a pharmaceutical agent or
10 composition to a subject, and includes, but is not limited to, administering by a medical professional and self-administering.

The term “*agent*” is used herein to denote a chemical compound, a small molecule, a mixture of chemical compounds and/or a biological macromolecule (such as a nucleic acid, an antibody, an antibody fragment, a protein or a peptide). Agents may be identified
15 as having a particular activity by screening assays described herein below. The activity of such agents may render them suitable as a “therapeutic agent” which is a biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

The term “*binding*” or “*interacting*” refers to an association, which may be a stable
20 association, between two molecules, *e.g.*, between a polypeptide and a binding partner or agent, *e.g.*, small molecule, due to, for example, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions.

As used herein, the terms “*interfering nucleic acid*,” “*inhibiting nucleic acid*” are used interchangeably. Interfering nucleic acids generally include a sequence of cyclic
25 subunits, each bearing a base-pairing moiety, linked by intersubunit linkages that allow the base-pairing moieties to hybridize to a target sequence in a nucleic acid (typically an RNA) by Watson-Crick base pairing, to form a nucleic acid:oligomer heteroduplex within the target sequence. Interfering RNA molecules include, but are not limited to, antisense molecules, siRNA molecules, single-stranded siRNA molecules, miRNA molecules and
30 shRNA molecules. Such an interfering nucleic acids can be designed to block or inhibit translation of mRNA or to inhibit natural pre-mRNA splice processing, or induce degradation of targeted mRNAs, and may be said to be “directed to” or “targeted against” a target sequence with which it hybridizes. Interfering nucleic acids may include, for

example, peptide nucleic acids (PNAs), locked nucleic acids (LNAs), 2'-O-Methyl oligonucleotides and RNA interference agents (siRNA agents). RNAi molecules generally act by forming a herteroduplex with the target molecule, which is selectively degraded or “knocked down,” hence inactivating the target RNA. Under some conditions, an interfering
5 RNA molecule can also inactivate a target transcript by repressing transcript translation and/or inhibiting transcription of the transcript. An interfering nucleic acid is more generally said to be “targeted against” a biologically relevant target, such as a protein, when it is targeted against the nucleic acid of the target in the manner described above.

The terms “*polynucleotide*”, and “*nucleic acid*” are used interchangeably. They refer
10 to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA,
15 ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the
20 polymer. A polynucleotide may be further modified, such as by conjugation with a labeling component. In all nucleic acid sequences provided herein, U nucleotides are interchangeable with T nucleotides.

The phrase “*pharmaceutically-acceptable carrier*” as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid
25 filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body.

“*Small molecule*” as used herein, is meant to refer to a composition, which has a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small
30 molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon-containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays described herein.

An oligonucleotide “*specifically hybridizes*” to a target polynucleotide if the oligomer hybridizes to the target under physiological conditions, with a T_m substantially greater than 45° C., or at least 50° C., or at least 60° C.-80° C. or higher. Such hybridization corresponds to stringent hybridization conditions. At a given ionic strength and pH, the T_m is the temperature at which 50% of a target sequence hybridizes to a complementary polynucleotide. Again, such hybridization may occur with “near” or “substantial” complementarity of the antisense oligomer to the target sequence, as well as with exact complementarity.

As used herein, the term “*subject*” means a human or non-human animal selected for treatment or therapy.

The phrases “*therapeutically-effective amount*” and “*effective amount*” as used herein means the amount of an agent which is effective for producing the desired therapeutic effect in at least a sub-population of cells in a subject at a reasonable benefit/risk ratio applicable to any medical treatment.

“*Treating*” a disease in a subject or “*treating*” a subject having a disease refers to subjecting the subject to a pharmaceutical treatment, *e.g.*, the administration of a drug, such that at least one symptom of the disease is decreased or prevented from worsening.

Cdh1

In certain embodiments, provided herein are methods of treating and/or preventing a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced expression or activity of FMRP through the inhibition of Cdh1-APC. Inhibition of Cdh1-APC can, for example, be via inhibition of Cdh1 protein activity or Cdh1 protein amount. For example, agents that inhibit Cdh1-APC include agents that reduce Cdh1 protein activity, agents that increase Cdh1 protein degradation, agents that inhibit transcription and/or translation of nucleic acids encoding Cdh1 protein and agents that increase degradation of nucleic acids encoding the Cdh1 protein. Cdh1-APC inhibition can also be accomplished by, for example, using agents that inhibit the formation of the Cdh1-APC complex, agents that inhibit Cdh1-APC activity, and agents that increase Cdh1-APC degradation.

Cdh1 is a regulatory and coactivating subunit of the anaphase-promoting complex (APC). Cdh1 is also known as fizzy-related protein homolog or FZR1. As disclosed herein, Cdh1-APC is the E3 ubiquitin ligase that targets the fragile X syndrome protein FMRP for proteasome-dependent degradation. Cdh1 is expressed as three isoforms, the

amino acid sequence of each of which is available at NCBI accession numbers NP_001129669.1, NP_001129670.1 and NP_057347.2, each of which is incorporated by reference herein. The nucleic acid sequence of the human Cdh1 isoform mRNA is available at NCBI accession numbers NM_001136197.1, NM_001136198.1, and NM_016263.3,
5 each of which is incorporated by reference herein.

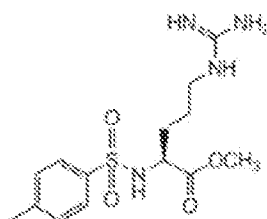
APC is a large protein complex of about 1.5 MDa in size that includes at least 14 core subunits: APC1, APC2, APC3, APC4, APC5, APC6, APC7, APC8, APC10, APC11, APC13, APC15, APC16 and CDC26 ("APC peptides"). APC is activated by association with either Cdc20 or Cdh1.

10 Small Molecule Agents

In certain embodiments, provided herein are methods of treating and/or preventing a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced expression or activity of FMRP through the inhibition of Cdh1-APC. These methods include administering an agent that inhibits Cdh1-APC. Such agents include those disclosed
15 below, those known in the art and those identified using the screening assays described herein.

In some embodiments, any agent that inhibits Cdh1-APC can be used to practice the methods disclosed herein. In some embodiments, the agent is a small molecule. For example, in some embodiments the agent is a cardiac glycoside. In some embodiments, is
20 tosyl-L-arginine methyl ester (TAME) or a TAME prodrug. In some embodiments the agent is a TAME prodrug comprising a TAME derivative in which a guanidine group is protected by a carbamate group. In some embodiments, the agent is a TAME prodrug comprising an esterase-activatable N,N'-bis(acyloxymethyl carbamate) derivative of TAME. In some embodiments, the agent is proTAME.

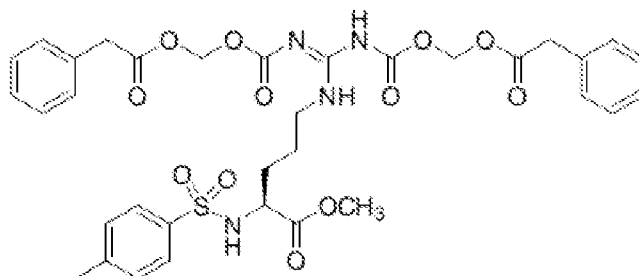
25 In some embodiments, the agent is TAME or an active derivative thereof. TAME has the following chemical structure:



Exemplary active TAME derivatives include tosyl-L-arginine amide (TAA), tosyl-
30 L-lysine methylester (TLME), tosyl-L-arginine (TAOH), Benzoyl-L-arginine amide

(BAA), tosyl-L-arginine-t-butyl-ester (TATE) and benzoyl-L-argininemethylester (BAME). Additional information on TAME and active TAME derivatives is provided in U.S. Pat. App. Pub. No. US/2012/0115948, the contents of which are hereby incorporated by reference.

- 5 In some embodiments, the agent is a prodrug of TAME or a TAME derivative. For example, in some embodiments the agent is an esterase-activatable N,N'-bis(acyloxymethyl carbamate) derivative of TAME. In some embodiments, the agent is proTAME. proTAME has the following chemical structure:



- 10 Additional information on prodrugs of TAME and active TAME derivatives is provided in U.S. Pat. App. Pub. No. US/2013/0230468, the contents of which are hereby incorporated by reference.

- In some embodiments, assays used to identify agents useful in the methods described herein include a reaction between an Cdh1 protein or a fragment thereof and/or an APC protein (*e.g.*, one or more proteins or polypeptides of APC the APC complex that interact with Cdh1 in Cdh1-APC) or a fragment thereof and a test compound. Agents identified *via* such assays, may be useful, for example, for treating or preventing neurodegenerative diseases.

- Agents useful in the methods disclosed herein may be obtained from any available source, including systematic libraries of natural and/or synthetic compounds. Agents may also be obtained by any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, *e.g.*, Zuckermann *et al.*, 1994, *J. Med. Chem.* 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited

to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of agents may be presented in solution (*e.g.*, Houghten, 1992, *Biotechniques* 13:412-421), or on beads (Lam, 1991, *Nature* 354:82-84), chips (Fodor, 1993, *Nature* 364:555-556), bacteria and/or spores, (Ladner, USP 5,223,409), plasmids (Cull *et al.*, 1992, *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith, 1990, *Science* 249:386-390; Devlin, 1990, *Science* 249:404-406; Cwirla *et al.*, 1990, *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici, 1991, *J. Mol. Biol.* 222:301-310; Ladner, *supra.*).

Agents useful in the methods disclosed herein may be identified, for example, using assays for screening candidate or test compounds which inhibit complex formation between Cdh1 and APC.

The basic principle of the assay systems used to identify compounds that inhibit complex formation between Cdh1 and APC involves preparing a reaction mixture containing a Cdh1 protein or fragment thereof and an APC protein or fragment thereof under conditions and for a time sufficient to allow the Cdh1 protein or fragment thereof to form a complex with the APC protein or fragment thereof. In order to test an agent for modulatory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition of the APC protein or fragment thereof and the Cdh1 protein or fragment thereof. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the APC protein or fragment thereof and the Cdh1 protein or fragment thereof is then detected. The formation of a complex in the control reaction, but less or no such formation in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the APC protein or fragment thereof and the Cdh1 protein or fragment thereof.

The assay for compounds that modulate the interaction of the APC protein or fragment thereof and the Cdh1 protein or fragment thereof may be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the APC protein or fragment thereof or the Cdh1 protein or fragment thereof onto a solid phase and detecting complexes anchored to the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the APC protein or fragment thereof and the Cdh1 protein or fragment thereof (*e.g.*, by competition) can be identified by conducting the reaction in the presence of the test substance, *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with the APC protein or fragment thereof and the Cdh1 protein or fragment thereof. Alternatively, test compounds that disrupt preformed complexes, *e.g.*, compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are briefly described below.

In a heterogeneous assay system, either the APC protein or fragment thereof or the Cdh1 protein or fragment thereof is anchored onto a solid surface or matrix, while the other corresponding non-anchored component may be labeled, either directly or indirectly. In practice, microtitre plates are often utilized for this approach. The anchored species can be immobilized by a number of methods, either non-covalent or covalent, that are typically well known to one who practices the art. Non-covalent attachment can often be accomplished simply by coating the solid surface with a solution of the APC protein or fragment thereof or the Cdh1 protein or fragment thereof and drying. Alternatively, an immobilized antibody specific for the assay component to be anchored can be used for this purpose.

In related assays, a fusion protein can be provided which adds a domain that allows one or both of the assay components to be anchored to a matrix. For example, glutathione-S-transferase/marker fusion proteins or glutathione-S-transferase/binding partner can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and either the non-adsorbed the APC protein or fragment thereof or the Cdh1 protein or fragment thereof, and the mixture incubated under conditions conducive to

complex formation (*e.g.*, physiological conditions). Following incubation, the beads or microtiter plate wells are washed to remove any unbound assay components, the immobilized complex assessed either directly or indirectly, for example, as described above.

5 A homogeneous assay may also be used to identify inhibitors of complex formation. This is typically a reaction, analogous to those mentioned above, which is conducted in a liquid phase in the presence or absence of the test compound. The formed complexes are then separated from unreacted components, and the amount of complex formed is determined. As mentioned for heterogeneous assay systems, the order of addition
10 of reactants to the liquid phase can yield information about which test compounds modulate (inhibit or enhance) complex formation and which disrupt preformed complexes.

 In such a homogeneous assay, the reaction products may be separated from unreacted assay components by any of a number of standard techniques, including but not limited to: differential centrifugation, chromatography, electrophoresis and
15 immunoprecipitation. In differential centrifugation, complexes of molecules may be separated from uncomplexed molecules through a series of centrifugal steps, due to the different sedimentation equilibria of complexes based on their different sizes and densities (see, for example, Rivas, G., and Minton, A.P., *Trends Biochem Sci* 1993 Aug;18(8):284-7). Standard chromatographic techniques may also be utilized to separate complexed
20 molecules from uncomplexed ones. For example, gel filtration chromatography separates molecules based on size, and through the utilization of an appropriate gel filtration resin in a column format, for example, the relatively larger complex may be separated from the relatively smaller uncomplexed components. Similarly, the relatively different charge properties of the complex as compared to the uncomplexed molecules may be exploited to
25 differentially separate the complex from the remaining individual reactants, for example through the use of ion-exchange chromatography resins. Such resins and chromatographic techniques are well known to one skilled in the art (see, *e.g.*, Heegaard, 1998, *J Mol. Recognit.* 11:141-148; Hage and Tweed, 1997, *J. Chromatogr. B. Biomed. Sci. Appl.*, 699:499-525). Gel electrophoresis may also be employed to separate complexed molecules
30 from unbound species (see, *e.g.*, Ausubel *et al* (eds.), In: *Current Protocols in Molecular Biology*, J. Wiley & Sons, New York. 1999). In this technique, protein or nucleic acid complexes are separated based on size or charge, for example. In order to maintain the binding interaction during the electrophoretic process, nondenaturing gels in the absence of

reducing agent are typically preferred, but conditions appropriate to the particular interactants will be well known to one skilled in the art. Immunoprecipitation is another common technique utilized for the isolation of a protein-protein complex from solution (see, e.g., Ausubel *et al* (eds.), In: *Current Protocols in Molecular Biology*, J. Wiley & Sons, New York. 1999). In this technique, all proteins binding to an antibody specific to one of the binding molecules are precipitated from solution by conjugating the antibody to a polymer bead that may be readily collected by centrifugation. The bound assay components are released from the beads (through a specific proteolysis event or other technique well known in the art which will not disturb the protein-protein interaction in the complex), and a second immunoprecipitation step is performed, this time utilizing antibodies specific for the correspondingly different interacting assay component. In this manner, only formed complexes should remain attached to the beads. Variations in complex formation in both the presence and the absence of a test compound can be compared, thus offering information about the ability of the compound to modulate interactions between the APC protein or fragment thereof and the Cdh1 protein or fragment thereof.

Interfering Nucleic Acid Agents

In certain embodiments, interfering (*i.e.*, inhibiting) nucleic acid molecules that selectively target Cdh1 are provided herein and/or used in methods described herein.

Interfering nucleic acids generally include a sequence of cyclic subunits, each bearing a base-pairing moiety, linked by intersubunit linkages that allow the base-pairing moieties to hybridize to a target sequence in a nucleic acid (typically an RNA) by Watson-Crick base pairing, to form a nucleic acid:oligomer heteroduplex within the target sequence.

Interfering RNA molecules include, but are not limited to, antisense molecules, siRNA molecules, single-stranded siRNA molecules, miRNA molecules and shRNA molecules.

Typically at least 17, 18, 19, 20, 21, 22 or 23 nucleotides of the complement of the target mRNA sequence are sufficient to mediate inhibition of a target transcript. Perfect complementarity is not necessary. In some embodiments, the interfering nucleic acid molecule is double-stranded RNA. The double-stranded RNA molecule may have a 2 nucleotide 3' overhang. In some embodiments, the two RNA strands are connected via a hairpin structure, forming a shRNA molecule. shRNA molecules can contain hairpins derived from microRNA molecules. For example, an RNAi vector can be constructed by cloning the interfering RNA sequence into a pCAG-miR30 construct containing the hairpin from the miR30 miRNA. RNA interference molecules may include DNA residues, as well

as RNA residues. In some embodiments, the interfering nucleic acid is a single-stranded antisense nucleic acid (*e.g.*, RNA).

Interfering nucleic acid molecules provided herein can contain RNA bases, non-RNA bases or a mixture of RNA bases and non-RNA bases. For example, interfering
5 nucleic acid molecules provided herein can be primarily composed of RNA bases but also contain DNA bases or non-naturally occurring nucleotides.

The interfering nucleic acids can employ a variety of oligonucleotide chemistries. Examples of oligonucleotide chemistries include, without limitation, peptide nucleic acid (PNA), linked nucleic acid (LNA), phosphorothioate, 2'O-Me-modified oligonucleotides,
10 and morpholino chemistries, including combinations of any of the foregoing. In general, PNA and LNA chemistries can utilize shorter targeting sequences because of their relatively high target binding strength relative to 2'O-Me oligonucleotides. Phosphorothioate and 2'O-Me-modified chemistries are often combined to generate 2'O-Me-modified oligonucleotides having a phosphorothioate backbone. See, *e.g.*, PCT
15 Publication Nos. WO/2013/112053 and WO/2009/008725, incorporated by reference in their entireties.

Peptide nucleic acids (PNAs) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone, consisting of N-(2-aminoethyl) glycine units to which pyrimidine or purine bases are attached. PNAs containing natural
20 pyrimidine and purine bases hybridize to complementary oligonucleotides obeying Watson-Crick base-pairing rules, and mimic DNA in terms of base pair recognition (Egholm, Buchardt et al. 1993). The backbone of PNAs is formed by peptide bonds rather than phosphodiester bonds, making them well-suited for antisense applications (see structure below). The backbone is uncharged, resulting in PNA/DNA or PNA/RNA duplexes that
25 exhibit greater than normal thermal stability. PNAs are not recognized by nucleases or proteases.

Despite a radical structural change to the natural structure, PNAs are capable of sequence-specific binding in a helix form to DNA or RNA. Characteristics of PNAs include a high binding affinity to complementary DNA or RNA, a destabilizing effect caused by
30 single-base mismatch, resistance to nucleases and proteases, hybridization with DNA or RNA independent of salt concentration and triplex formation with homopurine DNA. PANAGENE.TM. has developed its proprietary Bts PNA monomers (Bts; benzothiazole-2-sulfonyl group) and proprietary oligomerization process. The PNA oligomerization using

Bts PNA monomers is composed of repetitive cycles of deprotection, coupling and capping. PNAs can be produced synthetically using any technique known in the art. See, *e.g.*, U.S. Pat. Nos. 6,969,766, 7,211,668, 7,022,851, 7,125,994, 7,145,006 and 7,179,896. See also U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262 for the preparation of PNAs. Further
5 teaching of PNA compounds can be found in Nielsen et al., Science, 254:1497-1500, 1991. Each of the foregoing is incorporated by reference in its entirety.

Interfering nucleic acids may also contain "locked nucleic acid" subunits (LNAs). "LNAs" are a member of a class of modifications called bridged nucleic acid (BNA). BNA is characterized by a covalent linkage that locks the conformation of the ribose ring in a
10 C3'-endo (northern) sugar pucker. For LNA, the bridge is composed of a methylene between the 2'-O and the 4'-C positions. LNA enhances backbone preorganization and base stacking to increase hybridization and thermal stability.

The structures of LNAs can be found, for example, in Wengel, et al., Chemical Communications (1998) 455; Tetrahedron (1998) 54:3607, and Accounts of Chem.
15 Research (1999) 32:301; Obika, et al., Tetrahedron Letters (1997) 38:8735; (1998) 39:5401, and Bioorganic Medicinal Chemistry (2008) 16:9230. Compounds provided hereon may incorporate one or more LNAs; in some cases, the compounds may be entirely composed of LNAs. Methods for the synthesis of individual LNA nucleoside subunits and their incorporation into oligonucleotides are described, for example, in U.S. Pat. Nos.
20 7,572,582, 7,569,575, 7,084,125, 7,060,809, 7,053,207, 7,034,133, 6,794,499, and 6,670,461, each of which is incorporated by reference in its entirety. Typical intersubunit linkers include phosphodiester and phosphorothioate moieties; alternatively, non-phosphorous containing linkers may be employed. One embodiment is an LNA containing compound where each LNA subunit is separated by a DNA subunit. Certain compounds are
25 composed of alternating LNA and DNA subunits where the intersubunit linker is phosphorothioate.

"Phosphorothioates" (or S-oligos) are a variant of normal DNA in which one of the nonbridging oxygens is replaced by a sulfur. The sulfurization of the internucleotide bond reduces the action of endo-and exonucleases including 5' to 3' and 3' to 5' DNA POL 1
30 exonuclease, nucleases S1 and P1, RNases, serum nucleases and snake venom phosphodiesterase. Phosphorothioates are made by two principal routes: by the action of a solution of elemental sulfur in carbon disulfide on a hydrogen phosphonate, or by the method of sulfurizing phosphite triesters with either tetraethylthiuram disulfide (TETD) or

3H-1, 2-benzodithiol-3-one 1, 1-dioxide (BDTD) (see, *e.g.*, Iyer et al., J. Org. Chem. 55, 4693-4699, 1990). The latter methods avoid the problem of elemental sulfur's insolubility in most organic solvents and the toxicity of carbon disulfide. The TETD and BDTD methods also yield higher purity phosphorothioates.

5 “2'-O-Me oligonucleotides” molecules carry a methyl group at the 2'-OH residue of the ribose molecule. 2'-O-Me-RNAs show the same (or similar) behavior as DNA, but are protected against nuclease degradation. 2'-O-Me-RNAs can also be combined with phosphothioate oligonucleotides (PTOs) for further stabilization. 2'-O-Me oligonucleotides (phosphodiester or phosphothioate) can be synthesized according to routine techniques in
10 the art (see, *e.g.*, Yoo et al., Nucleic Acids Res. 32:2008-16, 2004).

 The interfering nucleic acids described herein may be contacted with a cell or administered to an organism (*e.g.*, a human). Alternatively, constructs and/or vectors encoding the interfering RNA molecules may be contacted with or introduced into a cell or organism. In certain embodiments, a viral, retroviral or lentiviral vector is used. In some
15 embodiments, the vector has a tropism for cardiac tissue. In some embodiments the vector is an adeno-associated virus.

 Typically at least 17, 18, 19, 20, 21, 22 or 23 nucleotides of the complement of the target mRNA sequence are sufficient to mediate inhibition of a target transcript. Perfect complementarity is not necessary. In some embodiments, the interfering nucleic acids
20 contains a 1, 2 or 3 nucleotide mismatch with the target sequence. The interfering nucleic acid molecule may have a 2 nucleotide 3' overhang. If the interfering nucleic acid molecule is expressed in a cell from a construct, for example from a hairpin molecule or from an inverted repeat of the desired sequence, then the endogenous cellular machinery will create the overhangs. shRNA molecules can contain hairpins derived from microRNA molecules.
25 For example, an RNAi vector can be constructed by cloning the interfering RNA sequence into a pCAG-miR30 construct containing the hairpin from the miR30 miRNA. RNA interference molecules may include DNA residues, as well as RNA residues.

 In some embodiments, the interfering nucleic acid molecule is a siRNA molecule. Such siRNA molecules should include a region of sufficient homology to the target region,
30 and be of sufficient length in terms of nucleotides, such that the siRNA molecule down-regulate target RNA. The term “ribonucleotide” or “nucleotide” can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions. It is not necessary that there be perfect

complementarity between the siRNA molecule and the target, but the correspondence must be sufficient to enable the siRNA molecule to direct sequence-specific silencing, such as by RNAi cleavage of the target RNA. In some embodiments, the sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double-strand character of the molecule.

In addition, an siRNA molecule may be modified or include nucleoside surrogates. Single stranded regions of an siRNA molecule may be modified or include nucleoside surrogates, *e.g.*, the unpaired region or regions of a hairpin structure, *e.g.*, a region which links two complementary regions, can have modifications or nucleoside surrogates.

Modification to stabilize one or more 3'- or 5'-terminus of an siRNA molecule, *e.g.*, against exonucleases, or to favor the antisense siRNA agent to enter into RISC are also useful. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis.

Each strand of an siRNA molecule can be equal to or less than 35, 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. In some embodiments, the strand is at least 19 nucleotides in length. For example, each strand can be between 21 and 25 nucleotides in length. In some embodiments, siRNA agents have a duplex region of 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotide pairs, and one or more overhangs, such as one or two 3' overhangs, of 2-3 nucleotides.

A "small hairpin RNA" or "short hairpin RNA" or "shRNA" includes a short RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNAs provided herein may be chemically synthesized or transcribed from a transcriptional cassette in a DNA plasmid. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC).

In some embodiments, shRNAs are about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, or are about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded shRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or about 20-24, 21-22, or 21-23 nucleotides in length, and the double-

stranded shRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, or about 18-22, 19-20, or 19-21 base pairs in length). shRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides on the antisense strand and/or 5'-phosphate termini on the sense strand. In some embodiments, the shRNA comprises a sense strand and/or antisense strand sequence of from about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, or 15-25 nucleotides in length), or from about 19 to about 40 nucleotides in length (*e.g.*, about 19-40, 19-35, 19-30, or 19-25 nucleotides in length), or from about 19 to about 23 nucleotides in length (*e.g.*, 19, 20, 21, 22, or 23 nucleotides in length).

Non-limiting examples of shRNA include a double-stranded polynucleotide molecule assembled from a single-stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; and a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions. In some embodiments, the sense and antisense strands of the shRNA are linked by a loop structure comprising from about 1 to about 25 nucleotides, from about 2 to about 20 nucleotides, from about 4 to about 15 nucleotides, from about 5 to about 12 nucleotides, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more nucleotides.

Additional embodiments related to the shRNAs, as well as methods of designing and synthesizing such shRNAs, are described in U.S. patent application publication number 2011/0071208, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, provided herein are micro RNAs (miRNAs). miRNAs represent a large group of small RNAs produced naturally in organisms, some of which regulate the expression of target genes. miRNAs are formed from an approximately 70 nucleotide single-stranded hairpin precursor transcript by Dicer. miRNAs are not translated into proteins, but instead bind to specific messenger RNAs, thereby blocking translation. In some instances, miRNAs base-pair imprecisely with their targets to inhibit translation.

In some embodiments, antisense oligonucleotide compounds are provided herein. In certain embodiments, the degree of complementarity between the target sequence and antisense targeting sequence is sufficient to form a stable duplex. The region of complementarity of the antisense oligonucleotides with the target RNA sequence may be as short as 8-11 bases, but can be 12-15 bases or more, *e.g.*, 10-40 bases, 12-30 bases, 12-25

bases, 15-25 bases, 12-20 bases, or 15-20 bases, including all integers in between these ranges. An antisense oligonucleotide of about 14-15 bases is generally long enough to have a unique complementary sequence.

In certain embodiments, antisense oligonucleotides may be 100% complementary to the target sequence, or may include mismatches, *e.g.*, to improve selective targeting of allele containing the disease-associated mutation, as long as a heteroduplex formed between the oligonucleotide and target sequence is sufficiently stable to withstand the action of cellular nucleases and other modes of degradation which may occur *in vivo*. Hence, certain oligonucleotides may have about or at least about 70% sequence complementarity, *e.g.*, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence complementarity, between the oligonucleotide and the target sequence. Oligonucleotide backbones that are less susceptible to cleavage by nucleases are discussed herein. Mismatches, if present, are typically less destabilizing toward the end regions of the hybrid duplex than in the middle. The number of mismatches allowed will depend on the length of the oligonucleotide, the percentage of G:C base pairs in the duplex, and the position of the mismatch(es) in the duplex, according to well understood principles of duplex stability.

Interfering nucleic acid molecules can be prepared, for example, by chemical synthesis, *in vitro* transcription, or digestion of long dsRNA by Rnase III or Dicer. These can be introduced into cells by transfection, electroporation, or other methods known in the art. See Hannon, GJ, 2002, RNA Interference, *Nature* 418: 244-251; Bernstein E et al., 2002, The rest is silence. *RNA* 7: 1509-1521; Hutvagner G et al., RNAi: Nature abhors a double-strand. *Curr. Opin. Genetics & Development* 12: 225-232; Brummelkamp, 2002, A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296: 550-553; Lee NS, Dohjima T, Bauer G, Li H, Li M-J, Ehsani A, Salvaterra P, and Rossi J. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nature Biotechnol.* 20:500-505; Miyagishi M, and Taira K. (2002). U6-promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnol.* 20:497-500; Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, and Conklin DS. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Dev.* 16:948-958; Paul CP, Good PD, Winer I, and Engelke DR. (2002). Effective expression of small interfering RNA in

human cells. *Nature Biotechnol.* 20:505-508; Sui G, Soohoo C, Affar E-B, Gay F, Shi Y, Forrester WC, and Shi Y. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* 99(6):5515-5520; Yu J-Y, DeRuiter SL, and Turner DL. (2002). RNA interference by expression of short-interfering
5 RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* 99(9):6047-6052.

In the present methods, an interfering nucleic acid molecule or an interfering nucleic acid encoding polynucleotide can be administered to the subject, for example, as naked nucleic acid, in combination with a delivery reagent, and/or as a nucleic acid comprising sequences that express an interfering nucleic acid molecule. In some embodiments the
10 nucleic acid comprising sequences that express the interfering nucleic acid molecules are delivered within vectors, *e.g.* plasmid, viral and bacterial vectors. Any nucleic acid delivery method known in the art can be used in the methods described herein. Suitable delivery reagents include, but are not limited to, *e.g.*, the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (*e.g.*, polylysine), atelocollagen,
15 nanoplexes and liposomes. The use of atelocollagen as a delivery vehicle for nucleic acid molecules is described in Minakuchi *et al.* *Nucleic Acids Res.*, 32(13):e109 (2004); Hanai *et al.* *Ann NY Acad Sci.*, 1082:9-17 (2006); and Kawata *et al.* *Mol Cancer Ther.*, 7(9):2904-12 (2008); each of which is incorporated herein in their entirety. Exemplary interfering nucleic acid delivery systems are provided in U.S. Patent Nos. 8,283,461,
20 8,313,772, 8,501,930, 8,426,554, 8,268,798 and 8,324,366, each of which is hereby incorporated by reference in its entirety.

In some embodiments of the methods described herein, liposomes are used to deliver an inhibitory oligonucleotide to a subject. Liposomes suitable for use in the methods described herein can be formed from standard vesicle-forming lipids, which generally
25 include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example, as described in Szoka *et al.* (1980), *Ann. Rev. Biophys. Bioeng.* 9:467; and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and
30 5,019,369, the entire disclosures of which are herein incorporated by reference.

The liposomes for use in the present methods can also be modified so as to avoid clearance by the mononuclear macrophage system ("MMS") and reticuloendothelial system

("RES"). Such modified liposomes have opsonization-inhibition moieties on the surface or incorporated into the liposome structure.

Opsonization-inhibiting moieties for use in preparing the liposomes described herein are typically large hydrophilic polymers that are bound to the liposome membrane.

5 As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, *e.g.*, by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer that significantly decreases the uptake of the liposomes by the MMS and RES;
10 *e.g.*, as described in U.S. Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference.

In some embodiments, opsonization inhibiting moieties suitable for modifying liposomes are water-soluble polymers with a number-average molecular weight from about 500 to about 40,000 daltons, or from about 2,000 to about 20,000 daltons. Such polymers
15 include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; *e.g.*, methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, *e.g.*, polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as
20 ganglioside GM1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, *e.g.*, galacturonic acid,
25 glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, *e.g.*, reacted with derivatives of carbonic acids with resultant linking of carboxylic groups. In some embodiments, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or
30 PEG-derivatives are sometimes called "PEGylated liposomes."

Pharmaceutical Compositions

In certain embodiments, provided herein is a composition, *e.g.*, a pharmaceutical composition, containing at least one agent described herein together with a

pharmaceutically acceptable carrier. In one embodiment, the composition includes a combination of multiple (*e.g.*, two or more) agents described herein.

As described in detail below, the pharmaceutical compositions disclosed herein may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; or (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation.

Methods of preparing these formulations or compositions include the step of bringing into association an agent described herein with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association an agent described herein with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Pharmaceutical compositions suitable for parenteral administration comprise one or more agents described herein in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

Regardless of the route of administration selected, the agents provided herein, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions disclosed herein, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Therapeutic Methods

Provided herein are methods for treating or preventing a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced expression or activity of FMRP. In some embodiments, the disease or disorder is fragile X syndrome, fragile X permutation, mental retardation, premature ovarian failure, autism, Parkinson's disease, or cognitive impairment. In some embodiments, the disease or disorder is fragile X syndrome.

The methods described herein can be used to treat any subject in need thereof. As used herein, a "*subject in need thereof*" includes any subject that has a disease or disorder associated with impairment of mGluR-dependent LTD and/or reduced expression or activity of FMRP, and well as any subject with an increased likelihood of acquiring a such a disease or disorder.

FMRP is encoded by the gene FMR1, which is located on the X chromosome. Thus, males have a single FMR1 copy, while females have two copies. In certain embodiments, the subject in need thereof carries a gene mutation in one or both copies of their FMR1 gene that reduces FMRP expression or activity. In some embodiments the subject has 45 or more CGG repeats in one or both copies of their FMR1 gene. In some embodiments, the subject has 55 or more CGG repeats in one or both copies of their FMR1 gene. In some embodiments, the subject has 100 or more CGG repeats in one or both copies of their FMR1 gene. In some embodiments, the subject has 200 or more CGG repeats in one or both copies of their FMR1 gene. In some embodiments, the methylation of one or both copies of the subject's FMR1 gene has led to reduced expression of FMRP.

The pharmaceutical compositions disclosed herein may be delivered by any suitable route of administration, including orally and parenterally. In certain embodiments the pharmaceutical compositions are delivered generally (e.g., via oral or parenteral administration). In certain other embodiments the pharmaceutical compositions are delivered locally through direct injection into a specific tissue (e.g., central nervous system tissue and/or peripheral nervous system tissue). In some embodiments, the agent is injected into the subjects cerebrospinal fluid through intrathecal injection.

The dosage of the subject agent may be determined by reference to the plasma concentrations of the agent. For example, the maximum plasma concentration (C_{max}) and the area under the plasma concentration-time curve from time 0 to infinity (AUC (0-4))

may be used. Dosages include those that produce the above values for C_{max} and AUC (0-4) and other dosages resulting in larger or smaller values for those parameters.

Actual dosage levels of the active ingredients in the pharmaceutical compositions may be varied so as to obtain an amount of the active ingredient which is effective to
5 achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular agent employed, the route of administration, the time of administration, the rate of excretion or metabolism of the particular compound being
10 employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine
15 and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could prescribe and/or administer doses of the agents employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

20 In general, a suitable daily dose of an agent described herein will be that amount of the agent which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

EXEMPLIFICATION

Experimental Procedures

25 Antibodies

The antibodies used include mouse anti-Cdh1 (Thermo Scientific, DH01), rabbit anti-FMRP (Abcam, Ab17722), mouse anti-FMRP (Millipore, 1C3), mouse anti-Flag (Sigma Aldrich, M2), rabbit anti-Flag (Sigma Aldrich, F7425), mouse anti-NeuroD1 (Abcam, Ab60704), mouse anti-ubiquitin (Millipore, FK2), mouse anti-actin (Santa Cruz,
30 C2), mouse anti- α -tubulin (Santa Cruz, B7), rat anti-HA (Roche, 3F10), mouse anti-HA (Covance, 101P), chicken anti-GFP (Abcam, Ab13970) and rabbit anti-DsRed (Clontech, 632496).

Plasmids

The expression plasmids for Flag-Cdh1, GFP-NES-Cdh1, GFP-NLS-Cdh1 are described in Stegmüller *et al.*, *Neuron* 50:389-400 (2006), which is hereby incorporated by reference. GFP-NES-Cdh1 and GFP-NLS-Cdh1 were subcloned into a pCAG vector
 5 (Matsuda and Cepko, *Proc. Natl. Acad. Sci. U.S.A.* 104:1027-2032 (2007)). FMR1 cDNA was amplified from mouse brain cDNA by PCR using the following primers: Forward: 5'-aaagaattcgaggagctggtggtggaagtg-3'; Reverse: 5'-aaactcgagttagggtactccattcaccag-3', and then cloned into pcDNA3-HA vector to generate pcDNA3-HA-FMRP. The D-box mutation of FMRP (pcDNA3-HA-FMRP-Dbm) was generated by site-directed mutagenesis using the
 10 following primers: Forward: 5'-gatgcagtgaaaaaggctgctagctttgctgaattgctgaagat-3'; Reverse: 5'-agcctttttcactgcattctgacatctctccat-3'.

Animals

Emx-Cre;Cdh1^{loxP/loxP} mice were mated with *Cdh1^{loxP/loxP}* mice to generate the conditional Cdh1 knockout mice (*Emx-Cre;Cdh1^{loxP/loxP}*) and control mice (*Cdh1^{loxP/loxP}*).
 15 Female *Fmr1^{-/+};Cdh1^{loxP/loxP}* mice were bred with male *Emx-Cre;Cdh1^{loxP/loxP}* mice to generate FMRP and Cdh1 double knockout mice (*FMR1^{-/-};Emx-Cre;Cdh1^{loxP/loxP}*), FMRP knockout mice (*FMR1^{-/-};Cdh1^{loxP/loxP}*), conditional Cdh1 knockout mice (*Emx-Cre;Cdh1^{loxP/loxP}*), and control mice (*Cdh1^{loxP/loxP}*).

Electrophysiology

20 Acute hippocampal slices were prepared as described in Auerbach *et al.* *Nature* 480:63-68 (2011) and Debanne *et al.*, *Nature Protocols* 3:1559-1568 (2008), with slight modifications. Three-week old age- and gender- matched littermate mice were used. Briefly, 350 µm slices of hippocampi were cut by vibratome (Leica VT1000S) in cold high-Mg²⁺ and low-Ca²⁺ slicing solution containing (in mM) NaCl 124, KCl 2.5, CaCl₂ 0.5,
 25 MgCl₂ 8, NaHCO₃ 26, and D-Glucose 17, and then allowed to recover for 1 hour at 35 °C in ACSF containing (in mM) NaCl 124, KCl 5, NaH₂PO₄ 1.25, NaHCO₃ 26, MgCl₂ 1, CaCl₂ 2, and D-Glucose 10, saturated in 95% O₂/5% CO₂. For recording, slices were transferred to a recording chamber, perfused with O₂-saturated ACSF at a rate of 2 ml/min, and maintained at 30 °C. Whole-cell patch-clamp recordings were performed with
 30 recording electrodes (3-4 MΩ), filled with internal solution containing (in mM) K-Gluconate 125, KCl 15, HEPES 10, ATP-Mg 2, GTP-Na₃ 0.3, Na₂-Phosphocreatine 10, and EGTA 0.2. Neurons were visualized using an Olympus BX61WI microscope with a 40x-water-immersion objective. Electrophysiological signals were acquired by Axon-700B

MultiClamp Amplifier, digitized at 10 kHz by Digidata 1440A D-A Converter, and Bessel filtered at 2 kHz.

In field recordings, electrodes ($<1\text{ M}\Omega$) were filled with ACSF. Tip of the recording electrode was placed in the CA1 stratum radiatum where the dendrites of CA1 neurons reside. Electrical stimulations were delivered from a stimulus isolator (WPI A360) through a concentric bipolar electrode (FHC, CBAEC75) placed on the Schaffer Collaterals.

Electrical stimulations consisted of 0.1 ms pulses with constant current in a range from 0.1-0.3 mA. The stimulation intensity was determined by the induction of around 50-60% of maximum response. Electrical stimulations with the same intensity were applied at 0.05 Hz (3 pulses/1 min) for the recording of baseline (20 min) and testing (60 min). NMDAR-LTP was induced by high frequency stimulation (100 pulses at 100 Hz for 1 s). NMDAR-LTD was induced by single-pulse low-frequency stimulation (900 pulses at 1 Hz for 15 min). mGluR-LTD was induced by bath application of 50 μM s-DHPG (Sigma Aldrich) for 10 min or by paired-pulse low-frequency stimulation (PP-LFS, 900 pairedpulses with 50 ms inter-pulse interval at 1 Hz for 15 min). In PP-LFS induced-LTD experiments, recordings were performed in the presence of NMDAR antagonist DL-AP5 (100 μM , Sigma Aldrich).

In utero electroporation

Female *Emx-Cre;Cdh1^{loxP/loxP}* mice mated with *Cdh1^{loxP/loxP}* male mice were used for hippocampal in utero electroporation. Briefly, pCAG-GFP-NES-Cdh1 or pCAG-GFP-NLSCdh1 plasmids (2 $\mu\text{g}/\mu\text{l}$) together with pCAG-mCherry (1 $\mu\text{g}/\mu\text{l}$) 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 tweezerrode (BTX, 45-0489) and a square wave pulse electroporator (BTX, ECM830).

Immunoprecipitation analyses

Immunoprecipitation analyses were performed as described (Lehtinen *et al. Cell* 125:987-1001 (2006), which is hereby incorporated by reference. Cells were lysed in a lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% nonyl phenoxypolyethoxylethanol 40 (NP40) and protease inhibitors (Sigma Aldrich). Lysates were incubated with the appropriate primary antibody overnight. The antibody-protein complexes were purified with Protein G Sepharose 4 Fast Flow beads (GE Healthcare). The immunoprecipitated protein complexes were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane for

immunoblotting analyses with the appropriate primary antibodies and HRP-conjugated secondary antibodies (Jackson ImmunoResearch).

Subcellular fractionation

Nuclear and cytoplasmic fractions were prepared from P18 mouse hippocampus.

- 5 Tissues were lysed in hypotonic buffer (10 mM Hepes-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, and protease inhibitors) with a 2 ml-Kontes Dounce grinder. 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 (20mM Hepes-KOH pH 7.9, 400 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM
- 10 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.

15 Immunohistochemistry and confocal imaging

- Immunohistochemistry analyses were performed as described (Yamada *et al.*, *Neuroscience* 33:4726-4740 (2013)). The following primary antibodies were used: rabbit anti-FMRP (Abcam Ab17722, 1:400 dilution), chicken anti-GFP (Abcam, 1:500 dilution), and rabbit anti-DsRed (Clontech, 1:500 dilution). Alexa Fluor 488- or Cy3-conjugated goat
- 20 anti-rabbit IgG or anti-mouse IgG was used as a secondary antibody. The DNA dye bisbenzimidazole (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.

Data analysis and statistics

- 25 Student's t-test was used to compare the means of two independent samples in analyses that contained only two samples. Analysis of variance (ANOVA) followed by the Bonferroni post-hoc test was used to compare means of two samples within multiple samples. The Kolmogorov-Smirnov test was used to compare the cumulative distribution of two samples. Data were presented as mean $\pm$ SEM. Statistical difference was displayed as
- 30 ***($P < 0.005$), **($P < 0.01$) and *($P < 0.05$), while ns indicates no significant difference. Electrophysiological data analysis was carried out with IGOR Pro or Clampfit 10.1 software. In Figures 1 and 4, rising slopes of synaptic responses were measured in field recording experiments. In Figure 2, peak amplitudes of synaptic responses were measured

in whole-cell recording experiments. Each data point was normalized to the average of baseline responses.

Example 1: Cdh1-APC plays an essential role in mGluR-dependent LTD

To determine the function of the ubiquitin ligase Cdh1-APC in the forebrain,
 5 conditional Cdh1 knockout mice were generated by crossing mice harboring a floxed allele of the *Cdh1* gene, (*Cdh1*^{loxP/loxP}) with mice carrying the recombinase Cre expressed under the control of the forebrain-specific driver *Emx*. In *Emx-Cre* mice, Cre is expressed in neocortical and hippocampal excitatory neurons, but not GABAergic interneurons, starting at embryonic day 10.5 (E10.5). In immunoblotting analyses, it was confirmed that
 10 disruption of the *Cdh1* gene in *Emx-Cre;Cdh1*^{loxP/loxP} mice (also referred to as Cdh1 cKO in figures) led to the loss of Cdh1 protein in the hippocampus (Figure 1A). Notably, Cdh1 downregulation in conditional knockout mice became marked after the second postnatal week and continued into adulthood (Figure 1A). Conditional knockout of Cdh1 had little or no effect on the proliferation of neural precursor cells in the ventricular zone of E15
 15 embryos in *Emx-Cre;Cdh1*^{loxP/loxP} mice (Figures 2A and 2B). Knockout of Cdh1 also had little or no effect on neuronal migration in the forebrain (Figure 2C). Consistent with these results, Cdh1 knockout did not appear to alter the structure of the forebrain including the hippocampus (Figure 1B).

To assess the role of Cdh1-APC in synaptic plasticity, LTP and LTD were
 20 characterized in the CA1 region of the hippocampus in 3-week old *Cdh1*^{loxP/loxP} and *Emx-Cre;Cdh1*^{loxP/loxP} littermate mice. By applying high frequency stimulation (HFS, 100 pulses at 100Hz) or low frequency stimulation (LFS, 900 pulses at 1 Hz) to Schaffer collaterals, LTP or LTD, respectively, were induced at CA1 synapses in the hippocampus in control *Cdh1*^{loxP/loxP} mice (Figures 1C and 1D). The induction of these forms of synaptic plasticity
 25 is thought to depend on activation of NMDA receptors. Conditional knockout of Cdh1 had little or no effect on HFS-induced LTP and LFS-induced LTD (Figures 1C and 1D), indicating that Cdh1-APC is dispensable for these forms of NMDAR dependent synaptic plasticity.

Whether Cdh1-APC might play a role in non-NMDAR-dependent synaptic
 30 plasticity was examined. Besides LFS-induced NMDAR-dependent LTD, another form of LTD is triggered upon activation of metabotropic glutamate receptors (mGluR-LTD). The selective group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) effectively induces mGluR-LTD. Notably, mGluR-LTD contributes to learning flexibility and is deregulated in

certain types of neurodevelopmental disorders of cognition. Therefore, mGluR-LTD was characterized in *Emx-Cre;Cdh1^{loxP/loxP}* mice and control *Cdh1^{loxP/loxP}* littermates.

Knockout of *Cdh1* significantly impaired DHPG-induced mGluR-LTD at hippocampal CA1 synapses (Figure 1E). Exposure of hippocampal slices from control
 5 *Cdh1^{loxP/loxP}* mice to DHPG at 50 μ M for 10 minutes induced robust long-term synaptic depression at CA1 synapses (75 $\pm$ 5% of baseline responses, 1 hour post-DHPG) (Figure 1E). However, in *Cdh1* conditional knockout mice, the level of DHPG-induced synaptic depression was substantially attenuated (90 $\pm$ 3% of baseline responses) (Figure 1E). In control experiments, expression of the recombinase Cre alone in *Emx-Cre* mice failed to
 10 impair mGluR-dependent LTD at CA1 synapses (74 $\pm$ 3% of baseline responses) (Figure 1E). These results indicate that *Cdh1* is required for mGluR-LTD in the hippocampus.

In addition to mGluR activation with DHPG, electrical stimulation of Schaffer collaterals by a paired-pulse low frequency paradigm (PP-LFS, 900 paired-pulses at 1 Hz with 50ms interpulse interval) also triggers mGluR-dependent LTD at CA1 synapses in the
 15 hippocampus (Massey and Bashir, 2007). PP-LFS triggered robust LTD in control *Cdh1^{loxP/loxP}* mice (68 $\pm$ 4% of baseline responses, 1 hour post-stimulus) (Figure 1F). By contrast, PP-LFS-induced LTD was profoundly reduced in conditional *Cdh1* knockout mice (99 $\pm$ 3% of baseline responses) (Figure 1F). Taken together, based on independent pharmacological and electrical modes of inducing synaptic plasticity, these results indicate
 20 that *Cdh1*-APC plays an essential role in mGluR-dependent LTD, but not NMDAR-dependent LTD or LTP, at CA1 synapses in the hippocampus.

A series of control experiments were performed to determine whether the impairment of mGluR-LTD in forebrain-specific conditional *Cdh1* knockout mice might be secondary to abnormalities in synaptic morphogenesis or basal synaptic transmission and
 25 intrinsic neuronal excitability. In morphological assays, the density, length and width of dendritic spines in CA1 hippocampal neurons was not altered in *Emx-Cre;Cdh1^{loxP/loxP}* mice as compared to control *Cdh1^{loxP/loxP}* mice (Figures 2D, 2E and 2F). In analyses of basal synaptic transmission, no significant differences were detected in the amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in conditional *Cdh1*
 30 knockout and control mice (Figures 3A and S3B). Likewise, there was little or no difference in synaptic strength in response to distinct electrical stimulation intensities at the population level (Figures 3C and 3D) or at the single synapse level (Figures 3E and 3F). In measures of intrinsic excitability of hippocampal CA1 neurons, *Cdh1* knockout had little or

no effect on the action potential firing rate and amplitude (Figures 3G-I). Collectively, these findings indicate that Cdh1-APC specifically drives mGluR-LTD independently of alterations of synapse morphology or basal synaptic neurotransmission.

Example 2: Cdh1-APC operates in the cytoplasm to regulate mGluR-LTD

5 The mechanism underlying the novel function of Cdh1-APC in the brain was determined. First, the subcellular site of action of Cdh1-APC in mGluR-LTD was characterized.

 In fractionated lysates of the forebrain, Cdh1 was present in both the nuclear and cytoplasmic fractions (Figure 4A). To determine whether the nuclear or cytoplasmic pool of
10 Cdh1-APC regulates synaptic plasticity, structure-function analyses of Cdh1 in the background of conditional Cdh1 knockout mice was performed. An in utero electroporation approach was used in *Emx-Cre; Cdh1^{loxP/loxP}* mice to express Cdh1 targeted to the cytoplasm using a nuclear export signal (GFP-NES-Cdh1) or Cdh1 targeted to the nucleus using a nuclear localization signal (GFP-NLS-Cdh1). Together with GFP-NES-Cdh1, GFP-NLS-
15 Cdh1, or the control vector, an expression plasmid encoding mCherry was included to identify the transfected neurons in the hippocampus. In utero electroporations at E15 and electrophysiological analyses in hippocampal slices from 3-week old mice were performed (illustrated in Figure 4B). First, it was confirmed that GFP-NES-Cdh1 and GFP-NLS-Cdh1 were expressed in the cytoplasmic and nuclear compartments, respectively, in CA1
20 pyramidal neurons in the hippocampus of 3-week old mice (Figure 4C). Notably, although GFP-NLS-Cdh1 displayed modest expression in the soma in addition to robust expression in the nucleus, GFP-NES-Cdh1 was restricted to the cytoplasm and excluded from the nucleus in CA1 neurons (Figure 4C).

 In whole-cell synaptic current recording analyses, just as in field potential recording
25 analyses (Figure 1E), conditional knockout of Cdh1 strongly impaired DHPG-induced mGluRLTD (Figure 4D). Importantly, in analyses of mCherry-positive CA1 neurons in *Emx-Cre; Cdh1^{loxP/loxP}* mice, expression of cytoplasmic Cdh1 effectively reversed the Cdh1 knockout induced mGluR-LTD deficit (Figure 4D). By contrast, expression of nuclear Cdh1 failed to reverse the mGluR-LTD deficit in conditional Cdh1 knockout mice (Figure
30 4D). In control analyses, electroporation of *Emx-Cre; Cdh1^{loxP/loxP}* mice with the pCAG expression vector had little or no effect on the impairment of mGluR-LTD (Figure 4D). Taken together, these data reveal that Cdh1-APC operates in the cytoplasm rather than the nucleus to promote mGluR-LTD in CA1 neurons in the hippocampus.

Example 3: Cdh1-APC triggers the ubiquitination and degradation of FMRP

The importance of the cytoplasmic localization of Cdh1-APC in the regulation of synaptic plasticity suggests that relevant targets of Cdh1-APC reside in the cytoplasm. Whether Cdh1-APC might regulate mGluR-LTD via the ubiquitination and degradation of FMRP was investigated.

Whether Cdh1 interacts with FMRP was first determined. In reciprocal coimmunoprecipitation analyses, Cdh1 formed a complex with FMRP in cells (Figures 5A and 5B). Whereas Cdh1 interacted strongly with FMRP (Figures 5A and 5B), the Cdh1-related APC coactivator Cdc20 failed to interact with FMRP (Figure 5C), indicating that FMRP specifically interacts with Cdh1-APC. Interrogation of FMRP by sequence gazing revealed a conserved Dbox motif (Figure 5D), which represents a Cdh1 recognition motif. Mutation of the D-box in FMRP (Dbm), whereby the sequence RSFLEFAED was changed to ASFAEFAED (Figure 5D), disrupted the ability of FMRP to interact with Cdh1 in coimmunoprecipitation analyses (Figures 5A and 5B). These results indicate that FMRP specifically interacts with Cdh1 via the FMRP D-box motif.

The identification of an interaction between Cdh1 and FMRP in a D-box-dependent manner indicated that FMRP represents a substrate of the ubiquitin ligase Cdh1-APC. Whether ubiquitination of endogenous FMRP is dependent on endogenous Cdh1-APC in neurons was investigated. In immunoprecipitation analyses of FMRP followed by immunoblotting with an antibody that recognizes ubiquitin, FMRP was ubiquitinated in DHPG-treated hippocampal slices from 3-week old control *Cdh1^{loxP/loxP}* mice (Figure 5D). Strikingly, the level of ubiquitinated FMRP was substantially reduced in DHPG-treated conditional Cdh1 knockout hippocampal slices (Figure 5E). These findings indicate that endogenous FMRP is ubiquitinated by endogenous Cdh1-APC in the hippocampus.

It was next examined whether Cdh1-APC triggers the degradation of endogenous FMRP in the hippocampus. Since FMRP undergoes both synthesis and degradation in response to mGluR stimulation, in order to facilitate the analyses of FMRP degradation, acute brain slices were pretreated with protein synthesis inhibitor, Anisomycin, to block protein synthesis. In immunoblotting assays, exposure of hippocampal slices from 3 week-old control *Cdh1^{loxP/loxP}* mice to DHPG induced the downregulation of FMRP (Figure 5F). By contrast, DHPG failed to induce the downregulation of FMRP in hippocampal slices from conditional Cdh1 knockout mice (Figure 5F). In immunohistochemical analyses, DHPG triggered the downregulation of endogenous FMRP in the CA1 region of the

hippocampus from 3 week-old control but not conditional Cdh1 knockout mice (Figure 5G). Taken together, these data indicate that Cdh1-APC triggers the ubiquitination and consequent degradation of endogenous FMRP in the hippocampus.

Example 4: FMRP operates downstream of Cdh1-APC in the regulation of mGluR-

5 dependent LTD

Whether the degradation of FMRP mediates the ability of Cdh1-APC to drive mGluR-dependent LTD was investigated. To address this question, Cdh1 and FMRP double knockout mice were generated. The ability of DHPG to induce mGluR-LTD in control (*Cdh1^{loxP/loxP}*), conditional Cdh1 knockout (*Emx-Cre;Cdh1^{loxP/loxP}*), FMRP knockout (10 *FMRI^{-/-};Cdh1^{loxP/loxP}*), and double knockout (*FMRI^{-/-};Emx-Cre;Cdh1^{loxP/loxP}*) mice, respectively, was compared. In FMRP knockout mice, mGluR-dependent LTD was exaggerated (Figures 6A and 6B). In conditional Cdh1 knockout mice, mGluR-LTD was substantially impaired (Figures 6A and 6B). In Cdh1 and FMRP double knockout mice, mGluR-LTD was exaggerated and indistinguishable from the phenotype 15 in FMRP knockout mice (Figures 6A and 6B). Thus, FMRP knockout completely suppressed the Cdh1 knockout-induced impaired mGluR-LTD phenotype. These findings support the conclusion that FMRP operates downstream of Cdh1-APC in mGluR-dependent LTD.

Incorporation by Reference

20 All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

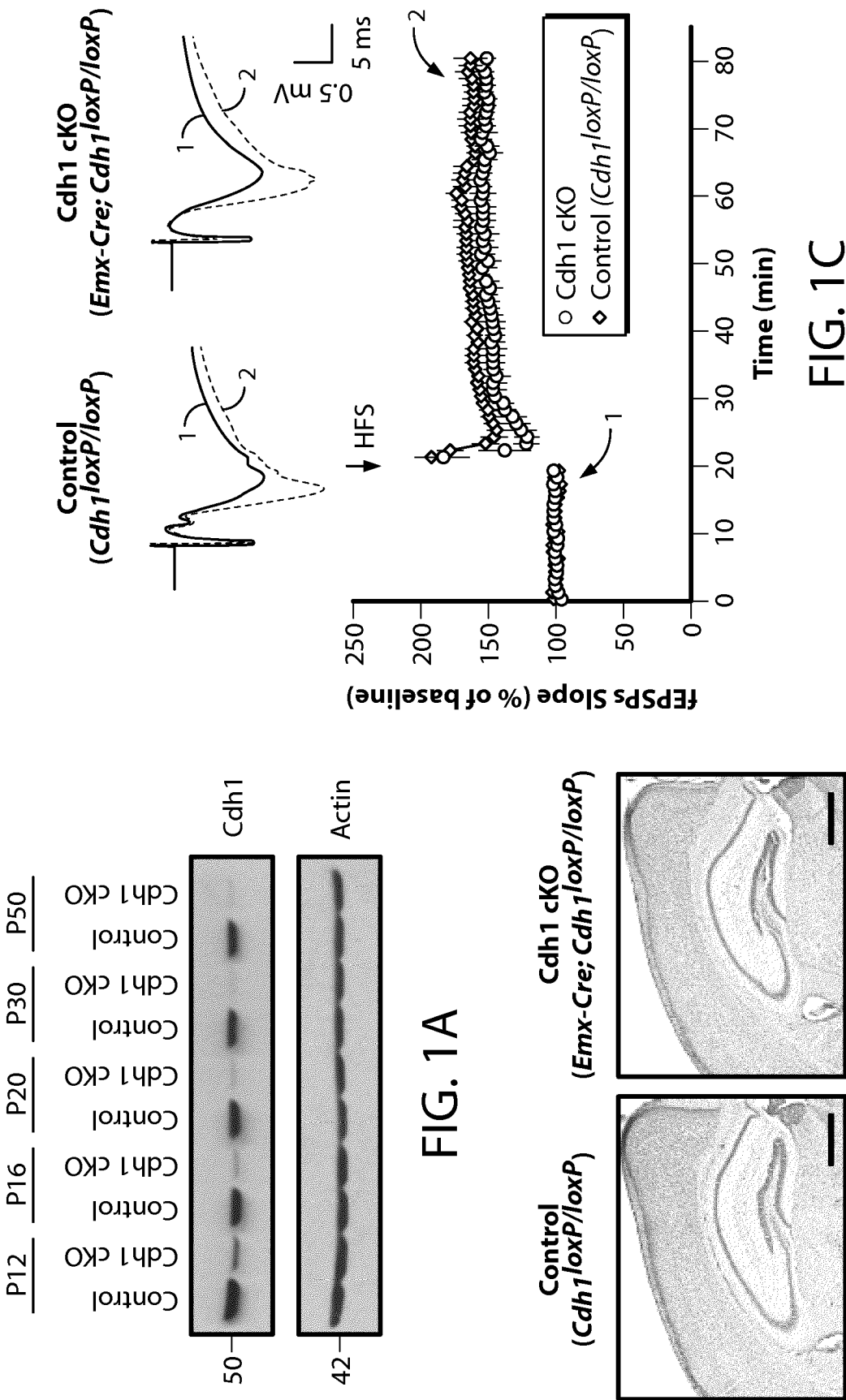
25 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method of treating a cognitive disorder comprising administering to a subject an agent that inhibits Cdh1-APC.
2. The method of claim 1, wherein the cognitive disorder is fragile X syndrome.
3. The method of claim 1 or 2, wherein the agent is a small molecule.
4. The method of claim 3, wherein the small molecule is tosyl-L-arginine methyl ester (TAME) or a TAME prodrug.
5. The method of claim 4, wherein the small molecule is a TAME prodrug comprising a TAME derivative in which a guanidine group is protected by a carbamate group.
6. The method of claim 4, wherein the small molecule is a TAME prodrug comprising an esterase-activatable N,N'-bis(acyloxymethyl carbamate) derivative of TAME.
7. The method of claim 4, wherein the small molecule is proTAME.
8. The method of claim 1 or 2, wherein the agent is an interfering nucleic acid molecule specific for Cdh1.
9. The method of claim 8, wherein the interfering nucleic acid molecule is an antisense molecule, an siRNA molecule, an shRNA molecule or a miRNA molecule.
10. The method of any one of claim 1 or 2, wherein the agent inhibits the formation of a complex between Cdh1 and APC.
11. A method of determining whether a test agent is a candidate therapeutic agent for the treatment of a cognitive disorder, the method comprising:
 - a) forming a test reaction mixture comprising:
 - a Cdh1 polypeptide or fragment thereof;
 - an APC polypeptide or fragment thereof; and
 - a test agent;
 - b) incubating the test reaction mixture under conditions conducive for the formation of a complex between the Cdh1 polypeptide or fragment thereof and the APC polypeptide or fragment thereof; and
 - c) determining the amount of the complex in the test reaction mixture;
 - wherein a test agent that reduces the amount of the complex in the test reaction mixture compared to the amount of the complex in a control reaction mixture is a candidate therapeutic agent for the treatment of a cognitive disorder.
12. The method of claim 11, wherein the test agent is a small molecule.

13. The method of claim 11, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture does not comprise a test agent.
14. The method of claim 11, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture comprises a placebo agent instead of a test agent.
15. The method of claim 11, wherein the test reaction mixture is formed by adding the test agent to a mixture comprising the Cdh1 polypeptide or fragment thereof and the APC polypeptide or fragment thereof.
16. The method of claim 11, wherein the test reaction mixture is formed by adding the APC polypeptide or fragment thereof to a mixture comprising the test agent and the Cdh1 polypeptide or fragment thereof.
17. The method of claim 11, wherein the test reaction mixture is formed by adding the Cdh1 polypeptide or fragment thereof to a mixture comprising the test agent and the APC polypeptide or fragment thereof.
18. The method of any one of claims 11-17, wherein the APC polypeptide or fragment thereof is anchored to a solid support in the test reaction mixture.
19. The method of claim 18, wherein the test reaction mixture is incubated under conditions conducive to the binding of the Cdh1 polypeptide or fragment thereof to the anchored APC polypeptide or fragment thereof.
20. The method of claim 19, further comprising the step of isolating Cdh1 polypeptide or fragment thereof bound to the APC polypeptide or fragment thereof from the Cdh1 polypeptide or fragment thereof not bound to the APC polypeptide or fragment thereof.
21. The method of claim 20, wherein the amount of complex in the test reaction mixture is determined by detecting the amount of APC polypeptide or fragment thereof bound to the Cdh1 polypeptide or fragment thereof.
22. The method of claim 18, wherein the Cdh1 polypeptide or fragment thereof is linked to a detectable moiety.
23. The method of any one of claims 11-17, wherein the Cdh1 polypeptide or fragment thereof is anchored to a solid support in the test reaction mixture.
24. The method of claim 23, wherein the test reaction mixture is incubated under conditions conducive to the binding of the APC polypeptide or fragment thereof to the anchored Cdh1 polypeptide or fragment thereof.

25. The method of claim 24, further comprising the step of isolating APC polypeptide or fragment thereof bound to the Cdh1 polypeptide or fragment thereof from the APC polypeptide or fragment thereof not bound to the Cdh1 polypeptide or fragment thereof.
26. The method of claim 25, wherein the amount of complex in the test reaction mixture is determined by detecting the amount of APC polypeptide or fragment thereof bound to the Cdh1 polypeptide or fragment thereof.
27. The method of claim 23, wherein the APC polypeptide or fragment thereof is linked to a detectable moiety.
28. The method of any one of claims 11-17, wherein the test agent is a member of a library of test agents.
29. The method of any one of claims 11-17, wherein the test agent is a small molecule.



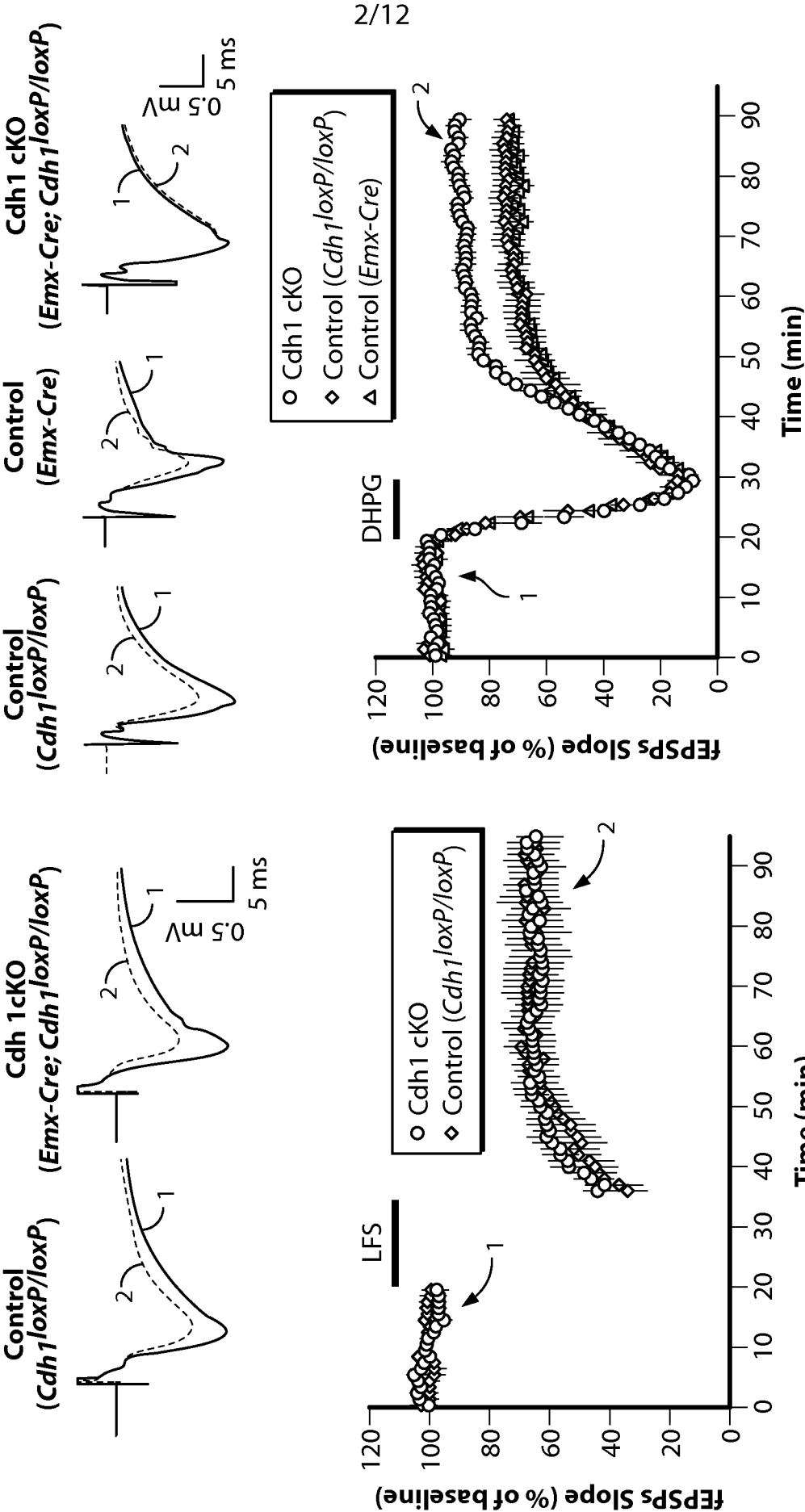


FIG. 1E

FIG. 1D

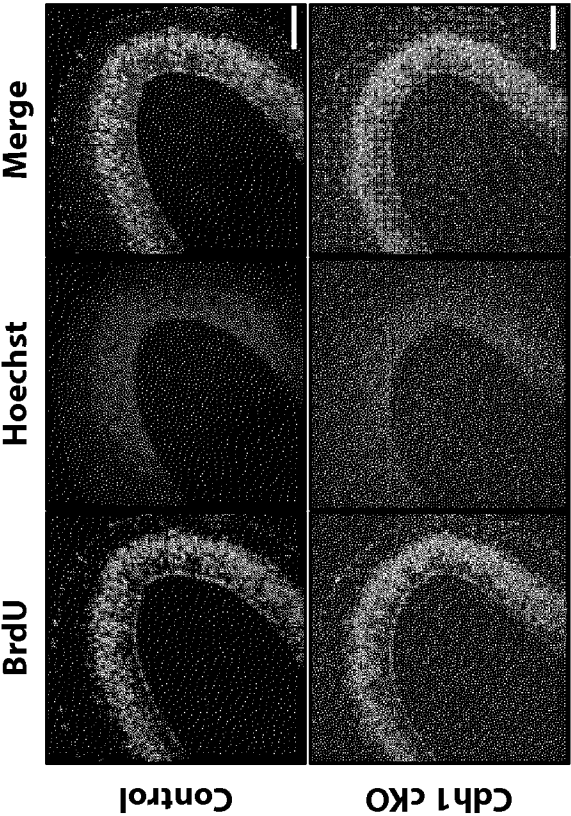


FIG. 2A

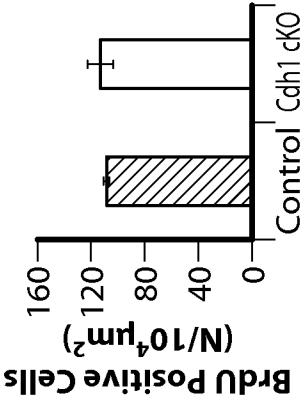


FIG. 2B

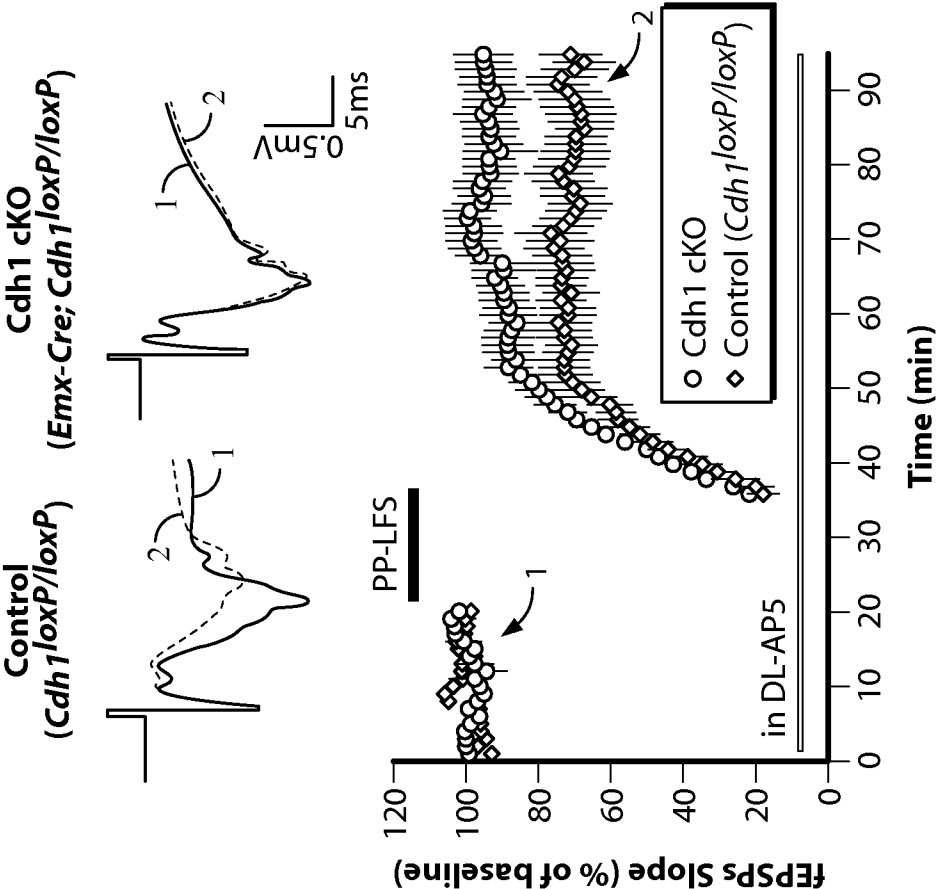


FIG. 1F

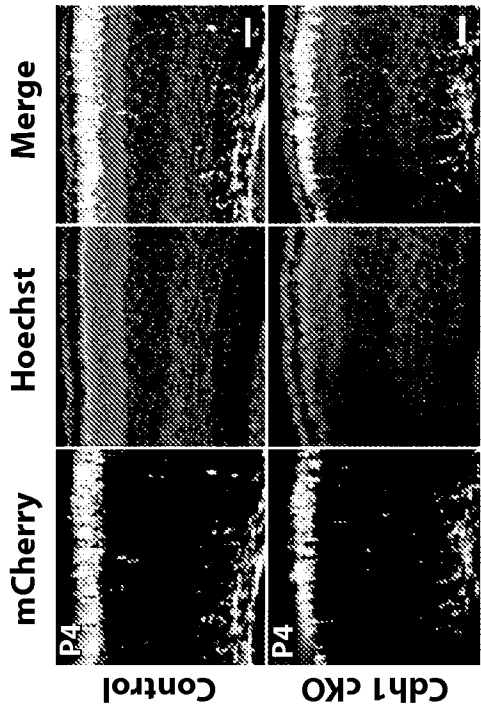


FIG. 2C

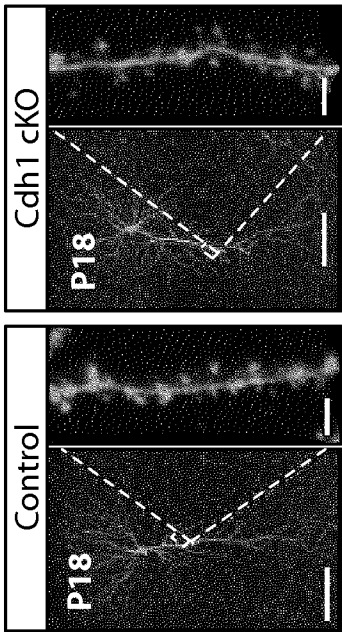


FIG. 2D

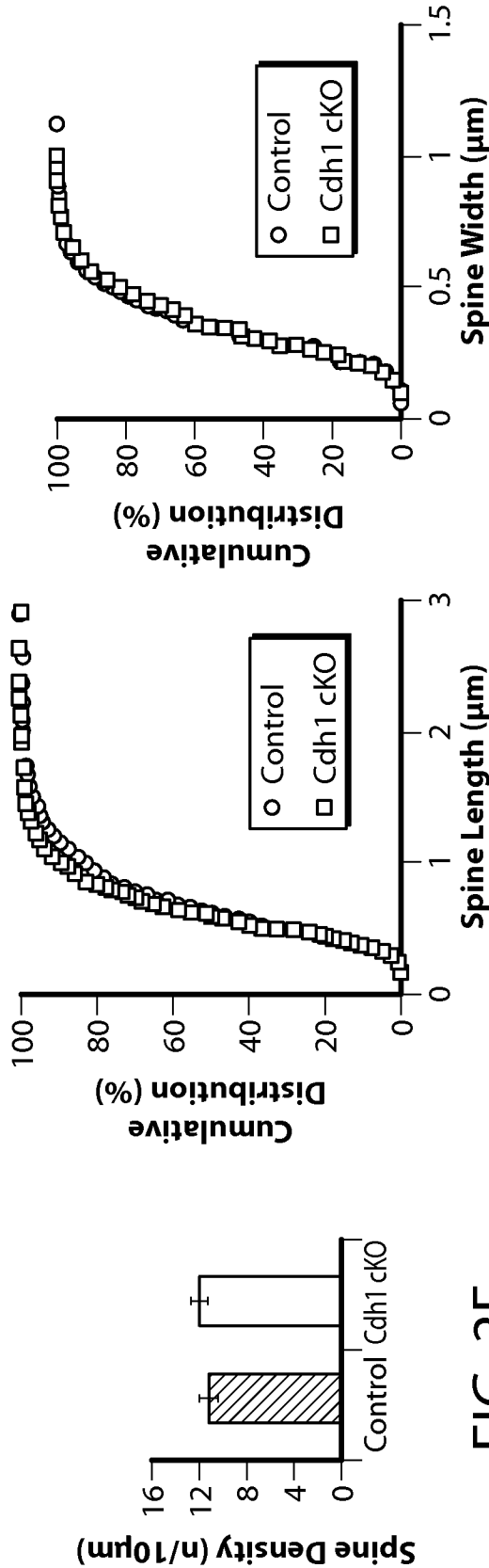


FIG. 2F

FIG. 2E

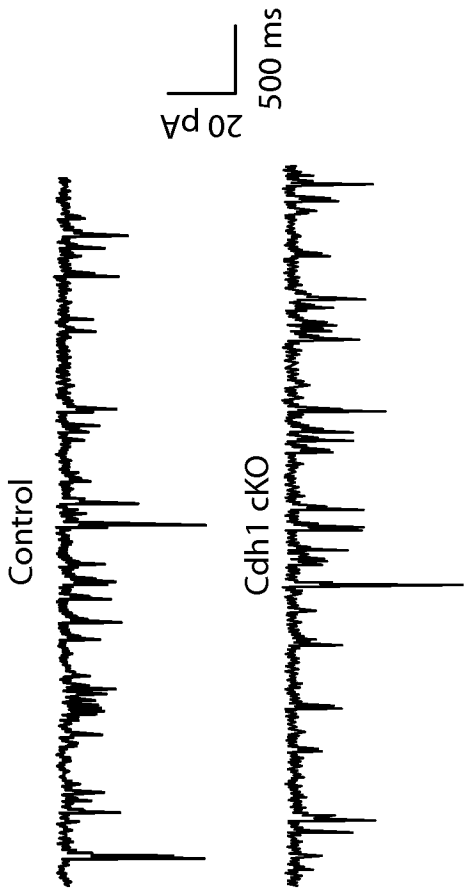


FIG. 3A

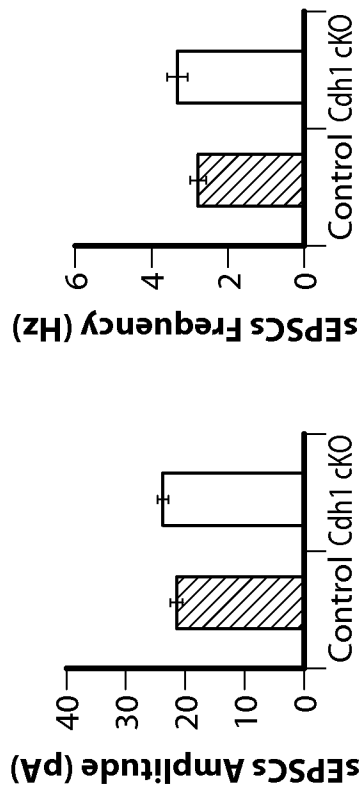


FIG. 3B

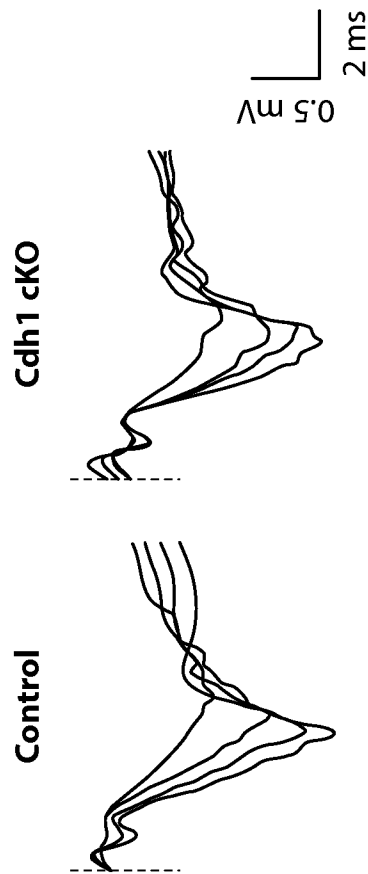


FIG. 3C

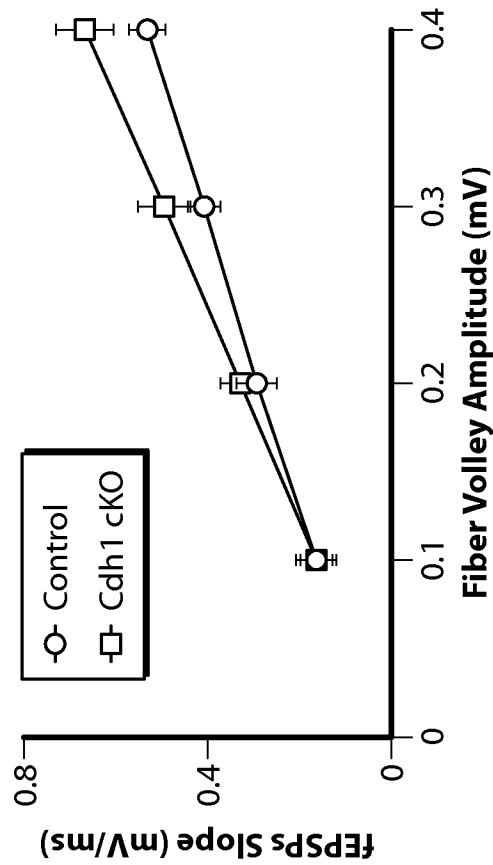


FIG. 3D

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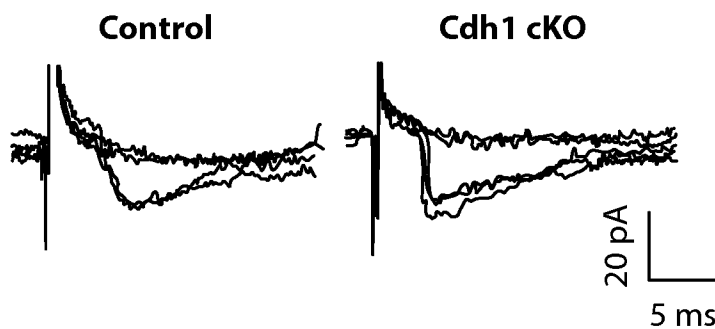


FIG. 3E

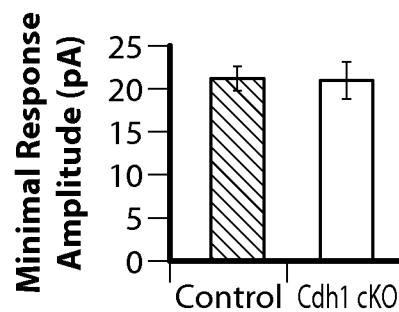


FIG. 3F

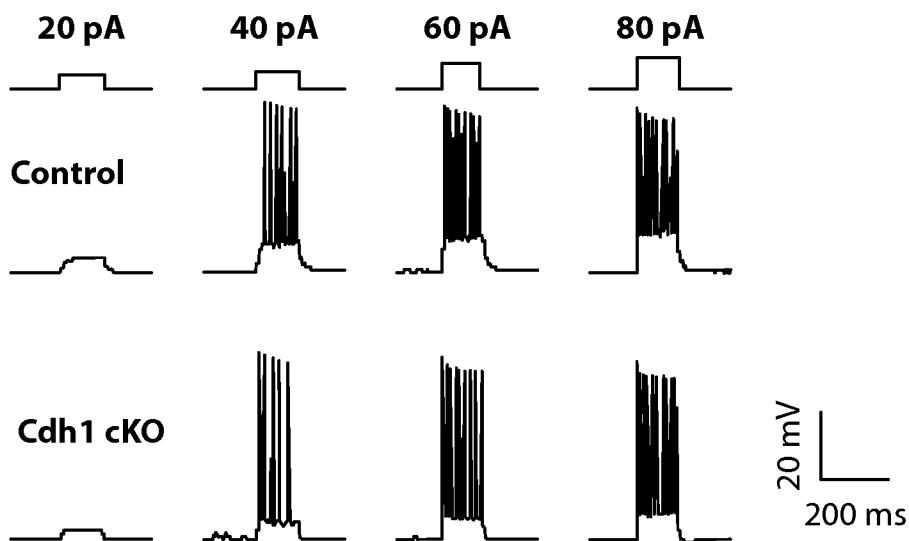


FIG. 3G

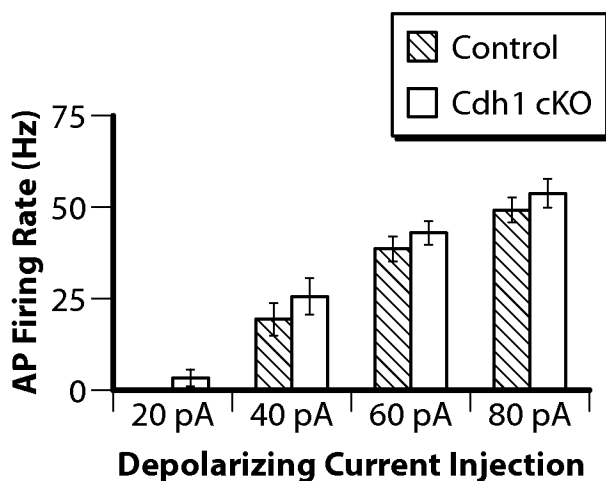


FIG. 3H

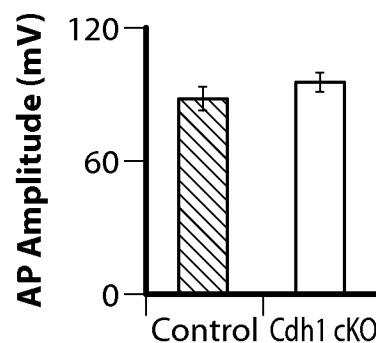


FIG. 3I

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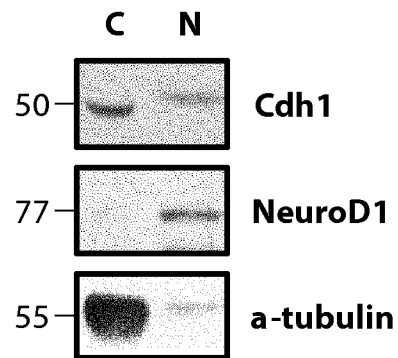


FIG. 4A

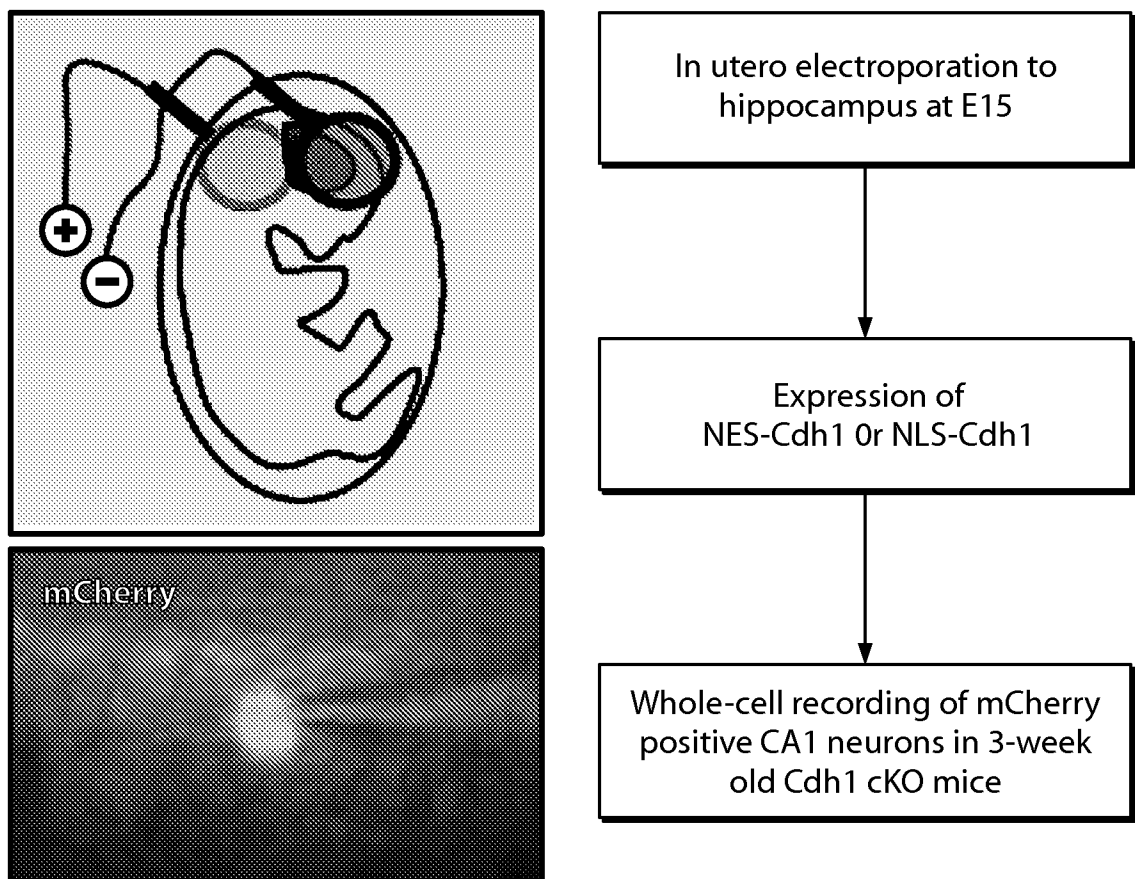


FIG. 4B

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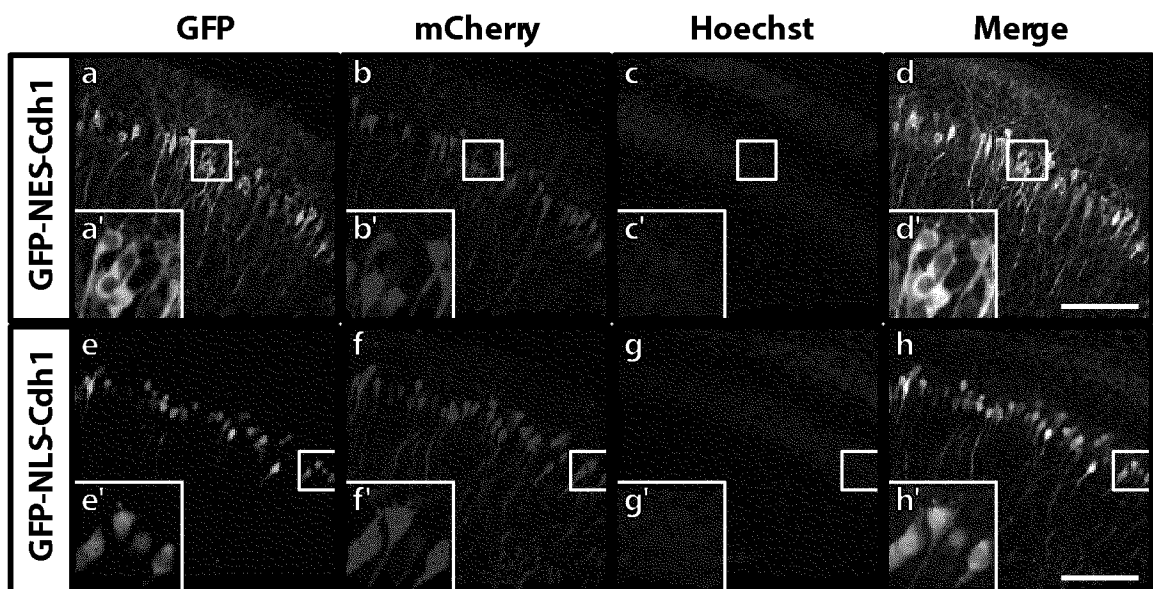


FIG. 4C

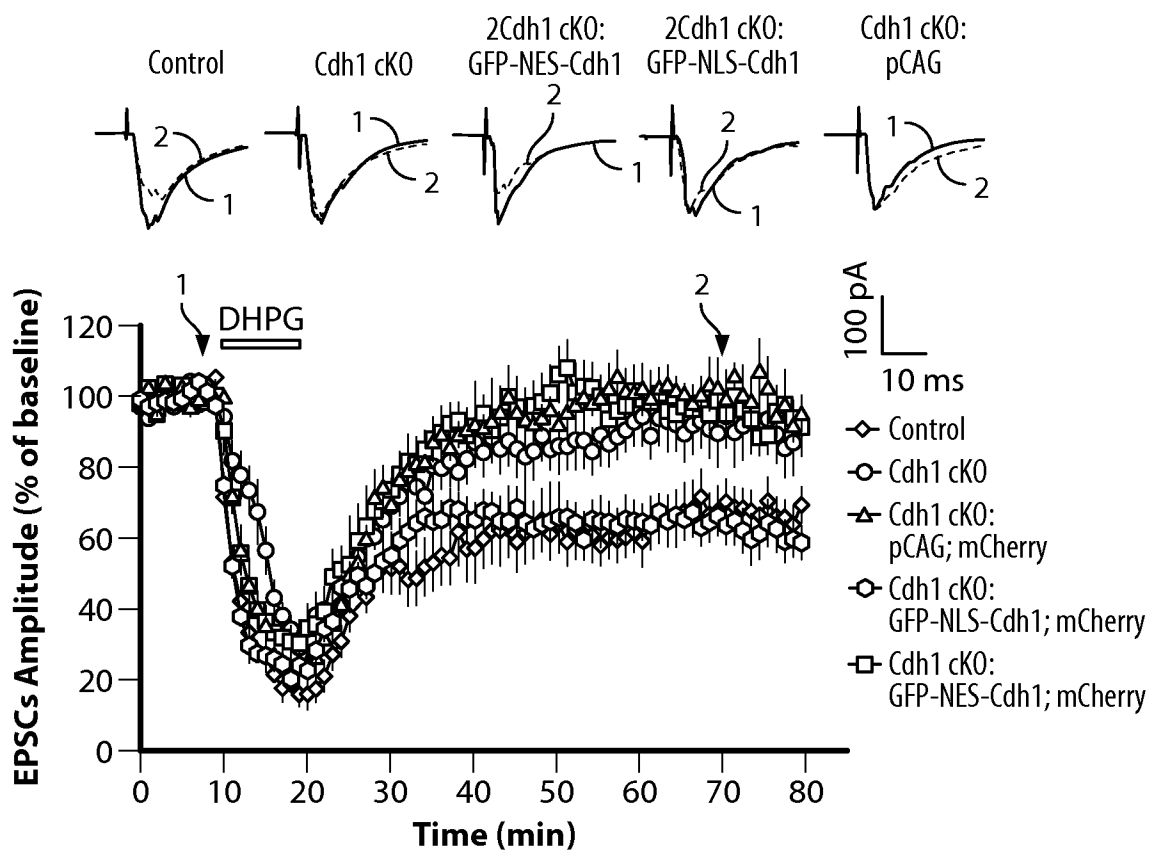


FIG. 4D

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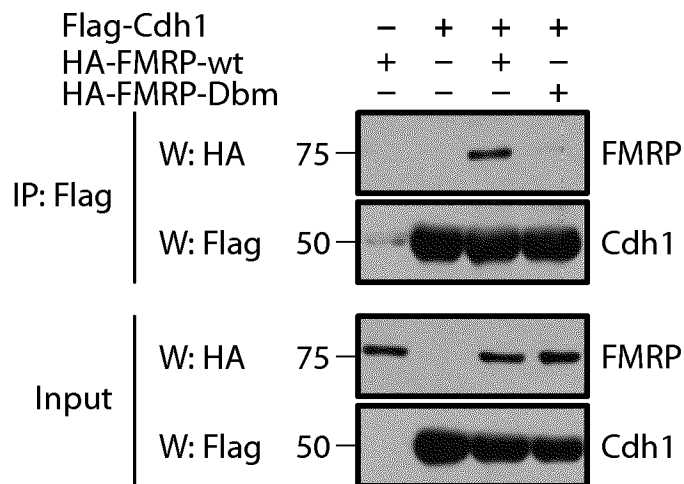


FIG. 5A

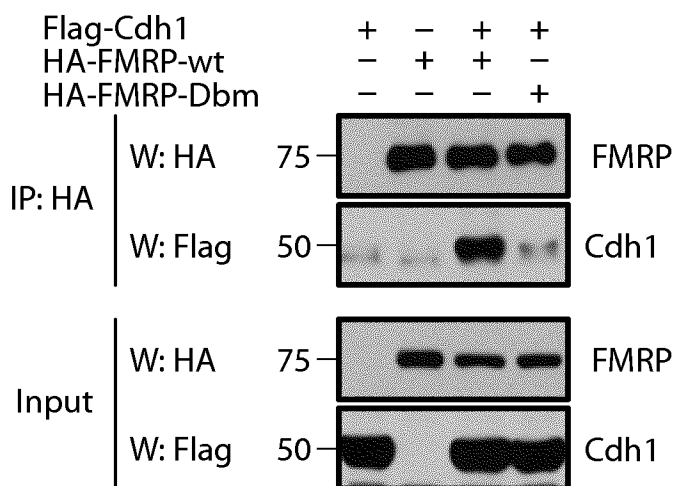


FIG. 5B

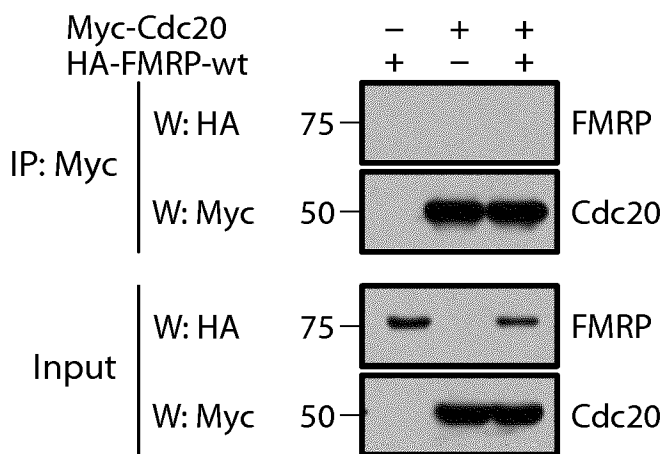


FIG. 5C

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FMRP D-box motif (aa276-284)	FMRP-Dbm
<i>Mouse:</i> QDAVKKARSFLEFAEDVIQVPRN	RSFLEFAED ⇒ ASFAEFAED
<i>Rat:</i> QDAVKKARSFLEFAEDVIQVPRN	
<i>Human:</i> QDAVKKARSFLEFAEDVIQVPRN	

FIG. 5D

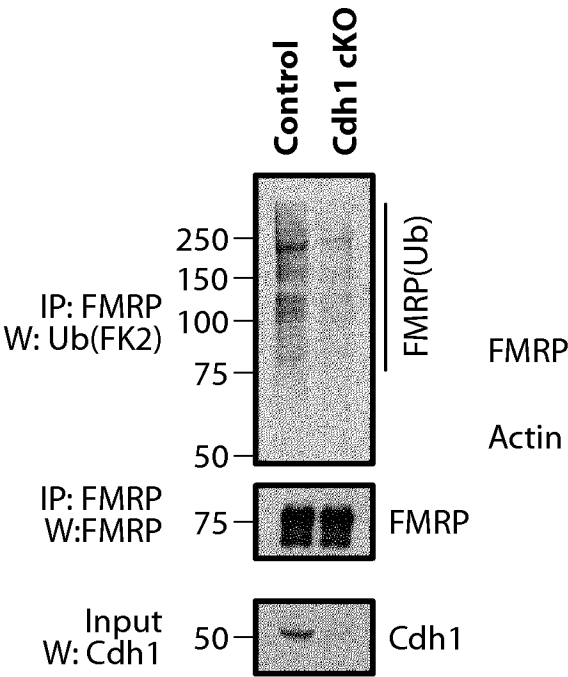


FIG. 5E

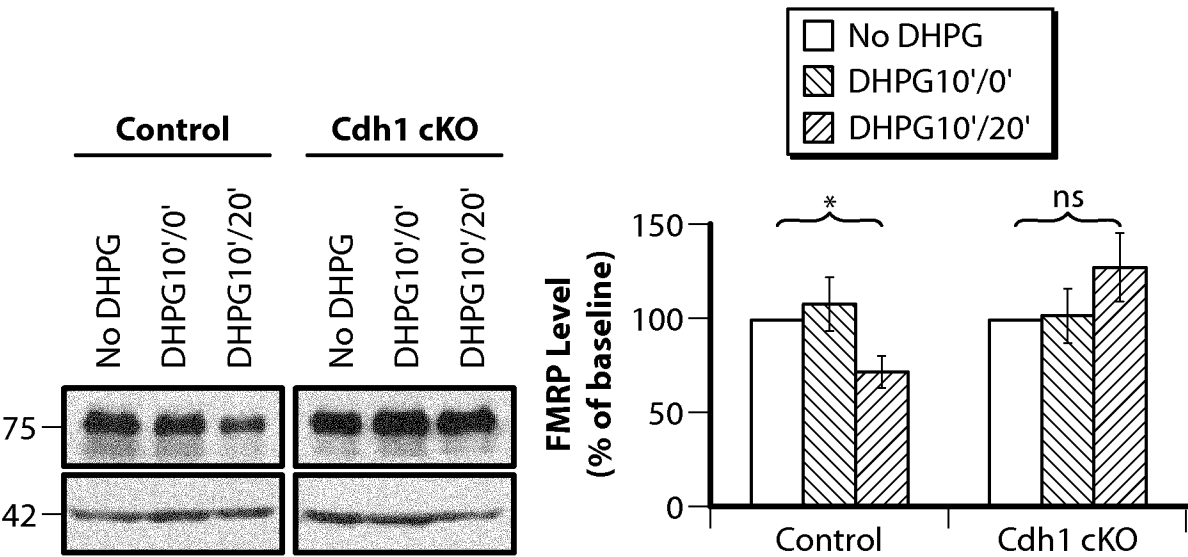


FIG. 5F

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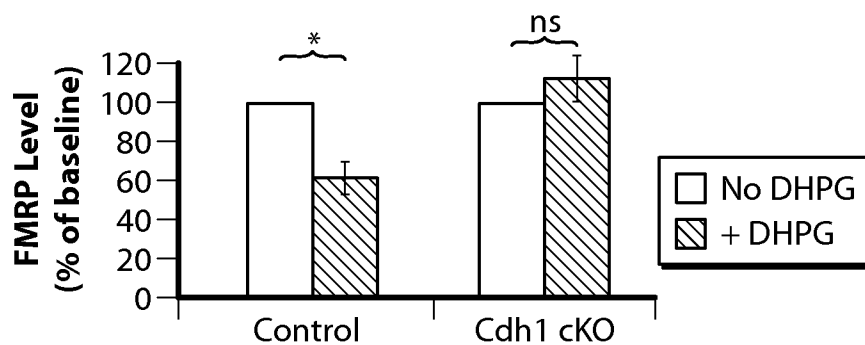
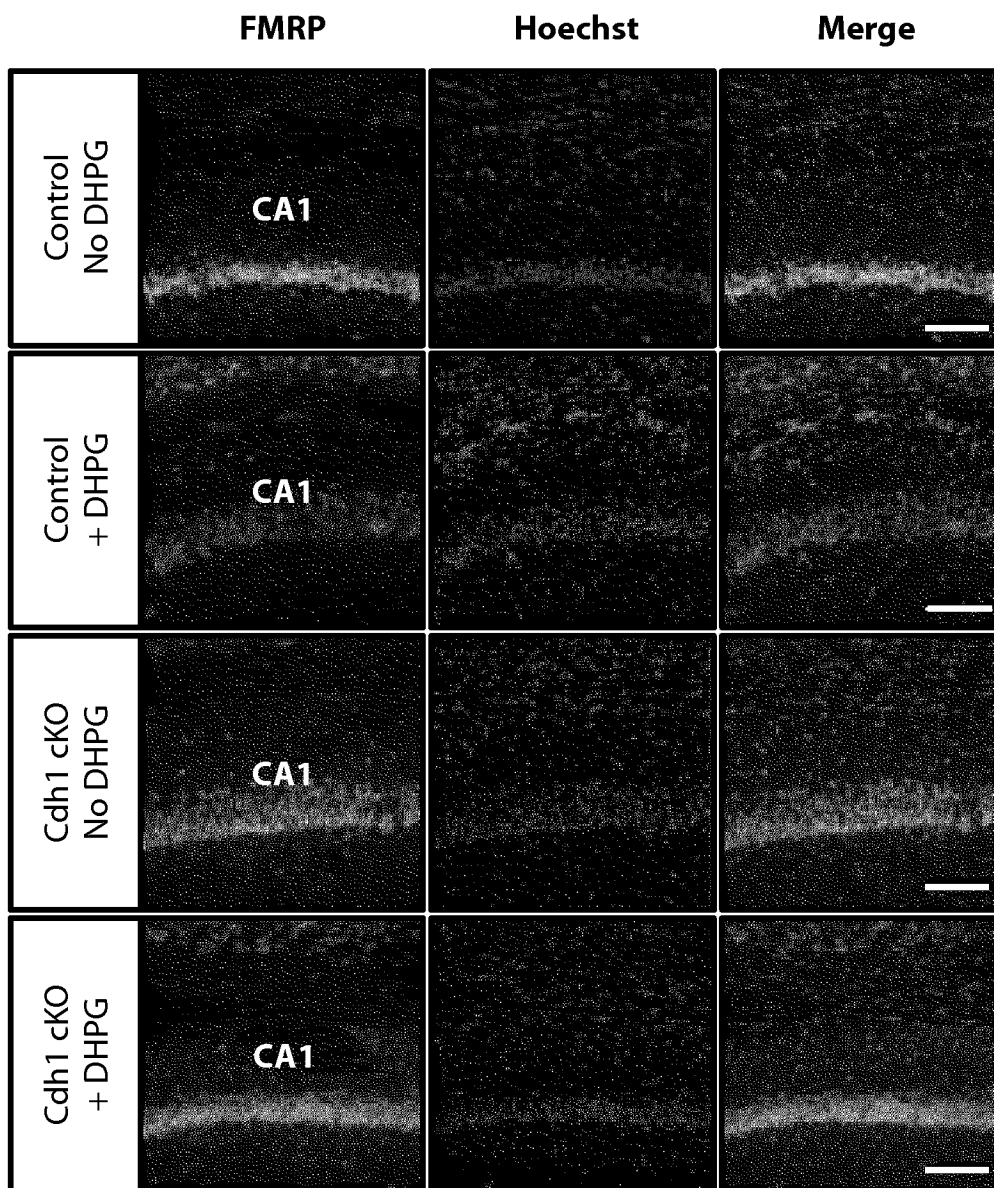


FIG. 5G

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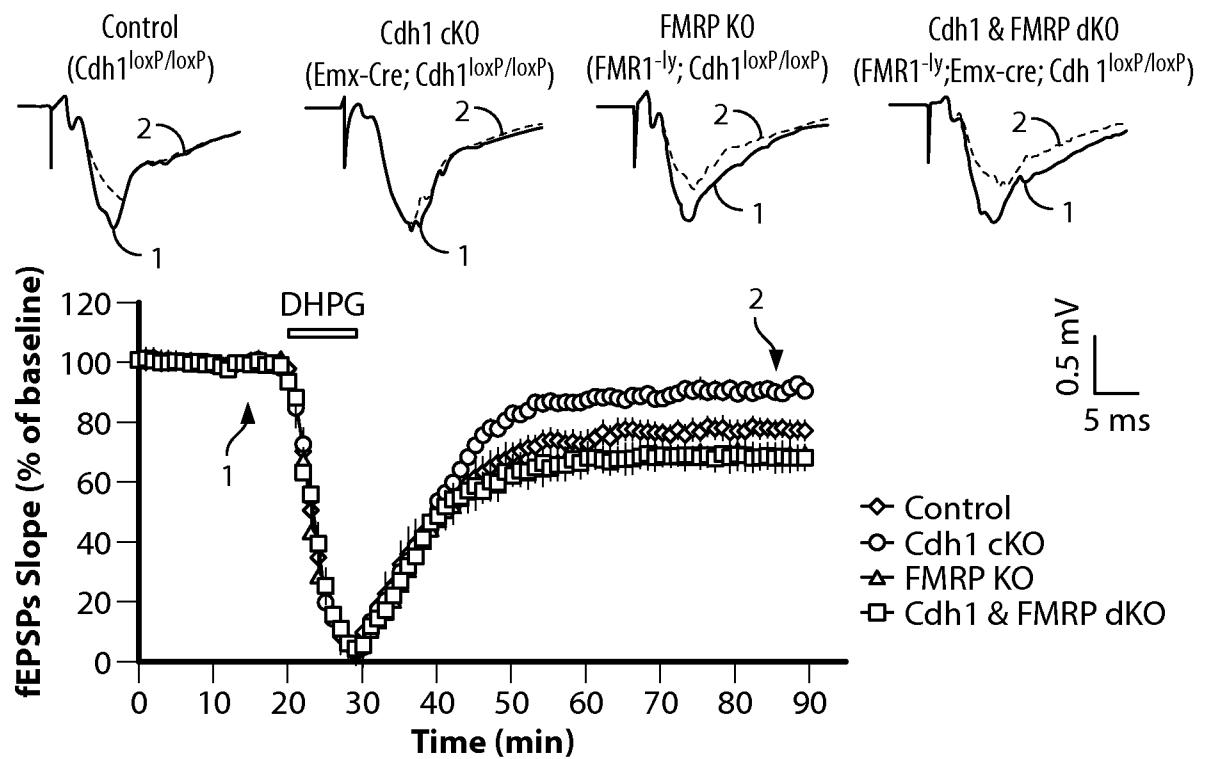


FIG. 6A

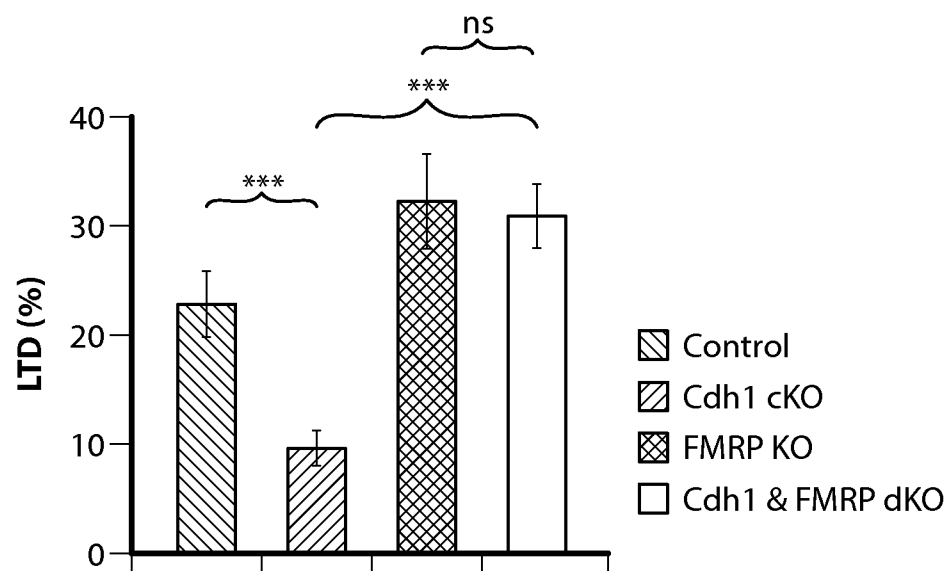


FIG. 6B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/34166

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/37; A61K 48/00, 31/24; A61P 43/00; C07C 307/02 (2015.01)

CPC - G01N 2500/02, 2500/00; A61K 48/005, 31/00; G01N 33/5058; C12N 2310/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12Q 1/37; A61K 48/00, 31/24; A61P 43/00; C07C 307/02

CPC: G01N 2500/02, 2500/00; A61K 48/005, 31/00; G01N 33/5058; C12N 2310/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, Other Countries (INPADOC), RU, AT, CH, TH, BR, PH); PubMed; Google; Google Scholar; Google Patents; 'Cdh1,' 'APC,' 'ubiquitin ligase,' 'fizzy-related protein homolog,' 'anaphase promoting complex,' 'cognitive disorder,' 'cognitive impairment,' 'Parkinson's,' 'autism, brain,' 'mental retardation,' 'fragile x syndrome protein,' 'FMRP,' 'tosyl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2008/066744 A2 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE); June 5, 2008; pages 4, 7, 12-16, 22, 24, 25	1, 3/1, 8/1, 9/8/1, 10/1, 11-13, 18/11-18/13, 19/18/11-19/18/13, 22/18/11-22/18/13, 23/11, 23/13, 24/23/11-24/23/13, 27/23/11-27/23/13 ----- 4/3/1, 5/4/3/1, 6/4/3/1, 7/4/3/1, 14-17, 18/14-18/17, 19/18/14-19/18/17, 20/19/18/11-20/19/18/17, 21/20/19/18/11-21/20/19/18/17, 22/18/14-22/18/17, 23/14-23/17, 24/23-24/23/17, 25/24/23/11-25/24/23/13, 26/25/24/23/11-26/25/24/17, 27/23/14-27/23/17, 28/11-28/13, 28/14-28/17, 29/14-29/17 ... Continued on Next Supplemental Page ...

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

 Date of the actual completion of the international search
 25 August 2015 (25.08.2015)

Date of mailing of the international search report

15 SEP 2015

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 Authorized officer
 Shane Thomas

 PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/34166

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0115948 A1 (KING, R et al.) May 10, 2012; paragraphs [0011], [0039], [0040]; Claim 30	14-17, 18/14-18/17, 19/18/14-19/18/17, 20/19/18/11-20/19/18/17, 21/20/19/18/11-21/20/19/18/17, 22/18/14-22/18/17, 23/14-23/17, 24/23/14-24/23/17, 25/24/23/11-25/24/23/13, 26/25/24/23/11-26/25/24/23/17, 27/23/14-27/23/17, 28/11-28/13, 28/14-28/17, 29/14-29/17
Y	US 2013/230458 A1 (KING, R et al.) September 5, 2013; paragraphs [0014], [0100], [0190]-[0192]	4/3/1, 5/4/3/1, 6/4/3/1, 7/4/3/1
A	YANG, Y et al. The Dynamic Ubiquitin Ligase Duo: Cdh1-APC and Cdc20-APC Regulate Neuronal Morphogenesis and Connectivity. Curr Opin. Neurobiol., February 2010, Vol. 20, No. 1, pages 92-99. doi:10.1016/j.conb.2009.12.004.	2, 3/2, 4/3/2, 5/4/3/2, 6/4/3/2, 7/4/3/2, 8/2, 9/8/2, 10/2
A	HEGDE, AN et al. The Ubiquitin-Proteasome Pathway And Synaptic Plasticity. Learning & Memory. 2010, Vol. 17, pages 314-327. DOI:10.1101/lm.1504010.	2, 3/2, 4/3/2, 5/4/3/2, 6/4/3/2, 7/4/3/2, 8/2, 9/8/2, 10/2
A	NALAVADI, VC, et al. Dephosphorylation-Induced Ubiquitination And Degradation Of FMRP In Dendrites: A Role In Immediate Early mGluR-Stimulated Translation. J Neurosci. 2012, February 22nd, Vol. 32, No. 8, pages 2582-2587. DOI: 10.1523/JNEUROSCI.5057-11.2012.	2, 3/2, 4/3/2, 5/4/3/2, 6/4/3/2, 7/4/3/2, 8/2, 9/8/2, 10/2