APC/C\textsuperscript{Cdh1}\,-Rock2 pathway controls dendritic integrity and memory

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Disruption of neuronal morphology contributes to the pathology of neurodegenerative disorders such as Alzheimer’s disease (AD). However, the underlying molecular mechanisms are unknown. Here, we show that postnatal deletion of Cdh1, a cofactor of the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase in neurons [Cdh1 conditional knockout (cKO)], disrupts dendrite arborization and causes dendritic spine and synapse loss in the cortex and hippocampus, concomitant with memory impairment and neurodegeneration, in adult mice. We found that the dendrite destabilizer Rock protein kinase 2 (Rock2), which accumulates in the brain of AD patients, is an APC/C\textsuperscript{Cdh1}\,-substrate in vivo and that Rock2 protein and activity increased in the cortex and hippocampus of Cdh1 cKO mice. In these animals, inhibition of Rock activity, using the clinically approved drug fasudil, prevented dendritic network disorganization, memory loss, and neurodegeneration. Thus, APC/C\textsuperscript{Cdh1}\,-mediated degradation of Rock2 maintains the dendritic network, memory formation, and neuronal survival, suggesting that pharmacological inhibition of aberrantly accumulated Rock2 may be a suitable therapeutic strategy against neurodegeneration.

Significance

Disruption of neuronal dendrites causes cognitive impairment in Alzheimer’s disease (AD). Rock2, a kinase of the rho family of proteins, is a dendrite destabilizer that accumulates in the AD brain. Here, we show that Rock2 protein stability is controlled by the ubiquitin ligase APC/C\textsuperscript{Cdh1}. Accordingly, APC/C\textsuperscript{Cdh1}\,-loss of function in adult neurons increases Rock2 protein and activity, causing dendrite disruption in the cortex and hippocampus, along with memory loss and neurodegeneration, in mice. These effects are abolished by inhibition of Rock2 activity. Thus, the APC/C\textsuperscript{Cdh1}\,-Rock2 pathway may be a novel therapeutic target against neurodegeneration.

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postnatal week in a subset of glutamatergic pyramidal neurons, including nearly all CA1 hippocampal neurons and in scattered cortical and other neurons throughout the forebrain (14). Immunoblotting analyses revealed depletion of Cdh1 levels in the cortex and hippocampus, but not in the cerebellum (Cbh), of cKO Cdh1 from postnatal day 25 and continued into adulthood (B and C). Genetic ablation of Cdh1 in the adult forebrain did not modify body weight (B) but significantly reduced brain weight from the age of 120 d (C). (D and E) Brain sections were immunostained with the neuronal marker NeuN. (D) Cerebral cortex from Cdh1 cKO mice failed to grow normally, resulting in a marked reduction of cortical thickness from 120 d after birth. White dashed lines mark cortex thickness. (Scale bars, 100 μm.) (E) The CA1 layer of the hippocampus was thinner in the Cdh1 cKO from 120 d of age, compared with age-matched control mice. (Scale bars, 200 μm; magnification: Insets, 20x.) Data are expressed as mean ± SEM; *P < 0.05 versus age-matched control mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 6 mice per group).

Whereas neurogenesis is the main determinant of embryonic brain growth (12, 15), dendrite length and dendritic arbor complexity are key determinants of adult brain size (16). We found a reduction in dendrite density in the cortex (Fig. 2A and Fig. S2 A and C) and hippocampal CA1 layer (Fig. 2B and Fig. S2 B and C) of Cdh1 cKO mice. Cdh1 knockdown (siCdh1) reduced the dendrite length of primary cortical neurons (Fig. S2D). Furthermore, the dendrite disruption in Cdh1 cKO mice at 120 d (Fig. 2A and B and Fig. S2C) was not observed in the cerebellum (Fig. S2F), where Cdh1 levels were unchanged (Fig. 1D and Fig. S2F). In addition, dendritic complexity (Fig. S2G) of cortical pyramidal neurons was greatly reduced in Cdh1 cKO mice. Thus, loss of Cdh1 triggers dendrite disruption and reduces dendrite arborization, suggesting that Cdh1 is essential for dendritic network integrity and stability in the adult brain.

Golgi impregnation analyses revealed that Cdh1 cKO mice displayed lower spine density than controls (Fig. 2C and Fig. S2H). Moreover, the presynaptic proteins vesicular glutamate transporter 1 (VGlut1) and synaptotagmin 1 (SYT1) and postsynaptic markers glutamate receptor subunit NR2B and postsynaptic density protein 95 (PSD95) were strongly reduced in the cortex and hippocampus of Cdh1 cKO mice, indicating synapse loss (Fig. 2D and Fig. S2I).

To evaluate the functionality of the neural pathway integrity, we recorded cortical electrical activity in the left hemisphere of mice after sciatric stimulation (17). A marked decrease in the amplitude of evoked potentials but not in latency was observed (Fig. 2F), reminiscent of the dysfunctional neural network connectivity that is observed during neurodegeneration (17). In agreement with this, Cdh1 deficiency induced neuronal apoptosis (Fig. S3 A and B).

Thus, Cdh1 deficiency in pyramidal neurons disrupts the dendritic network, leading to dendritic spine and synapse loss, impaired functional brain connectivity, and neurodegeneration.

Next, we assessed whether Cdh1 cKO mice show impaired learning, memory, cognition, and anxiety. We observed no differences in motor coordination (Fig. S4A), but learning and memory, as judged by validated tests (18), were impaired in Cdh1 cKO mice (Fig. S4 B and C). These results indicate that Cdh1 loss in the adult cortex and hippocampus triggers learning and spatial memory deficits. Because psychiatric disorders and dementia include anxiety (19), we next performed tests (20, 21) and found that Cdh1 cKO mice showed impaired locomotion/exploratory activity and higher levels of anxiety (Fig. S4 D and E). Altogether, these data indicate that Cdh1 depletion in pyramidal neurons of the adult brain impaired hippocampus-dependent spatial learning and memory, reduced locomotion and exploration activities, and increased levels of anxiety, all of which are consistent clinical signs of psychiatric diseases and AD (2, 3). Thus, Cdh1 loss-mediated dendrite arbor disruption in the adult brain may be involved in the pathogenesis of these neurological disorders.
Nucleus-cytosol fractionation of primary cortical neurons revealed that Rock2 is present in the nucleus and, more abundantly, in the cytosol; however, Rock1 is exclusively present in the cytosol (Fig. S5E). Interestingly, APC3 and Cdh1 were found in the nucleus, but not in the cytosol (Fig. S5E). The modestly shifted band of Cdh1 in the cytosol (Fig. S5E) likely reflects a hyperphosphorylated—inactive—form of Cdh1 (27). Furthermore, APC3 immunoprecipitation in the nuclear and cytosolic neuronal fractions, followed by immunoblotting against Rock1 and Rock2, revealed that the interaction between APC3 and Rock2 only occurred in the nucleus; however, no interaction between APC3 and Rock1 occurred either in the nucleus or the cytosol (Fig. S5E). Together, these data indicate that, at least in primary neurons and in the in vivo brain, Rock2, but not Rock1, is an APC/C\(^{C_{\text{ub}}}{\text{h}}\) substrate.

To ascertain whether APC/C\(^{C_{\text{ub}}}{\text{h}}\) targets Rock2 protein for degradation, cortical and hippocampal slices from wild-type mice were first incubated in the presence of the proteasome inhibitor MG132, which resulted in Rock2 protein accumulation (Fig. S5F), indicating that Rock2 normally undergoes proteolysis. Rock2 immunoprecipitation confirmed substantial Rock2 ubiquitination in the hippocampus and cortex of wild-type mice; however, the levels of ubiquitinated Rock2 were significantly lower in cKO Cdh1 mice (Fig. 3C). To confirm Rock2 ubiquitination, we then performed in-cell (HEK293T) ubiquitination assays. Rock2 showed a smeared pattern of ubiquitinated bands that was substantially attenuated in the presence of proTAME (Fig. 3D). Expression of mutant Rock2, in which the KEN motif was substituted by an AAA sequence (Rock2-mut), disrupted the interaction of Rock2 with Cdh1 (Fig. 3F) and attenuated the smeared pattern of ubiquitinated bands in immunoprecipitated Rock2 (Fig. 3F), indicating the direct participation of the KEN box in Rock2 interaction with Cdh1. Altogether, these data demonstrate that, by recognizing the KEN box, APC/C\(^{C_{\text{ub}}}{\text{h}}\) ubiquitates Rock2, targeting it for proteasomal degradation.

### APC/C\(^{C_{\text{ub}}}{\text{h}}\) Controls Dendritic Network Integrity via the Regulation of Rock2 Activity

Given that APC/C\(^{C_{\text{ub}}}{\text{h}}\) regulates Rock2 levels and activity, we next were prompted to investigate whether the control of dendritic network integrity by APC/C\(^{C_{\text{ub}}}{\text{h}}\) occurred via Rock2 activity. First, we aimed to ascertain whether the increased protein levels of Rock in the brain of Cdh1 cKO mice correlated with Rock2 activity, as measured by its ability to specifically phosphorylate Thr853 of myosin phosphatase myosin-binding subunit (MBS) (23). As shown in Fig. S5G, neuronal Cdh1 loss triggered Rock2 activation in the hippocampus and cortex of the adult brain, indicating that APC/C\(^{C_{\text{ub}}}{\text{h}}\) regulates Rock2 levels and activity in the adult brain. Next, we took advantage of fasudil, a clinically approved drug that, by inhibiting Rock activity, improves the clinical outcome of ischemic stroke patients (28). We found that i.p. administration of fasudil for 2 mo starting at postnatal day 30 strongly inhibited Rock2 activity in the cortex and hippocampus, as judged by its ability to fully prevent Thr853 MBS phosphorylation (Fig. S5G).

Immunostaining against the neuronal markers NeuN and Map2 of brain sections of Cdh1 cKO mice revealed that fasudil treatment partially rescued the reduced thickness and neuronal number of the cerebral cortex (Fig. 4A and C and Fig. S6A) and hippocampal CA1 layer (Fig. 4B and D and Fig. S6A). Furthermore, fasudil prevented Cdh1 depletion-induced dendrite disruption in both the cortex and CA1 layer of the hippocampus (Fig. 4E and Fig. S6B) of the adult brain, as revealed by Map2 immunostaining. To confirm these results using a more sensitive technique, Cdh1 cKO mice were cross-bred with mice expressing YFP as a volume label in pyramidal neurons of the hippocampus and in layer 5 of the cerebral cortex (29). Immunostaining against GFP confirmed the dendrite disruption and neuronal loss in the cortex (layer 5) and hippocampus (CA1 layer) (Fig. 4F and Fig. S6C) of Cdh1 cKO mice, which were partially prevented by fasudil. Finally, we observed that fasudil also prevented dendritic spine loss in pyramidal neurons in the cortex of Cdh1 cKO mice (Fig. 4G and Fig. S6D). Interestingly, we noticed...
that fasudil caused a slight loss of neurons in control mice, as revealed by GFP staining in the cortex and hippocampus (Fig. 4F and Fig. S6C), likely reflecting that trace amounts of Rock activity are essential for optimal neural structure integrity. In addition, it should be noted that fasudil inhibits both Rock1 and Rock2 activities. However, selective silencing of Rock2 (Fig. S5A and Fig. S6F) abolished the neurite disruption observed in Cdh1–knocked-down primary neurons (Fig. S6E), as judged by quantification of the average neurite length (Fig. S6F) and number (Fig. S6G) per neuron. This suggests that the impact of Rock1, which is present in Rock2-silenced neurons (Fig. S5A), on Cdh1 loss-mediated neurite disruption is negligible, at least in primary neurons. In contrast, selective silencing of Rock1 (Fig. S5A) had no effect on Cdh1 loss-mediated neurite disruption (Fig. S6E–G). Thus, although we cannot unambiguously disregard a possible effect of Rock1 inhibition by fasudil in vivo, our data suggest that its effect on dendritic integrity is specifically mediated by Rock2 inhibition. Altogether, our data indicate that Cdh1 depletion in pyramidal neurons of the adult brain increases Rock2 protein and activity, leading to dendrite disruption, dendritic spine loss, and neurodegeneration in the adult brain. Interestingly, fasudil mitigated the locomotion/exploratory activity impairment and attenuated anxiety caused by Cdh1 loss (Fig. 5A and B and Fig. S6H), as well as improved the learning and memory impairment caused by Cdh1 loss (Fig. 5C). It should be noted that fasudil slightly worsened locomotion and anxiety (Fig. 5A and B and Fig. S6H) and learning and memory (Fig. 5C) in control mice, confirming the importance of baseline Rock activity for neural integrity and function. Together, our data indicate that depletion of Cdh1 in pyramidal neurons increases Rock2 protein levels and activity in adulthood, causing dendrite network disruption, anxiety, and impaired learning and memory.

Discussion

We describe a signaling pathway in which the E3 ubiquitin ligase APC/C-Cdh1 controls the stability and integrity of neuronal dendrites in the adult brain by destabilizing the Rho kinase protein Rock2. Thus, knockout of Cdh1, specifically in the pyramidal neurons of the cerebral cortex and hippocampus, induces dendrite structure disruption and dendritic spine and synapse loss, resulting in impaired neuronal connectivity, deficits in learning and memory, and neurodegeneration. Furthermore, we identify that the mediator of dendrite destabilization, Rock2, is an APC/C-Cdh1 substrate in vivo by direct interaction between Cdh1 and Rock2 through its KEN box. Thereby, depletion of Cdh1 in pyramidal neurons triggers accumulation and activation of Rock2 in the affected areas of the adult brain. Finally, administration of the clinically approved Rock inhibitor fasudil prevents dendrite disruption, impaired learning and memory, and neuronal loss induced by Cdh1 knock-out. Our results therefore demonstrate an APC/C-Cdh1-Rock2 signaling pathway that regulates structural and functional integrity of the dendritic network in the adult forebrain.
Interestingly, at postnatal day 25, Cdh1 cKO mice exhibited normal, mature dendrite structure and branching in cortical and hippocampal CA1 pyramidal neurons. This contrasts with the reduced cortical size and microcephaly phenotype upon deletion of Cdh1 seen at embryonic stages (12). Thus, in the developing brain, the stabilization of dendrites critically depends on synapse formation, whereas in the mature nervous system, dendritic network stability depends on the microtubule cytoskeleton (1). Nevertheless, the normal dendritic structure observed in Cdh1 cKO mice at postnatal day 25 was not maintained afterward, when dendrite disruption, loss of dendritic branches, and dysfunction of neural network connectivity occurred. These changes caused a thinning of anatomical layers in the cortex and hippocampus in adult mice, as it has been reported that dendrite disruption and reduced dendritic arbor complexity determine brain size in adult animals (16). Furthermore, the reduction in dendritic spine density and synapses altered synaptic connectivity that, in the hippocampus, likely contributed to the anxiety and impaired learning, cognition, and memory phenotypes that recapitulate the alterations seen in a variety of psychiatric and neurodegenerative disorders (2, 3). Thus, APC/C\(^{\alphaCdh1}\) maintains structural and functional integrity of dendritic networks, suggesting that Cdh1 is important for the molecular pathogenesis of memory disorders.

The identification of the microtubule-stabilizing protein Rock2 as an APC/C\(^{\alphaCdh1}\) substrate may have important implications for our understanding of the mechanism behind dendrite stability in the adult brain. Dendritic microtubules are enriched in microtubule-associated protein Map2, which promotes microtubule polymerization and dendrite arbor stabilization (1, 30, 31). Even though Rock2 disrupts dendrite architecture through several possible mechanisms, it is known that loss of Map2-mediated microtubule instability is an important contributing factor in Rock2-induced dendrite arbor disruption (1, 5). Thus, Rock2 phosphorylates Map2 at Ser1796 (32), a critical residue at the microtubule-binding region, thus reducing its ability to bind microtubules for correct assembly (32, 33). We show that conditional KO of Cdh1 promoted an age-dependent reduction in Rock2 levels, thus correlating with Map2 loss and dendrite disruption. Notably, reduction of dendritic arbor complexity and loss of dendritic spines were prevented by administration of the Rock2 inhibitor fasudil to Cdh1 cKO mice. Therefore, we can conclude that APC/C\(^{\alphaCdh1}\) promotes the degradation of Rock2 to ensure proper stability of dendrites and maintenance of the dendritic cytoskeleton in the adult brain.

Plasticity of neural circuits relies on dynamic changes in the cytoskeleton of dendritic spines (34), in which Rock2 plays a pivotal role. The RhoA-Rock2 pathway mediates remodeling of dendritic spine morphology and density downstream of glutamate receptor activation (35, 36). Therefore, the APC/C\(^{\alphaCdh1}\)-Rock2 signaling pathway is consistent with the previously described effect of APC/C\(^{\alphaCdh1}\) on synaptic plasticity, including long-term potentiation, mGlurR-dependent long-term depression, and homeostatic synaptic plasticity (10, 37–39).

Human neuropathology data reveal that dendritic defects in AD—including dendrite disruption, reduced arbor complexity, and loss of spines—are widespread and occur early in the disease (40). Interestingly, we have observed these features in Cdh1 cKO mice. It has also been suggested that the pathological outcome of neurodegenerative conditions is not necessarily originated by neuronal loss but by subtle changes in dendrites and spines and/or synapses that limit neuronal functionality within the network (3). Moreover, the gradual loss of microtubule mass in neurons is thought to occur through destabilization and depolymerization of microtubules, leading to dendrite disruption (41). Our findings that APC/C\(^{\alphaCdh1}\) controls the stability of Rock2 in neurons support this notion, and identify a potential therapeutic target against AD. Interestingly, although no effective therapy is known to combat the progression of AD, studies in animal models provide strong evidence that maintaining dendritic network integrity may ease the symptoms and slow down disease progression (3, 42). Furthermore, Rock2 levels increase in the very early stages of AD and remain elevated throughout the course of the disease (43), and Rock2 inhibition reduces amyloid-\(\beta\) levels (43, 44) and attenuates amyloid-\(\beta\)-induced neurodegeneration (45). Therefore, the reduction in dendrite disruption, and the improvement in learning and memory observed after fasudil administration to Cdh1 cKO mice, further supports the notion that this clinically approved Rock inhibitor drug should be considered as a drug to treat AD.

In conclusion, here we describe an APC/C\(^{\alphaCdh1}\)-Rock2 signaling pathway that regulates structural and functional integrity of the dendritic network in the adult forebrain, which may have important implications for the pathology of psychiatric and neurodegenerative disorders. Importantly, we have previously described that glutamate receptor overactivation, a hallmark of neurodegenerative diseases, promotes Cdk5-induced Cdh1 phosphorylation (9, 27) and inactivation, which may explain the accumulation of Rock2 that has been found in these disorders (43). Therefore, pharmacological inhibition of aberrantly accumulated Rock2 with fasudil treatment may be a suitable therapeutic strategy against these neurological diseases. Beyond neural tissue, it is known that Rock activity, through its actions on cytoskeletal dynamics, promotes tumor cell invasion and metastasis (46, 47). Whether the APC/C\(^{\alphaCdh1}\)-Rock2 pathway also represents a new mechanism in cancer progression is a tempting possibility that remains to be elucidated.

Materials and Methods

**Camk2a\^{lox/lox}\text{-Mediated Cdh1 Conditional Knockout Mice.** The Cdh1\^{lox/lox}\_transgenic mouse model (13) was crossed with transgenic mice carrying the gene encoding Cre recombinase under the control of the Camk2a\^{cre} promoter (14) (**SI Materials and Methods**).

All animals were bred and maintained at the Animal Experimentation Service of the University of Salamanca in accordance with Spanish legislation (RD 53/2013). Procedures and protocols have been approved by the research Bioethics Committee of the University of Salamanca.

**Cell cultures and transfections.** Primary cultures of cortical neurons (12) and transfections (27) were prepared as previously described (**SI Materials and Methods**). **Communoprecipitation assay.** Immunoprecipitation of endogenous (39) and exogenous (27) proteins was performed as previously described (**SI Materials and Methods**).

**Rock2 ubiquitination assay.** Tissue slices were incubated with MG132 (20 \(\mu\)M) for 1 h, and cortex and hippocampus were micro dissected, lysed, and immunoprecipitated with anti-Rock2 antibody, followed by SDS-PAGE and immunoblotting with anti-ubiquitin antibody (39) (**SI Materials and Methods**).

**Immunohistochemistry.** Immunohistochemistry was performed according to a previously published protocol (48) (**SI Materials and Methods**).

**Neuronal counting.** Brain areas of interest were identified as in Paxinos and Watson (49) in NeuN-stained 40-\(\mu\)m-thick sections and used for cell counting by an author blinded to genotype and treatment (50, 51) (**SI Materials and Methods**).

**Behavioral studies.** One hundred and twenty-day-old mice were handled for 3 d to acclimate them to the experimenter before subjecting them to the experimental procedures. All behavioral procedures were video-recorded and scored by an individual blind to the genotype of the mouse (**SI Materials and Methods**).

**Statistical Analysis.** The results are expressed as means \(\pm\) SEM. A one-way ANOVA followed by the Bonferroni post hoc test was used for pairwise comparisons within multiple samples. The Student’s \(t\) test was used to compare the means of two independent groups. In all cases, \(P\) values < 0.05 were considered significant. Statistical analysis was performed using SPSS Statistics 22.0 for Macintosh.

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Supporting Information

SI Materials and Methods

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CamKIIalpha-Cre–Mediated Cdh1 Conditional Knockout Mice. The Cdh1-loxP-loxP-targeted mouse model has been described previously (13). For the conditional inactivation of Cdh1 in glutamatergic pyramidal neurons of the cortex and hippocampus of the adult brain, we used transgenic mice carrying the gene encoding Cre recombinase under the control of the CamK2α-Cre promoter [B6.Cg-Tg(Camk2α-cre)T29-1Stl/J; The Jackson Laboratory] (14). To generate Cdh1flp+/flpCdh1α cre (referred to as Cdh1 cKO) and Cdh1flp/flpPAPα cre control mice, mice harboring a Cdh1flp+/flpPAPα cre were crossed with Thy1-YFP mice. When indicated (control-YFP and Cdh1 cKO-YFP), double-transgenic mice Cdh1flp+/flpPAPα cre were crossed with Thy1-YFP, control mice, and recombination was analyzed. Cell cultures and transfections. Primary cultures of cortical neurons were prepared from embryonic day 14.5 mouse embryo cortices and incubated at 37 °C in a humidified 5% CO2-containing atmosphere. Neurons grown on glass coverslips were fixed with 4% (vol/vol; in PBS) paraformaldehyde for 7 min and quenched with 1.25 M glycine/PBS. The supernatants were collected and stored at −80 °C until use. The amount of protein in each sample was measured using the BCA Protein Assay Kit (Pierce, Thermo Scientific).

Western blotting. Aliquots of tissue lysates (40 to 60 μg protein) were subjected to SDS/PAGE on 6, 8, 10, or 15% acrylamide gels (Mini Protean; Bio-Rad) including BenchMark (Invitrogen, Thermo Scientific) or Dual Color Standards (Bio-Rad) as prestained protein markers. The resolved proteins were transferred electrophotoherically to nitrocellulose membranes (Hybond ECL, GE Healthcare Life Sciences). Membranes were blocked with 5% (wt/vol) low-fat milk in 20 mM Tris, 500 mM NaCl, and 0.1% (wt/vol) Tween 20 (pH 7.5) for 1 h. After blocking, membranes were immunoblotted with anti-Cdh1 (AR38; a gift from J. Gannon, Cancer Research UK, London), anti-GAPDH (6C5; Ambion), anti-VGlut1 (317G6; Synaptic Systems), anti-SYT (Synaptic Systems), anti-PSD95 (6G6-1C9; Affinity BioReagents), anti-NR2B (a gift from A. Fernández-López, University of León, León, Spain), anti-NeuN (A-60; Merck Millipore), anti-MAP-2 (AP-20; Sigma-Aldrich), anti-Tuj1 (Abcam), anti-active caspase-3 (Asp175; Cell Signaling Technology), anti-RhoA (26C4; Santa Cruz Biotechnology), anti-Rock1 (H-85; Santa Cruz Biotechnology), anti-Rock1 (H-85; Santa Cruz Biotechnology), anti-Rock1-2 (85–22C7; BD Pharmingen, BD Biosciences), anti-ubiquitin (Abcam), anti-hemaggulutinin (HA; 2-2.2.14; Thermo Scientific), anti-myc (Sigma-Aldrich), anti-MBS (BioLegend), anti-phospho(mSer853)-MBS (MyBioSource), and anti-GFP (Sigma-Aldrich) antibodies, at dilutions ranging from 1:500 to 1:1,000, overnight at 4 °C. GAPDH was used as loading control. After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Thermo Scientific) or goat anti-mouse IgG (Bio-Rad), membranes were immediately incubated with enhanced chemiluminescence SuperSignal West Dura (Pierce) for 5 min before exposure to Kodak X-AR-5 film for 1 to 5 min, and the autoradiograms were scanned. Band intensities were quantified using ImageJ software (27).

Coimmunoprecipitation assay. For immunoprecipitation of endogenous proteins, tissue slices were first cross-linked with 1.2% formaldehyde for 7 min and quenched with 1.25 M glycine/PBS. The cortex (Cx) and hippocampus (Hy) were microdissected and lysed in ice-cold lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40, supplemented with the phosphatase and protease inhibitors cited above). Lysates were incubated with anti-APC3 (1 μg) for 4 h at 4 °C, followed by the addition of 30 μL protein G Sepharose (GE Healthcare Life Sciences) for 2 h at 4 °C.
Immunoprecipitates were extensively washed with lysis buffer and detected by Western blotting analysis (39).

For immunoprecipitation of exogenous proteins, 293T cells were transfected with plasmids encoding Cdh1 fused to HA (HA-Cdh1) (11) and wild-type Rock2 fused to myc (Myc-Rock2-wt; a gift from M. Olson, Beatson Institute for Cancer Research, Glasgow, United Kingdom) or KEN box-mutated Rock2 fused to myc (Myc-Rock2-mut) using Lipofectamine 2000 (Invitrogen). Cells were cross-linked and lysed as described above. Lysates were incubated with anti-HA agarose beads (Invitrogen) for 2 h at 4 °C and extensively washed with lysis buffer. Immunoprecipitated proteins were detected by Western blot analysis (27).

**Rock2 ubiquitination assay.** Tissues slices were incubated with MG132 (20 μM) for 1 h, and cortex and hippocampus were microdissected and lysed in buffer containing 0.1% SDS, 1% Nonidet P-40, 0.5% Na-deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 40 mM N-ethylmaleimide, and 1 mM DTT, supplemented with the phosphatase and protease inhibitors cited above (39). Immunoprecipitation of endogenous Rock2 was performed with anti-Rock2 antibody, followed by immunoblotting with anti-ubiquitin antibody.

HEK293T cells were cotransfected with HA-ubiquitin (HA-Ub) and Myc-Rock2-wt or Myc-Rock2-mut (KEN box mutated to AAA) for 24 h. Cells were pretreated with MG132 (20 μM) for 2 h either in FBS-containing medium or presence of the APC/C inhibitor proTAME (10 μM) and immunoprecipitated with anti-Myc agarose beads followed by immunoblotting with Myc and HA antibodies (39).

**Immunohistochemistry.** Animals were deeply anesthetized by i.p. injection of a mixture (1:4) of xylazine hydrochloride (Rompun; Bayer) and ketamine hydrochloride/chlorbutol (Imalgene; Merial) with 1 mL of the mixture per kg of body weight, and then perfused intraaortically with 0.9% NaCl followed by 5 mL/g body weight of Somogy’s fixative [4% wt/vol paraformaldehyde, 0.2% wt/vol picric acid in 0.1 M phosphate buffer, pH 7.4]. After perfusion, brains were dissected out sagitally in two parts and postfixed, using Somogy’s fixative, overnight at 4 °C. Brain blocks were then rinsed successively for 10 min, 30 min, and 2 h with 0.1 M phosphate buffer solution (PBS; pH 7.4) and sequentially immersed in 10, 20, and 30% (wt/vol) sucrose in PBS until they sank. After these counting frame areas, we first established to sample 250 sites per brain region per animal to give a coefficient of error (CE) <0.1 using the smoothness factor m = 1. The total number of neurons (N) was defined as V_{ref}×N_{ref} (50). These analyses were carried out using a Leica TSC-SL microscope equipped with a 4× objective lens (for V_{ref}) and a 60× objective lens with a 1.4 numerical aperture condenser (for N_{ref}) and NIS-Elements AR software (version 4.20.00; Nikon). The microscope was also equipped with a motor-driven stage to move within the x and y axes and an attached microcator to determine the z axis.

Neuron number was also quantified using the isotropic fractionator method (51). The cortex and CA1 layer of the hippocampus were fixed and disrupted by homogenization. The nuclear suspension obtained after centrifugation (4,000 × g) was collected and immunostained with NeuN antibody (1:300) followed by incubation with the secondary antibody for 3 h at room temperature. Aliquots from the nuclear suspension were introduced into a hemocytometer (Neubauer chamber), and the average neuronal nucleus (NeuN^+) density was quantified using a fluorescence microscope (Provis AX70; Olympus).

**Golgi staining.** Animals were deeply anesthetized and intraaortically perfused as described above. Brains were incubated in a dichromate Colomniier solution (3% K₂Cr₂O₇ (wt/vol), 3% glacial acetic acid (vol/vol)) for 7 d followed by cleaning with distilled water, and then impregnated by immersion in 0.75% AgNO₃ (wt/vol) for 3 d. Coronal sections (100 μm) were obtained using a vibratome and mounted onto slides and coverslipped with an Entellan solution (Merck Millipore). Golgi staining was quantified using ImageJ. Grayscale 8-bit images were acquired using a microscope (Nikon; inverted microscope) equipped with a B/W CCD digital camera (Hamamatsu). Images were exported into ImageJ in tiff format and brightness/contrast was adjusted using the ImageJ “auto” function. Golgi staining was thresholded using the “auto setting threshold” (default method) function and subsequently quantified as percent area (area fraction) using the “analyze-measure” function, which represents the percentage of pixels in the image that have been

CA1 layer, using the NIH image-processing package ImageJ (version 1.47). Values are mean ± SEM from 20 measurements from four different animals (n = 4).

The degeneration of dendrites in the cortex and CA1 layer of the hippocampus was assayed by analyzing the density of Map2-positive dendrites in three sections per animal. Fluorescence 8-bit images were acquired as z stacks using an HCX Plan Apo CS2 63x oil objective and an inverted confocal microscope. Images were exported into ImageJ in tiff format for processing. Before image analysis, a maximum-intensity projection over z-series projections spanning 18 to 19 μm was performed. Images were converted to grayscale 8-bit images and brightness/contrast was adjusted using the ImageJ “auto” function. All Map2-positive dendrites were automatically delineated using the “auto setting threshold” (default method) and “dark background” functions of ImageJ. Thresholded images were subsequently quantified as percent area (area fraction) using the “analyze-measure” function, which represents the percentage of pixels in the image that have been highlighted (% area) (53). Values are mean ± SEM from 20 measurements from four different animals (n = 4).

**Stereological counting.** Areas of interest in the cortex and hippocampal CA1 layer were identified as in Paxinos and Watson (49) in NeuN-stained 40-μm-thick sections and used for cell counting by an author blinded to genotype and treatment. Neurons (NeuN^+) cells were quantified using the Cavalieri method—which determines the reference volume contained in the cells of interest (V_{ref})—and the optical dissector method—which determines the density of neurons (i.e., the number of NeuN^+ cells per μm^3) within V_{ref} (N_{ref}). V_{ref} was determined every 6th section, and N_{ref} every 12th section. The size of the counting frame was 50 μm × 50 μm (cortex) or 25 μm × 25 μm (hippocampal CA1 layer) in a dissector height of 12 μm. With these counting frame areas, we first established to sample ~200 to 250 sites per brain region per animal to give a coefficient of error (CE) <0.1 using the smoothness factor m = 1. The total number of neurons (N) was defined as V_{ref}×N_{ref} (50). These analyses were carried out using a Leica TSC-SL microscope equipped with a 4× objective lens (for V_{ref}) and a 60× objective lens with a 1.4 numerical aperture condenser (for N_{ref}) and NIS-Elements AR software (version 4.20.00; Nikon). The microscope was also equipped with a motor-driven stage to move within the x and y axes and an attached microcator to determine the z axis.

Neuron number was also quantified using the isotropic fractionator method (51). The cortex and CA1 layer of the hippocampus were fixed and disrupted by homogenization. The nuclear suspension obtained after centrifugation (4,000 × g) was collected and immunostained with NeuN antibody (1:300) followed by incubation with the secondary antibody for 3 h at room temperature. Aliquots from the nuclear suspension were introduced into a hemocytometer (Neubauer chamber), and the average neuronal nucleus (NeuN^+) density was quantified using a fluorescence microscope (Provis AX70; Olympus).
highlighted (% area) (53). Values are mean ± SEM from 15 measurements from three different animals (n = 3).

**Terminal deoxynucleotidyl transferase dUTP nick end-labeling assay.** TUNEL assay was performed in brain sections following the manufacturer’s protocol (Roche Diagnostics). Brain sections, fixed as above, were preincubated in TUNEL buffer containing 1 nM CoCl2, 140 mM sodium cacodylate, and 0.5% Triton X-100 in 30 mM Tris buffer (pH 7.2) for 30 min. After incubation at 37°C with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (800 U/mL) and nucleotide mixture (1 µM) for 90 min, sections were rinsed with PBS and counterstained with Cy3-streptavidin (Jackson ImmunoResearch) (48).

**Electrophysiological measurements.** One hundred and twenty-day-old mice were anesthetized and placed in a mouse stereotaxic frame (model 1900; Kopf) with a digital coordinate readout system (Wizard 550 Readouts; Anilam). Once the surgical area was disinfect, a small cranial Burr hole was drilled (model 1911; Kopf) with a 0.50-mm drill bit through the skull over the left primary motor cortex (1.98 mm anterior and +2.0 mm lateral to bregma), where the recording concentric tungsten electrode (1 Mohm; Hz; World Precision Instruments) was placed. The sciatic nerve in the contralateral paw was dissected to place a self-building stimulation bipolar electrode. We recorded both spontaneously, to record the cortical activity over 1 min and the evoked potential after stimulation of the sciatic nerve. Square-wave pulses of 0.1 nA and 0.1 ms with a frequency of 0.5 Hz were administered by using a stimulator (Master-8; AMPI Equipment) and an isolator (ISO-Flex; AMPI Equipment) at the sciatic nerve with the appropriate stimulation electrode. At the same time, cortical activity was recorded, from 60 to 90 stimulation cycles. Digital treatment of the signal was identical in both cases: an initial band-pass filter between 0.3 Hz and 10 kHz and analog-to-digital conversion with a sampling frequency of 6,250 Hz with an interface (Power1401-Plus; CED Products). A minimum of 30 sweeps was averaged out to obtain evoked potentials. Amplitudes and latencies of evoked potentials were obtained from the averages of recordings. Latencies were calculated as the time elapsed between the onset of stimulus and the peak of the evoked response, whereas amplitude was measured as the waves’ peak-to-peak voltage difference. Latency mainly reflects nerve demyelination in the pathway and amplitude correlates with neurite and neural pathway integrity (17).

**Behavioral studies.** One hundred and twenty-day-old mice were handled for 3 d to acclimate them to the experimenter before subjecting them to the experimental procedures. Mice were placed in the experiment room at least 60 min before beginning any behavioral protocol. Unless indicated otherwise, all experimental environments were thoroughly cleaned with 70 and 30% ethanol between trials and allowed to dry. All behavioral procedures were video-recorded and scored by an individual blind to the genotypic of the mouse.

**Rotarod test.** Motor balance and coordination were analyzed using the rotarod test. Mice were trained for 3 d. All determinations were carried out at the same time each day and the test was performed during a four-trial testing session. Mice were allowed to stay for 300 s on a five-lane accelerating rotating rod (model 47600; Ugo Basile) with a continuous accelerating rotation speed from 4 to 40 rpm, increasing 4 rpm every 30 s and reaching a final speed at 270 s. The latency to fall off the rotarod was measured during this period, annotating the time the animal stayed on the rotating rod. Data from tests in which animals completed three turns without walking were disregarded (48).

**Lashley III maze test.** Animals were tested in the Lashley III maze for analysis of learning and memory (54). This maze consists of a start box, four interconnected alleys, and a goal box. On 3 successive days, animals received a day of acclimation to adapt to the maze, followed by 2 training days. On the acclimation day, each mouse was confined in each of the first two alleys of the maze for 4 min, and in the final alley (close to the goal box) for 6 min. On the training day, each animal was placed in the start box and allowed to freely navigate the maze. To initiate testing, each animal was picked up by the tail and placed in the start box of the maze. Each animal’s home cage was placed at the end of the goal box, with the acrylic door raised so that the animal could enter it after traversing the maze. The trial was initiated by raising the start box door when an animal approached it. The trial was finished when the animal entered its home cage and then the door was lowered and the home cage was returned to the cart. Latency to enter the goal box and number of errors (a wrong turn or a retracing of the animal’s pathway was considered an error) were recorded. The frequencies of defection were also recorded. Mice were given one trial per week for 11 weeks. Some mice were sacrificed 4 d after the final training session in order to analyze the histological changes in the brain. The results are expressed as means ± SEM. A one-way ANOVA followed by the Bonferroni post hoc test was used for pairwise comparisons within multiple samples. The Student’s t test was used to compare the means of two independent groups. In all cases, P values < 0.05 were considered significant. Statistical analysis was performed using SPSS Statistics 22.0 for Macintosh.
Conditional knockout of Cdh1 from the adult forebrain reduced neuronal number in the cerebral cortex and CA1 hippocampal layer. 

(A) Cdh1 depletion in the adult forebrain did not modify body weight. 

(B and C) The cortex (B) and CA1 layer of the hippocampus (C) were thinner in the Cdh1 cKO from 120 d of age, compared with age-matched control mice. 

(D) Stereological counting of neurons (NeuN+ cells) revealed that Cdh1 ablation reduced neuron number in both the cortex and hippocampal CA1 layer (reference volume: control: cortex, 13.79 mm³; CA1, 10.9 \times 10^{-2} \text{ mm}³; Cdh1 cKO: cortex, 8.51 mm³; CA1, 6.39 \times 10^{-2} \text{ mm}³). Data are expressed as mean ± SEM. 

(A–C) *P < 0.05 versus age-matched control mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 6 mice per group). 

(D) *P < 0.05 versus control mice (Student's t test; n = 4 mice per group).
Fig. S2. Cdh1 depletion induced dendrite disruption in the adult brain. (A and B) Cdh1 loss triggered dendrite disruption in both the cortex (A) and CA1 layer of the hippocampus (B) in 360-d-old mice, as revealed by immunostaining for the dendritic marker Map2. (Scale bars, 50 μm.) (C) Cdh1 depletion caused reduction in dendrite density in the cortex and CA1 layer of the hippocampus of 120-d-old mice, as revealed by quantification of Map2 staining. Representative images are shown in Fig. 2A and B. (D) Quantification of primary dendrite length revealed that silencing of Cdh1 (siCdh1) in primary cortical neurons induced dendrite length reduction and disruption. White arrowheads (Lower) show primary dendrites considered for length measurements. (Scale bars, 10 μm.) (E) Dendrite disruption was not observed in the cerebellum. (Scale bars, 50 μm.) (F) Conditional KO of Cdh1 reduced Map2 and Tuj1 levels in the cortex and hippocampus of 120-d-old mice but not in 25-d-old mice, as revealed by immunoblotting. (G and H) Golgi impregnation of brain sections showed that Cdh1 depletion reduced dendrite density (G) and spine number (H) in the cortex of 120-d-old mice. (Scale bars, 20 μm.) (I) Results of the relative protein abundance quantification of synaptic proteins showed in Fig. 2D. Data are expressed as mean ± SEM. (C, G, and H) *P < 0.05 versus control mice (Student’s t test; n = 3 or 4 mice per group). (D) *P < 0.05 versus siControl (Student’s t test; n = 3 neuronal cultures). (F and I) *P < 0.05 versus 25-d-old control mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 4 mice per group).
Fig. S3. Cdh1 depletion caused neuronal apoptosis in the adult brain. (A) Conditional KO of Cdh1 induced caspase-3 activation in the cortex and hippocampus. *P < 0.05 versus 25-d-old control mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 4 mice per group). (B) Cdh1 depletion induced neuronal apoptosis in the cortex and hippocampus of 120-d-old mice, as showed by TUNEL assay. (Scale bars, 20 μm.) Data are expressed as mean ± SEM. *P < 0.05 versus 25-d-old control mice (Student’s t test; n = 4 mice per group). Cx, cortex; Hy, hippocampus.
Cdh1 deficiency triggers memory and learning deficits and impairs explorative behavior. Behavioral tests were performed in 120-d-old animals. (A) Cdh1 loss did not affect motor coordination, as shown by the performance of Cdh1 cKO and control mice in the accelerated rotarod test. (B) In contrast to control mice, latency to enter the box and the number of errors in the Lashley III maze test did not decrease across trials in Cdh1 cKO mice. (C) Deficiency of Cdh1 increased latency to enter into the eight arms of the radial maze task and prevented the reduction in the number of working and reference memory errors across trials, in comparison with control mice. (D) Representative images showing the explorative behavior of Cdh1 cKO and control mice in the open-field task. Compared with control mice, Cdh1 cKO mice spent less time in the center of the arena and conversely more time in the border area. Total distance moved and number of rears were reduced and time spent immobile (freezing) was increased in Cdh1 cKO mice. (E) Representative images showing the explorative behavior of Cdh1 cKO and control mice in the hole-board test. Conditional KO of Cdh1 reduced the distance moved and number of head dips into holes. Data are expressed as mean ± SEM; *P < 0.05 versus control mice [(A–C) one-way ANOVA followed by the Bonferroni post hoc test; (D and E) Student’s t test; n = 6 to 8 mice per group].
Fig. S5. Cdh1 knockdown induced neuronal Rock2 accumulation. (A) The specificity of Rock1 and Rock2 antibodies was validated in Rock1− and Rock2-knockdown neurons in primary culture. (B) Results of the relative protein abundance quantification of proteins shown in Fig. 4A. (C) Cdh1 knockdown or inhibition of APC/C activity with proTAME promoted Rock2 accumulation in primary cortical neurons. (D) Cortical and hippocampal slices from control and Cdh1 cKO mice were pretreated with the APC/C inhibitor proTAME (10 μM for 1 h). Immunoblotting analysis revealed that proTAME induced Rock2 accumulation, whereas Rock1 remained unchanged. (E) Nucleus-cytosol fractionation in primary cortical neurons followed by Western blotting revealed that, although Rock2 is present in the nucleus and cytosol, Rock1 is exclusively present in the cytosol. APC3 immunoprecipitation in the nuclear and cytosolic neuronal fractions, followed by immunoblotting against Rock1 and Rock2, revealed that the interaction between APC3 and Rock2 only occurred in the nucleus, but no interaction was found between APC3 and Rock1. (F) Cortical and hippocampal slices from control and Cdh1 cKO mice were pretreated with the proteosomal inhibitor MG132 (20 μM for 1 h). MG132 promoted Rock2 accumulation. (G) The Rock inhibitor fasudil (20 mg/kg) was injected intraperitoneally every other day into 30-d-old mice for 60 d. Rock2 activity was increased in the cortex and hippocampus of 120-d-old Cdh1 cKO mice, as revealed by determination of phospho-MBS levels. Treatment of animals with fasudil blocked Rock2 activity in the cortex and hippocampus. Data are expressed as mean ± SEM [(B) *P < 0.05 versus 25-d-old control mice; one-way ANOVA followed by the Bonferroni post hoc test; n = 4 mice per group; (C) *P < 0.05 versus control neurons; one-way ANOVA followed by the Bonferroni post hoc test, n = 3 neuronal cultures; (D) *P < 0.05 versus control mice; Student’s t test; n = 3 mice per group; (F) *P < 0.05 versus control mice; Student’s t test; n = 3 mice per group]. Cx, cortex; Hy, hippocampus; IB, immunoblotting; IP, immunoprecipitation.
**Fig. S6.** Rock2 knockdown prevented the reduction in neurite length caused by Cdh1 loss. The Rock inhibitor fasudil (20 mg/kg) was injected intraperitoneally every other day into 30-d-old mice for 2 mo. (A) Fasudil treatment prevented the reduced thickness of the cerebral cortex and hippocampal CA1 layer induced by Cdh1 depletion in 120-d-old mice. Representative images are shown in Fig. 4A and B. (B) Fasudil treatment prevented Cdh1 loss-induced dendrite density reduction in the cortex and hippocampal CA1 layer of 120-d-old mice, as revealed by quantification of Map2 staining. Representative images are shown in Fig. 4E. (C) Fasudil treatment prevented Cdh1 loss-induced dendrite disruption in the cortex and CA1 layer of the hippocampus of 120-d-old mice, as revealed by quantification of GFP fluorescence. Representative images are shown in Fig. 4F. au, arbitrary units. (D) Treatment with fasudil prevented dendritic spine loss caused by Cdh1 depletion in cortical pyramidal neurons of 120-d-old mice. (E) Primary cortical neurons were cotransfected with pSuper-neo-gfp-Cdh1 (shCdh1) together with siRock2 or siRock1 for 3 d. After GFP immunostaining, neurites were traced and labeled as primary (emanating directly from the soma), secondary (branching from a primary), or tertiary (branching from a secondary), and the average of primary, secondary, and tertiary neurite length and number per neuron was measured using the NeuronJ plugin of ImageJ. (Scale bars, 25 μm.) (F and G) GFP immunostaining revealed that Rock2 knockdown (siRock2), but not Rock1 knockdown (siRock1), prevented the reduction in the average neurite length (F) and number per neuron (G) induced by Cdh1 silencing. (H) Representative images showing the explorative behavior of mice in the hole-board test. Data are expressed as mean ± SEM; *P < 0.05 versus Cdh1 cKO mice [one-way ANOVA followed by the Bonferroni post hoc test; (A–D) n = 4 to 6 mice per group; (F and G) n = 3 neuronal cultures].