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 Rho protein kinase 2 (Rock2), which accumulates in the brain of AD patients, is an APC/C-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-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.

APC/C-Cdh1 | Rock | dendrite | memory | neurodegeneration

The correct formation and long-term maintenance of the dendritic network are essential for the normal functioning of the brain. In the adult brain, dendrite stability confers mature neurons with the ability to maintain long-term dendritic arbor integrity and integration within networks (1). Loss of dendrite stability is associated with psychiatric disorders and neurodegenerative diseases. Dendritic disruption and loss of dendritic spines and synapses have been reported in schizophrenia and depression, as well as in neurodegenerative conditions, including Alzheimer’s disease (AD), and after an excitotoxic insult during stroke (2, 3). The serine/threonine Rho protein kinase (Rock), an effector of the RhoA GTPase (4), is a central regulator of the microtubule cytoskeleton in neurons. Rock is known to modify the number, morphology, and stability of dendrites in a variety of neuronal cell types, including cortical neurons (1, 5). The overactivation of Rock signaling antagonizes dendrite stability, whereas Rock inhibition promotes microtubule assembly and restores dendritic arbor complexity (6–8). Consistent with these results, Rock has been considered a promising drug target for central nervous system disorders (4). However, the molecular mechanism that regulates Rock abundance and activity in neurological disorders is unknown.

The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit E3 ubiquitin ligase that plays a critical role in controlling both cell-cycle progression and important functions in postmitotic neurons (9, 10). APC/C is activated by two alternative regulatory subunits, namely Cdh1 and Cdc20. In the developing brain, APC/C is involved in the regulation of neuronal differentiation and survival, glial differentiation and migration, axonal growth and patterning, and synapse formation and plasticity (9, 10). In mature neurons, Cdh1 is the main activator of the APC/C ligase (11). However, whereas in brain development the functions of APC/C-Cdh1 are well-understood (9, 10), its potential functions in the adult brain are largely unknown.

Here we describe that conditional knockout (cKO) of Cdh1 in the pyramidal neurons of the cortex and hippocampus of the adult brain induces dendrite arbor and structure disruption and dendritic spine and synapse loss, which results in impaired learning and memory as well as neurodegeneration. We also found that the expression level and biochemical activity of Rock2, but not Rock1, was increased in damaged brain areas of Cdh1 cKO mice. Furthermore, we show that APC/C-Cdh1 targets Rock2, but not Rock1, for degradation in the brain. Administration of the clinically approved Rock inhibitor fasudil to Cdh1 cKO mice prevented dendrite disruption, dendritic spine loss, impaired memory and learning, and neurodegeneration. Together, these data reveal an APC/C-Cdh1-Rock2 pathway that regulates structural stability and functional integrity of dendrites, thus posing Cdh1 as a key molecular factor in the pathogenesis of neurodegenerative disorders.

Results

Cdh1 Deficiency Causes Dendritic Network Disruption and Impaired Neuronal Connectivity, Leading to Memory and Learning Impairment in the Adult Brain. Cdh1 is essential for neurogenesis and cortical size during brain development (12). To study the function of Cdh1 in the adult brain, here we generated Cdh1 cKO mice by mating mice harboring a floxed allele of the Cdh1 gene (13) with CaMKIIα-Cre mice, which express Cre recombinase from the third

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. However, why Rock2 aberrantly aggregates, causing neuronal integrity loss, is unknown. Here, we show that Rock2 protein stability is controlled by the ubiquitin ligase APC/C-Cdh1. Accordingly, APC/C-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-Cdh1-Rock2 pathway may be a novel therapeutic target against neurodegeneration.

Author contributions: A.A. designed research; V.B.-J., M.D.-E., J.A., I.S.-M., A.d.l.F., J.Y., and Angeles Almeida performed research; U.V.N., J.C., J.P.B., and A.A. analyzed data; and A.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/1616024114/DCSupplemental.
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 (Cdh1 cKO 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.) C. 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. S2A and C) and hippocampal CA1 layer (Fig. 2B and Fig. S2 B and C) of Cdh1 cKO mice. Cdh1 knockout (sCdhh1) reduced the dendrite length of primary cortical neurons (Fig. S2D). Furthermore, the dendritic disruption in Cdh1 cKO mice at 120 d (Fig. 2A and B and Fig. S2C) was not observed in the cerebellum (Fig. S2E), where Cdh1 levels were unchanged (Fig. 1A 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 sciatic 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/explorative 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.

![Image](https://example.com/image.png)
also increased Rock2, but not Rock1, protein abundances.

neurons (Fig. S5).

mice, an effect that was correlated with dendrite disruption (Fig. 3).

the cortex and hippocampus of wild-type mice (Fig. 3).

ubiquitination by the ubiquitin ligase APC/CCdh1 (9).

destabilization of Protein Rock2.

of ARC/Cdh1 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.

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, ARC/Cdh1 ubiquitinates Rock2, targeting it for proteasomal degradation.

ARC/Cdh1 Controls Dendritic Network Integrity via the Regulation of Rock2 Activity. Given that ARC/Cdh1 regulates Rock2 levels and activity, we next were prompted to investigate whether the control of dendritic network integrity by ARC/Cdh1 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 phosphor-

ulate Thr583 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 ARC/Cdh1 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 Thr583 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).

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 immunopre-

cipitation 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 ARC/Cdh1 substrate.

Fig. 3. APC/C<sup>Cdh1</sup> triggers the ubiquitination of Rock2. (A) Cdh1 depletion in the cortex and hippocampus did not alter Rheo and Rock1 expression, but age-
dependent decreases in Rock2 levels and decreases in Map2 levels from 120 d of age were seen. (B) Endogenous coimmunoprecipitation analyses revealed that the core subunit of APC/C, APC3, interacts with endogenous Rock2 and Cdh1, but not with Rock1, in the hippocampus and cortex from 120-d-old control mice. (C) Immunoprecipitation of Rock2 followed by immunoblotting with the anti-ubiquitin (Ub) antibody revealed that endogenous Rock2 was ubiquitinated in the hippocampus and cortex from control mice. (D) Lysates of 293T cells transfected with hemagglutinin-ubiquitin (HA-Ub) and wild-type Rock2 (Myc-Rock2-wt) and pretreated with proTAME (10 μM) and MG132 (20 μM) for 2 h were immunoprecipitated with anti-Myc agarose beads followed by immunoblotting with HA and Myc antibodies. (E) 293T cells were transfected with Myc-Rock2-wt or Myc-Rock2-mut (KEN box mutated to AAA), together with HA-Cdh1, and were immunoprecipitated with HA agarose beads followed by immunoblotting with Myc and HA antibodies. Coimmunoprecipitation analysis revealed that Cdh1 formed a complex with wild-type Rock2 but not with the KEN box mutant of Rock2. (F) 293T cells transfected as in E and pretreated with MG132 (20 μM for 2 h) were immunoprecipitated with anti-Myc agarose beads followed by immunoblotting with HA and Myc antibodies. IB, immunoblotting; IP, immunoprecipitation.

**Neuroscience**

**Fig. 3. APC/C<sup>Cdh1</sup> triggers the ubiquitin-Dependent Degradation of the Dendrite-Destabilizing Protein Rock2.** The activation of Rho signaling through Rock plays the role of a central mediator of dendrite destabilization (1, 4, 22). We noticed that both Rock isoforms, Rock1 and Rock2, contain a conserved KEN box motif, which targets proteins for ubiquitination by the ubiquitin ligase APC/C<sup>Cdh1</sup> (9). Hence, we analyzed Rock1 and Rock2 protein levels in the brains of Cdh1cko and wild-type mice using specific and validated antibodies (Fig. S5A) (25). We found both Rock1 and Rock2 proteins to be expressed in the cortex and hippocampus of wild-type mice (Fig. 3A). However, Rock2, but not Rock1, increased at 120 and 360 d in Cdh1 cKO mice, an effect that was correlated with dendrite disruption (Fig. 3D and Fig. S5B). Because this result suggests that Cdh1 may regulate levels of Rock2 but not those of Rock1, we next silenced Cdh1 in primary neurons. siCdh1 caused an increase in Rock2 but not in Rock1 (Fig. S5C). Inhibition of APC/C activity in cortical primary neurons (Fig. S5C) and cortical and hippocampal slices (Fig. S5D) also increased Rock2, but not Rock1, protein abundances.

Next, we ascertained whether Rock2 was regulated by APC/C<sup>Cdh1</sup> activity via ubiquitination and proteasomal degradation. Immunoprecipitation of the APC/C core subunit APC3, in Cdh1 cKO or control mice, followed by immunoblotting against Rock1 or Rock2, revealed that APC/C<sup>Cdh1</sup> forms a complex with Rock2 but not with Rock1 (Fig. 3B). Because the APC/C<sup>Cdh1</sup> recognition motif, the KEN box, is conserved both in Rock1 and Rock2, we next aimed to resolve this apparent paradox. In cancer cells, Rock1 is present in the cytosol and Rock2 both in the nucleus and cytosol (23, 24); however, APC is present only in the nucleus (25, 26).
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 Rock1 (Fig. S5A) had no effect on Cdh1 loss-mediated neurite disruption (Fig. S6A–D), on Cdh1 cKO mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 4 mice per group).

Fig. 4. Rock2 inhibition with fasudil improves dendrite disruption in the Cdh1 cKO cortex and hippocampus. The Rock inhibitor fasudil (20 mg/kg) was injected intraperitoneally every other day into 30-d-old mice for 2 mo. (A and B) Brain sections were immunostained for the neuronal marker NeuN. Fasudil treatment prevented the reduced thickness of the cerebral cortex (A) (white dashed lines mark cortex thickness) and hippocampal CA1 layer (B) induced by Cdh1 depletion in 120-d-old mice. (Scale bars, 100 μm (A) and 200 μm (B); magnification: B, Insets, 20x.) (C and D) The cortex (C) and CA1 layer of the hippocampus (D) from one hemisphere of 120-d-old mice were dissected and the number of neurons was estimated using the isotropic fractionator method. Cdh1 loss induced neuronal loss, which was partially prevented by fasudil treatment. (E) Brain sections were immunostained for the dendritic marker Map2. Fasudil treatment prevented Cdh1 depletion-induced dendrite disruption in the cortex and CA1 layer of the hippocampus. (Scale bars, 20 μm.) (F) Immunostaining for GFP in brain sections of control-YFP and Cdh1 cKO-YFP tripletransgenic mice revealed that Cdh1 depletion triggered neurite disruption and loss of pyramidal neurons in the cortex (layer 5) and hippocampus (CA1 layer), which were partially prevented by fasudil treatment. (Scale bars, 100 μm (layer 5) and 20 μm (CA1 layer).) (G) Golgi impregnation of brain sections showed that fasudil treatment prevented dendritic spine loss caused by Cdh1 depletion in cortical pyramidal neurons of 120-d-old mice. (Scale bars, 10 μm.) Data are expressed as mean ± SEM; *P < 0.05 versus Cdh1 cKO mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 4 mice per group).

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 catalytic domain (Fig. 6A and 6B). Thus, Rock2 signaling pathway that regulates structural and functional integrity of the dendritic network in the adult forebrain.

Fig. 5. Treatment with the Rock2 inhibitor fasudil improves behavioral deficits in Cdh1 cKO mice. Fasudil (20 mg/kg) was injected intraperitoneally every other day into 30-d-old mice for 2 mo. (A) Representative images showing the explorative behavior of mice in the open-field task. Fasudil partially prevented the reduction in total distance moved and number of rears and the increase in time spent immobile (freezing) caused by Cdh1 loss. (B) Cdh1 depletion-mediated reduction in distance moved and number of head dips into holes in the hole-board test was improved by fasudil treatment. (C) Rock2 inhibition by fasudil partially prevented the increase in the latency to enter the goal box (100 cm) and the number of errors across trials in the Lashley III maze test found in cKO Cdh1 mice. Data are expressed as mean ± SEM; *P < 0.05 versus Cdh1 cKO mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 6 mice per group).
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<sup>Cdc10</sup> 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-destabilizing protein Rock2 as an APC/C<sup>Cdc10</sup> 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<sup>Cdc10</sup> promotes the degradation of Rock2 to ensure proper stability of dendrites and maintenance of dendritic cytoskeleton (34) 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 Rhod-A-Rock2 pathway mediates remodeling of dendritic spine morphology and density downstream of glutamate receptor activation (35, 36). Therefore, the APC/C<sup>Cdc10</sup>-Rock2 signaling pathway is consistent with the previously described effect of APC/C<sup>Cdc10</sup> on synaptic plasticity, including long-term potentiation, mGlur-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<sup>Cdc10</sup> 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-β levels (43, 44) and attenuates amyloid-β–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<sup>Cdc10</sup>-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<sup>Cdc10</sup>-Rock2 pathway also represents a new mechanism in cancer progression is a tempting possibility that remains to be elucidated.
Supporting Information

Bobo-Jiménez et al. 10.1073/pnas.1616024114

SI Materials and Methods

CamKIIalpha-Cre–Mediated Cdh1 Conditional Knockout Mice. The Cdh1loxP/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;1Sst/J; The Jackson Laboratory] (14). To generate Cdh1loxFlox/PfpPfpCamk2α-Cre (referred to as Cdh1 cKO) and Cdh1loxFdpFdpCamk2α-Cre control mice, mice harboring a Cdh1loxFdpFdp allele were crossed with Camk2α-Cre mice. When indicated (control-YFP and Cdh1 cKO-YFP), double-transgenic mice Cdh1loxFdpFdpCamk2α-Cre (Cdh1 cKO) were crossed with Thy1-YFP-H [B6.Cg-Tg(Thy1-YFP);H2Jrs/J; The Jackson Laboratory]. The resulting progeny selectively express yellow fluorescent protein (YFP) in pyramidal neurons in the hippocampus and in layer 5 of the cerebral cortex. YFP fills the entire dendritic tree, providing a Golgi-like stain (29). Mice were kept on a C57BL/6J background. Tail DNA was analyzed by PCR using the following primer sets: Cdh1 mutant mice (286 bp amplified), forward primer 5′-AGC-ATGGTGACCGCTTCATCC-3′ and reverse primer 5′-TGGGCTGTTGACCTTCACTTCCC-3′ (13); CamKIIα transgene (150 bp amplified), forward primer 5′-CCGGTATTCACTT-GCCACC-3′ and reverse primer 5′-CTGATTACCGTGCGATACCC-3′ and reverse primer 5′-CCGGTATTCACTTCCC-3′; and Thy1-YFP transgene (415 bp amplified), forward primer 5′-ACACAGACCAACCCAGGACA-3′ and reverse primer 5′-GGGTGTTGAGATGAACTT-3′. PCR products were separated on agarose gels (3%) and Midori Green Advance-stained DNA fragments (Nippon Genetics Europe) were analyzed under a UV source, using the Bio-Rad Universal Hood II Molecular Imager System. All animals were bred and maintained at the Animal Experimental 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.

To inhibit Rock activity, a dosage of 20 mg/kg body weight fasudil (Selleck Chemicals) was injected intraperitoneally every other day into 30-d-old mice. Mice were killed at 60 d after the treatment began. Control mice received saline solution (43).

Cell cultures and transfections. Primary cultures of cortical neurons were prepared from embryonic day 14.5 mouse embryo cortices (12). Cells were seeded at 2.0 × 10^5 cells per cm² in DMEM (Sigma) supplemented with 10% (vol/vol) FCS (Roche Diagnostics) and incubated at 37 °C in a humidified 5% CO₂-containing atmosphere. At 4 h after plating, the medium was replaced with Neurobasal medium (Invitrogen, Thermo Scientific) supplemented with 2% B27 (Invitrogen) and glutamine (4 mM; Invitrogen). At 8 to 9 d in culture, neurons were transfected with plasmids (27) and/or siRNA using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions, and cells were used after 72 h. Specific knockdown of proteins was achieved by using small interfering double-stranded ribonucleic acid (siRNA) to target the coding sequence of the mouse Cdh1 (27), Rock1, and Rock2 (52) mRNA. When indicated, specific knockdown of Cdh1 was performed by using pSuper-neo.gfp (Oligoengine) including the small hairpin sequences for luciferase (shControl) or Cdh1 (shCdh1) (27).

Immunocytochemistry. Neurons grown on glass coverslips were fixed with 4% (vol/vol) in PBS) paraformaldehyde and immunostained with anti-Map2 (1:100; AP-20; Sigma-Aldrich) and anti-GFP (1:1,000; Sigma-Aldrich). Immunolabeling was detected by using Alexa 568-conjugated goat anti-mouse (1:500; Jackson ImmunoResearch) or Alexa 568-conjugated goat anti-rabbit (1:500; Molecular Probes, Invitrogen). Coverslips were washed, mounted in SlowFade Light Antifade Reagent (Invitrogen) on glass slides, and examined using a spectral laser confocal microscope (TSC-SDL; Leica Microsystems). Quantification of the average dendrite length (Map2 staining) and neurite length and number (GFP staining) per neuron was performed using the plugin NeuronJ 1.4.0 (ImageJ, version 1.47; National Institutes of Health). Values are mean ± SEM from 60 neurons per group measured in three different neuronal cultures.

Western blotting. Aliquots of tissue lysates (40 to 60 μg protein) were subjected to SDS/PAGE on 6, 8, 10, or 15% acrylamide gels (MiniPROTEAN; Bio-Rad) including BenchMark (Invitrogen, Thermo Scientific) or Dual Color Standards (Bio-Rad) as prestained protein ladders. The resolved proteins were transferred electrotherophetically 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-Rock2 (H-85; Santa Cruz Biotechnology), anti-Rock1 (H-85; Santa Cruz Biotechnology), anti-APC3 (35/DCDC2; BD Pharmingen, BD Biosciences), anti-ubiquitin (Abcam), anti-hemagglutinin (HA; 2-2.2.14; Thermo Scientific), anti-myc (Sigma-Aldrich), anti-MBS (BioLegend), anti-phospho(Thr853)-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 XAR-5 film for 1 to 5 min, and the autoradiograms were scanned. Band intensities were quantified using ImageJ software (27).

Communoprecipitation 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.

Bobo-Jiménez et al. www.pnas.org/cgi/content/short/1616024114
Immuno precipitates were extensively washed with lysis buffer and detected by Western blotting analysis (39). For immuno precipitation 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. Immuno precipitated 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 the absence 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) using 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 sagittally 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 cryoprotection, 40-μm-thick sagittal sections were obtained with a freezing-sliding cryostate (Leica; CM1950 AgProtect) and collected in 10% sucrose in PBS. Sections were rinsed in 0.1 M PBS three times each for 10 min and then incubated in (1) 1:1,000 anti-NeuN (A-60; Merck Millipore) or 1:500 anti-Map2 (AP-20; Sigma-Aldrich) in 0.2% (wt/vol) Triton X-100 (Sigma-Aldrich) and 5% goat serum (Jackson ImmunoResearch) in 0.1 M PBS for 72 h at 4 °C; (ii) phospho-conjugated secondary antibodies (Jackson ImmunoResearch) in 0.05% Triton X-100 and 2% goat serum in 0.1 M PBS for 2 h at room temperature; or (iii) 0.5 μg/mL DAPI in PBS for 10 min at room temperature (48). After rinsing with PBS, sections were mounted with Fluoromount (Sigma-Aldrich) aqueous mounting medium.

Imaging. Sections were examined with epifluorescence and appropriate filter sets using a microscope (Nikon; Eclipse Ti-E inverted microscope) equipped with a precentered fiber illuminator (Nikon; Intensilight C-HGFI) and B/W CCD digital camera (Hamamatsu; ORCA-ER). Confocal images were acquired using a spectral laser confocal microscope (TCS-SP5; Leica Microsystems) and a commercial inverted confocal microscope (TCS SP5; Leica Microsystems). Large fields of view were acquired with an HCX Plan Apo CS2 40× oil objective (N.A. 1.30) with a pixel size of ~300 nm at a scan speed of 400 Hz using three line averages. High-resolution images were acquired using an HCX Plan Apo CS2 63× oil objective (N.A. 1.40) with a pixel size of 100 nm and a z-step size of 290 nm at a scan speed of 400 Hz using three line averages. Immunohistochemistry digital images (RGB images) were used for measuring the thickness of the cortex and hippocampal 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 63× 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 (Vref)—and the optical dissector method—which determines the density of neurons (i.e., the number of NeuN+ cells per μm3) within Vref (Nref). Vref was determined every 6th section, and Nref 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 Vref × Nref (50). These analyses were carried out using a Leica TSC-SL microscope equipped with 4× objective lens (for Vref) and a 60× objective lens with a 1.4 numerical aperture condenser (for Nref) 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 Colonier solution [3% K2Cr2O7 (wt/vol), 3% glutaraldehyde (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
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 dissected, a small cranial bur 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; 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 genotype 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 during 4 consecutive weeks.

**Radial arm maze test.** The eight-arm radial maze (Stoelting) was used to evaluate spatial learning and memory, including short-term working memory and long-term reference memory (18). During acclimation, training, and testing phases of the procedure, animals had 18 h of food deprivation to increase the saliency of food pellets located at the end of each baited arm. During acclimation (1 d), animals were allowed to explore the eight-arm radial maze with randomly placed food pellets throughout the maze. During the subsequent training day, animals were placed in the maze facing arm 1 and the four baited arms were located such that two of these arms were adjacent (1, 2, 4, and 7) and the other two arms were 90° apart from these arms (3, 5, 6, and 8), which were closed. The same four of eight arms were baited, and the other four arms were never baited. The training session lasted until all food pellets had been retrieved or 5 min had elapsed. During testing, all arms of the radial maze were open. One testing session occurred each week for each animal and lasted until all food reward was retrieved or 5 min had elapsed. Latency to enter eight arms was recorded. Arm entry was counted when all four legs of an animal entered an arm. Entries into unbaited arms were counted as reference memory errors, and reentries into previously baited arms were counted as working memory errors. In contrast to spatial working (short-term) memory, spatial reference memory has more capacity and lasts longer (18). Mice received one trial per week for 4 consecutive weeks (four trials in total).

**Open-field test.** Mice were assessed for novelty-induced locomotor activity/exploration in an open field (40 cm × 40 cm × 35 cm high) divided into two regions (64% border and 36% center) and placed in an ANY-box base (Stoelting) including a camera to track the animals and a height-adjustable, infrared photobeam array to detect rearing behavior. The animals were placed in the center of the apparatus and video-recorded for 5 min. The percentage of time spent in each region, distance moved, freezing (immobility), and number of rears (raising the forepaws, denoting vertical exploration) were scored in 5-min sessions (20).

**Hole-board test.** Mice were assessed for exploratory behavior in a black box (40 cm × 40 cm × 35 cm high) with 16 holes (3-cm diameter) equally spaced in the floor and placed in the ANY-box base (Stoelting), which includes a camera to track the animals and an infrared photobeam array to detect automatically nose pokes through the holes. Mice were placed facing one corner of the apparatus and observed for 5 min. The exploratory behavior was measured as the number of head dips into holes. Distance moved was also recorded (21).

**Statistical Analysis.** 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.
Fig. S1. 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 x 10^-2 mm³; Cdh1 cKO: cortex, 8.51 mm³; CA1, 6.39 x 10^-2 mm³). 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.
Fig. S4. 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-knocked-down 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 proteasomal 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. 4 A 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 hippocampal 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].