



APC/C^{Cdh1}-Rock2 pathway controls dendritic integrity and memory

Verónica Bobo-Jiménez^{a,b}, María Delgado-Esteban^{a,b}, Julie Angibaud^c, Irene Sánchez-Morán^{a,b}, Antonio de la Fuente^d, Javier Yajeya^d, U. Valentin Nägerl^c, José Castillo^e, Juan P. Bolaños^{a,b,f}, and Angeles Almeida^{a,b,1}

^aInstitute of Biomedical Research of Salamanca, University Hospital of Salamanca, University of Salamanca, Consejo Superior de Investigaciones Científicas, 37007 Salamanca, Spain; ^bInstitute of Functional Biology and Genomics, University of Salamanca, CSIC, 37007 Salamanca, Spain; ^cInterdisciplinary Institute for Neuroscience, University of Bordeaux, CNRS UMR 5297, 33077 Bordeaux, France; ^dDepartment of Physiology and Pharmacology, Faculty of Medicine, University of Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain; ^eClinical Neurosciences Research Laboratory, Department of Neurology, University Hospital, Health Research Institute of Santiago de Compostela, University of Santiago de Compostela, 15706 Santiago de Compostela, Spain; and ^fCentro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable, 37007 Salamanca, Spain

Edited by Masatoshi Takeichi, RIKEN Center for Developmental Biology, Kobe, Japan, and approved March 16, 2017 (received for review September 26, 2016)

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 CaMKII α -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 A.A. 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.

¹To whom correspondence should be addressed. Email: aaparra@usal.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1616024114/-DCSupplemental.

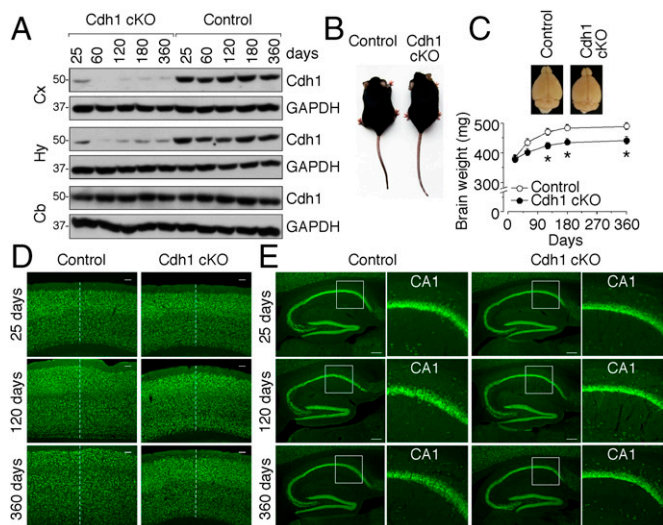


Fig. 1. Conditional knockout of *Cdh1* from the adult forebrain reduced thickness of the cerebral cortex and CA1 hippocampal layer. (A) Immunoblotting analyses reveal *Cdh1* down-regulation in the cortex (Cx) and hippocampus (Hy), but not in the cerebellum (Cb), of cKO *Cdh1* from postnatal day 25 and 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 μ m.) (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 μ m; magnification: Insets, 20 \times .) Data are expressed as mean \pm SEM; * P < 0.05 versus age-matched control mice (one-way ANOVA followed by the Bonferroni post hoc test; n = 6 mice per group).

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). Consistent with the temporal activity of the *CamKII α* promoter (14), immunoblotting analyses revealed depletion of *Cdh1* levels in the cortex and hippocampus, but not in the cerebellum, from postnatal day 25 and continued into adulthood (Fig. 1A). As from postnatal day 120 the brain weight was reduced (Fig. 1B and C and Fig. S1A), as was the cortex (Fig. 1D and Fig. S1B) and the CA1 layer of the hippocampus, which were thinner in *Cdh1* cKO (Fig. 1E and Fig. S1C). This effect was a consequence of decreased number, not soma size, of neurons, as judged by stereological counting of NeuN⁺ cells (Fig. S1D). Thus, inactivation of APC/C^{*Cdh1*} in neurons alters age-related growth of the cortex and hippocampus.

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. S2B 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. 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

(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. S3A 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. S4B 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. S4D 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.

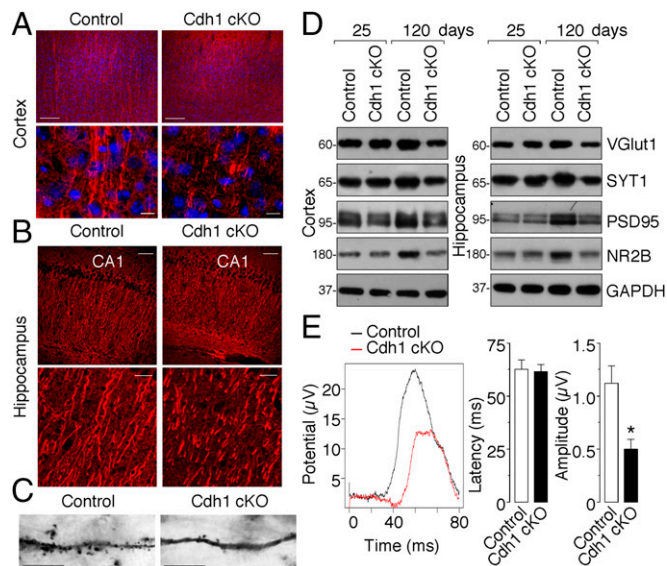


Fig. 2. *Cdh1* deficiency triggers dendrite disruption and loss of dendritic spines and synapses in the adult brain. (A and B) *Cdh1* loss triggered dendrite disruption in both the cortex (A) and hippocampal CA1 layer (B) of 120-d-old mice, as revealed by immunostaining for the dendritic marker Map2. [Scale bars, 50 μ m (A, Top), 10 μ m (A, Bottom), 50 μ m (B, Top), 15 μ m (B, Bottom).] (C) Golgi impregnation of brain sections showed dendritic spine loss in cortical pyramidal neurons of 120-d-old *Cdh1* cKO mice, compared with control mice. (Scale bars, 10 μ m.) (D) *Cdh1* depletion triggered synapse loss, as revealed by the reduction in levels of the presynaptic proteins vesicular glutamate transporter 1 and synaptotagmin 1 and postsynaptic markers glutamate receptor subunit NR2B and postsynaptic density protein 95 in the cortex and hippocampus. (E) *Cdh1* deficiency caused a marked decrease in evoked potential amplitude, whereas latency was not modified. Data are expressed as mean \pm SEM; * P < 0.05 versus control mice (Student's *t* test; n = 4 to 6 mice per group).

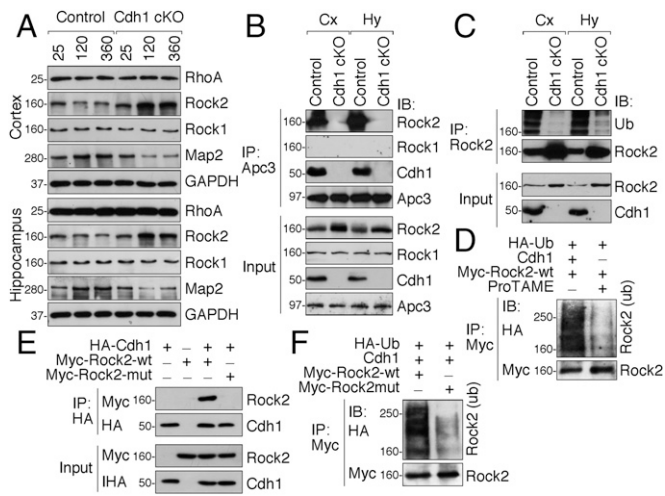


Fig. 3. APC/C^{Cdh1} triggers the ubiquitination of Rock2. (A) Cdh1 depletion in the cortex and hippocampus did not alter RhoA and Rock1 expression, but age-dependent increases in Rock2 levels and decreases in Map2 levels from 120 d of age were seen. (B) Endogenous coimmunoprecipitation analyses revealed 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 μ M) and MG132 (20 μ M) 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 μ M for 2 h) were immunoprecipitated with anti-Myc agarose beads followed by immunoblotting with HA and Myc antibodies. IB, immunoblotting; IP, immunoprecipitation.

APC/C^{Cdh1} 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^{Cdh1} (9). Hence, we analyzed Rock1 and Rock2 protein levels in the brains of Cdh1 cKO and wild-type mice using specific and validated antibodies (Fig. S5A) (23). 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. 3A 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^{Cdh1} 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^{Cdh1} forms a complex with Rock2 but not with Rock1 (Fig. 3B). Because the APC/C^{Cdh1} 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).

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^{Cdh1} substrate.

To ascertain whether APC/C^{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. 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. 3E) 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^{Cdh1} ubiquitinates Rock2, targeting it for proteasomal degradation.

APC/C^{Cdh1} Controls Dendritic Network Integrity via the Regulation of Rock2 Activity.

Given that APC/C^{Cdh1} regulates Rock2 levels and activity, we next were prompted to investigate whether the control of dendritic network integrity by APC/C^{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 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^{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 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

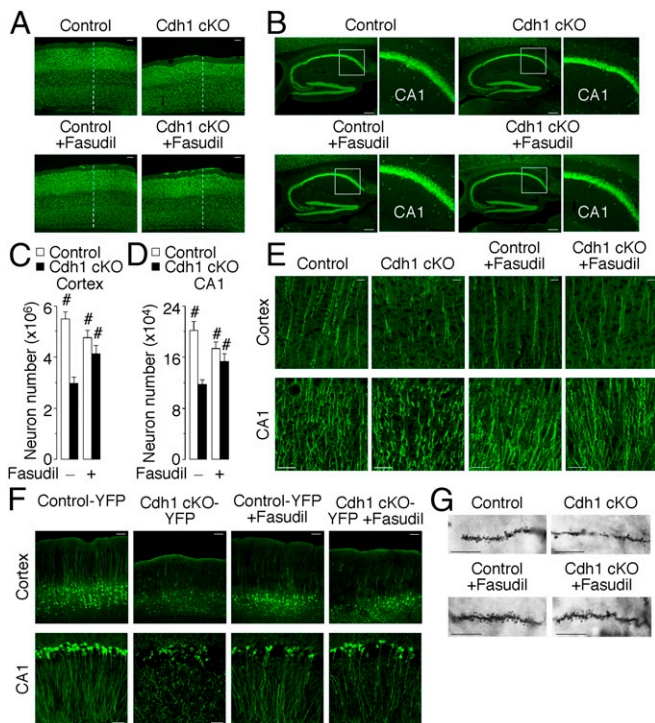


Fig. 4. Rock2 inhibition with fasudil improves dendrite disruption in the Cdh1 cKO cortex and hippocampus. The Rock inhibitor fasudil (20 mg/kg) was injected intraperitoneally every other day into 30-d-old mice for 2 mo. (A and B) Brain sections were immunostained for the neuronal marker NeuN. Fasudil 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 μ m (A) and 200 μ m (B); magnification: B, Insets, 20 \times .] (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 μ m.) (F) Immunostaining for GFP in brain sections of control-YFP and Cdh1 cKO-YFP triple-transgenic 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 μ m (layer 5) and 20 μ m (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 μ m.) Data are expressed as mean \pm SEM; $^{\#}P < 0.05$ versus Cdh1 cKO mice (one-way ANOVA followed by the Bonferroni post hoc test; $n = 4$ mice per group).

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) 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 dendrite 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. S6 E-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/explorative activity impairment and attenuated anxiety caused by Cdh1 (Fig. 5 A 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. 5 A 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 knockout. 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.

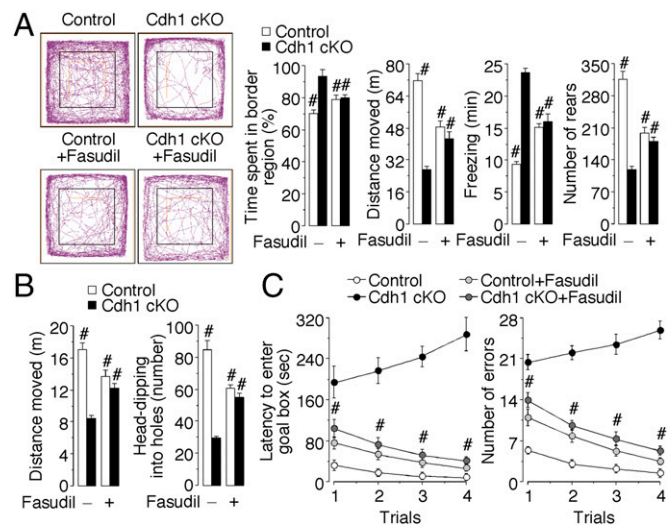


Fig. 5. Treatment with the Rock2 inhibitor fasudil improves behavioral deficits in Cdh1 cKO mice. Fasudil (20 mg/kg) was injected intraperitoneally every other day into 30-d-old mice for 2 mo. (A) Representative images showing the explorative behavior of mice in the open-field task. Fasudil partially 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 and the number of errors across trials in the Lashley III maze test found in cKO Cdh1 mice. Data are expressed as mean \pm 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 dendrite 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^{Cdh1} 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^{Cdh1} 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 dendritic 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^{Cdh1} promotes the degradation of Rock2 to ensure proper stability of dendrites and maintenance of dendritic arbors and synapses 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^{Cdh1}-Rock2 signaling pathway is consistent with the previously described effect of APC/C^{Cdh1} 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 by destabilization and depolymerization of microtubules, leading to dendrite disruption (41). Our findings that APC/C^{Cdh1} 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^{Cdh1}-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^{Cdh1}-Rock2 pathway also represents a new mechanism in cancer progression is a tempting possibility that remains to be elucidated.

Materials and Methods

CamKIIalpha-Cre-Mediated Cdh1 Conditional Knockout Mice. The Cdh1^{lox/lox}-targeted mouse model (13) was crossed with transgenic mice carrying the gene encoding Cre recombinase under the control of the Camk2 α -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*).

Coimmunoprecipitation 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 μ M) for 1 h, and cortex and hippocampus were microdissected, lysed, and immunoprecipitated with anti-Rock2 antibody, followed by 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- μ 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.

ACKNOWLEDGMENTS. We thank Prof. Sergio Moreno (Institute of Functional Biology and Genomics, CSIC) for the generous supply of the Cdh1^{lox/lox}-targeted mouse model. We also appreciate the technical assistance of Monica Resch and Monica Carabias. The confocal images of fixed samples were taken at the Bordeaux Imaging Center, which is a service unit of CNRS, INSERM, and the University of Bordeaux and a member of the national infrastructure France BioImaging. This work was funded by Instituto de Salud Carlos III Grants PI15/00473, BAE14/0005, RD12/0014/0007, and RD16/0019/0018 (to A.A.) and RD12/0014/0001 and RD16/0019/0001 (to J.C.); FEDER (European Regional Development Fund); Ministerio de Economía y Competitividad Grant SAF2016-78114-R (to J.P.B.); and European Union's Horizon 2020 Research and Innovation Programme Grant 686009 (to A.A. and J.C.). V.B.-J. (PI15/00473) and M.D.-E. (CP0014/00010) are supported by the Instituto de Salud Carlos III. I.S.-M. is supported by the Junta de Castilla y León (Spain).

1. Koleske AJ (2013) Molecular mechanisms of dendrite stability. *Nat Rev Neurosci* 14: 536–550.
2. Kulkarni VA, Firestein BL (2012) The dendritic tree and brain disorders. *Mol Cell Neurosci* 50:10–20.
3. Cochran JN, Hall AM, Roberson ED (2014) The dendritic hypothesis for Alzheimer's disease pathophysiology. *Brain Res Bull* 103:18–28.
4. Hensel N, Rademacher S, Claus P (2015) Chatting with the neighbors: Crosstalk between Rho-kinase (ROCK) and other signaling pathways for treatment of neurological disorders. *Front Neurosci* 9:198.
5. Amano M, Nakayama M, Kaibuchi K (2010) Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton* 67:545–554.
6. Li Z, Van Aelst L, Cline HT (2000) Rho GTPases regulate distinct aspects of dendritic arbor growth in *Xenopus* central neurons in vivo. *Nat Neurosci* 3:217–225.
7. Nakayama AY, Harms MB, Luo L (2000) Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J Neurosci* 20:5329–5338.
8. Chen H, Firestein BL (2007) RhoA regulates dendrite branching in hippocampal neurons by decreasing cypin protein levels. *J Neurosci* 27:8378–8386.
9. Almeida A (2012) Regulation of APC/C-Cdh1 and its function in neuronal survival. *Mol Neurobiol* 46:547–554.
10. Huang J, Bonni A (2016) A decade of the anaphase-promoting complex in the nervous system. *Genes Dev* 30:622–638.
11. Almeida A, Bolaños JP, Moreno S (2005) Cdh1/Hct1-APC is essential for the survival of postmitotic neurons. *J Neurosci* 25:8115–8121.
12. Delgado-Esteban M, García-Higuera I, Maestre C, Moreno S, Almeida A (2013) APC/C-Cdh1 coordinates neurogenesis and cortical size during development. *Nat Commun* 4:2879.
13. García-Higuera I, et al. (2008) Genomic stability and tumour suppression by the APC/C cofactor Cdh1. *Nat Cell Biol* 10:802–811.
14. Minichiello L, et al. (1999) Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* 24:401–414.
15. Depaape V, et al. (2005) Ephrin signalling controls brain size by regulating apoptosis of neural progenitors. *Nature* 435:1244–1250.
16. Jan YN, Jan LY (2010) Branching out: Mechanisms of dendritic arborization. *Nat Rev Neurosci* 11:316–328.
17. Zhou R, Abbas PJ, Assouline JG (1995) Electrically evoked auditory brainstem response in peripherally myelin-deficient mice. *Hear Res* 88:98–106.
18. Sharma S, Rakoczy S, Brown-Borg H (2010) Assessment of spatial memory in mice. *Life Sci* 87:521–536.
19. Porter VR, et al. (2003) Frequency and characteristics of anxiety among patients with Alzheimer's disease and related dementias. *J Neuropsychiatry Clin Neurosci* 15: 180–186.
20. Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. *Eur J Pharmacol* 463:3–33.
21. Takeda H, Tsuji M, Matsumiya T (1998) Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. *Eur J Pharmacol* 350:21–29.
22. Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. *Nature* 472:100–104.
23. Zhanaka T, et al. (2006) Nuclear Rho kinase, ROCK2, targets p300 acetyltransferase. *J Biol Chem* 281:15320–15329.
24. Ma Z, et al. (2006) Interaction between ROCK II and nucleophosmin/B23 in the regulation of centrosome duplication. *Mol Cell Biol* 26:9016–9034.
25. Tugendreich S, Tomkiel J, Earnshaw W, Hieter P (1995) CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell* 81:261–268.
26. Meghini F, et al. (2016) Targeting of Fzr/Cdh1 for timely activation of the APC/C at the centrosome during mitotic exit. *Nat Commun* 7:12607.
27. Maestre C, Delgado-Esteban M, Gomez-Sanchez JC, Bolaños JP, Almeida A (2008) Cdk5 phosphorylates Cdh1 and modulates cyclin B1 stability in excitotoxicity. *EMBO J* 27: 2736–2745.
28. Shibuya M, Hirai S, Seto M, Satoh S, Ohtomo E; Fasudil Ischemic Stroke Study Group (2005) Effects of fasudil in acute ischemic stroke: Results of a prospective placebo-controlled double-blind trial. *J Neurol Sci* 238:31–39.
29. Feng G, et al. (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28:41–51.
30. De Camilli P, Miller PE, Navone F, Theurkauf WE, Vallee RB (1984) Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. *Neuroscience* 11:817–846.
31. Huber G, Matus A (1984) Differences in the cellular distributions of two microtubule-associated proteins, MAP1 and MAP2, in rat brain. *J Neurosci* 4:151–160.
32. Amano M, et al. (2003) Identification of Tau and MAP2 as novel substrates of Rho-kinase and myosin phosphatase. *J Neurochem* 87:780–790.
33. Yamamoto H, Fukunaga K, Tanaka E, Miyamoto E (1983) Ca²⁺- and calmodulin-dependent phosphorylation of microtubule-associated protein 2 and tau factor, and inhibition of microtubule assembly. *J Neurochem* 41:1119–1125.
34. Hotulainen P, Hoogenraad CC (2010) Actin in dendritic spines: Connecting dynamics to function. *J Cell Biol* 189:619–629.
35. Cerri C, et al. (2011) Activation of Rho GTPases triggers structural remodeling and functional plasticity in the adult rat visual cortex. *J Neurosci* 31:15163–15172.
36. Martino A, et al. (2013) Rho GTPase-dependent plasticity of dendritic spines in the adult brain. *Front Cell Neurosci* 7:62.
37. Li M, et al. (2008) The adaptor protein of the anaphase promoting complex Cdh1 is essential in maintaining replicative lifespan and in learning and memory. *Nat Cell Biol* 10:1083–1089.
38. Fu AK, et al. (2011) APC(Cdh1) mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. *Nat Neurosci* 14:181–189.
39. Huang J, Ikeuchi Y, Malumbres M, Bonni A (2015) A Cdh1-APC/FMRP ubiquitin signaling link drives mGluR-dependent synaptic plasticity in the mammalian brain. *Neuron* 86:726–739.
40. Adlard PA, Vickers JC (2002) Morphologically distinct plaque types differentially affect dendritic structure and organization in the early and late stages of Alzheimer's disease. *Acta Neuropathol* 103:377–383.
41. Conde C, Cáceres A (2009) Microtubule assembly, organization and dynamics in axons and dendrites. *Nat Rev Neurosci* 10:319–332.
42. Jiao SS, et al. (2015) Edaravone alleviates Alzheimer's disease-type pathologies and cognitive deficits. *Proc Natl Acad Sci USA* 112:5225–5230.
43. Herskowitz JH, et al. (2013) Pharmacologic inhibition of ROCK2 suppresses amyloid- β production in an Alzheimer's disease mouse model. *J Neurosci* 33:19086–19098.
44. Zhou Y, et al. (2003) Nonsteroidal anti-inflammatory drugs can lower amyloidogenic Abeta42 by inhibiting Rho. *Science* 302:1215–1217.
45. Song Y, Chen X, Wang LY, Gao W, Zhu MJ (2013) Rho kinase inhibitor fasudil protects against β -amyloid-induced hippocampal neurodegeneration in rats. *CNS Neurosci Ther* 19:603–610.
46. Rath N, Olson MF (2012) Rho-associated kinases in tumorigenesis: Re-considering ROCK inhibition for cancer therapy. *EMBO Rep* 13:900–908.
47. Ridley AJ (2015) Rho GTPase signalling in cell migration. *Curr Opin Cell Biol* 36: 103–112.
48. Rodriguez C, et al. (2017) Neovascularization and functional recovery after intracerebral hemorrhage is conditioned by the Tp53 Arg72Pro single-nucleotide polymorphism. *Cell Death Differ* 24:144–154.
49. Paxinos G, Watson C (2007) *The Rat Brain in Stereotaxic Coordinates* (Academic/Elsevier, Boston), 6th Ed.
50. Schmitz C, Hof PR (2005) Design-based stereology in neuroscience. *Neuroscience* 130: 813–831.
51. Herculano-Houzel S, Lent R (2005) Isotropic fractionator: A simple, rapid method for the quantification of total cell and neuron numbers in the brain. *J Neurosci* 25:2518–2521.
52. Park YH, Wood G, Kastner DL, Chae JJ (2016) Pypin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. *Nat Immunol* 17:914–921.
53. Komnig D, Schulz JB, Reich A, Falkenburger BH (2016) Mice lacking Faim2 show increased cell death in the MPTP mouse model of Parkinson disease. *J Neurochem* 139:848–857.
54. Matzel LD, et al. (2003) Individual differences in the expression of a "general" learning ability in mice. *J Neurosci* 23:6423–6433.