Cdh1-APC Controls Axonal Growth and Patterning in the Mammalian Brain

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Supporting Online Material

Materials and Methods

Cerebellar Granule Neuron Cultures

Granule neuron cultures were prepared as described (S1). Briefly, cerebellar granule neurons were cultured from P6 Long-Evans rat pups. Neurons were placed on polyornithine coated plates and grown in basal medium Eagle (BME, Sigma) supplemented with calf serum (10%) (Hyclone), 25 mM KCl, 2mM glutamine, penicillin, and streptomycin. One day after cultures were prepared (P6+1DIV) they were treated with the anti-mitotic agent cytosine-β-D arabinofuranoside (AraC, 10 µM) to prevent the proliferation of non-neuronal cells. AraC was used whenever serum was present in the medium as in Figs. S1 and S2. Cultures were transfected with a modified calcium phosphate transfection method as described (S1). After transfection, cultures were placed in medium containing BME supplemented with glutamine, penicillin, glucose, and insulin (10 µg/ml) unless stated otherwise. To determine the role of Cdh1 in neuronal morphogenesis, we transfected primary granule neurons with the control U6 plasmid or the U6/shcdh1 plasmid together with a plasmid encoding green fluorescent protein (GFP) at the time of plating, when these neurons just begin to grow axons (S2). To eliminate any potential unforeseen effect of our genetic manipulations in granule neurons on cell survival, we included an expression plasmid encoding the anti-apoptotic protein Bcl-xl in all experiments in which we monitored axonal morphogenesis except for experiments in Figs. S2 (S3). While Bcl-xl expression promoted survival of granule neurons, it failed to promote axonal growth (S4).
RNA Interference

A DNA-based template method was used to express hairpin RNAs (S5). The sequences ggtgccgccatcgagaaggt and gggcgtgccctggaatgctg were used to construct the U6/shcdh1 and U6/shcdh1-b plasmids.

Morphometry

To analyze axonal and dendritic morphology of transfected granule neurons in primary cultures or in slice overlay assays, images of individual granule neurons were captured randomly and in a blinded manner on a NIKON eclipse TE2000 epifluorescence microscope at 10X, 20X, or 40X magnification using a digital CCD camera (DIAGNOSTIC instruments) and imported into the SPOT imaging software. In the experiments in Fig. 4, pairs of parallel fibers were randomly selected and the separation between the two fiber was measured by a blinded observer in an orthogonal plane to the parallel fibers at sequential points (20 to 100 µm from a randomly determined reference point) and normalized to the separation of the parallel fibers at the reference point. Distance from reference point (x-axis) and difference in the separation of the pairs of parallel fibers (y-axis) were measured in 3 different animals (mean +/- SD, a total of 60 pairs of parallel fibers were analyzed in each condition). The parallel fiber index (PFi) was calculated at each point along the x-axis by analyzing the mean +/- SEM of the value of [+1SD-(-1SD)] of the change in separation between pairs of parallel fibers in Fig. 4K and plotted in Fig. 4L.

Cerebellar Slice Overlay Assay

The slice overlay assay was performed based on a modification of the method described in Polleux and Ghosh (S6). Postnatal cerebellar slices from P8 or P9 rat pups were cut using a McIlwain Tissue Chopper. 400 µm slices were cultured on 0.4 µm membranes using medium-air interface method (MEM/25 mM HEPES/25% horse serum/6.5mg/ml D-glucose/1ml/100ml PSG) for 24 hours at 36ºC/5% CO₂ before coculture with granule neurons. Granule neurons
were prepared from P6 rats as described above, transfected 3 hours later in suspension (2.5x10^6 cells/2 ml DMEM) using a modified calcium phosphate method as described (SI) with the control U6 plasmid that also contained an expression cassette encoding GFP (U6-cmvGFP) or the U6/shcdh1-cmvGFP together with an expression plasmid encoding Bcl-xl. Transfection reaction was terminated by adding a large volume of DMEM. Cells were pelleted and then plated on top of the cerebellar slices and cocultured for 3 days. Slices were then subjected to immunostaining using the GFP and Calbindin antibodies. Slice integrity was assessed with Hoechst and Calbindin staining.

In vivo Electroporation
In vivo electroporation into postnatal rat cerebellum was performed by modification of the method recently developed in the retinal cell system (S7). Briefly, a plasmid DNA-containing solution (3-4 µl/animal) diluted in PBS containing 0.3% fast green was injected into the cerebellar cortex of P3 rat pups using a Hamilton syringe with 30-gauge needle. In Fig. 4A-D, 3 µg/µl of the GFP expression plasmid and 2 µg/µl of the Bcl-xl expression plasmid were injected. In Fig. 4E-J, 4 µg/µl of the U6 or U6/shcdh1 plasmid together with 1 µg/µl of GFP and 1 µg/µl Bcl-xl expression vectors were injected. Following DNA injection, animals were subjected to electric pulses (5 pulses of 160V for 50 ms with intervals of 950 ms).

Myelin
Myelin was prepared from adult rat brain as described (S8). Polyornithine-coated glass coverslips were coated with 3µg myelin/150µl PBS overnight at 4ºC. Cerebellar granule neurons were plated on either polyornithine- or polyornithine/myelin-coated glass coverslips and transfected 8 hours later using a modified calcium phosphate method as described (SI).
Subcellular fractionation
Cerebellar granule neurons (P6+3DIV) were washed twice with PBS, and harvested with the hypotonic buffer (10 mM Hepes-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors). After 20 min incubation on ice, cells were homogenized 50 times using a dounce homogenizer. Homogenates were centrifuged at 500g for 5 minutes to isolate nuclei. The pelleted nuclei were washed and resuspended in a nuclear lysis buffer (20 mM Hepes-KOH pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors). After 20 min incubation in the nuclear lysis buffer at 4°C, the nuclear fraction was centrifuged at 14000 rpm for 10 min at 4°C, and nuclear lysate fraction was obtained. The homogenate that remained after isolating and pelleting the nuclei was centrifuged at 500g for 5 minutes to eliminate contaminated nuclei and the supernatant was used as the postnuclear supernatant (PNS). Protein concentration of extracts was determined by a Bradford assay, and equal amount of protein was subjected to immunoprecipitation or immunoblotting analyses using antibodies to Cdh1 (Pharmingen), Cdc27 (Santa Cruz), 14-3-3 (Santa Cruz, sc-1657), and SP-1 (Upstate Biotechnology). SP-1 and 14-3-3 proteins served respectively as markers of the nuclear and PNS fractions. In the experiments in Fig. 2A, additional experiments were performed in which Ponceau S staining was used to validate the presence of proteins in both the nuclear and postnuclear supernatant fractions (S4).

Assay of APC activity
Immunoprecipitation of Cdh1 and Cdc27 and detection of endogenous APC activity were done as described (S9). The APC activity assay was carried out in the reaction mixture (5 mM Tris pH 7.5, 1 mM MgCl2, 2 mM ATP, 0.1 mM EGTA, 30 U/ml creatine kinase, 7.5 mM phosphocreatine, 0.3 U/ml pyrophosphatase) together with 12.5 µg ubiquitin, 400 ng ubiquitin-activating enzyme (E1), 500 ng UbcH10 (Boston Biochem), and 35S-labeled substrate at 37°C. For the substrate, Flag-tagged D-box sequence derived from the N-terminal portion of human
Cyclin B was fused to GFP, and expressed in reticulocyte lysates followed by immunopurification using the anti-Flag antibody (Sigma).

Modified Stripe Assay
A modified stripe assay was performed as described (S10). Briefly, glass coverslips were coated with polyornithine and allowed to dry. Myelin that was isolated from adult rat brain as described above (13 µg/ml) was coated overnight at 4°C on top of the polyornithine. A plastic pipette tip was used to remove myelin coating in a pattern so as to generate myelin-laden and myelin-free areas in stripes as shown in Fig. S4A. The myelin and polyornithine stripe pattern was confirmed by immunocytochemical analysis of the coverslips with a mouse monoclonal antibody to the myelin protein CNPase. Cerebellar granule neurons were plated on top of the coverslips and transfected 8 hours later with the control U6-cmvGFP or U6/shcdh1-cmvGFP plasmid together with expression plasmids encoding Bcl-xl and GFP. After 3 days in culture, transfected cells were fixed and subjected to immunocytochemical analysis using the GFP antibody. Hoechst staining revealed that granule neuron cell bodies clearly preferred the myelin-free stripes. Transfected granule neurons on polyornithine were counted and the direction of the outgrowing axon was determined. Axons were categorized into two groups: those whose growth was restricted to the polyornithine stripes and those that crossed the polyornithine-myelin borders and extended over the myelin stripes.

Statistical analyses
All statistical analyses were done using the software program Statview. For pairwise comparisons, the student’s t-test was used. For comparisons within multiple data sets, ANOVA was used, and P values were calculated using the Fischer’s PLSD post-hoc test.
FIGURE LEGENDS

Figure S1: Cdh1 RNAi induces the knockdown of Cdh1 in COS cells and primary neurons. (A) Lysates of COS cells that were transfected with the control U6 or the U6/shcdh1 plasmid (0.5 µg in lanes 1 and 3, 1.0µg in lane 2 and 4) together with expression plasmids encoding Flag-14-3-3 and Flag-Cdh1 were immunoblotted with an antibody to Flag (Sigma). (B) Cerebellar granule neurons were transfected with the U6 (a, c) or U6/shcdh1 (b, d) plasmid together with plasmids encoding Cdh1-GFP and β-galactosidase (beta-gal). Four days after transfection, granule neurons were fixed and subjected to immunocytochemistry using antibodies to β-galactosidase (Promega) and to GFP (Molecular Probes). Arrowheads point at the cell body of granule neurons. Calibration bar equals 50 µm. (C) Cdh1 RNAi does not affect survival or death of granule neurons. Cerebellar granule neurons were transfected at 2DIV with the U6 or U6/shcdh1 plasmid together with the β-galactosidase expression plasmid. After 3 days in full survival medium (serum + 30 mM KCl), cultures were switched to full survival medium or were deprived of membrane depolarization (5 mM KCl) with and without serum withdrawal for 2 days and then subjected to immunocytochemistry using the mouse monoclonal β-galactosidase antibody and analyzed for cell survival as described (S1). Briefly, cell survival and death were assessed in transfected neurons expressing β-galactosidase based on the integrity of neurites and integrity of the nucleus as determined by the DNA dye bisbenzimide (Hoechst 33258). Neurons with intact nuclei and neurites were counted as surviving, and neurons with disintegrated neurites and fragmented or pyknotic nuclei were counted as apoptotic (S1). The percentage of cell survival within the population of transfected β-galactosidase-positive neurons is shown on the y-axis. At least 300 neurons were counted per condition.

Figure S2: Cdh1 knockdown promotes axonal growth in granule neurons in full survival medium in the absence of Bcl-xl expression. Cerebellar granule neurons were transfected with the U6 or U6/shcdh1 plasmid together with the GFP expression plasmid. After 3 days in full survival medium containing serum, cultures were fixed, subjected to immunocytochemistry with
the GFP antibody and axonal length was measured in GFP-positive neurons. The basal length of axons in these cultures was higher than in serum-free medium (Figs. 1 and 2). Nevertheless, Cdh1 knockdown significantly induced axonal growth (P<0.001, t-test). A total of 107 neurons was counted.

**Figure S3: Cdh1 knockdown overrides myelin-inhibition of axonal growth.** (A) Granule neurons from P6 rat pups were transfected in suspension with the U6-cmvGFP or U6/shcdh1-cmvGFP plasmid and then plated on top of P9 cerebellar slices. Slices were analyzed 72 hours later with immunohistochemistry using an antibody to GFP to label transfected neurons and Hoechst to reveal the anatomical organization of the cerebellar cortex. The majority of control U6-transfected granule neurons extended axons characteristically along the molecular layer and EGL or toward these layers. By contrast, many of the shcdh1-expressing neurons extended axons that did not respect the layer boundaries and many of these axons even grew over the myelin-laden white matter. The abbreviations EGL, ML, GCL, and WM refer respectively to external granule layer, molecular layer, granule cell layer, and white matter. Calibration bar equals 300 µm. (B) P6 granule neurons were transfected with the U6 or U6/shcdh1 plasmid together with the GFP expression plasmid and placed on polyornithine substrate in the presence or absence of myelin. Quantitation of these results is shown in Fig. 4M. In other experiments, we found that the percent increase in axonal growth induced upon the expression of shcdh1 as compared to control U6-transfected neurons was twice as great on myelin than on polyornithine (S4). (C) Closer examination of U6/shcdh1-expressing and U6-transfected neurons on myelin revealed that whereas U6-transfected neurons extended very short axons (arrow) when faced with islands of high myelin concentration, U6/shcdh1-expressing neurons (arrow) extended long axons even along these areas of high myelin concentration (indicated by double asterisks). Calibration bar equals 200 µm.
Figure S4: Cdh1 knockdown overrides myelin-inhibition of axon growth in a modified stripe assay. (A) Schematic of a myelin-coated glass coverslip with a grid pattern of myelin-free stripes. Immunofluorescence staining using a monoclonal antibody to CNPase confirms the sharp borders of myelin-laden stripes (M) and myelin-free polyornithine stripes (P). (B) Granule neurons that were transfected with the control U6-cmvGFP (upper two rows) or U6/shcdh1-cmvGFP (bottom two rows) plasmids together with the Bcl-xl plasmid were subjected to immunoctyochemistry using the GFP antibody (left and right panels) and Hoechst staining (middle panels). The outlines of myelin-laden and myelin-free polyornithine stripes were superimposed in the images shown in the right panels. Axons of GFP-positive granule neurons whose cell bodies were on polyornithine stripes were examined for evidence of growth over polyornithine or myelin stripes. Asterisks indicate cell bodies of individual cells, arrowheads indicate axons whose growth was restricted to polyornithine stripes and respecting the polyornithine-myelin borders, and arrows indicate axons that crossed the polyornithine-myelin borders and extended over myelin. (C) Quantitation of the results in (B) revealed that while only 15.7% of control U6-transfected granule neurons crossed over the polyornithine-myelin borders, 41.5% of shcdh1-expressing granule neurons extended axons well into the myelin stripes. A total of 91 neurons were measured. These results corroborated those in Fig. S3C.
Figure S1

A

\[ \text{Flag-Cdh1} \]

\[ \text{Flag-14-3-3} \]

B

C

Figure S2

\[ \text{Axon length (µm)} \]

\[ \text{Survival of neurons (%)} \]

\[ \text{Serum KCl 10% 30 mM 10% 5 mM 0% 5 mM} \]
References and Notes


S4. Y. Konishi, J. Stegmüller, A. Bonni, unpublished observations.


S11. For materials and methods, please see the first section of the Supporting online material.