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Supplemental Data

Activity-Induced Protocadherin Arcadlin Regulates Dendritic Spine Number by Triggering N-Cadherin Endocytosis via TAO2β and p38 MAP Kinases

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Construction of Expression Vectors

Expression vectors for *N-cadherin* mutants were constructed by modifying pCXN2-*Ncad-myc*, in which *N-cadherin* fused with 5x or 6x*myc*-tag is ligated downstream of *β-actin* promoter (Okamura et al., 2004; Shan et al., 2000). *NcadΔE-myc* and *Ncad-TM-myc* were constructed by replacing the *N-cadherin* coding sequence between EcoRI and XhoI sites with the N-terminal signal- and pre-sequences fused with the transmembrane-cytoplasmic regions and a transmembrane domain alone of mouse *N-cadherin*, respectively. The inserted cDNA fragment was amplified with pfu-polymerase (Promega). Point mutations were generated by amplifying the wild-type cDNAs with mutated primers and pfu DNA polymerase. The expression vector for wild-type *arcadlin* is described (Yamagata et al., 1999). *Arcadlin-egfp* was constructed by ligating pfu-amplified full-length *arcadlin* cDNA into *pEGFP-N1* (Clontech) between XhoI and EcoRI sites. *Acad-CP-EGFP* was constructed by ligating the cytoplasmic sequence of *arcadlin* into *pEGFP-C1* (Clontech). The integrity of the constructs was verified by sequencing. For the purification of arcadlin extracellular domain protein (Acad-EC), an *arcadlin* fragment corresponds to amino acids 31-696 was ligated to *BamHI-NotI* sites in pET32(c) expression vector (Novagen).

Immunohistochemistry of Rat Brain Slices

Brains of male Sprague Dawley rats (170 g) were freshly dissected 4hr after MECS (30 mA; 60 Hz; pulse width, 1 msec; duration, 0.1 sec), frozen in powdered dry ice and mounted with OCT compound (-20 ˚C). Sections (25 μm) were cut using a cryostat, mounted on silica-coated slide glasses, treated with graded series of methanol (25%, 50%, 75%, 100%, 75%, 50%, 25%, respectively, in PBS), blocked and permeabilized for 15 min in BL solution (5% normal goat serum, 0.1% Triton X-100, 0.02% sodium azide in PBS). The sections were then incubated overnight with primary antibodies (mouse anti-N-cadherin, 12.5 μg/ml, Transduction lab., rabbit anti-arcadlin cytoplasmic region, 1:200 (Yamagata et al., 1999)) in BL at 4 ˚C. Immunoreactivity was visualized using species-specific fluorochrome-conjugated secondary antibodies.

Neuron Cultures

Neuron cultures were prepared from hippocampi of embryonic day 18 Sprague Dawley rats as described (Banker and Goslin, 1991). Cells were plated at a density of 5,400 cells/cm^2 for microscopic analyses and 14,400 cells/cm^2 for biochemical analyses. For biotinylation analyses, neurons were plated at density of 5.0 x 10^4 cells/cm^2 onto polyethyleneimine (PEI) -coated dishes. They were maintained for up to 5 weeks in MEM containing N2 supplements (Bottenstein and Sato, 1979), 1 mM sodium pyruvate, 0.1% ovalbumin, and 5 μM cytosine arabinoside with glial feeder layer. Similar experimental results were obtained with the neurons maintained in Neurobasal medium (GibcoBRL) with B27 supplement (GibcoBRL) without the feeder layer. The neurons from *arcadlin/papc* mutant mice (*acad* $^{-/-}$) (Yamamoto et al., 2000) were collected from brains of P0 pups generated by crossing of *acad*$^{+/-}$ parents. Neurons collected from individual hippocampi were separately cultured, while determining the genotype of each pool of the neurons. Comparisons of mutant and wild-type neurons were made among cultures derived from a single littermate.

Transfection of cDNA and siRNA into Cultured Neurons

Hippocampal neurons (7000 cells/cm^2) attached on 18 mm diameter coverslips cultured in 6 cm diameter dishes supplied with Neurobasal/B27 were transfected with cDNAs and siRNAs by using Lipofectamine 2000 (Invitrogen) according to manufacture's instructions.

Preparation of Anti-TAO2β Antibody

TAO2 antisera were generated by immunizing two rabbits with HisTrx-TAO2β(398-1056) fusion protein. To obtain TAO2β-specific antibodies, antisera were passed through GST-TAO2β(398-751)-bound beads twice. Immunoblot, immunocytochemistry and immunoprecipitation were performed with this TAO2β-specific flow through serum.

Immunoprecipitation

Hippocampi were dissected from control or MECS-treated (4hr) rats (Sprague Dawley, 170 g, male), homogenized in Ca^{2+}-lysis buffer (10 mM Hepes-NaOH [pH7.4], 120 mM NaCl, 2 mM $CaCl_2$, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml aprotinin, 0.2 mM PMSF) with teflon-glass homogenizer, and centrifuged for 1hr to obtain clear protein extracts. Mature neurons cultured for 4-5 weeks (14,400 cells/cm^2 x 8 cm^2) were depolarized with 25 mM KCl for 1 min followed by recovery in the normal medium for 15-120 min. For the stimulation of cAMP-mediated pathway, 50 μM forskolin and 250 μM IBMX were added to the culture medium simultaneously, and incubated for 15-120 min. The neurons were then harvested in Ca^{2+}-lysis buffer and clarified as above. N-cadherin-associated protein complex was immunoprecipitated from these samples with 10 μl of rabbit anti-pan-cadherin antiserum (Sigma). Although the antiserum is named "pan-cadherin", this is raised against the C-terminal 24 amino acids of chicken N-cadherin, and we confirmed that the antibody recognized mouse N-cadherin exclusively but not E-cadherin or R-cadherin. The arcadlin-associated protein complex was immunoprecipitated from these samples with 5 μl of rabbit anti-arcadlin antibody. The precipitated protein complex was immunoblotted with anti-N-cadherin (Transduction lab, mouse, 1:100), anti-arcadlin (1:2000), anti-cadherin-11 (Zymed, mouse, 1:800) and anti β-Catenin (Zymed, rabbit, 1:1000) antibodies. Other immunoblots were probed with anti-synaptophysin (Zymed, rabbit, 1:50), anti-α-catenin (Transduction lab, 1:250), anti-p120 (Transduction lab, 1:1000) and anti-plakoglobin (Transduction lab, 1:2000) antibodies.

COS7 cells (2.0 x 10^5) were plated on 35 mm diameter dish, cultured for 16 hr, transfected with the mixture of 0.3 μg of *N-cadherin-myc* and 0.7 μg of *arcadlin* (or *arcadlin-EGFP*) constructs, and 3 μl of FuGENE 6 (Roche), and cultured further for 3 days

before harvest. Each sample was immunoprecipitated either with 2 μg of mouse anti-c-myc antibody (Calbiochem) or 2 μl of rabbit anti-GFP serum (Molecular Probe), and the precipitated protein complex was analyzed by immunoblot using anti-c-myc (Calbiochem, 1:1000), anti-GFP, anti-N-cadherin and anti-arcadlin antibodies.

HEK293T cells were maintained as described previously (Sugiura et al., 2004). Cells were plated at 70-80% densities onto polyethyleneimine-coated dishes. Cells were transfected with plasmids using PolyFect or SuperFect (Qiagen) according to manufacture's instructions. Immunoprecipitation of TAO2β and arcadlin from HEK293T cells and hippocampi was performed as described previously (Irie et al., 2000; Sugiura et al., 2004). Cells or rat hippocampi were extracted with TNE homogenize buffer (20 mM Tris-HCl [pH 7.6], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and 10 μg/mL antipain). The extract was precleared by incubation for 1 hr at 4°C with protein A Sepharose and divided into two parts. Antibodies to arcadlin or TAO2β were added to one part, whereas preimmune serum was added to the other part, and the lysates were incubated for 1 hr. The immune complexes were precipitated with 10 μl of protein A Sepharose (GE Healthcare) for overnight at 4°C and eluted with 20 μl of SDS sample buffer. The precipitated protein complex was immunoblotted with anti-GFP (1:2000, Clontech), anti-arcadlin (1:2000) and anti-TAO2β (1:2000) antibodies.

Measurement of Spine Density

Neurons were visualized by transfecting with *pEGFP-N1*, each coverslip was scanned from one end to another, and every transfected neuron was serially photographed with 40x objective. Any type of protrusions on the proximal 50-μm region of the thickest dendrite of each neuron were counted. The first 3-10 neurons from one end of the coverslip were counted. The counting was performed with at least three coverslips from independent preparations. Two independent blinded investigators performed photographing and counting, respectively.

Quantification of Protein Co-localization

For quantifying co-localizations of arcadlin with various synaptic markers, Pearson's correlation coefficient (r) was calculated from a typical image out of at least 3 independent

preparations by using the Intensity Correlation Analysis program in ImageJ software.

Animal Care

All experiments were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience and the Osaka University Medical School Animal Care Committee, and carried out in accordance with the Guidelines for Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience, 2000; Osaka University Medical School, 2003).

Statistics

The data were statistically analyzed by unpaired two-tailed Student's t test.

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SUPPLEMENTAL FIGURES

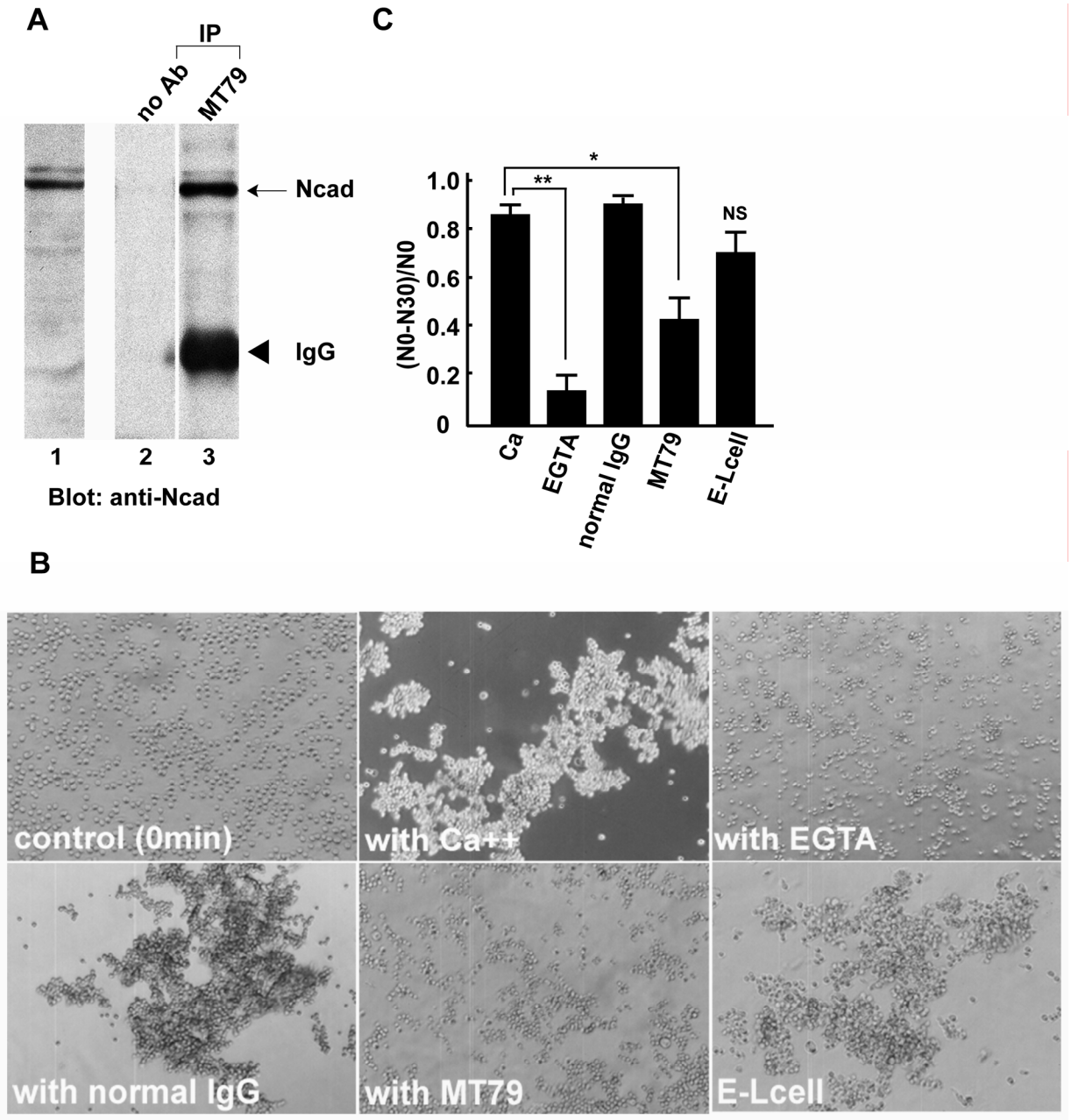

Figure S1. Production and Characterization of MT79 Serum against the Extracellular Domain of N-cadherin

MT79 serum was obtained from the rabbit immunized with full extracellular domain of N-cadherin fused with the Fc-portion of human immunoglobulin G (Shan et al., 2004). The

IgG fraction of the serum was purified for experimental use.

(A) Mouse brain homogenate was subjected to immunoblot probed with MT79 (lane 1). A 127 kDa band corresponded to N-cadherin. Brain homogenate was incubated without (lane 2) or with (lane 3) MT79, and the resultant immunoprecipitate was subjected to immunoblot probed with anti-N-cadherin antibody that recognizes the intracellular domain of N-cadherin (Tanaka et al., 2000). Arrow, N-cadherin; arrowhead, immunoglobulin heavy chain.

(B) Cell aggregation assay was performed using L929 cells (Takeichi, 1977). *N-cadherin* transfected cells formed aggregates with the existence of extracellular Ca^{++} (upper panels). Addition of MT79 specifically inhibited the aggregation of N-cadherin expressing cells (with MT79), but did not the aggregation of E-cadherin expressing cells as control (E-Lcell). Normal IgG as a control did not show inhibitory effect on N-cadherin-mediated cell aggregation (with normal IgG).

(C) Cell aggregation was quantified by counting the number of aggregates at time 0 (N_0) and 30 min (N_{30}).

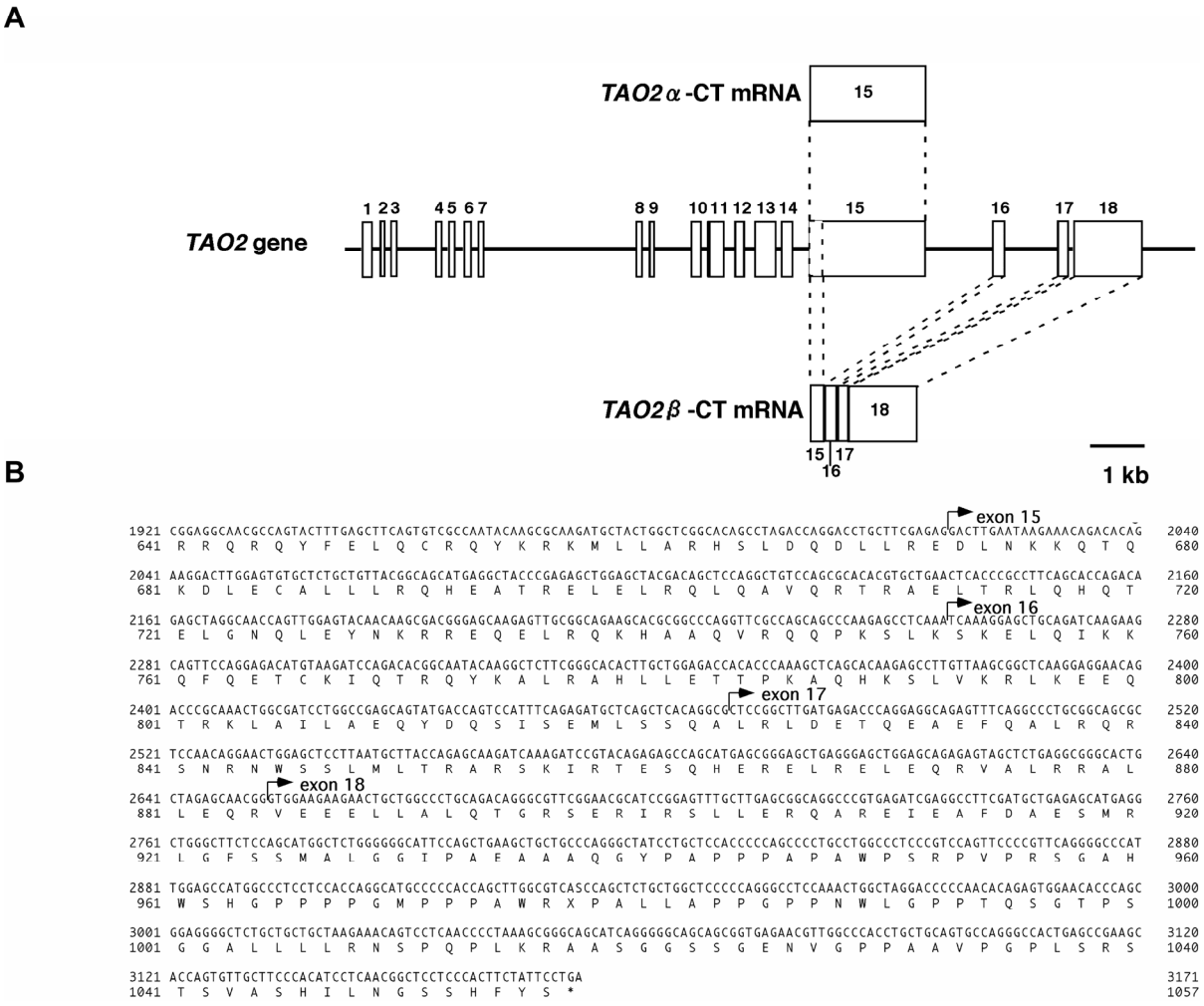

Figure S2. Structure of TAO2β Gene

(A) (Middle) Organization of the *TAO2* coding region exons. Both *tao2α* and *β* mRNAs utilize the identical exons 1-14 other than exon 11, of which *TAO2β* is 18 base longer than that of *tao2α* (thick line). (Top and bottom) Diagrams of the structure of *TAO2α* and *β* mRNAs encoding the C-terminal domains, respectively. The C-terminal region of TAO2β is encoded by a mRNA portion transcribed from a part of exon 15 and entire exons 16-18, whereas exon 15-derived mRNA covers the whole C-terminal region of TAO2α.

(B) Nucleotide and amino acid sequences of TAO2β C-terminal region. The junction of each exon is indicated.

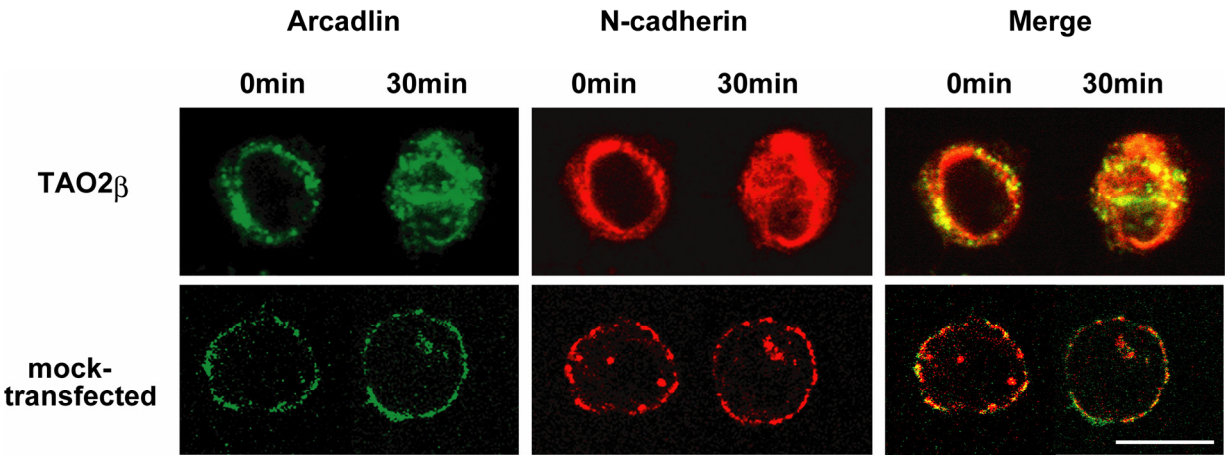

Figure S3. Acad-EC-Dependent Internalization of N-cadherin Requires TAO2β

(Top) HEK293T cells were transfected with *arcadlin-EYFP*, *tao2β*, *MEK3*, *p38 MAPK* and *N-cadherin-ECFP*, and then treated with Acad-EC for 30 min. The distribution of arcadlin-EYFP (left) and N-cadherin-ECFP (right) were analyzed by time-lapse confocal imaging. Both the proteins largely distributed on the cell surface as well as a small amount of intracellular vesicular fraction in the resting cells (0 min). In 30 minutes of the treatment, both the arcadlin-EYFP and N-cadherin-ECFP were vigorously internalized in the cytosolic vesicular compartments (30 min).

(Bottom) The Acad-EC induced internalization of N-cadherin was not observed without the co-transfection of *tao2β*. Scale bar: 10 μm

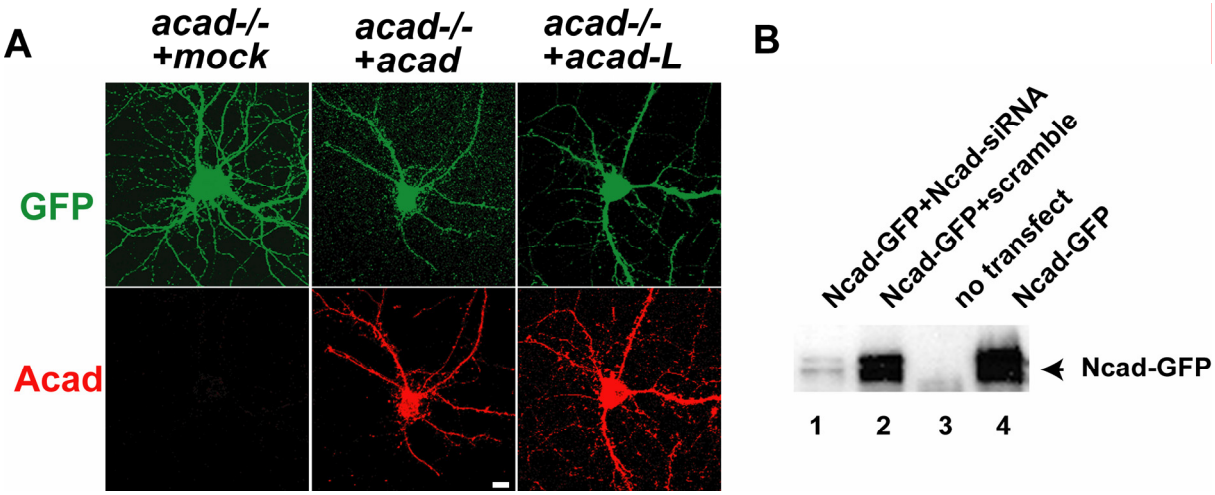

Figure S4. Arcadlin Overexpression and N-cadherin Knockdown to Verify the Role of Arcadlin in the Regulation of Spine Density

(A) Cultured *acad*$^{-/-}$ neurons were doubly transfected with *EGFP* and *mock*, *arcadlin*, or *arcadlin-l* at 6 DIV, and visualized with GFP at 15 DIV. EGFP positive neurons (green, top) were also positive for transfected arcadlin (middle) or arcadlin-L (right), as examined by immunocytochemistry (red, bottom). Left, mock transfected.

(B) HEK293T cells were doubly transfected with mouse *N-cadherin-EGFP* and *N-cadherin-siRNA* (lane 1) or *scramble-siRNA* (lane 2), and subjected to immunoblot for mouse N-cadherin-GFP (GFP epitope). Lane 3, no transfection. Lane 4, *N-cadherin-EGFP* single transfection. The upper band of the N-cadherin-GFP doublet is supposed to be an immature peptide without truncation of the N-terminal prodomain.

Scale bar: 10 μm

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