Supporting Information

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SI Materials and Methods

Hippocampal Neuronal Cultures and Transfection. Cultures of hippocampal neurons were prepared from embryonic day 15 (E15) or E16 mice as previously described (1). Briefly, after treatment with papain (0.5 mg/mL) and DNase I (0.6 μg/mL) for 20 min at 37 °C and mechanical dissociation, hippocampal cells were plated on glass coverslips or plastic dishes coated with poly-DL-ornithine (0.5 mg/mL) and laminin (5 μg/mL). The cells were cultured in Neurobasal medium with 25 μM glutamine, 1% penicillin–streptomycin, and B-27 supplement (Invitrogen) under a 5% CO_2/10% O_2 atmosphere at 37 °C. The cultures were transfected at 12 DIV using the calcium phosphate method as previously described (1, 2). The levels of expression of WT cofilin-GFP, cofilin^{S3A}-GFP, and cofilin^{S3D}-GFP in WT and β-arrestin KO neurons were evaluated by measuring GFP fluorescence in proximal dendrites of the neurons before treatment. Although the expression levels of GFP-tagged cofilin were slightly variable among different neurons within the same group, the mean expression levels (GFP fluorescence) were not significantly different between WT and KO neurons (one-way ANOVA followed by Tukey's multiple-comparison posttest). Mean fluorescence intensity of cofilin^{S3A}-GFP was 32.57 ± 23.71 in WT neurons, 39.55 ± 25.39 in β-arrestin-1 KO neurons, and 57.66 ± 14.81 in β-arrestin-2 KO neurons. Mean fluorescence intensity of cofilin^{S3D}-GFP was 43.54 ± 56.12 in WT neurons, 38.24 ± 36.46 in β-arrestin-1 KO neurons, and 53.74 ± 26.85 in β-arrestin-2 KO neurons. Neurons with similar levels of expression of various cofilin constructs were selected from each group for treatment and dendritic spine analysis.

Pharmacology and Cell Treatments. To induce a form of "chemical LTD" as previously described (3–5), the NMDA receptor was activated with *N*-methyl-D-aspartic acid (NMDA) (Sigma-Aldrich; 50 μM) in Mg^{2+}-Free Hank's Balanced Salt Solution (Invitrogen) containing 1.8 mM $CaCl_2$ and 1 μM glycine. Inhibition of the NMDAR was achieved by applying (+)-MK-801 Hydrogen Maleate (MK801, 10 μM; Sigma-Aldrich) concurrently with NMDA. Cyclosporin A (Sigma-Aldrich; 50 μM, 2 h pretreatment) was used for inhibition of calcineurin phosphatase and LY294,002 (Sigma-Aldrich; 50 μM, 10 min pretreatment) for inhibition of PI3-kinase.

Solubilized human Aβ monomer was kindly provided by Doug Ethell (Western University of Health Sciences, Pomona, CA). Briefly, dried $Aβ_{1–42}$ peptide (Biomer Technology) was suspended in water and titered with 1 N NaOH until the peptide dissolved completely and the solution became clear. That solution was diluted with 10× PBS to produce a final working concentration of 225 nM $Aβ_{1–42}$ in 1× PBS. For oligomer formation, the working solution was incubated in PBS at 37 °C for 5 d. GFP-expressing 13 DIV hippocampal neurons were then treated with 225 pM oligomeric Aβ for 24 h.

Biochemical Analysis. Fourteen DIV hippocampal neurons (~1.2 million cells per 10-cm dish) were rinsed quickly with Hank's Balanced Salt Solution (Invitrogen) containing 0.493 mM $MgCl_2$ and 0.407 mM $MgSO_4$ and then treated with the appropriate reagent (in Hank's Balanced Salt Solution with 1.8 mM $CaCl_2$ and 1 μM glycine, with or without $MgCl_2$ and $MgSO_4$) under 5% CO_2/10% O_2 at 37 °C. Following treatment, the dishes were placed on ice and immediately washed with ice-cold PBS and then scraped from the plate in 800 μL lysis buffer (25 mM Tris·Cl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 5 mM EDTA, pH 8.0) containing 1× protease inhibitor mixture (Sigma-Aldrich) and 2 mM sodium vanadate. After 30 min rotation at 4 °C, the cell lysate was centrifuged for 15 min at 14,000 × *g* at 4 °C. The supernatant was mixed 1:1 with 2× Laemmli loading buffer (Sigma-Aldrich), boiled for 10 min, and loaded onto an 8–16% Tris-glycine SDS/PAGE gel (Invitrogen). The contents of the gel were transferred onto a Nitrocellulose membrane (Perkin-Elmer), which was blocked for 1 h in 5% milk/TBS-Tween20 (0.2%), and specific primary antibodies (1:1,000) were applied overnight at 4 °C in 3% BSA/TBS-Tween20. Secondary HRP-conjugated antibodies were applied (1:100,000) for 1 h at room temperature in TBS-Tween20. The primary antibodies were rabbit anti–phospho-cofilin (Cell Signaling Technology) and rabbit anti-total cofilin (Cytoskeleton). The secondary antibodies used were HRP-conjugated goat anti-rabbit (0.08 μg/mL; Jackson ImmunoResearch) and HRP-conjugated donkey anti-mouse (0.08 μg/mL; Jackson ImmunoResearch). Signal was detected on film using the ECL Plus detection kit from GE Healthcare. Phospho-cofilin levels were quantified by densitometry (Adobe Photoshop) and normalized to total cofilin levels. Five to 10 independent experiments were performed for each condition. Statistical differences were compared using Student's *t* test.

Binding Assay. Recombinant GST-tagged human WT cofilin, cofilin^{S3A}, and cofilin^{S3D} in pGEX-4T1 were produced. GST tags were removed by thrombin cleavage, and 2 μg of each untagged cofilin protein was incubated overnight in microplates coated with mouse anticofilin antibodies. Unbound protein was removed and analyzed by SDS/PAGE to ensure that equal amounts of protein (normally 1 μg) remained bound to the plates. Plates were washed, and increasing concentrations from 1 nM to 5 μM of either GST alone or GST-tagged β-arrestin-2 were added. Plates were washed, probed with anti–GST-IR800 for 1 h, washed again, and scanned using the LICOR Odyssey. Integrated intensity corresponding to bound GST protein was determined.

Immunocytochemistry. Fourteen DIV hippocampal neurons (~50,000 cells per 10-mm coverslip) were treated with the appropriate reagent under 5% CO_2/10% O_2 at 37 °C and then quickly rinsed with PBS and fixed in 2% paraformaldehyde, permeabilized in 0.1% TritonX-100, and blocked in PBS containing 5% normal goat serum and 1% BSA. Primary antibodies were applied in blocking solution for 16 h at 4 °C, and secondary antibodies were applied in PBS-Tween20 (0.2%) for 1 h at room temperature. Coverslips were mounted in Vectashield antifade medium with DAPI (Vector Labs), sealed with Cytoseal 60 (Fisher), and then viewed under a confocal laser scanning microscope (model LSM 510; Carl Zeiss MicroImaging).

The primary antibodies used were as follows (1:100): mouse anti-synaptophysin SVP-38 (Sigma-Aldrich), mouse anti–PSD-95 (Affinity BioReagents), rabbit anti-NR2A/B (Millipore), and mouse anti-GluR2 (Millipore). The secondary antibodies used were as follows (1:500): HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch), HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), Alexa Fluor 594-conjugated anti-mouse IgG, and Alexa Fluor 660-conjugated anti-mouse IgG and anti-rabbit IgG (Invitrogen).

Confocal Microscopy and Fluorescence Intensity. Fluorescence was analyzed using a confocal laser-scanning microscope (model LSM 510; Carl Zeiss MicroImaging). A series of five high-resolution

optical sections (1,024 × 1,024-pixel format) were taken for each neuron with a 63× water-immersion objective (1.2 numerical aperture), with 1× zoom at 0.5-μm step intervals (*z*-stack). All images were acquired under identical conditions. Each *z*-stack was collapsed into a single image by projection (Zeiss LSM Image software), converted to a tiff file, and analyzed using Image J Software. Seven to 10 neurons were randomly selected for each experimental group, and three to four proximal dendrites per each neuron were analyzed. For antibody analysis, projected stacks were thresholded to identical levels using Image J software, and puncta ranging from 6 to 25 pixels were counted.

Live Imaging and Fluorescence Intensity. Time-lapse images of live cells were captured at 1-min intervals for 1 h under an inverted fluorescent microscope (model TE2000; Nikon) with a 40× oil Fluor objective, a 12-bit CCD camera (model ORCA-AG; Hamamatsu), and Image-Pro software (Media Cybernetics). During imaging, the cultures were maintained in Hank's Balanced Salt Solution (Invitrogen) supplemented with 1.8 mM $CaCl_2$ and 1 μM glycine, at 37 °C with 5% CO_2. For quantification of GFP-tagged cofilin levels in spine heads vs. dendrites, the GFP fluorescence levels were measured in each spine head and in an equally sized region of the dendrite at the base of each spine, and these values were normalized to the DsRed fluorescence levels in the same regions of interest (Adobe Photoshop). The head/base ratio was then determined for each spine.

Adult Brain Slices. Age-matched adult male mice were anesthetized with isoflurane and perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA). Brains were extracted, postfixed for 3 h in 4% PFA at 4 °C, and sectioned coronally into 300-μm slices on a vibratome. Sections were biolistically labeled with 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate (DiI) using a gene gun, incubated in PBS at 4 °C for 3 d, and then mounted onto slides in PBS and sealed with Cytoseal 60 (Fisher). Pyramidal neurons from CA1 were imaged using confocal microscopy (model LSM 510; Carl Zeiss MicroImaging), and dendritic spines were analyzed using Image J software.

Radial Arm Water Maze. Mice were housed in a 12:12 h light:dark cycle with food and water ad libitum and were tested during the light cycle between 0700 h and 1100 h. Age-matched adult WT and β-arrestin-$2^{-/-}$ mice in a C57BL/6 background were individually housed for 5 d before the start of testing. An insert containing six arms was placed inside a 100-cm diameter pool, which was filled to a depth of 30 cm with water containing nonfat dry milk (Kroger) at 21 °C. A clear platform was submerged and invisible 0.5 cm below the milk's surface at the far end of the assigned arm, which was altered each day of the test. A mouse was placed in a starting arm that was chosen semirandomly for each trial (the starting arm for trial 4 each day was always the same as the starting arm for trial 5). Each mouse was given four consecutive 60-s trials, each separated by a 30-s stay on the platform. The mouse was guided to the platform if it was not found during the 60-s trial. A fifth 60-s retention trial occurred 30 min after trial 4. Each time the mouse entered an incorrect arm, it was replaced into the starting arm for that trial and an error was scored. A video camera recorded the trials from the ceiling directly above the maze, and TopScanLite software (Clever Sys) was used to analyze the videos. Mice were subjected to five trials per day for 11 d, such that performance during the later days of testing is an indicator of long-term spatial memory.

Electrophysiology. Mice postnatal age 17–21 d were anesthetized by isofluorane inhalation and decapitated. Brains were removed from the skull and immediately immersed in chilled dissection buffer containing 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, 10 mM dextrose, 1.3 mM ascorbic acid, 7 mM $MgCl_2$, and 0.5 mM $CaCl_2$, bubbled with 95% O_2/5% CO_2. Transverse hippocampal slices (400 μm thick) were prepared from β-arrestin-2 KO and WT C57BL/6J mice, using a Vibratome (Leica VT1200S). Slices were allowed to recover for 45 min in a 35 °C submersion chamber filled with oxygenated artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 15 mM dextrose, 1.3 mM $MgCl_2$, and 2.5 mM $CaCl_2$ and then for an additional 2 h at room temperature. For long-term potentiation (LTP) experiments, a previously described protocol (6) was used. Briefly, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in CA1 stratum radiatum 50 μm below the pyramidal cell layer with glass electrodes (1 MΩ) filled with ACSF. Stimuli were delivered to the commissural/Schaffer collateral afferents with a concentric bipolar electrode positioned parallel to the recording electrode 500 μm away. Input/output curves were generated by stepping the stimulation amplitude from 5 to 80/120 μA. The basal stimulation intensity for our experiments was set at 50% of the intensity that evoked maximal fEPSP amplitude and was delivered at a frequency of 0.033 Hz. The half-maximal intensity of stimulation ranged between 18 (lowest level) to 40 μA (highest level). After 15 min of stable baseline recording (<5% drift), two trains of 1-s, 100-Hz pulses were applied to induce LTP. LTP was sampled for 60 min after induction, and potentiation was calculated by dividing the average slope of 45- to 60-min postinduction responses by the average slope of 0- to 15-min preinduction baseline responses.

For LTD, a low-frequency stimulation (LFS) consisting of 900 pairs of stimuli (distance 50 ms) at 1 Hz was used (7). LTD was sampled for 60 min after induction, and depression was calculated by dividing the average slope of 45- to 60-min postinduction responses by the average slope of 0- to 15-min preinduction baseline responses.

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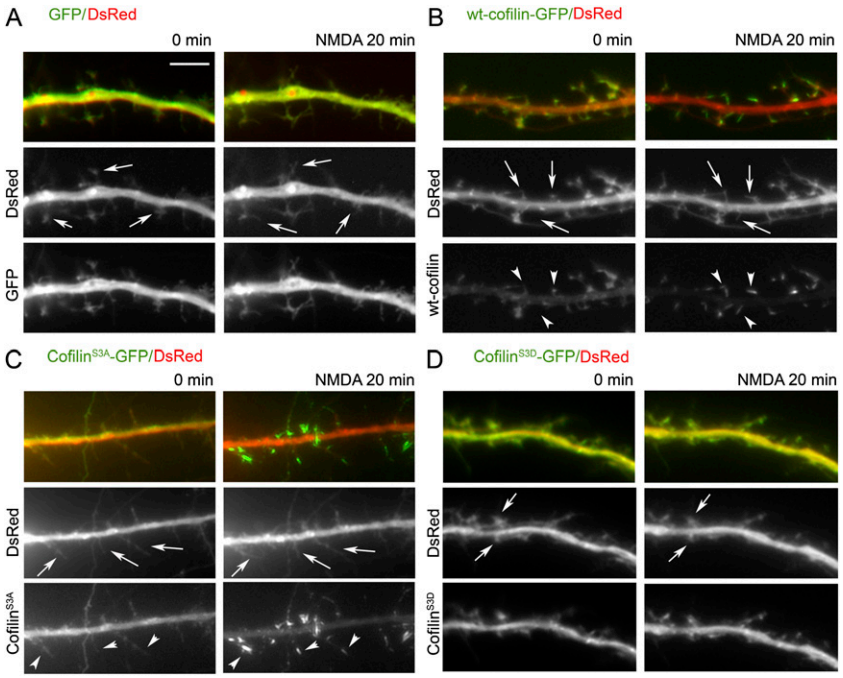


Fig. S1. NMDAR activation induces dendritic spine remodeling. (*A–D*) Time-lapse fluorescent images showing the dendrites of 14 DIV hippocampal neurons expressing DsRed (red) and GFP (*A*), WT cofilin-GFP (*B*), cofilin^{S3A}-GFP (*C*), or cofilin^{S3D}-GFP (*D*), before (0 min) or after treatment (NMDA, 20 min). Time-lapse imaging of live neurons was performed under an inverted fluorescent microscope (model TE2000; Nikon) with 40× oil Fluor objectives and monitored by a 12-bit CCD camera (model ORCA-AG; Hamamatsu), using Image-Pro software (Media Cybernetics). Images were captured at 1-min intervals for 1 h. Time-lapse imaging revealed cofilin translocation into dendritic spines as soon as 1 min after NMDA application, followed by a significant increase in cofilin levels in dendritic spines at 5 min. Changes in dendritic spine length and head area were first detected at 5 min, became significant at 20 min, and were most pronounced at 60 min following NMDAR activation. The time point of 20 min was chosen to show both cofilin translocation to dendritic spines and subsequent changes in the morphology of those spines. Arrows denote spines before and after NMDA treatment. Arrowheads denote clusters of GFP-tagged WT cofilin (*B*) or cofilin^{S3A} (*C*) that translocate to dendritic spines following NMDA treatment. (Scale bar, 10 μm.)

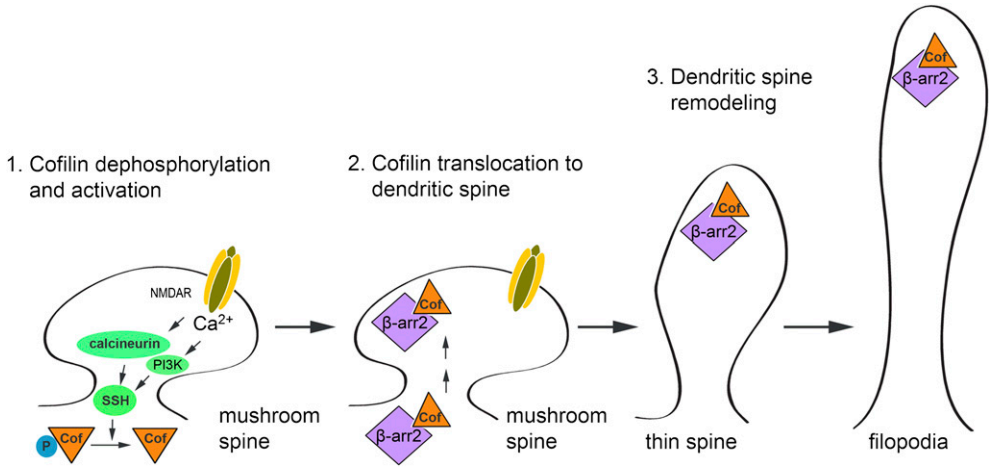
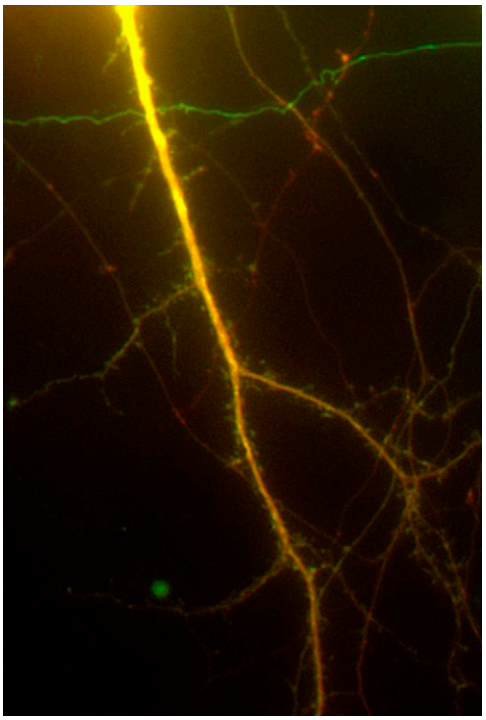
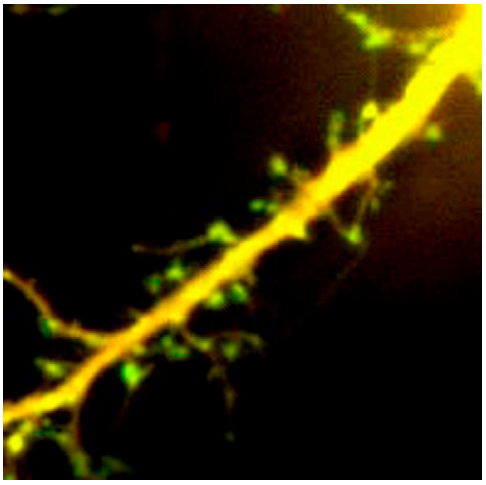


Fig. S2. NMDAR activation leads to β-arrestin-2–dependent cofilin translocation into dendritic spines and their remodeling. NMDAR activation and Ca^{2+} influx promote an intracellular signaling cascade that leads to (*1*) cofilin activation through dephosphorylation. (*2*) β-Arrestin-2 binds and translocates active cofilin to dendritic spines. (*3*) The actin-severing activity of cofilin leads to remodeling of mature mushroom spines into immature thin spines and filopodia. Abbreviations: Cof, cofilin; β-arr2, β-arrestin-2; NMDAR, *N*-methyl-D-aspartate receptor.



Movie S1. NMDAR activation induces rapid translocation of constitutively active cofilin^{S3A} to dendritic spines in WT neurons. Digital recording is shown of 14 DIV WT hippocampal neuron expressing cofilin^{S3A}; frames were captured every minute for 20 min, starting 1 min before (first frame) NMDA application (50 μM).

Movie S1



Movie S2. NMDAR activation fails to induce the translocation of constitutively active cofilin^{S3A} to dendritic spines in β-arrestin-2 KO neurons. Digital recording is shown of 14 DIV β-arrestin-2 KO hippocampal neuron expressing cofilin^{S3A}; frames were captured every minute for 20 min, starting 1 min before (first frame) NMDA application (50 μM).

Movie S2