Supporting Information

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SI Text

Purification and Identification of ARPP-90. A low salt extract was prepared by the homogenization of 75 rat brains (Pel-freeze Biologicals) in 350 mL of ice-cold homogenization buffer (10 mM Tris/1 mM EGTA/1 mM EDTA/1 mM DTT/1 mM PMSF/10 μg/mL leupeptin/4 μg/mL pepstatin). Efforts were taken to work quickly, as ARPP-90 degraded rapidly in the homogenate. After centrifugation for 30 min at 100,000 $\times$ *g*, the extract was immediately loaded on a 500-mL DEAE column equilibrated in buffer A [10 mM Tris (pH 8)/1 mM DTT] and washed with buffer A + 80 mM NaCl, and bound proteins were eluted directly in buffer A + 200 mM NaCl. The eluate was diluted 4-fold with buffer A, loaded on a MonoQ 16/10 column (GE Healthcare), and washed with buffer A + 100 mM NaCl. Bound proteins were eluted with a 450-mL linear gradient from 100–400 mM NaCl at 3 mL/min. Even fractions were analyzed for the presence of ARPP-90 by incubating 20 μL of each fraction with 10 μL reaction mix containing 5 mM $MgCl_2$, [γ-^{32}P]ATP (1 μCi/nmol), and $\approx$10 ng PKA catalytic subunit for 2 min at room temperature (RT). Peak fractions were pooled and concentrated by adding solid ammonium sulfate to 50% (wt/vol) followed by centrifugation. The protein pellet was resuspended in 300 μL buffer A and separated on a Superose 6 gel filtration column (GE Healthcare) at 0.2 mL/min while collecting 0.5-mL fractions. Fractions were assayed by phosphorylation with PKA, and the peak of ARPP-90 was concentrated by TCA precipitation, separated by SDS/PAGE, and transferred to a PVDF membrane. Proteins were visualized by staining with Coomassie G-250 and the ARPP-90 band was cut out and subjected to tryptic digestion and LC/MS/MS analysis (Rockefeller University DNA/Protein Technology Center).

Phosphopeptide Mapping. Excised SDS/PAGE gel pieces containing ^{32}P-labeled Rap1GAP were destained with 50% methanol/10% acetic acid in water, washed twice with 50% methanol, and digested overnight with 1 μg proteomics grade trypsin (Sigma) in 200 μL 50 mM NH_4HCO_3 (pH 8.0) at 37 °C. The supernatants containing the soluble phosphopeptides were concentrated to dryness using a speed-vac (Savant) and spotted on 20 $\times$ 20-cm flexible cellulose sheets (Polygram CEL400, Macherey–Nagel). Electrophoresis was performed at pH 3.5 (10% acetic acid/1% pyridine) for 90 min at 400 V, followed by ascending chromatography using pyridine/1-butanol/acetic acid/water (15:10:3:12). Phosphopeptides on dried sheets were detected by autoradiography.

Neostriatal Slice Studies. Male C57BL/6 mice at 6–10 weeks old (Charles River Labs) were anesthetized with halothane followed by decapitation. The brains were rapidly removed and placed in ice-cold, oxygenated Krebs-HCO_3 buffer (124 mM NaCl/4 mM KCl/26 mM $NaHCO_3$/1.5 mM $CaCl_2$/1.25 mM KH_2PO_4/1.5 mM $MgSO_4$/10 mM d-glucose, pH 7.4). Coronal slices (350 μm) were prepared using a vibrating blade microtome (VT1000S, Leica Microsystems). The dorsal and ventral striata were dissected from the slices in ice-cold Krebs-HCO_3 buffer. Slices were incubated in fresh Krebs-HCO_3 buffer and allowed to recover for 30 min under constant oxygenation with 95% O_2/5% CO_2 at 30 °C. Buffer was replaced with fresh prewarmed Krebs-HCO_3 buffer and incubated an additional 90 min at 30 °C before treatment. After drug treatment, slices were transferred to new tubes and frozen in liquid nitrogen. For immunoblotting, frozen slices were sonicated in 150 μl sample buffer and boiled for 5 min before separation by SDS/PAGE.

In Situ Hybridization. A mouse brain cDNA library was used as a template for PCR using the following primers: 5′-ATCCAGAGCTCTCAGAATGGAT-3′ (forward) and 5′-ACCCTATCCACAACCAAGGATA-3′ (reverse). The PCR product was cloned into pCR-TOPO II (Invitrogen) and used as a template for RNA synthesis in the presence of [α-^{33}P]UTP. Fresh frozen brains were sectioned in a cryostat at 16 μm and mounted on slides. Slides were fixed in 4% paraformaldehyde, pretreated with acylation solution, and dehydrated. Slides were hybridized with the labeled probe at 60 °C overnight. After hybridization, slides were washed with SSC and dehydrated before exposure to film.

Immunohistochemistry. Mice were perfused with PBS followed by 4% paraformaldehyde in PBS (pH 7.3). The brains were removed and incubated in 4% paraformaldehyde for 4–6 h at 4 °C and then transferred into 25% sucrose in PBS for 1–2 days. The tissues were frozen and sectioned into 45 μm-thick slices with a cryostat and collected into PBS. Sections were washed 3 $\times$ 5 min with PBS and soaked in blocking buffer [5% normal goat serum (NGS)/1% BSA/0.1% Triton X-100 in PBS] for 1 h at RT and then incubated with anti-Rap1GAP antibody (Epitomics Y134; 1:250) in blocking buffer overnight at 4 °C. After washing 3 times with PBS + 0.1% Triton X-100, the sections were incubated with secondary antibody (goat anti-rabbit 1:500 Alexa fluor 488, Molecular Probes) in blocking buffer. Following 3 $\times$ 5-min washes with PBST and H_2O, the sections were mounted onto microscopy slides and analyzed using a Zeiss confocal microscope.

Neuronal Culture, Transfection, and Immunostaining. Hippocampal primary neuronal cultures were prepared essentially as described (1) from embryonic day 18 (E18) rat embryos. Dispersed cells were plated in 24-well dishes at medium density (500 cells/mm^2) on coverslips coated with poly(D)-lysine (BD Biosciences). One day after plating, the culture media were changed to neurobasal medium supplemented with 2% B27/0.5 mM glutamine/12.5 μM glutamate/FDU (16.47 μg of uridine, 6.7 μg of 5-fluoro-2-deoxyuridine/ml). One-half of the media was exchanged with fresh media every 3–4 days. Neurons were transfected at 18 DIV using 0.3 μg of each plasmid DNA with 0.3 μL Lipofectamine 2000 per well for 4 h. After 48 h, coverslips were fixed (3.5% formaldehyde and 10% sucrose in PBS) for 20 min and permeabilized/blocked (10% NGS, 0.2% Triton X-100 in PBS) for 1 h at RT. Neurons were stained with rabbit anti-GFP (1:500; Invitrogen) and mouse anti-V5 tag (5 μg/mL; Invitrogen) antibodies in PBS + 2% NGS for 4 h at RT. After washing with PBS 3 $\times$ 10 min, coverslips were incubated with Alexa fluor secondary antibodies for 1 h. After washing as before, coverslips were mounted on slides (ProLong Gold antifade reagent, Invitrogen), dried overnight, and sealed.

In Vitro Rap1 GAP Assay. Recombinant Rap1A fused to 6xHis tag was expressed in *Escherichia coli* (BL21) and purified using Ni-NTA Agarose (Qiagen). Purified 6xHis-Rap1A was loaded with 5000 μCi/pmol [γ-^{32}P]GTP in 50 mM Hepes (pH 7.5)/5 mM EDTA/100 mM NaCl/1 mM DTT for 1 h at RT. Unbound GTP was removed by desalting into reaction buffer [50 mM Hepes (pH 7.5)/100 mM NaCl/5 mM $MgCl_2$/3 mM DTT], using a NICK

desalting column (GE Healthcare). Samples (5 μL) were mixed with 20 μL (5 μg) GTP-loaded Rap1 and incubated 10 min at RT. Reactions were terminated by the addition of 200 μL 75 mM phosphoric acid and 5% (wt/vol) activated charcoal and vortexing. After centrifugation for 10 min at 12,000 $\times$ *g*, the supernatant was transferred to a new tube and counted by Cerenkov scintillation.

1. Kawasaki H, *et al.* (1998) A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proc Natl Acad Sci USA* 95:13278–13283.

Fig. S1. Identification of ARPP-90 as Rap1GAP by mass spectrometry. The protein sequence of rat Rap1GAP is shown with identified tryptic peptides underlined.

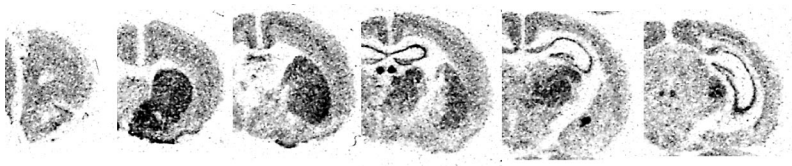

Fig. S2. Analysis of Rap1GAP expression in coronal sections of mouse brain by in situ autoradiography.

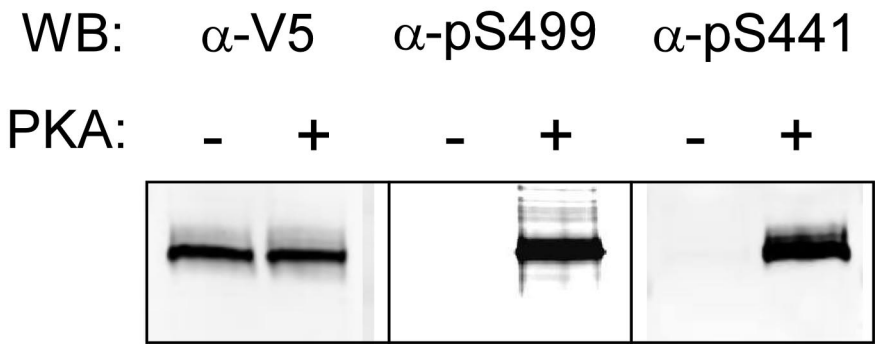

Fig. S3. Evaluation of phosphorylation-state specific antibodies for Rap1GAP Ser-441 and Ser-499. Affinity-purified sera were immunoblotted against 6xHis/V5-Rap1GAP purified from transiently transfected HEK293 cells without or with phosphorylation in vitro, using PKA catalytic subunit.

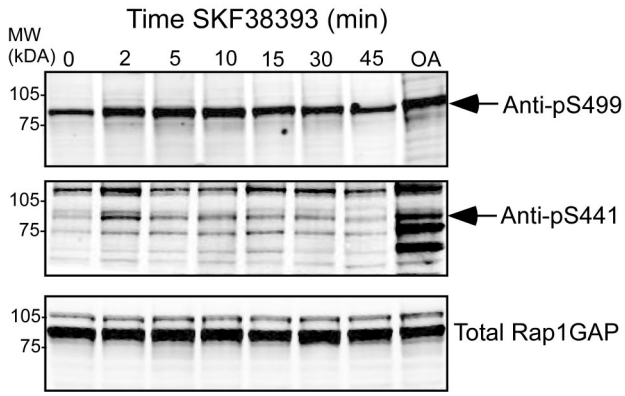

Fig. S4. Mouse neostriatal slices were treated for the indicated times with SKF38393 (10 μM). Slices were also treated with okadaic acid (1 μM, OA) for 30 min. Samples were then analyzed by SDS/PAGE and immunoblotting using antibodies specific for total Rap1GAP or for Rap1GAP phosphorylated at Ser-499 or Ser-441.

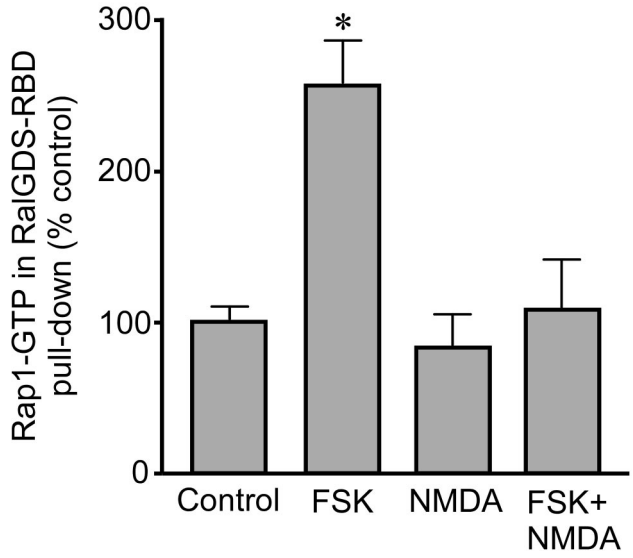

Fig. S5. Striatal slices were incubated for 10 min without or with forskolin (10 μM), NMDA (50 μM), or forskolin plus NMDA (NMDA added 10 min before forskolin). Rap1 activity was measured by a RalGDS pull-down assay as shown in Fig. 5*B*. Data are means ± SEM, $n = 3$. *, $P < 0.05$ vs. control.