Supplementary Methods

Antibodies
All primary antibodies were monoclonal: Pan-14-3-3 antibody (SC-629, Santa Cruz Biotech., Santa Cruz, CA, 1:500 dilution), myc antibody (clone 9E10, 1-500 dilution), RIM1 antibody (clone 21, 1:500 dilution, BD Biosciences Pharmagen, San Diego, CA), and GST antibody (clone GST-2, Sigma-Aldrich, St. Louis, MO, 1:1,000 dilution) were used. Secondary anti mouse antibodies were either horse radish peroxidase coupled (1:5,000 to 1: 20,000 dilution, ICN/Cappel, Aurora, OH) or 125I labeled (1:1000 dilution, Amersham Biosciences, Piscataway, NJ). Super Signal West Pico ECL reagent was from Pierce (Rockford, IL).

Plasmids
pGEX-KG-RimAP[377–516] wt, S413A, S413D vectors were previously described (Lonart et al., 2003; RIM1 residue numbers in brackets). V415P mutant RIM1 was made by site directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) using sense primer: 5’-GC CAC AGC GAC CCA GCG CTC CCG CAC ACC-3’. Base substitutions are in bold, BseY I restriction digest site, introduced to accelerate identification of mutant clones, is underlined. pCDA3.1-14-3-3-wt, pCDA3.1-14-3-3-DN, pCDA3.1/myc-14-3-3-wt, and pGEX-14-3-3 were kindly provided by Andrey S. Shaw, Washington University, St. Louis, MO. To make a myc-tagged pCNDA3.1-14-3-3-DN, the EcoRI-XhoI fragment of pCDA3.1-14-3-3-DN was cloned into the same sites of pCDA3.1/myc-14-3-3-wt.
Expression of Fusion Proteins, Phosphorylation of GST-RIM1 AP and Rodent Brain Lysate, and Binding Studies.

GST-fusion proteins were expressed in bacterial BI21 Gold cells as recommended by the manufacturer (Stratagene, La Jolla, CA). Myc-tagged 14-3-3 constructs were transfected into 293T cells using a calcium phosphate method. Cells were harvested 48 hrs later, frozen at -80 °C, and lysed with M-PER non-denaturing buffer (Pierce, Rockford, IL) immediately before use.

Fifty µg of fusion proteins bound to glutathione-beads were phosphorylated in a reaction buffer (250 µl) containing, 20 mM DTT, 2 mM MgCl₂, 1 mM EDTA, 100 µM ATP, 10 mM HEPES NaOH (pH 7.4), 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, and 60 U of the catalytic subunit of PKA (Sigma) for 30 min at 37°C. Reactions were stopped with ice cold reaction buffer that contained no ATP and MgCl₂ and was supplemented with 10 mM NaF, 10 mM Na₄P₂O₇, and 1 mM Na₃VO₄. Beads were washed 3 times with a washing buffer containing the same components plus 0.5% Triton-X-100, and used for subsequent binding studies.

Fresh rodent brain lysate for the Sp-cAMPS and okadaic acid (both from Biomol, Plymouth Meeting, PA) treatment and subsequent binding experiment was homogenized by a motorized pestle (900 rpm, 12 strokes) in ice-cold lysis buffer containing 20 mM DTT, 100 µM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, 10 mM HEPES NaOH (pH 7.4). Aliquots were treated with 100 µM Sp-cAMP, 1 µM okadaic, or the combination of the two for 5 or 30 min at 37 °C in the presence of 100 µM ATP, 2 mM MgCl₂, 1 mM Na₃VO₄, 1 mM NaF. Control sample was treated the same manner except for the presence of Sp-cAMPS, okadaic acid, ATP, and MgCl₂. After centrifugation at
15,000 X g for 20 min at 4 °C, pellet was solubilized by adding 20% SDS and 5 M NaCl to a final concentration of 1% and 150 mM, respectively. Nine volumes of 1.1% Triton-X-100 in 10 mM HEPES (pH 7.4) was added to dilute SDS to 0.1% final concentration. Insoluble particles were removed by centrifugation at 18,500 X g for 20 min at 4 °C and the supernatant was used for binding studies.

Brain lysate for 14-3-3 binding studies was prepared by homogenizing frozen rodent brains in 4 volumes of buffer containing 25 mM HEPES NaOH (pH 7.4), 125 mM potassium acetate, 5 mM MgCl₂, 220 mM sucrose, 1% Triton-X-100, 1 mM PMSF followed by centrifugation (20,000 x g, 20 min 4 °C) to separate insoluble material.

To perform binding assays, cell or brain lysates were added to 15-50 µg GST-tagged recombinant proteins immobilized to glutathione-agarose beads and incubated for 1 hr at 4 °C, washed three times with buffer containing 25 mM HEPES NaOH (pH 7.4), 125 mM potassium acetate, 5 mM MgCl₂, 220 mM sucrose, 1 mM PMSF, 0.5% Triton-X-100. For analyzing 14-3-3 protein binding, elution from beads was performed with 100 µl 1X SDS-PAGE sample buffer, and boiled for 5 min at 95 °C. For analyzing RIM1αbinding, elution from agarose beads was performed with 100 µl 1X SDS-PAGE sample buffer supplemented with 8 M urea, followed by heating for 20 min at 60 °C. Samples were centrifuged for 2 min at room temperature and 10 to 30 µl of supernatant was loaded for SDS-PAGE analysis, followed by Western blotting. Blots were developed by either applying ECL reagents and exposure to film, or for quantitative results, blots were exposed Phosphoimager screen (Molecular Dynamics, Sunnyvale, CA).
Transfection and Electrophysiological Analysis of Cultured Cerebellar Neurons

Neurons and glia from embryonic mouse cerebellum were cultured as described previously (Linden and Ahn, 1999). At 4–6 days in vitro, a fraction of gold particles (0.6 µm diameter, 25 mg) coated with 1 µg of EGFP plasmid (Clontech) and 5 µg of RIM1α or 14-3-3 expression vector were delivered by particle-mediated gene transfer using the Helios Gene Gun System (Linden and Ahn, 1999). Cultures were returned to the incubator and used for recordings 20–50 hr later. Transfected granule cells were identified by imaging EGFP signals. Whole-cell recordings were made from granule cell-Purkinje cell pairs as previously described (Linden, 2001; Wang and Linden, 2000). Cultures were superfused with a rate of 0.5 ml/min with a solution that contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES NaOH (pH 7.35), 10 mM glucose, and 0.2 mM picrotoxin. In some experiments, the GABAβ receptor antagonist 2-hydroxysaclofen was added at a concentration of 100 µM. For interneuron-Purkinje cell pairs, picrotoxin was excluded and the external saline was supplemented with 20 µM NBQX and 100 µM D-AP5. The electrode for Purkinje cell recording contained 120 mM CsCl, 10 mM HEPES, and 10 mM Cs4-BAPTA adjusted to pH 7.35 with CsOH. Sp-8CPT-cAMP-S was purchased from Alexis Biochemicals, Cs4-BAPTA from Molecular Probes, 2-hydroxysaclofen from Tocris Cookson and all other compounds were from Sigma. Patch electrodes were pulled from N51A glass and yielded a resistance of 3–5 MΩ. For stimulation/recording of granule cells or interneurons, slightly smaller electrodes (5–6 MΩ) were fabricated and filled with external saline for the loose-patch configuration. Membrane currents were recorded at room temperature with an Axopatch 200A amplifier in resistive voltage-clamp mode, filtered at 2 kHz, and digitized at 10 kHz. Rseries was compensated at 60%–75%. Cell pairs in which Rinput or Rseries varied by more than 15% were excluded from the analysis.