SUPPLEMENTARY METHODS

Antibodies and Plasmids: Mouse anti-GFP (Clontech, Palo Alto, CA), rabbit anti-phospho-p38 (NEB, Beverly, MA), mouse anti-pan-p38, mouse anti-Rac1 (Transduction Labs, Lexington, KY) and mouse anti-RhoA (sc-418, Santa Cruz Biotech, Santa Cruz, CA) were used with secondary reagents from various sources (Santa Cruz, UBI). pEGFP-C1 was from Clontech (Palo Alto, CA). pEBG-p38α was from Bruce Mayer (New Haven, USA). pCMV and pGEX-KG were from Sander van den Heuvel and John M. Kyriakis (MGH, Boston, MA). pGEX-PAK-CRIB-domain (amino acids 56-272), pGEX-Rhotekin-Rho-binding domain (amino acids 7–89), pRK5-Rac1-T17N, pRK5-RhoQ63L and pEF-C3 were from Alan Hall. pCGN-Rho-Q63L and pCGN-Rho-Q63L-VA were from Lawrence A. Quilliam (Indianapolis, USA). pRaichu-RBD was from Michiyuki Matsuda (Osaka, Japan), pYC2.12 and pYC3.60 were from Atsushi Miyawaki (RIKEN, Saitama, Japan). pSRE-luc was from Eric Metzger (Freiburg, Germany), pCMV-p115RGS was from Thomas Wieland (Heidelberg, Germany), pcDNA3-Gα13QL was from Manabu Negishi (Kyoto, Japan), pRc/CMV-CaMKIIδ wt and KD were from Harold A Singer (Danville, PA, USA) and pEBS7-Bcl2 was from Marja Jäättelä (Danish Cancer Society, Copenhagen). pVenus-H2B was prepared by inserting the coding sequence of Venus from YC2.12 into pECFP-H2B, which was obtained from Jan Ellenberg (EMBL).

Production of immobilised GST, GST-PAK-CD and GST-Rhotekin fusion proteins: Escherichia coli BL21(DE3) cells transformed with pGEX-KG (generous gift of John Kyriakis) pGEX-PAK-CRIB-domain (amino acids 56-272) or pGEX-Rhotekin-Rho-binding domain (amino acids 7–89) (generous gifts of Alan Hall) constructs were grown
at 37°C to an absorbance of 0.7 OD$_{595\text{nm}}$. Expression of recombinant protein was induced by 0.3 mM isopropylthiogalactoside for 4 h at 25°C, and purified by affinity chromatography on glutathione sepharose.

*Rho and Rac1 pull-down assay:* Rho and Rac activity were measured by the previously described Rhotekin and PAK-CRIB pull-down assays$^{1,2}$. 8 days after plating in 10-cm dishes, cerebellar granule neuron cultures were treated with or without glutamate 50 µM as above. After 3 min of glutamate addition, cells were rinsed with ice cold PBS once and lysed in 800 µl lysis buffer (50 mM Tris pH 7.2, 500 mM NaCl, 1% (v/v) Triton X-100, 5 mM MgCl$_2$, 1 mM DTT and protease inhibitors). Homogenized lysates were precleared and supernatants were incubated with 10 µg immobilised GST bound to glutathione-sepharose to remove non-specific binding. Beads were spun out and lysates rotated with 10 µg of immobilised GST-Rhotekin at 4°C for 30 min followed by two washes in 50 mM Tris pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 5 mM MgCl$_2$ and 1 mM DTT. The aspirated pellets were boiled at 95°C in laemmli sample buffer, and active RhoA was detected by immunoblotting with monoclonal anti-RhoA antibody. Supernatants precleared with immobilised GST and GST-Rhotekin and adjusted to PAK-binding buffer (50 mM Tris pH 7.2, 100 mM NaCl, 1% (v/v) Triton X-100, 2 mM MgCl$_2$, 1 mM DTT and protease inhibitors), or in the case of Fig S2 cells lysed directly in PAK-binding buffer and precleared with immobilised GST, were incubated with 20 µg of immobilised GST-PAK-CRIB-domain fusion protein at 4°C for 30 min and washed 2 times with lysis buffer. Protein was eluted in sample buffer and blotted with anti-Rac1 monoclonal antibody.

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Assay of p38α activity in transfected cells: 7 days after plating in 35 mm dishes, cerebellar granule neurons were transfected as previously described\(^3\). Cortical neurons were transfected as described\(^4\) and hippocampal neurons were transfected similarly. They were co-transfected with the plasmids pEBG-p38α together with either empty vector pCMV, pEF-toxin-C3, pRK5-Rac1-T17N, pRK5-RhoQ63L as indicated (generous gifts of Bruce Mayer, Sander van den Heuvel and Alan Hall respectively). Neuro2A cells, maintained in MEM, 10% (v/v) FCS, 1:100 nonessential amino acids (Life Technologies BRL) and antibiotics, were transfected with plasmids as indicated, using Polyfect (Qiagen) according to the manufacturer’s protocol. 24 h after transfection, cells were treated as described for 5 min or left untreated as indicated, rinsed once with ice-cold PBS and lysed in 500 µl of lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM benzamidine, 1 µg/ml aprotinin, leupeptin, and pepstatin, and 100 µg/ml PMSF). Homogenized and precleared supernatants were incubated with 10µl (bed volume) pre-washed S-Hexylglutathione-agarose beads (Sigma, St. Louis, MO) for 3 hr at 4°C. Beads were spun out and washed 3 times in lysis buffer followed by boiling the drained pellet in 40µl 1 x laemml sample buffer and immunoblotting. GST-p38α ran at about 65kD, and anti-phospho-p38 and anti-pan-p38 immunoreactive bands shown migrate at this molecular weight.

Luciferase reporter assay: COS7 cells at 50-70 % confluence were transfected with pSRE-luc reporter pEGFP-C1 as transfection marker, pRL-TK as internal control and expression plasmids for constitutively active Ga13 (Ga13QL), RGS domain of

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p115RhoGEF (p115RGS) and pEF-C3 exoenzyme as indicated. After 24 h, cells were lysed and promoter activity was quantitated and graphed as previously described\textsuperscript{35}.

Pyknosis assay for transfected cerebellar neurons and neuroblastoma: 7 day in vitro cerebellar granule cultures and Neuro2A neuroblastoma cultures were co-transfected with marker plasmid together with either empty vector pCMV, pEF-toxin-C3, pRK5-Rac-T17N, pRK5-Rho-T19N, pRK5-Rho-Q63L, pCGN-Rho-Q63L or pCGN-Rho-Q63L-VA as indicated in the figures. When tested, co-transfection efficiency was near 100\%\textsuperscript{5}. The marker plasmid used was EGFP-C1 for pyknosis assays 3 h after glutamate, or pHistone2B-Venus for pyknosis assays at 24 h when membrane integrity may be lost. Twenty-four h after transfection, cells were treated where indicated with or without glutamate as described above. 3 h after stimulation, cells are fixed with 4\% paraformaldehyde, rinsed with ice-cold PBS and stained with Hoechst 33342. For transfected neurons, fluorescence image fields of GFP emission using 450-490 nm excitation light and a 20x air objective was taken to locate transfected cells, and the corresponding image of Hoechst fluorescence was examined to determine whether the transfected cell has a pyknotic nucleus. Four evenly spaced fields were counted per sample, or a single central field per sample in the case of the more efficiently transfected neuroblastomas. Imaging of DNA dyes and transfected fluorescent proteins was carried out with a cooled CCD and Olympus microscope with appropriate filter cubes. Data presented are means +/- SEMs of the number independent experiments indicated. Statistical analysis used the Student’s two-tailed t-test. The number of cells counted in all cytometry-based experiments is shown by figure part in supplementary table 1.
**Focal ischaemia model:** Ischaemia was induced in adult Balb/cA mice (Taconic, ~1 year old, 20–25 g) under deep anesthesia by middle cerebral artery occlusion, electrocoagulating a main branch of the middle cerebral artery close to its origin at the junction with the olfactory branch). After 30 min, brains were rapidly removed and snap-frozen in liquid nitrogen.

**Viability assay of transfected hippocampal neurons:** Hippocampal neurons were transfected at 7 DIV with C3 expression plasmid (pEF-C3) or empty vector (pCMV) together with expression plasmid for soluble fluorescent protein marker (Venus). After 24 h, cells were exposed to 100 μM NMDA or left untreated as indicated. After 24 h, cells were washed and fixed with 4% paraformaldehyde. Hoechst and propidium iodide were added 30 min before fixation as described. Fluorescence images were captured with an IX70 microscope with 10x objective and the number of Venus-positive cells was counted from 4 fields per coverslip. Viability data shown was calculated as the mean number of cells per normalised to the number of cells in fields from untreated cultures. The exclusion of propidium iodide was checked to validate that Venus-positive cells had an intact plasma membrane. Pyknosis was not used as a marker for these cells because they are cultures of mixed neuronal types which exhibit considerably greater heterogeneity of initial nuclear size than the single neuron-type cerebellar granule cultures, and we wished to avoid the possible confusion between a healthy cell with small nucleus and a the shrunken nucleus of a cell which initially had a large nucleus. Data and statistical
analysis were as for the pyknosis assays. Data and statistical analysis were as for the pyknosis assays.


