SUPPLEMENTARY MATERIAL

METHODS

Primary antibodies

The following primary antibodies were used: Affinity-purified rabbit polyclonal antibody against βgc (Quiroga et al., Proc. Natl. Acad. Sci. USA 92: 4309-4312, 1995), diluted 1:200; rat monoclonal antibody against tyrosinated α-tubulin (clone YL 1/2), diluted 1:200; anti-c-myc antibody produced in rabbit (Sigma), 1:800; antibody to the phosphorylated (tyr) binding motif of p85 PI3k (Cell Signalling), diluted 1:200; PY20 monoclonal antibody (Santa Cruz Biotechnology), diluted 1:500; mouse monoclonal antibody against TrkB (Santa Cruz Biotechnology), used at 1:200 for immunofluorescence and at 1:100 for Western blot; mPar3 antibody was a generous gift from Dr. T. Pawson (Mount Sinai Hospital, Toronto, Canada); rabbit antibody to phosphorylated IFG-1R (Cell Signalling), diluted 1:50; mouse monoclonal antibody to the insulin receptor β-subunit (Chemicon International), diluted 1:200; antibody to the axonal marker tau-1 (Calbiochem) diluted 1:200.

Animals

All animal procedures were done using approved protocols by the Board of Animal Welfare, School of Chemical Sciences, National University of Córdoba.

Cell Culture
Dissociated hippocampal pyramidal cells were prepared from embryonic rat brain and cultured as follows: Cells were plated onto polylysine-coated glass coverslips and maintained in DMEM plus 10% horse serum for 1h. The coverslips with the attached cells were transferred subsequently to 60-mm Petri dishes containing serum-free medium plus the N2 mixture. To allow for neuronal survival and growth, this medium contains a high level of insulin sufficient to stimulate the receptors for insulin as well as IGF-1. In some experiments (where indicated) we additionally included in the tissue culture medium 50 ng/ml BDNF to stimulate TrkB receptors or 50 ng/ml NGF to stimulate TrkA receptors. Cultures were maintained in a humidified 37° incubator with 5% CO₂. Shortly after plating, hippocampal neurons first extend lamellipodia (stage 1) and afterwards several minor neurites that are initially indistinguishable (stage 2). Then, at stage 3, one of these initially equivalent neurites grows more rapidly than the others and becomes the axon, whereas the other neurites subsequently develop into dendrites (stage 4). Neurons are considered to be at stage 3 when the length of the axon exceeds that of the average minor neurite by at least 20 m (Craig and Banker, Annu. Rev. Neurosci. 17, 267-310, 1989).

Cell transfection

By screening a fetal rat brain expression library with an anti-βgc antibody prepared in our laboratories (Quiroga et al., Proc. Natl. Acad. Sci. USA 92: 4309-4312, 1995) we cloned a 150-bp sequence that is essentially identical to a segment of the published IGF-1R. The cloned sequence encodes a peptide close to the extracellular amino terminus of the IGF-1R β subunit. Because there is only one known IGF-1R gene and the different receptor variants thus are generated by alternative splicing, we used a published siRNA sequence (biotinylated) to suppress IGF-1R synthesis (GCCCAUGUGUGAGAAGACCTT; Bohula et al., J Biol Chem
This sequence did indeed silence expression of IGF-1R containing the \( \beta_{gc} \) antigen, which is abundant in developing neurons and enriched at the growth cone (Quiroga et al., Proc. Natl. Acad. Sci. USA 92: 4309-4312, 1995; Mascotti et al., J. Neurosci. 17: 1447:1459, 1997). RNA oligonucleotides (106 nM) were mixed with Lipofectamine 2000 and added to the neurons 2 h after plating. In order to reveal transfection we used a FITC-conjugated mouse monoclonal antibody to biotin. Transfection efficiencies were about 50 to 60 \%. In some experiments neurons were co-transfected with fast cycling (fc) or negative dominant (nd) forms of cddc-42 (Chuang et al., Dev. Cell 9: 75-86, 2005).

**Blocking IGF-1R with antibodies**

Neurons were grown in the presence of a mouse monoclonal antibody (clone aIR3 GR11L, Calbiochem) that blocks activation of the IGF-1R (concentration, 1 \( \mu \)g/ml). Clone alpha IR3 binds an epitope within the alpha-subunit adjacent to the ligand-binding site and blocks IGF-I binding to its receptor without affecting either the insulin or IGF-II receptors (Kull, F.C., et al. 1983. J. Biol. Chem. 258, 6561; Rohlik, Q.T., et al. 1987. Biochem. Biophy Res. Comm. 149, 276), cross-reacts with the alpha-subunit of the rat IGF-1 receptor (Syroid, DE. et al., 1999 J Neurosci 19:2059; Chung, YH. et al., 2002 Brain Res 946:307) and has been extensively used in IGF-1 receptor blocking experiments (see e.g. Linseman et al., 2002 J Biol Chem 277:25546). Fresh antibody was added to the culture medium every 8 h.

**Immunofluorescence**
Cells were fixed for 1 h at room temperature with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) containing 4% (wt/vol) sucrose. Cultures were washed with PBS, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 30 minutes, and again washed in PBS. After labelling with a first primary antibody (1-3 h at room temp.) and washing with PBS, cultures were incubated with fluorescent secondary antibody (conjugated to Alexa fluor 488, 546 or 633; 1 h at 37°C) and washed with PBS. The same procedure was repeated for the second and third primary and secondary antibodies. The cells were observed with a Zeiss Axiovert microscope equipped with epifluorescence optics. Fluorescence images were captured with a CCD camera (Hamamatsu), digitized directly into a Metamorph/Metafluor Image Processor (Universal Imaging Corporation, West Chester, PA), and printed using Adobe PhotoShop.

**Immunofluorescence to the active IGF-1R**

Cells were cultured as described. After 6 or 12 hs in culture, cells were deprived of growth factors for 4 h and challenged for 2 m with 10 nM IGF-1, fixed, and processed for immunofluorescence as described, using an antibody selective for the phosphorylated form of the receptor (See Fig. 1 Supplementary material -online).

**Gel Electrophoresis and Western Blot**

Proteins were separated by SDS-polyacrylamide gel electrophoresis. The concentration of acrylamide of the resolving gel varied from 7.5 to 11%. The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes in Tris-glycine buffer containing 20% methanol. The membranes were either first dried, washed with Tris-buffered saline
(TBS; 10 mM Tris pH 7.5, 150 mM NaCl) and then blocked, or directly blocked for 1 h in TBS containing 5% BSA. The blots were incubated with the primary antibodies in PBS containing 0.05% Tween 20, for 2 h at room temperature. After washing with TBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Promega Corp., Madison WI) for 1 h at room temperature. After washing the blots were developed using a chemiluminescence detection kit (ECL, Amersham Life Sciences Inc., Arlington Heights, IL).

**Cdc42 Activity Assays**

Cdc42 activity was measured in protein extracts of siRNA- or ssRNA-transfected neurons with the “Cdc42 Activation Assay” (Cytoskeleton, Denver, CO). Briefly, the GTP-bound form of Cdc42 was affinity-purified on GST-agarose containing the Cdc42-binding domain of Pak1, and Cdc42 levels were determined by immunoblot as described.