

SUPPLEMENTARY INFORMATION TO VILAR ET AL.

MATERIALS AND METHODS

T7 phage display

A T7 phage display cDNA library was prepared from differentiated PC12 cells according to manufacturer's instructions (Novagen). Briefly, poly(A) RNA was purified from differentiated PC12 cells 5 d after NGF treatment using a mRNA purification kit from Invitrogen. Randomly primed cDNA was synthesized, ligated with adapters, and unidirectionally cloned into the T7Select1-1b phage using the T7Select1-1 Cloning Kit according to the manufacturer's protocols (Novagen). The cDNA was size-selected by agarose gel electrophoresis to exclude fragments < 500 bp. The library was amplified once in liquid culture before use.

The intracellular domain of p75^{NTR} (p75^{ICD}) was produced and purified as previously described (Liepinsh et al., 1997). Purified p75^{ICD} was biotinylated using the EZ-link reagent from Pierce, and immobilized onto streptavidin-coated paramagnetic beads (Promega) by incubating 1 mg of beads with 50 mM biotinylated p75^{ICD} in phosphate buffer saline (PBS) supplemented with 0.1% Nonidet P-40 (PBS-N) for 30 min at room temperature with gentle mixing. Beads were then washed four times in PBS-N, resuspended in 1 ml Superblock-PBS solution (Pierce) before biopanning.

Phage particles for biopanning were prepared by infecting *E. coli* BL5615 cells (Novagen) with 1×10^{10} p.f.u. of library stock. After complete lysis, the lysate was cleared by centrifugation and supplemented with *E. coli* protease inhibitor cocktail (Sigma-Aldrich, P-8465) and Pan Mix buffer (Novagen). Phages were then incubated with 0.1 ml beads coated with p75^{ICD} during 30 min at room temperature or overnight at 4°C. Beads collected on a magnetic support were washed 4 times by complete resuspension in 1.5 ml PBS supplemented with 0.5% Nonidet P-40, and then eluted with 0.1 ml of 1% SDS for 15 min at room temperature. The eluate was used to infect BL5615 cells which were then plated

overnight until the appearance of plaques. Phages recovered by elution in PBS were supplemented with Pan Mix and used in a second round of biopanning. After a third round, serially diluted phage eluate was used to infect BL5615 cells for isolation of monoclonal phages. DNA inserts were amplified by PCR using primers described by the manufacturer, and analyzed by automatic DNA sequencing. Details of the complete T7 phage display protocol are available from the Novagen website at <http://www.emdbiosciences.com/html/NVG/home.html>. Micropanning experiments using the isolated Bex1 phage and unrelated controls confirmed the specificity of the interaction between this phage and the intracellular domain of p75^{NTR} (data not shown).

Site-directed mutagenesis

Site-directed mutagenesis of various constructs was done using the Quick-Change method (Stratagene). All epitope taggings of Bex1 (HA, Flag, GFP and GST) were done at the N-terminus of the protein. GST-Bex1 was expressed in the pGEX1 vector (Promega), and produced and purified in *E. coli* following standard procedures.

Cell culture

PC12 cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal calf serum (FCS), and gentamycin, unless otherwise indicated. HEK293T cells were cultured in DMEM supplemented with 10% FCS and gentamycin. Schwann cells were extracted from the newborn rat sciatic nerve by collagenase treatment and expanded in DMEM supplemented with 10% FCS, 10 ng/ml basic FGF and 10 μ M forskolin. Non-proliferative cultures were obtained by removing FGF and forskolin for 48 h. SVZ-derived neurospheres were grown in DMEM:F12 supplemented with B27 (Life Technologies), bFGF and EGF. After 5 days, they were dissociated, transfected, and allowed to grow in suspension for

another 5 days. Neuronal differentiation was initiated by plating on poly-D-Lysine-coated dishes in the absence of mitogens.

***In situ* hybridization**

Rat brains from embryonic day (E) 13.5 and E19.5 were removed and rapidly frozen in OCT (optimal cutting temperature) compound at -70°C . In situ hybridization was performed on 14 μm cryosections with a ^{35}S -labeled rat Bex1 riboprobe using standard procedures. Briefly, sections were first fixed in 4% PFA, deproteinized in 0.2 M HCl, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, and dehydrated in increasing concentrations of ethanol. Slides were incubated overnight in a humidified chamber at 53°C with 106 cpm of probe in 200 μl of hybridization cocktail, washed, dehydrated, air-dried, and dipped in NTB-2 photoemulsion (Kodak). After 8 week exposure, slides were developed and counterstained with thionin.

Immunoprecipitation, immunoblotting and metabolic labeling

Total cell lysates for immunoprecipitation were obtained by extracting cell monolayers with 1% Triton X-100, 60 mM β -octylglucoside, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA supplemented with a protease inhibitor cocktail (Roche) for 60 min at 4°C . For rapid cell lysis that did not require subsequent immunoprecipitation, cell monolayers were directly lysed in 10% trichloroacetic acid (TCA), centrifuged and boiled in SDS-PAGE sample buffer. Immunoprecipitations were performed by overnight incubation at 4°C with the corresponding antibodies followed by precipitation with gamma-bind protein-G beads (Amersham), and elution by boiling in sample buffer. SDS-PAGE and immunoblotting were performed using standard procedures. After processing, PVDF filters were developed by Enhanced Chemifluorescence (ECF, Amersham) and scanned in a STORM 840 fluorimager (Molecular Dynamics). For metabolic labeling, cell monolayers were pre-incubated in phosphate-free

DMEM supplemented with dialyzed FCS, followed by 1 h incubation with 1 mCi/ml of ^{32}P orthophosphate in phosphate-free DMEM. After washing with PBS, cells were lysed as above.

Antibodies

Antibodies were obtained from various sources as follows: anti-phospho-ERK (Thr-202/Tyr-204), anti-AKT, anti-phospho-AKT and anti-phospho-AKT substrate from New England Biolabs; monoclonal anti-HA from Covance; anti-Flag, anti-actin and anti- α -tubulin from Sigma; anti-myc from Santa Cruz; anti-p75^{NTR} extracellular domain MC192 mouse monoclonal from Chemicon; anti-GST, secondary anti-mouse and anti-rabbit IgG conjugated to peroxidase from Amersham. Rabbit polyclonal anti-serum against the intracellular domain of p75^{NTR} (9992) was kindly provided by Dr. Moses Chao.

BrdU incorporation and gene reporter assays

BrdU was used at 10 μM for 30 min or 5 h. Anti-BrdU monoclonal antibody was from DAKO. BrdU positive nuclei were counted under epifluorescence illumination and expressed as the percentage of the total number of nuclei by counter-staining with DAPI. For gene reporter assays of NF- κ B activity, cells were transfected with a plasmid carrying the firefly luciferase gene under the control of a promoter sensitive to NF- κ B (Clontech) and the Renilla luciferase plasmid pRL-TK (Promega) to control transfection efficiency. One day after transfection, cultures were treated with 100 ng/ml NGF for 4 h and processed with the Dual-Luciferase Reporter Assay kit from Promega. The values obtained for firefly luciferase activity in each well were normalized with the corresponding values of Renilla luciferase activity.

siRNA and Bex1 knock-down

The hairpin DNA sequence used in the Bex1 siRNA construct was as follows: 5'-GCAAAAATTGTGCGCCTAGAGTCTCTTGAACTCTAGGCGCACAAATTTTTGC-3',

with the loop region underlined. The following protocol was used to assess the effects of the Bex1 siRNA construct on neuronal differentiation. Forty eight hours after transfection with the siRNA plasmid construct, PC12 cells were synchronized by 24 h serum starvation. Serum was then replaced (i.e. 72 h after transfection) to re-initiate the cell cycle, and neuronal differentiation was induced 12 h later —i.e. during the S phase of the cell cycle— by addition of 100 ng/ml NGF. Neuronal differentiation was then assessed at 12, 36 and 60 h following NGF addition by scoring the proportion of cells bearing neurites longer than 1- or 2-cell diameters.

REFERENCES

Liepinsh, E., Ilag, L.L., Otting, G. and Ibáñez, C.F. (1997) NMR structure of the death domain of the p75 neurotrophin receptor. *EMBO J.*, **16**, 4999-5005.

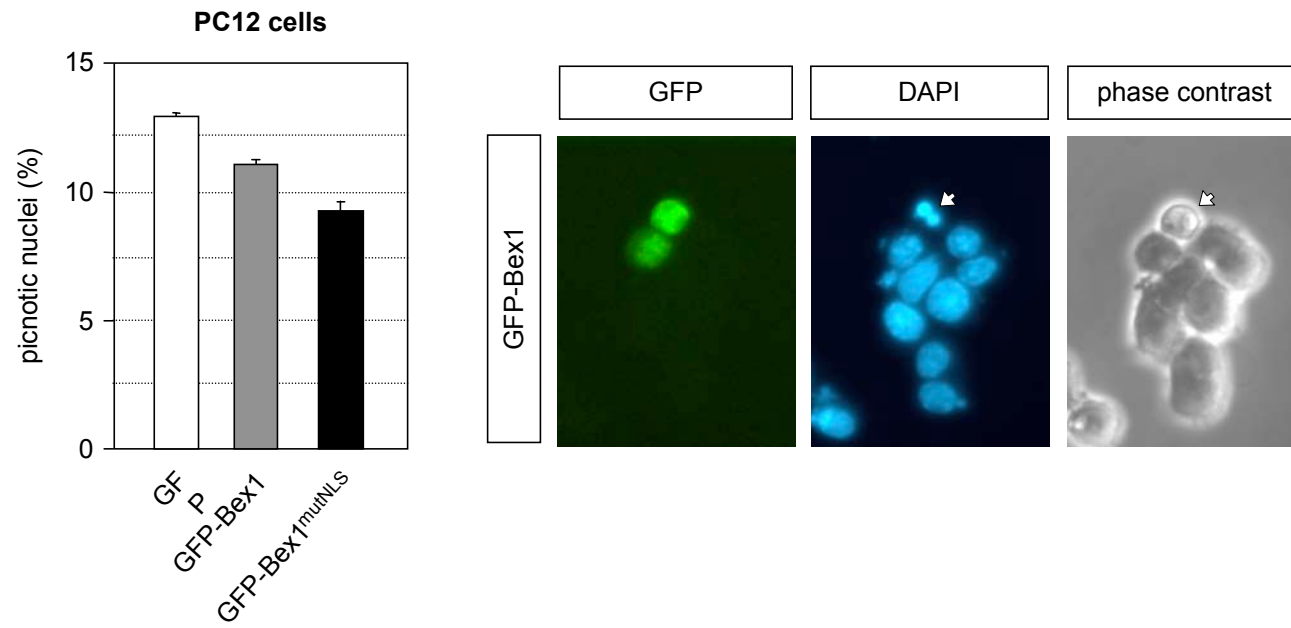
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Bex1 overexpression does not affect PC12 cell survival.

The percentage of picnotic nuclei were quantified in PC12 cells 24 h after transient transfection with the indicated plasmid combinations. Shown are averages of three independent experiments (each performed in triplicate) \pm SEM. Micrographs to the right show two cells transfected with GFP-Bex1 (GFP panel), one of which showed a picnotic nucleus (arrow, DAPI panel).

Supplementary Figure 2.

Flow cytometry analysis of serum-starved cultures of parental PC12 cells and clone C4 overexpressing Bex1. Cell cycle phases are indicated. Note the enlarged proportion of cells in S phase in clone C4 compared to parental cells.



24h serum free

