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Technical Communication

Adenovirus vector-directed expression of the neurotrophin-3 receptor (TrkC) in mouse astrocytes

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In the present article we report the generation of a neurovirological reagent, an adenovirus vector that efficiently delivers the gene for the Neurotrophin-3 (NT-3) receptor, TrkC. Using this AdTrkC vector, we examined the induction of the expression of the above neurotrophin receptor in pure cultures of mouse astrocytes, a glial cell type that does not constitutively express this gene. Infection of astrocytes at an optimal dose of 100±200 plaque forming units (p.f.u.) per cell, induced expression of specific mRNA, as demonstrated by RT± PCR and Northern blot. This mRNA was translated to produce a mean of 20,157 biologically active receptor molecules per astrocyte with a K_d of 4.1×10^{-11} M, as demonstrated by ^{125}I-NT-3 binding. After 2D electrophoresis, the mature glycoprotein and some precursors were recognised by antibodies raised against the carboxy-terminal peptide of Trk. Binding of the ligand induced autophosphorylation of TrkC and ^{3}H-thymidine incorporation in transduced cells. These results demonstrate that our AdTrkC vector efficiently mediates the expression of high-levels of biologically active NT-3 receptors. *Journal of NeuroVirology* (2001) **7,** 72–81.

Keywords: astrocytes; adenoviral vector; NT-3; TrkC

Introduction

Neurotrophins mediate their trophic activities through the Trk family of tyrosine kinase receptors (Barbacid, 1993). The TrkC gene product, gp145^{trkC}, is a high affinity receptor for neurotrophin-3 (NT-3), a member of the neurotrophin factors family (Lamballe *et al*, 1991, 1993). The TrkC locus is expressed in both the embryonic and adult central and peripheral nervous systems (Lamballe *et al*, 1991). Nevertheless, when chicken E11 sympathetic neurons are cultured in the presence of NT-3, p75^{NTR} is the only detectable NT-3 receptor (Dechant *et al*, 1997), and in the absence of TrkC, NT-3 uses TrkA instead (Cordon-Cardo *et al*, 1991).

Although astrocytes produce NT-3 mRNA (Rudge *et al*, 1992) and it is suggested that might secrete NT-3 (Barres *et al*, 1994), no full-length TrkC and only a low level of expression of the truncated form of TrkC, incapable of signal transduction, is found in those cells (Rudge *et al*, 1994).

NT-3 gene targeting induces severe sensory and sympathetic deficits (Ernfors *et al*, 1994; Fariñas *et al*, 1994), as well as death of spinal sensory neurons in mice (Fariñas *et al*, 1996). Furthermore, disruption of the TrkC gene eliminates group Ia muscle afferents and results in abnormal movements of 'knockout' mice (Klein *et al*, 1994). An important role in oligodendrocyte development has been also proposed for NT-3 (Barres *et al*, 1994). In addition, deficiencies in astrocyte development were observed in NT-3 and TrkC double 'knockout' mice, suggesting that both play some role in the proliferation of such glial population (Kahn *et al*, 1999).

Successful gene transfer in the central nervous system (CNS) is dependent on efficient methods of introducing a particular transgene into the appropriate population of neuronal or glial cells. This poses an exceptional challenge since the CNS contains a wide range of cell types and post-mitotic neural cells are refractory to transduction with classical retroviral vectors or are infected with limited efficiency (Luskin *et al*, 1988). Gene transfer to several neural cells can be achieved by using adenoviral vectors. These vectors are not dependent

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on host cell replication and allow expression of a foreign gene in a wide range of post-mitotic cells, including neurons and astrocytes (Akli *et al*, 1993; Bajocchi *et al*, 1993; Blömer *et al*, 1996; Davidson *et al*, 1993; Hermens *et al*, 1997; Kaplitt and Makimura, 1996; Le Gal La Salle *et al*, 1993; Neve, 1993; Ridoux *et al*, 1994; Sabate *et al*, 1995; Smith *et al*, 1995).

This methodology can be used to modify the physiology of neurons and glial cells *in vitro* and *in vivo* (Horellou *et al*, 1994; Lawrence *et al*, 1995; Xu *et al*, 1994). For instance, the adenoviral vector-mediated expression of NGF has been shown to reduce the consequences of aging (Castel-Barthe *et al*, 1996). In addition, astrocytes infected with an adenoviral vector encoding NT-3 increased survival of cultured neurons (Smith *et al*, 1996) and adenovirus-directed NT-3 expression in rat dorsal root ganglions induced neurite outgrowth (Dijkhuizen *et al*, 1997).

The purpose of the present study was to generate an adenoviral vector encoding TrkC and to demonstrate that this vector can be employed to induce astrocytes to express the receptor for NT-3 in amounts that are sufficient to result in a biological response in the presence of the ligand.

Results

Construction of the adenoviral vector

A recombinant adenovirus carrying a TrkC expression cassette was generated by cotransfection of *Cla*I and *Xba*I restriction digested Ad5 genomic DNA with the plasmid pNR2 (Figure 1). Cotransfection was done in 293 cells. Homologous recombination resulted in the formation of several plaques, which were further purified. To confirm the presence of TrkC cDNA in the adeno vector, plaques were subjected to PCR with specific primers, as stated under Materials and methods. A stock of an AdTrkC selected, PCR-positive plaque, was prepared and cesium chloride purification was carried out. The titer of this purified virus was 1×10^{10} p.f.u./ml as determined on 293 cells. This stock was employed in all studies reported here.

Adenoviral infection of astrocytes

In order to determine whether our primary astrocyte cultures were susceptible to adenovirus infection, we infected them with a control AdRSVβGal vector at different m.o.i. for 24–48 h. Incubation with the X-gal substrate labelled in blue the nuclei of around 75% of the cultures (not shown). The staining was nearly 100% after a 48 h incubation period in the presence of the mitotic inhibitor cytosine arabinofuranoside (Sigma), at a concentration of 10 μM, demonstrating that almost all the cells were susceptible to gene transfer with adenovirus vectors. Therefore, we concluded that under our experimental conditions astrocytes were fully sensitive to adenovirus infection, as previously stated by other authors (Le Gal La Salle *et al*, 1993; Ridoux *et al*, 1994).

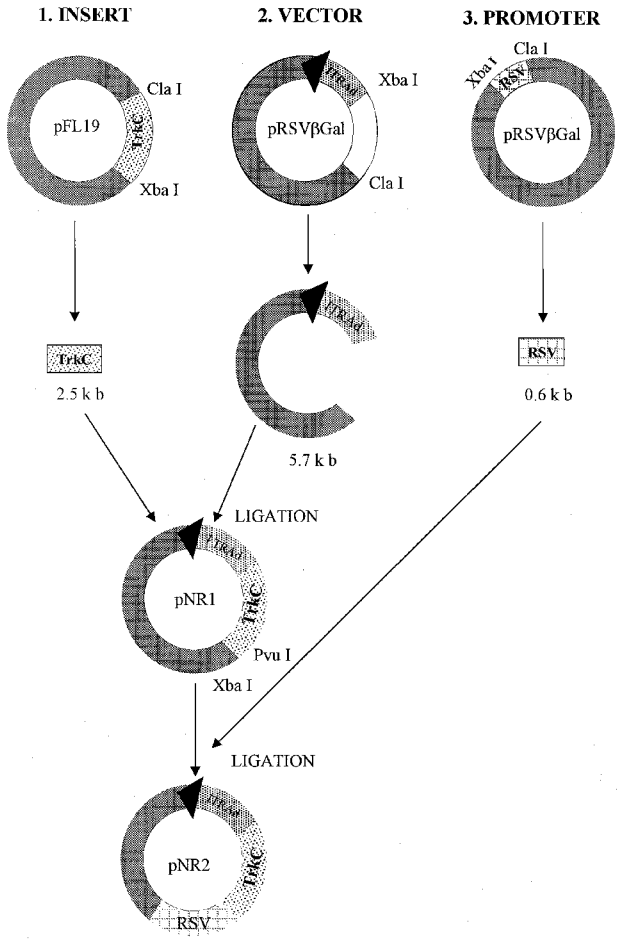

Figure 1 Construction of the adenovirus targeting plasmid pNR2. It contains the TrkC expression cassette flanked upstream by the adenoviral inverted terminal repeat (ITRAd) and downstream by the Rous sarcoma virus (RSV) promoter. To construct the AdTrkC adenoviral vector, the above plasmid was linearized by *Ase*I digestion and cotransfected with *Cla*I/*Xba*I restriction-digested Ad5dl309, into 293 cells. Homologous recombination between viral and plasmid DNA results in the formation of the AdTrkC vector.

Reverse transcriptase-PCR analysis

The presence of mRNA specific for full-length TrkC in cultures of mouse astrocytes, either mock-infected or infected at different m.o.i. with the AdTrkC vector for 24–48 h, was studied using an RT–PCR strategy. The specific amplimers described under Materials and methods amplified discrete sequences from the plasmid Bluescript pFL19 (Lamballe *et al*, 1991) which encodes full-length TrkC (Figure 2). This 703 bp fragment encodes the extracellular, transmembrane and tyrosine kinase domains of the 145 000 dalton glycoprotein $gp145^{trkC}$, the receptor for NT-3.

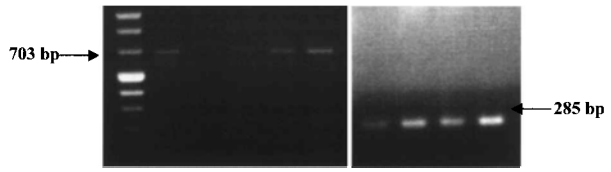

Figure 2 Expression of TrkC mRNA in AdTrkC-infected astrocytes. Plasmid pFL19 amplified by PCR with specific amplimers produces a fragment of 703 bp (Lane+). RNA from astrocytes mock-infected (0) or infected at a m.o.i. of 10, 100 and 200 were reverse transcribed and PCR amplified by using the same amplimers for TrkC or β-Actin primers from Promega. (Lane M): DNA molecular weight markers (pBR322 DNA-*Msp*I digest, New England Biolabs).

Uninfected astrocytes do not express physiologically active TrkC, as suggested previously by the fact that NT-3 does not influence DNA synthesis in these cells (Barres *et al*, 1994). Nevertheless, mRNA is fully reverse-transcribed and amplified from cells infected with 10, 100 or 200 p.f.u./cell of our adenovirus vector AdTrkC (Figure 2). The optimal dose, as detected by TrkC fragment amplification, is a m.o.i. of 200. Negative controls where the Moloney murine leukaemia virus reverse transcriptase was omitted, did not produce any band in the chain reaction, demonstrating that we were actually amplifying mRNA. β-Actin amplification was shown as positive control message amplification (Figure 2).

Northern blot analysis

Northern blot analysis was used to confirm the presence of transcripts coding for $gp145^{trkC}$. Using the ^{32}P-labelled TrkC 2500 bp insert from pFL19 as a probe, again we demonstrated the presence of TrkC mRNA in infected cells, and as seen with PCR, peaking at a m.o.i. of 200 (Figure 3). A unique transcript of 6.1 kb was found and the absence of constitutive expression of the gene was again demonstrated (Figure 3, lane 0). Controls for equal RNA loading was performed with a β-actin probe.

Previous attempts to induce the expression of TrkC mRNA by astrocyte stimulators including TNF, IL-1 and cAMP failed after treatment with different doses and periods of time (not shown).

Western blots of 2D gel electrophoresis

Urea-extracted proteins from uninfected astrocytes or astrocytes transduced with the AdRSVβGal or the AdTrkC vectors were separated by 2D gel electrophoresis. Figure 4, upper panel, shows the separation of proteins from astrocytes transduced by the adenovirus vector AdTrkC and stained with silver.

Western blots were developed with an anti-pan-Trk antiserum prepared by immunising rabbits with a synthetic peptide corresponding to the 14 carboxy-terminal residues of TrkA (Martin-Zanca *et al*, 1989). Four different proteins, two of them with two isoforms, showing isoelectric points between 5.2 and 6.7 and molecular weights between 145 000 and 80 000 dalton, were recognised by the anti-Trk antibody (Figure 4, lower panel, arrows). No antibody staining was observed with the proteins extracted from mock-infected astrocyte cultures or from cultures transduced with the control AdRSVβGal (not shown).

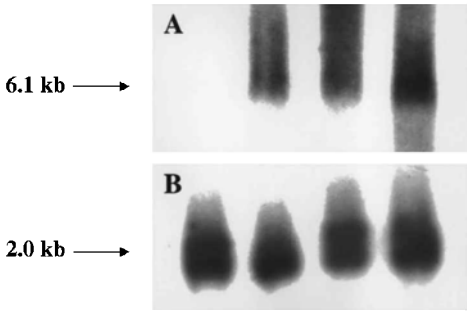

Figure 3 TrkC mRNA expression in astrocytes demonstrated by Northern blot analysis (**A**). Blots of mock-infected cultures (Lane 0) or cultures infected with AdTrkC at a m.o.i. of 10, 100 or 200 are shown. mRNA was extracted and probed with the pFL19 TrkC insert. Internal controls for equal total RNA loading were performed with a human β-actin cDNA probe from Clontech (**B**).

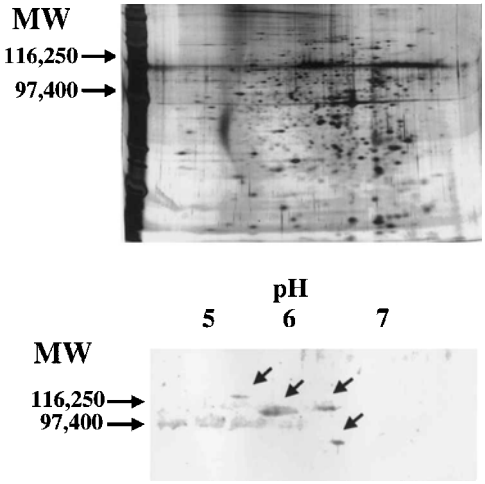

Figure 4 Silver stained 2D gel electrophoresis of the proteins (10 μg) extracted from AdTrkC-transduced astrocytes (upper panel). The proteins (50 μg) from a gel run in parallel were transferred onto nitrocellulose membranes and developed with a rabbit antibody against the synthetic peptide corresponding to the 14-carboxy-terminal residues of TrkA (arrows, lower panel). The broad range SDS–PAGE molecular weight standards from Bio Rad were used to determine molecular weights.

The 145 000 dalton single form could be the mature, biologically active receptor. Both isoforms of around 110 000 dalton could correspond to intermediate glycosylated forms and the 80 000 dalton molecule to the protein backbone, as happens for TrkA (Martin-Zanca *et al*, 1989).

Binding of ^{125}I-NT-3 to transduced astrocytes

The binding experiments were performed at 4°C in order to avoid complex kinetics resulting from internalisation following binding. Transduced astrocytes were incubated with ^{125}I-NT-3 for 60 min, allowing equilibrium to be reached. Figure 5 shows the results obtained with ^{125}I-NT-3 at concentrations between 1 and 16 $\times 10^{-12}$ M. Analysis of the linear transformation resulting from the binding data, performed with the GraphPad Prism™ program, revealed a unique high affinity saturable receptor with an equilibrium dissociation constant (K_d) of 4.1 $\times 10^{-11}$ M and further showed that there were a mean of 20 157 binding sites per cell. Our binding curve and data are similar to those reported for the high-affinity NT-3 binding sites on embryonic chicken sensory neurons (11 126 receptors per cell with a K_d of 1.8 $\times 10^{-11}$ M) (Rodríguez-Tebar *et al*, 1992) or the 45 000 p75 receptors acting with high-affinity on sympathetic neurons, with a K_d of 6 $\times 10^{-11}$ M (Dechant *et al*, 1997).

As shown also in Figure 5, no specific binding could be detected in astrocytes transduced with the AdRSVβGal negative control vector or in mock-infected cells, despite that a low level of expression of the truncated form of TrkC has been reported by Rudge *et al*, 1994. Therefore, it is likely that the binding sites found correspond to gp145^{trkC} receptors in the AdTrkC transduced cells.

Induction of DNA synthesis

To determine if the transduced astrocytes were responsive to NT-3 via the TrkC receptor, we examined DNA synthesis by tritiated thymidine incorporation. As shown in Table 1, addition of foetal calf serum to quiescent astrocytes infected 24 h before with AdTrkC, induced these cells to enter the S phase. Cells cultured in the absence of FCS or of any ligand (no addition) did not incorporate thymidine. Addition of different amounts of NGF and BDNF failed to elicit any detectable mitogenic response. The lower c.p.m. incorporation in the presence of NGF and BDNF was not statistically significant when compared with the untreated astrocytes (Table 1). However, when NT-3 was added to AdTrkC-transduced astrocytic cells, DNA synthesis was initiated, peaking at 20 ng/ml (Table 1). Negative control cultures, provided by AdRSVβGal-transduced astrocytes, do not incorporate significant amounts of tritiated thymidine when cultured in the presence of NT-3 (10–50 ng/ml). These results clearly indicate that the AdTRkC-directed expression of TrkC confers mitogenic responsiveness to NT-3 to transduced astrocytes.

NT-3 binding elicits autophosphorylation of gp145^{trkC}

We next examined whether the interaction of NT-3 with transduced gp145^{trkC} receptors elicits further functional responses as autophosphorylation, in

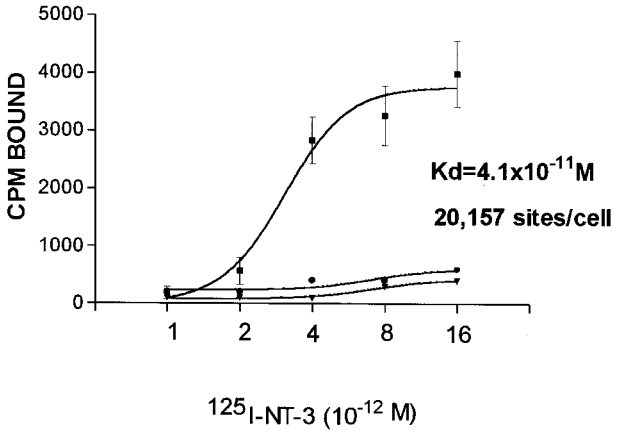

Figure 5 Saturation binding of ^{125}I-NT-3 to AdTrkC-transduced astrocytes (■——■). Specific binding of various concentrations (1–16 picomoles) of ligand at 4°C are shown. Non-specific binding was measured in the presence of a 100-times excess of unlabelled NT-3. Specific binding was determined by subtraction of non-specific binding from total binding. Negative controls were provided by mock-infected (●——●) or AdRSVβGal-transduced (▼——▼) astrocytes.

Table 1 H^3-TdR incorporation in astrocytes infected with AdTrkC

Additions	*c.p.m.*
None	731[a]
FCS	
20%	20.334*[b]
10%	17.230*
5%	9.933*
2%	4.611*
NT-3	
50 ng/ml	2.568*
20	4.047*[c]
10	2.350*
NGF	
50 ng/ml	235
20	437
10	282
BDNF	
50 ng/ml	725
20	432
10	493

[a]All figures represent the means of reproducible triplicate determinations. The significance of the differences was calculated by Student's *t*-test. Statistically significant values ($P<0.05$) were marked with *. [b]27.8 times increase. [c]5.5 times increase.

addition to ^{3}Hthymidine incorporation. To do so, we used a monoclonal antibody that recognises phosphorylated tyrosine residues in Western analysis of cell homogenates. As shown in Figure 6, incubation of isolated membrane from astrocytes transduced at different m.o.i. with 50 ng/ml NT-3 induced the rapid autophosphorylation of tyrosine residues in gp145^{trkC}. In contrast, we did not observe gp145^{trkC} phosphorylation when we used negative control AdRSVβGal-infected astrocytic cell membranes (Figure 6 o). Maximal phosphorylation was found in cells infected at a m.o.i. of 100. No phosphotyrosine-positive band in the 145 KDa area was observed in transduced astrocytes (m.o.i. of 100) in the absence of the NT-3 ligand (not shown).

Discussion

During nervous system development, neuronal populations reach a balance between the final number of neurons and the sizes of its target territories. These target zones secrete limiting amounts of several neurotrophins that mediate survival, differentiation and function of neurons. The action of the neurotrophin family of neurotrophic factors is mediated mainly by the tyrosine kinase Trk receptors. These interactions are very specific; NGF binds TrkA, BDNF and NT-4 binds TrkB and NT-3 binds TrkC (Barbacid, 1995). Binding of the different ligands to Trk receptors induces autophosphorylation and activation of several signalling pathways such as *ras*, PI-3 kinase, MAP kinases, etc, promoting differentiation and survival.

Due to the postmitotic state of most of the cells in the adult nervous tissue, viral vectors dependent of host cell replication, such as retrovirus, cannot be used for gene transfer. This situation could be overcome by the use of safe adenovirus vectors. Wild-type serotype 5 (Ad5) adenovirus infects resting cells in which it induces lytic productive cycles. The use of replication-defective adenovirus vectors represents a valuable tool for gene transfer

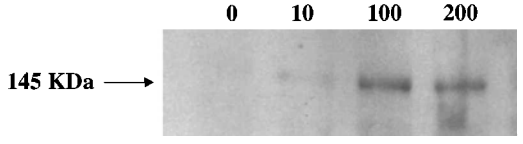

Figure 6 NT-3 induces autophosphorylation of tyrosine residues in transduced gp145^{trkC}. Isolated membranes from AdRSVβGal-infected cells at a m.o.i. of 100 (o), or from AdTrk-infected cells at a m.o.i. of 10, 100 or 200, were incubated for 10 min at 22°C in the presence of 50 ng/ml of NT-3. After 8% SDS-polyacrylamide gel electrophoresis, samples were transferred to a nitrocellulose membrane and blotted with an anti-phosphotyrosine monoclonal antibody (mAb PY20) and a secondary horseradish peroxidase-coupled anti-mouse IgG, as described under Material and methods. The migration of gp145^{trkC} is indicated by an arrow.

to a variety of terminally differentiated nervous system cell types (Akli *et al*, 1993; Bajocchi *et al*, 1993; Davidson *et al*, 1993; Neve, 1993; Sabate *et al*, 1995).

This article evaluates the use of an adenovirus gene transfer vector for the *in vitro* delivery of the TrkC gene, coding for the high affinity receptor for NT-3, into a nervous system cell population, such as the astrocytes. Our data demonstrate that the vector we constructed, AdTrkC, can efficiently transfer and drive the expression of the transgene in these glial cells maintained in tissue culture.

Following infection with our adenoviral vector at levels ranging from m.o.i. of 10 to 200 for 24 – 48 h, we demonstrated that synthesis of TrkC-specific mRNA occurs by using reverse transcriptase-PCR and Northern blot analysis (Figures 2 and 3). Bands migrating at the correct position in gels were found in AdTrkC-infected cells, but not in mock-infected astrocytes. When we separated by 2D electrophoresis the proteins from transduced astrocytes and further used an anti-Trk antibody as a probe in Western blot, we detected the presence of a 145 000 dalton molecule, with an isoelectric point of 5.3, that is the putative gp145^{trkC} NT-3 receptor (Figure 4, lower panel). In addition to that, the polypeptide backbone of around 80 000 dalton and several isoelectrically heterogeneous molecules, that could correspond to intermediate stages of glycosylation, were detected by the antibody directed to the carboxy-terminal tail of Trk protein. Glycosylation of TrkA has been demonstrated to occur also at two different stages, presumably during its transport to the plasma membrane (Martin-Zanca *et al*, 1989).

A genuine fully active NT-3 receptor protein is translated from this mRNA as specific binding of ^{125}I-NT-3 could be measured in transduced cells. Conversely, no specific binding could be found in control mock-infected or AdRSVβGal-infected astrocytes (Figure 5). In order to determine whether transduced astrocytes follow similar signal transduction pathways as cells expressing TrkC naturally, we studied whether the presence of NT-3 in the culture medium induces DNA synthesis and/or autophosphorylation of the *de novo* created gp145^{trkC}. Indeed, a functional and specific response is elicited as addition of recombinant NT-3 stimulates thymidine incorporation (Table 1), as well as rapid phosphorylation of tyrosine residues in gp145^{trkC} (Figure 6). This functional response is fully specific as NGF and BDNF do not induce thymidine incorporation (Table 1). No phosphorylation is detected either in astrocytes transduced by AdRSVβGal (Figure 6 o) or in AdTrkC-transduced cells in the absence of NT-3 (not shown).

No apparent toxic effects were detected on the cultures due to infection. Furthermore, confluent non-dividing astrocyte cultures express TrkC, as detected by RT – PCR, up to 1 month, the longest time tested.

The rationale of our choice of astrocytes instead of primary neurons is to induce fully active TrkC in nervous cells that never express it. Therefore, we do not expect that expression of TrkC in astrocytes would induce profound biological responses as is the case for induction of new neurotrophin receptor expression in neurons. We have detected (a) changes in DNA synthesis and (b) receptor tyrosine phosphorylation, but not dramatic changes as migration, morphology changes or potent survival stimuli must be expected. Nevertheless, previous studies with NT-3 and TrkC knockout mice demonstrated a reduction in astrocyte cell markers *in vivo*. This fact suggests a role for NT-3 and its receptor as key regulators of normal astrocytic cell development (Kahn *et al*, 1999).

There is an increasing interest in adenovirus-mediated gene delivery as a tool for basic neuroscience research and treatment of several kinds of neurological disorders. Our results suggest that adenoviral vectors encoding neurotrophin receptors of the Trk family could be applied in the future for the local expression of receptors that, in combination with exogenous or endogenous neurotrophins, could promote regeneration of injured nervous tissue. Additional biological studies are undertaken to know if our vector could also assist in preventing the degeneration of some populations of neurons. Furthermore, some reports relate the expression of TrkC and good prognosis of tumours of nervous tissue origin (Ryden *et al*, 1996; Yamashiro *et al*, 1997; Grotzer *et al*, 2000); opening the possibility of the use of AdTrkC in the treatment of neuroblastomas and medulloblastomas.

Materials and methods

Construction of the adenoviral vector
The plasmid pFL19, generated in pBluescript (Stratagene, La Jolla, CA, USA) and encoding the full length TrkC gene of porcine origin (Lamballe *et al*, 1991, 1993), was grown and purified by using the Qiagen Plasmid Midikit (Qiagen Inc, Santa Clarita, CA, USA). The 2500 bp insert, coding for the 145 000 dalton gp145^{trkC} protein, was purified from agarose gels after digestion with *Xba*I and *Cla*I (Figure 1, 1). The vector containing the ITR-Ad was purified from agarose gels after *Xba*I and *Cla*I digestion from pRSVβGal (Stratford-Perricaudet *et al*, 1992) (Figure 1, 2). The Rous sarcoma virus (RSV) promoter was purified also from pRSVβGal after *Xba*I and *Cla*I digestion (Figure 1, 3). The recombinant plasmid pNR1 was constructed by T4 ligation of the TrkC insert with the vector containing the ITR-Ad. After linearization with *Xba*I and *Pvu*I, the promoter was ligated with T4 ligase to obtain the final plasmid pNR2.

Construction of the recombinant adenoviral vector AdRSVTrkC (AdTrkC) was done by cotransfecting 293 cells with linearized pNR2 and *Cla*I/*Xba*I restricted Ad5dl309 DNA by homologous recombination. Five μg of each partner DNA, Ase 1-linearized pNR2 and *Cla*I and *Xba*I restriction digested Ad5 DNA (Yeh *et al*, 1996), were cotransfected into near confluent 293 cells by the $Ca_3(PO_4)_2$ method (Graham *et al*, 1977). All restriction enzymes were purchased from Life Technologies Sarl, Cergy Pontoise, France.

Viral stock preparation
Viral stocks of AdRSVβGal and AdTrkC were prepared from infected 293 cells by standard procedures (Stratford-Perricaudet *et al*, 1992). Briefly, cells were infected and infection was allowed to proceed until complete detachment of the cell monolayer occurred (8–12 days postinfection). The culture medium was then harvested, clarified and concentrated by ultrafiltration. Viruses were subsequently purified by isopycnic CsCl ultracentrifugation, desalted on PD-10 desalting columns (Pharmacia Biotech, Uppsala, Sweden) and eluted in phosphate buffered saline (PBS) before being aliquoted and stored at -70°C in PBS-10% glycerol. All viral stocks were titrated on 293 monolayers and their clonality was assessed by PCR.

Quantification of β-galactosidase activity
Infection controls were systematically included by counting the number of cells that scored positive following infection with AdRSVβGal and X-gal staining. Monolayers of astrocytes were infected at a multiplicity of infection (m.o.i.) of 100 with the control AdRSVβGal virus and incubated in the absence or presence of the chromogenic substrate X-gal (Sigma Chemical Co, St Louis, MO, USA), at a final concentration of 50 μg/ml, to score blue staining.

Astrocyte cultures
Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex of newborn Balb/c mice (Rubio *et al*, 1990). The cortex was isolated under a dissecting microscope and cleaned of choroid plexus and meninges. Cell suspensions were filtered through 80 μm pore mesh into Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum (FCS) and gentamicin (Flow Laboratories, Irvine, UK). After centrifugation, cells were filtered through a 20 μm mesh sieve, plated in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) and cultured at 37°C. The medium was changed after 4 days of culture and subsequently three times a week for the entire culture period. Cultures were enriched in astrocytes by removal of less adherent oligodendrocytes by shaking for 18 h at 37°C and 250 r.p.m. in a G24 environmental shaker (New Brunswick Scientific, Edison, NJ, USA). Cellular confluence was obtained 10 days after plating, and a polygonal flat cell

morphology was observed. A mean of 98% astrocytes was confirmed by indirect immunofluorescence staining of methanol-fixed cultures with rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (Dakopatts, Glostrup, Denmark) and fluorescein-labelled goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN, USA). The lack of noticeable oligodendrocytes and microglial/macrophage cells was determined by using a guinea-pig anti-myelin basic protein (MBP) antiserum prepared as described elsewhere (Rubio and Cuesta, 1989) and a monoclonal anti-Mac-1 antibody (Serotec, Oxford, UK). Secondary fluorescein-labelled antibodies against guinea-pig and rat IgG were purchased from Sigma.

PCR analysis

Total RNA was reverse-transcribed by using Moloney murine leukaemia virus reverse transcriptase (RT) (Promega, Madison, WI, USA) and the 3′ amplifier as template/primer. The amplimers used in the PCR reaction for TrkC amplification were: 5′: (5′-GGGTGTGTGGTGATGGTGACCCACTCATCATGGTC-3′); 3′: (5′-GCCAAGAATGTCCAGGTAGATCGGGGTGGCCTTCC-3′). Primer pairs for β-Actin were supplied by Promega.

PCR reactions were carried out for 40 cycles at an annealing temperature of 55°C using a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, CA, USA). The resulting PCR products were purified by using S-400 MicroSpin columns (Pharmacia Biotech) and electrophoresed in 2% NuSieve agarose gels (FMC Bio Products, Rockland, ME, USA) in TAE buffer, stained with ethidium bromide and photographed in a Gelstation System (TDI, Barcelona, Spain).

RNA isolation and Northern analysis

Astrocyte cell monolayers were infected in 75 cm^2 flasks. Total RNA was purified by using RNeasy Mini or Midi purification kits (Qiagen). Ten micrograms of RNA per lane were denatured in formamide and formaldehyde and electrophoresed in MOPS (morpholinepropanesulphonic acid) buffer through 1% agarose-0.66 M formaldehyde gels and capillary blotted to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Hercules, CA, USA). UV-cross linking, prehybridisation, hybridisation, and washing of the membranes were carried out as recommended by the supplier. Probes were labelled with ^{32}P-dCTP (Amersham Life Science, UK) by using the random-primed method kit (Boehringer Mannheim, Germany). Unincorporated radiolabel was removed with S-300 MicroSpin columns (Pharmacia Biotech). The blots were exposed to Kodak X-OMAT film with an intensifying screen (Hyperscreen, Amersham) at −75°C. A human β-actin cDNA probe (Clontech, Palo Alto, CA, USA) was systematically included as a loading and transfer control. To ensure maximum homogeneity among the RNA samples, all RNA preparations to be compared were carried out in parallel.

The probe used for TrkC blotting was the 2500 bp insert purified from pFL19 after *Xba*I and *Cla*I digestion, as previously stated. After electrophoresis in 2% agarose, the DNA band was excised from the agarose gel with a scalpel and purified using the QIAEX II kit provided by Qiagen.

Two-dimensional gel electrophoresis

Proteins from cultured cells were solubilized with urea-4% NP-40 and subjected to two-dimensional, high resolution isoelectric focusing (IEF) and electrophoresis (2D), as originally described by O'Farrell (1975). Briefly, first-dimension gels in glass capillary tubes (4% acrylamide, 2% bisacrylamide, 9.5 M urea, 2% NP-40, 10.5 μl TEMED and 500 μl ampholines (LKB, pH 3.5–10 and 5–7 in a 1:1 ratio) per 10 ml of acrylamide solution) were polymerized by addition of 13 μl of 10% ammonium persulphate. The lower (anodic) reservoir contained 0.01 M H_3PO_4 and the upper (cathodic) reservoir contained 0.02 M NaOH. Proteins were subjected to electrophoresis toward the anode at 400 V for 15 min and 750 V for 3.5 h. When the IEF run is completed, the gels were extracted from the tubes and the proteins were electrophoresed in a second-dimension 10% acrylamide-SDS slab gel according to Laemmli (Laemmli, 1970).

Western blots of 2D gels

2D electrophoresis gels were blotted to nitrocellulose filters (Trans-Blot, Bio-Rad). Rabbit antiserum anti-pan-Trk raised against a synthetic peptide corresponding to the 14 carboxy-terminal residues of the deduced TrkA sequence (Ala Leu Ala Gln Ala Pro Pro Val Tyr Leu Asp Val Leu Gly) (Martin-Zanca *et al*, 1989) was used in a 1:300 dilution and horseradish peroxidase (HRP)-coupled secondary donkey anti-rabbit antibodies (Amersham) were diluted 1:2000. Blocking and incubation with antibodies were carried out in a solution of 5% powder milk in TNT (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween). Detection of the antibody was done with the ECL Western blot detection system (Amersham), according to the manufacturer's instructions.

Binding assay

Human recombinant NT-3 (Promega, Madison, WI, USA) was labelled with lactoperoxidase (Sigma) and ^{125}I from Amersham, as described previously (Rodríguez-Tebar *et al*, 1992). Astrocytes cultured in 35 mm dishes (Costar) were used in the binding experiments. Three dishes were used for the determination of unspecific binding (preincubated for 30 min with a 100-fold excess of unlabelled NT-3) and three for the determination of total binding at each point. Specific binding was determined by subtraction of unspecific from total binding. Bind-

ing was performed with gentle horizontal shaking at 4°C. The buffer used was Hank's Balanced Salt Solution (Flow) containing 0.2% bovine serum albumin (BSA) from Sigma. After washing three times with ice-cold buffer and drying at room temperature, cells were detached from the plastic surface with 2% SDS and counted in an LKB-Wallac 1282 Compugamma counter (Pharmacia Biotech). Scatchard plot analysis of the data from binding assays was performed with the GraphPad Prism™ program (GraphPad Software, San Diego, CA, USA).

Thymidine incorporation

Cells in 96-well clusters (Costar) were infected with AdTrkC as previously stated and made quiescent by washing twice with serum-free DMEM followed by a 24 h incubation in DMEM containing 0.25% FCS. After adding the corresponding growth factors and incubating for 18 h, the cultures were pulsed with 1 μCi of [^{3}H]thymidine per well (5 Ci/mmol, Amersham) for an additional 6 h period, washed once with cold PBS, trypsinized, and the amount of [^{3}H]thymidine incorporated into DNA determined by filtration through glass filters (Schleicher & Schuell, Dassel, Germany) with an automatic sample harvester (Skatron Inc, Sterling, VA, USA). Filter-bound [^{3}H]thymidine was measured by liquid scintillation counting in 1 ml of Aquasol-2 cocktail (New England Nuclear, Boston, MA, USA) in a LKB-Wallac 1410 liquid scintillation counter (Pharmacia Biotech).

Autophosphorylation of TrkC in vitro

Mouse astrocytes mock-infected or infected with AdTrkC at m.o.i. of 10, 100 and 200 were washed in Ca^{2+} and Mg^{2+}-free PBS, scraped and homogenised by 10 strokes of a Dounce homogeniser using a tight-fitting pestle (Wheaton Scientific, Millville, NJ, USA) in 1.5 ml of homogenising buffer (20 mM HEPES buffer, pH 7.4, containing 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF and 10 μg/ml leupeptin) at 4°C. The lysates were centrifuged at 1500 ×g for 10 min at 4°C and the resulting pellets were rehomogenised in 1 ml of homogenising buffer and recentrifuged as stated. The two supernatants of each membrane sample were pooled and further centrifuged at 30 000 ×g for 60 min at 4°C. The pellets were resuspended in 100 μl of precooled 20 mM 2-N-morpholinoethanesulphonic acid (MES) buffer (pH 7, containing 100 μg/ml leupeptin) and the protein concentration measured by the Bradford method (Bradford, 1976) using bovine IgG as standard. Membrane samples (25 μl, 25 μg of protein/sample) were incubated with 10 μl of NT-3 (final concentration, 50 ng/ml) in the presence of 10 μl of incubation buffer (120 mM HEPES, pH 7.5, 6 mM $MnCl_2$, 60 μM $ZnCl_2$, 180 μM Na_3VO_4) for 10 min at 22°C and then phosphorylated for 10 min at 4°C with 5 μl of 50 mM ATP. The reaction was stopped by addition of 12 μl of 5 ×Laemmli's sample buffer. Samples were heated for 3 min in a boiling water bath, electrophoresed on an 8% PAGE–SDS gel and subjected to Western blotting on nitrocellulose filters. The primary antibody used was anti-phospho-Tyr mAb PY20 (Santa Cruz Inc., Santa Cruz, CA, USA) and the secondary antibody, horseradish peroxidase coupled-antimouse IgG mAb (Sigma). Staining was made with the ECL system (Amersham) following the instructions of the manufacturer.

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