The Neurotrophin Receptor p75 Binds Neurotrophin-3 on Sympathetic Neurons with High Affinity and Specificity

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High-affinity neurotrophin-3 (NT3) receptors have been identified on nerve growth factor (NGF)-dependent sympathetic neurons, but their occupancy by NT3 does not lead to neuronal survival. The molecular nature of these NT3 binding sites was investigated in this study. With freshly dissociated embryonic day 11 (E11) chick sympathetic neurons, cross-linking experiments revealed that the main receptor responsible for high-affinity specific binding was the neurotrophin receptor p75 (p75^{NTR}), with only a small fraction corresponding to trkC. When E11 sympathetic neurons were cultured in the presence

of NGF, *trkC* transcripts became undetectable, but high-affinity specific NT3 binding persisted. Cross-linking and antibody inhibition experiments indicated that p75^{NTR} was the only detectable NT3 receptor protein. These characteristics were not observed when p75^{NTR} was expressed in transformed cells. We conclude that p75^{NTR} can exist in neurons in a configuration conferring hitherto unrecognized properties to this receptor.

Key words: neurotrophins; neurotrophin receptors; sympathetic neurons; neurotrophin-3; p75^{NTR}; *trkC*

Neurotrophin-3 (NT3) shows a unique pattern of receptor interactions, because it binds to all identified neurotrophin receptors. Like all neurotrophins, NT3 interacts with the p75^{NTR} receptor, but it also binds and activates the receptor tyrosine kinases *trkA*, *trkB*, and *trkC*. *In vivo*, binding of NT3 to *trkC* is clearly of relevance, because there are striking similarities in the phenotypes of *NT3* (–/–) and *trkC* (–/–) mice (for review, see Snider, 1994; Lewin and Barde, 1996). With regard to neuronal survival, however, the loss of *trkC* function is less dramatic than that of NT3, and detailed analyses with neurons isolated from *trk* (–/–) mice have revealed that in the absence of *trkC*, NT3 uses *trkA* and *trkB* instead (Davies et al., 1995). Although remarkably useful for examining the *in vivo* relevance of neurotrophin receptor genes, such studies do not define the various molecular components participating in neurotrophin binding at the surface of neurons. *In vitro* studies, notably with PC12 cells, have helped to define the molecular nature of high-affinity specific NGF binding sites (Chao et al., 1986; Radeke et al., 1987; Hempstead et al., 1991). These studies led to the conclusion that p75^{NTR}, expressed at suitable levels, increases the ability of the NGF receptor *trkA* to detect low concentrations of NGF (Benedetti et al., 1993; Barker and Shooter, 1994) and confers increased specificity of NGF binding for *trkA* (Davies et al., 1993; Lee et al., 1994). With regard to neurotrophins other than NGF, only a few studies to date have examined the molecular nature of neurotrophin binding sites on neurons. With BDNF and NT3, previous cross-

linking experiments have identified *trkB* and *trkC* as components of high-affinity receptors on embryonic neurons (Escandón et al., 1993; Rodríguez-Tébar et al., 1993; Escandón et al., 1994), but the participation of p75 in the formation of such sites, if any, remains unclear.

The chick sympathetic neurons are one population of neurons that are amenable to receptor composition analyses. Like dissociated chick retinal cells, used previously for similar purposes (Rodríguez-Tébar et al., 1993), they can be isolated in sufficient numbers. Sympathetic neurons can be studied both after acute dissociation and in cell culture after they have formed long processes. This is of interest, because *in vivo*, neurotrophins are more likely to encounter neurotrophin receptors on processes than on cell bodies. In a previous study, high-affinity specific NT3 binding sites were described on embryonic day 11 (E11) sympathetic neurons (Dechant et al., 1993b). Such sites can usually be correlated with neuronal survival, but such was not the case with these neurons. The same neurons co-express high-affinity sites for NGF, and occupancy of the latter led to neuronal survival, indicating that all the components necessary to link tyrosine kinases with the prevention of cell death machinery were functional in these neurons (Dechant et al., 1993b). The results presented here unexpectedly reveal that the main component for NT3 high-affinity specific binding on sympathetic neurons and their processes can be accounted for by p75.

MATERIALS AND METHODS

Reverse transcription-coupled PCR. Total RNA was obtained from sympathetic ganglia and neuronal cell cultures, with use of the RNeasy kit (Qiagen, Hilden, Germany), and treated with DNase. One microgram of total RNA was reverse-transcribed with 0.5 μ g of oligo-dT_{12–18} (Pharmacia, Piscataway, NJ) and Superscript II Reverse Transcriptase (Life Sciences, Hialeah, FL) for 1 hr at 45°C. Transcripts of 30–60 ng total RNA were amplified (94°, 62°, 72°C, 30 min each) with 0.1 μ M primer (see below), 0.33 mM dNTP (Pharmacia), and 0.4 μ l (2 U) Ampli-Taq (Perkin-Elmer, Emeryville, CA) in a total volume of 60 μ l. SYBR Green

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(Biomol, Plymouth Meeting, PA) stained amplicates were analyzed by 1% agarose gel electrophoresis. *TrkC* amplicates were transferred onto Hybond N membrane (Amersham-Buchler, Braunschweig, Germany) and hybridized against a ³²P-labeled *trkC*-specific cDNA probe. The linear range of the amplification reactions was determined for all primer combinations. Chick glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts were amplified for 18–20 cycles with the primers 5'-TGGAAGCTTACTGGAATGGC-3' and 5'-GCAGGATGCAGAACTGAGCG-3', chick *trkC* full-length and kinase truncation transcripts (Garner and Large, 1994) for 24–26 cycles with 5'-AGCCCCACCAATGACAAAATGC-3' and 5'-CTCCAGAGGATCACCCCG-3', chick *trkC* kinase deletion transcripts (Garner and Large, 1994) for 34–36 cycles with 5'-CCAATAAGCCTCCCTGGACATTCC-3' and 5'-CCCTGTTATCTGGTGACAGAAAACC-3', and chick *p75* (Large et al., 1989) for 31–33 cycles with 5'-CCTGGCTGGACAGTGTGAC-C-3' and 5'-TCTGCCAGGGTGGTGGCC-3'. Amplified *trkC* fragments were isolated from agarose gels and sequenced with the amplification primers on a sequencing automate (Applied Biosystems 373A; Applied Biosystems, Foster City, CA).

Neurotrophins and antibodies. Recombinant mouse NT3 and BDNF produced in a Vaccinia virus-based expression system were used for iodination (Götz et al., 1992). Chinese Hamster Ovary (CHO)-derived human recombinant protein was used for radiolabeling of NGF. For biological assays and inhibition of binding, neurotrophins from the following sources were used: mouse NGF from salivary glands of adult male mice (purified according to Suda et al., 1978), recombinant human NGF (Genentech, San Francisco, CA), Vaccinia-derived mouse recombinant BDNF, human recombinant BDNF expressed in CHO cells (Genentech), Vaccinia-derived mouse recombinant NT3, and human recombinant NT4/5 (CHO, Genentech). The rabbit polyclonal serum (Chex) directed against the extracellular domain of chick p75^{NTR} was a gift from Drs. Weskamp and Reichardt.

Preparation of neurons. Fertilized chicken eggs were obtained from various commercial sources. For the preparation of sympathetic chains, embryos were staged according to Hamburger and Hamilton (1951) and E11 (stage 37/38) animals were removed from the egg, washed in PBS, and dissected under a stereomicroscope. Paravertebral sympathetic chains were taken from the thoracolumbar portion of the embryos, freed from connective tissue, and collected in ice-cold PBS. The chains were treated with 0.1% trypsin (Worthington, Freehold, NJ) at 37°C in PBS for 15–20 min. The ganglia were washed once in PBS and resuspended in Ham's F14 (Life Sciences) supplemented with 10% horse serum (Boehringer Mannheim) and 5% fetal calf serum (Life Sciences). Chains were dissociated by gentle trituration in a siliconized Pasteur pipette. The cell suspension was then filtered through a 40 µm nylon mesh (Falcon) and incubated on plastic cell culture dishes (Nunc, Dannstadt, Germany) for 2 hr in an incubator at 37°C in a 3% CO₂ atmosphere. During this preplating period non-neuronal cells settle onto the plastic dish, whereas neurons attach only loosely. The cell suspension, containing >95% neurons, was collected by aspiration, and the number of cells was counted in a hemocytometer. For binding or cross-linking experiments, cells were centrifuged (200 × g, 5 min) and resuspended in ice-cold Ca²⁺/Mg²⁺-free Krebs-Ringer's solution–HEPES buffer (KRH) containing 5 mg/ml BSA (Sigma, St. Louis, MO) and 0.1 mg/ml horse heart cytochrome *c* (binding buffer; Serva Feinbiochemica, Heidelberg, Germany).

Cell culture. Dissociated neurons were plated on dishes previously coated with poly-DL-ornithine (Sigma) and RN22 conditioned medium (Palm and Furcht, 1983) and incubated in a 3% CO₂ atmosphere at 37°C (Dechant et al., 1993b). For binding studies and cross-linking experiments on cell culture dishes, neurons were plated at a density of 3–10 × 10⁴ cells/3 cm dish (Falcon) or 3.5–5.0 × 10⁵ cells/6 cm dish (Nunc). Mouse L-cells overexpressing the rat p75^{NTR} gene (PCNA cells) (Radeke et al., 1987) were grown in DMEM containing 10% heat-inactivated fetal calf serum at 5% CO₂. Cells were removed from the dishes by gentle treatment with 0.01% trypsin (Worthington) for 10 min at 37°C. The reaction was stopped by the addition of fetal calf serum (Life Sciences). The cells were centrifuged (200 × g, 5 min) and resuspended in ice-cold binding buffer.

Iodination of neurotrophins, binding studies, and cross-linking experiments. NGF, NT3, and BDNF were iodinated using lactoperoxidase and ¹²⁵I (Amersham IMS30) as described previously (Rodríguez-Tébar et al., 1992; Dechant et al., 1993a). The specific radioactivity was 125–175 cpm/pg neurotrophin, and radiolabeled material was used within 1 week after the reaction.

Binding experiments with neurons from freshly dissected ganglia were performed at 4°C in a shaking water bath. For separation of free and bound radioactivity, a centrifugation method was used as described (Dechant et al., 1993a). Inhibition of binding experiments was performed as described previously (Dechant et al., 1993a). Binding on dishes was performed according to Rodríguez-Tébar and Rohrer (1991). Of six culture dishes per experimental point, three were used for the determination of unspecific binding and three for the determination of total binding. Before the experiments, neuronal cultures were incubated for 2 × 2 hr in fresh serum-containing medium to deplete the neuronal neurotrophin receptors from ligand. Binding was performed at room temperature in 1.5 ml binding buffer per dish. The dishes were gently agitated on a horizontal shaker (Cello Shaker, Renner). Dishes for the determination of unspecific binding were preincubated with 500 ng of unlabeled NT3 for 15 min. Antibodies were preincubated for 60 min. Radiolabeled NT3 was then added for an additional 60 min to all plates. After the incubation, the supernatant was removed by aspiration, and the dishes were washed once with 2 ml of KRH containing 1 mg/ml BSA for 15 min and left to dry. The bound radioactivity was eluted in 1 ml of 2% SDS solution and counted on a gamma counter with >70% counting efficiency (Canberra Packard Cobra). Free radioactivity in all experiments was determined using 3 × 10 µl binding solution from each experimental point. No significant depletion of free ligand was observed during the binding. Specific binding was determined by subtraction of unspecific binding from total binding, and data were analyzed using the Graphit software package (Erythacus Software London).

Incubations for cross-linking experiments were performed at 4°C in a water bath with freshly dissociated neurons (5 × 10⁵–1 × 10⁶ cells in 1 ml) or at room temperature with cell cultures as described above, with the modification that after the incubation with radioligand cross-linking reagents, either 10 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Pierce, Rockford, IL) or 1 mM [bis [sulfo succinimidyl] suberate (BS³) (Pierce) was added for 30 min. The reaction was quenched by the addition of 40 mM Tris/HCl, pH 8.0, for 10 min. Cell suspensions were centrifuged at 3000 rpm for 1 min, and the supernatant was aspirated. The pellets were washed once in ice-cold KRH containing protease inhibitors (aprotinin 10 µg/ml, leupeptin 1 µg/ml, PMSF 1 mM) and lysed by boiling in 4 × concentrated Laemmli gel loading solution containing 6% 2-mercaptoethanol for 5 min. Cell cultures were washed two times in KRH and lysed with 4 × Laemmli solution on the culture dish. Lysates were centrifuged at 20,000 × g for 20 min, and aliquots of the supernatant containing equal amounts of protein were analyzed by 7.5% SDS-PAGE. The dried gels were exposed for 2–14 d to autoradiographic film (Kodak X-omat AR).

RESULTS

p75^{NTR} and *trkC* gene expression in chick sympathetic neurons

Several alternative transcripts of the *trkC* gene identified previously in embryonic chick brain (Fig. 1A) (Garner and Large, 1994) were amplified by RT-PCR from RNA isolated from E11 sympathetic ganglia (Fig. 1B,C). The use of a single set of tyrosine kinase specific primers resulted in the simultaneous amplification of two bands that were isolated and sequenced (Fig. 1B). The larger DNA fragment encoded the expected fragment of the full-length, enzymatically active form of *trkC*. The smaller amplicon coded for an alternatively spliced variant of *trkC* with a truncation in the second half of the tyrosine kinase domain (Garner and Large, 1994). *TrkC* transcripts encoding tyrosine kinase insertion isoforms (data not shown) or isoforms with a deletion of the entire tyrosine kinase domain (Fig. 1C) are weakly expressed in sympathetic neurons. No evidence for differential expression of the kinase deletion, kinase truncation, and full-length *trkC* transcripts was obtained between E7 and E11 (data not shown). The expression of *trkC* in freshly isolated ganglia was compared with neurons cultured in the presence of NGF for 5 d (Fig. 1C). All *trkC* transcripts were strongly downregulated in culture in comparison to *GAPDH* or p75^{NTR} gene expression (Fig. 1C).

Figure 1. NT-3 receptor gene expression in sympathetic ganglia. *A*, Schematic representation of the protein isoforms encoded in *trkC* transcripts of chick sympathetic ganglia. At least four different *trkC* transcripts are generated by alternative splicing (Garner and Large, 1994) in sympathetic chick ganglia, of which the one encoding the enzymatically active full-length form is most abundant (*C*). *B*, Sequences corresponding to the full-length receptor and an isoform with a truncation in the tyrosine kinase domain can be amplified simultaneously with primers flanking the deleted exon. The kinase truncation isoform is detectable by Southern blotting of the amplificate after 38 cycles when the reaction is already saturated for the full-length form. *C*, NT-3 receptor gene expression in sympathetic ganglia and neuronal cell cultures. cDNAs from embryonic stage 37/38 animals (Hamburger and Hamilton, 1951) were amplified with primers specific for the neurotrophin receptor genes *p75*^{NTR} (31 cycles), *trkC* full length (26 cycles), or *trkC* kinase deletion (36 cycles) (Garner and Large, 1994). The kinase truncated variant (*B*) and the kinase insertion form (data not shown) are present at very low abundance. The cDNA content of the samples was controlled by amplification of GAPDH (20 cycles). The expression of the *trkC* and *p75*^{NTR} genes was compared in the sympathetic chains (*in vivo*) and in sympathetic neuronal cultures (*in vitro*, 5 d in the presence of 20 ng/ml NGF). *TrkC* but not *p75*^{NTR} transcripts were strongly downregulated in culture.

Figure 2. Cross-linking of NT3 and NGF to their receptors on sympathetic neurons. Cell suspensions of neurons freshly isolated from E11 chick embryos were incubated with radiolabeled NT3 or NGF at a concentration of 3×10^{-11} M (–), in the presence of a 130-fold excess of identical unlabeled factor, or with a 1:2000 dilution of an antiserum that prevents binding of neurotrophins to *p75*^{NTR} (Chex) (Weskamp and Reichardt, 1991). Proteins were chemically cross-linked with BS³ and neurotrophin receptor complexes identified by autoradiography of SDS polyacrylamide gels.

NT3 receptors on the cell bodies of E11 sympathetic neurons

Cell suspensions of neurons freshly isolated from E11 chick sympathetic ganglia were incubated with radiolabeled NT3 at 3×10^{-11} M, a concentration preferentially filling high-affinity NT3 binding sites (Rodríguez-Tébar et al., 1992). After cross-linking with the bifunctional hydrophilic cross-linker BS³, the neurotrophin receptor complexes were analyzed by gel electrophoresis and autoradiography. Three bands were observed with apparent molecular masses of 60, 80, and 170 kDa (Fig. 2). Binding was completely inhibited by the addition of a 130-fold molar excess of unlabeled NT3. The Chex anti-*p75*^{NTR} antiserum (Weskamp and Reichardt, 1991) abolished binding to the 60 and 80 kDa bands (Fig. 2). Both bands could also be precipitated with other antisera against *p75*^{NTR}, confirming that *p75*^{NTR} is part of both complexes (data not shown). The 60 kDa band is unlikely to result from proteolysis of the 80 kDa monomer, because it is absent in material cross-linked with EDC from identical preparations (data not shown) but present when BS³ is used to cross-link NT3 to a cell line expressing *p75*^{NTR} (see below). After incubation with Chex anti-*p75*^{NTR} antiserum, a high molecular weight band persisted, and its size (170 kDa) is consistent with the molecular mass expected for an NT3/*trkC* complex. This band was immu-

Figure 3. Ligand specificity of the NT3 and NGF receptors on E11 sympathetic neurons. Neuronal cell suspensions isolated from E11 sympathetic ganglia were incubated with 2×10^{-11} M radiolabeled NT3 or NGF alone (–) or in the presence of a 150-fold excess of unlabeled neurotrophins NGF, NT3, BDNF, or NT4/5. Cross-linked receptor–ligand complexes were analyzed by SDS-PAGE followed by autoradiography of the dried gels.

noprecipitated with a pan-trk antiserum (Schröpel et al., 1995) (data not shown). Thus, both p75^{NTR} and full-length trkC are involved in NT3 high-affinity binding on freshly dissociated neurons. Similar results were obtained with NGF, although the NT3/p75^{NTR} complexes were more abundant than NGF/p75^{NTR} under identical experimental conditions, independent of the use of BS³ (Fig. 2) or EDC as cross-linkers (data not shown). Also, the molecular weight of the NGF/trkA complexes (160 kDa) (Schröpel et al., 1995) is slightly lower than that of the NT3/trkC complex (Fig. 2). To test the ability of neurotrophins other than NT3 to compete with NT3 high-affinity binding, neurons freshly isolated from E11 sympathetic ganglia were incubated with 2×10^{-11} M radiolabeled NT3 in the presence of a 150 × molar excess of unlabeled NGF, BDNF, and NT4/5. Analysis of the cross-linking products revealed that binding of radiolabeled NT3 to p75^{NTR} was only incompletely inhibited by NGF and BDNF at 3×10^{-9} M (Fig. 3); however, human NT4/5 was essentially as effective as NT3 itself. In addition, binding of 125 I-NT3 to trkC was markedly antagonized by NT4/5, but only slightly reduced by NGF and not significantly by BDNF. Parallel experiments with 125 I-NGF indicated that its binding to p75^{NTR} was inhibited equally and completely by all neurotrophins. In addition, binding to trkA was substantially blocked by NT3, somewhat less by NT4/5, and not by BDNF (Fig. 3).

NT3 receptors on cultured E11 sympathetic neurons

Because the above results indicate that p75^{NTR} is a major component of NT3 high-affinity specific binding, it was of interest to perform similar cross-linking experiments with cultured neurons; the expression levels of trkC mRNA are negligible compared with intact sympathetic chains (Fig. 1). Sympathetic neurons were cultured for 4 d in the presence of NGF, and cross-linking experiments were performed with 125 I-NT3 as above. Major bands

Figure 4. Chemical cross-linking of NT3 to E11 sympathetic cultures and PCNA cells. Neurons were cultured in the presence of NGF for 4 d. The cultures were incubated with 3×10^{-11} M radioiodinated NT3 (–), and the binding of the radioactive ligand was chased by a 10- to 1000-fold molar excess of unlabeled neurotrophins NT3, BDNF, or NGF (top). These results are compared with an identical experiment with PCNA cells, a fibroblastic cell line overexpressing the p75^{NTR} gene in the absence of endogenous neurotrophin binding sites (bottom).

of 60 and 80 kDa were observed (Fig. 4), whereas the NT3-trkC complex could not be detected. A weak high molecular weight complex of ~210 kDa was also observed, which corresponds to the previously reported dimeric form of p75^{NTR} (Jing et al., 1992). Competition experiments indicate that as with freshly dissociated sympathetic neurons, unlabeled NT3 is a much better competitor for binding to p75^{NTR} compared with BDNF and NGF. The ability of p75^{NTR} to selectively bind NT3 is thus a property that can be observed in the absence of detectable cross-linking to trkC (Fig. 4). When similar experiments were performed with the fibroblastic cell line PCNA overexpressing p75^{NTR} receptors, identical band patterns were observed (60, 80, and 210 kDa), but prevention of binding by BDNF, NGF, or NT3 was roughly equally efficient (Fig. 4). Binding of 125 I-NT3 to p75^{NTR} on sympathetic neurons but not PCNA cells was already massively reduced at a 10-fold excess of unlabeled NT3. These cross-linking experiments thus reveal that on cultured sympathetic neurons, p75^{NTR} is the only detectable receptor for NT3 and its binds NT3 at very low concentrations and with high ligand specificity.

Binding experiments with E11 sympathetic neuronal cultures

To obtain quantitative information, binding experiments were then performed with cultured neurons. In steady-state binding

Figure 5. Inhibition of binding of ^{125}I -NT3 and ^{125}I -BDNF to neurons and PCNA cells under equilibrium conditions. The E11 sympathetic neuronal cultures for these experiments were grown in the presence of NGF for 4 d. Under these conditions, >90% of the specific binding can be blocked by Chex anti-p75^{NTR} antiserum at 1:1000 dilution (■ in B). **A**, Binding of ^{125}I -NT3 (3×10^{-11} M) to sympathetic neurons (▲) or PCNA cells (△) inhibited by increasing concentrations of unlabeled NT3. **B**, Inhibition of binding of ^{125}I -NT3 (3×10^{-11} M) to sympathetic neuronal cultures by NT3 (○), BDNF (●), or NGF (□). **C**, Inhibition of binding of ^{125}I -BDNF (3×10^{-11} M) to sympathetic neuronal cultures by NT3 (○), BDNF (●), or NGF (□).

experiments, we found that cultured E11 sympathetic neurons express $4.5 \pm 1.5 \times 10^4$ NT3 binding sites/cell binding with a K_d of $6 \pm 2 \times 10^{-11}$ M (data not shown). This affinity is similar to that observed previously for NT3 high-affinity binding sites on both sensory and sympathetic neurons ($1\text{--}3 \times 10^{-11}$ M). P75^{NTR} accounts for most of this binding, because incubation of the cultured cells with the anti-p75^{NTR} antiserum (1:1000) blocked $92 \pm 4\%$ of the specific binding of ^{125}I -NT3 to cultures of E11 sympathetic neurons (Fig. 5B). This reduction is less pronounced ($\sim 50\%$; data not shown) when the experiment is performed with freshly isolated neurons, in line with the cross-linking data indicating an involvement of trkC when binding components are analyzed on these neurons (Fig. 2). The unique binding characteristics of p75^{NTR} on cultured cells were then quantified and compared with those of p75^{NTR} expressed on PCNA cells (Fig. 5A). With unlabeled NT3, 50% inhibition of binding of radiolabeled NT3 (used at 3×10^{-11} M) was observed at 8×10^{-11} M for sympathetic neurons and at 1.2×10^{-9} M with PCNA cells, reflecting the higher degree of receptor occupancy on sympathetic neurons. With cultured sympathetic neurons, ^{125}I -NT3

binding was blocked by BDNF with an IC_{50} of 6×10^{-10} M and 2.5×10^{-9} M for NGF (Fig. 5B). BDNF binds to p75^{NTR} on E11 sympathetic neurons. At a concentration of 3×10^{-11} M, specific binding of ^{125}I -BDNF was markedly reduced compared with ^{125}I -NT3, consistent with the lower affinity of BDNF and a correspondingly lower occupancy of p75^{NTR}. NT3 very efficiently inhibited binding of ^{125}I -BDNF to p75^{NTR} ($\text{IC}_{50} = 3 \times 10^{-11}$ M), in comparison with BDNF ($\text{IC}_{50} = 1 \times 10^{-9}$ M) and NGF ($\text{IC}_{50} = 3 \times 10^{-9}$ M) (Fig. 5C). This result also indicates that high-affinity specific binding of NT3 for neuronal p75^{NTR} is independent of the use of radiolabeled NT3.

DISCUSSION

The characterization of the binding affinity of neurotrophin receptors on neurons has been useful, in particular with regard to neurotrophin responsiveness and selectivity of binding (for review, see Dechant et al., 1994). In this context, the NT3 binding sites described previously on E11 chick sympathetic neurons are intriguing because they display traditional characteristics of high affinity and selectivity, but their occupancy does not induce survival (Dechant et al., 1993b). The present study reveals that p75^{NTR} is the molecule explaining the high-affinity selective binding of NT3 to these neurons and their processes.

Neuronal p75^{NTR} forms high-affinity selective NT3 receptors

The evidence identifying p75^{NTR} as the high-affinity receptor on sympathetic neurons comes from cross-linking experiments performed with radioiodinated NT-3 at concentrations filling high-affinity but not low-affinity sites. Complexes of ~ 60 and 80 kDa were detected, and identical bands were obtained with PCNA cells overexpressing the p75^{NTR} gene. These bands were undetectable in the presence of antibodies blocking the binding of neurotrophins to p75^{NTR}, and binding of NT3 to the 60 and 80 kDa components was inhibited by all four neurotrophins, albeit only at high concentrations of NGF and BDNF. In contrast, NT4/5 can also bind efficiently to the NT3 high-affinity sites (Fig. 3), in line with previous studies using neurotrophin mutants (Rydén et al., 1995) and retrograde transport *in vivo* (Curtis et al., 1995), indicating a selective interaction of NT4/5 with p75^{NTR}.

A K_d of 6×10^{-11} M was determined for the interaction of ^{125}I -NT3 with p75^{NTR}. This value is similar to the affinities of the NT3 high-affinity receptors on other neuronal populations (Rodríguez-Tébar et al., 1992) and for a proportion of the sites formed by recombinant trkC-expressing cell lines (Tsoulfas et al., 1993). trkC was not detectable in cultured neurons, however, indicating that this receptor cannot play a significant role in p75^{NTR} high-affinity binding, which therefore seems to be independent of the formation of a ternary complex with trkC. Also, trkC protein is not very abundant on rodent sympathetic neurons (Belliveau et al., 1997). Because sympathetic neurons form an extensive network of processes in culture, it is likely that much of the NT3 binding occurs on nerve fibers, with a negligible contribution of the cell bodies. Finally, experiments with ^{125}I -BDNF in which NT3 very efficiently inhibited binding to p75^{NTR} (IC_{50} of 3×10^{-11} M) confirmed that high-affinity binding is also observed with unlabeled NT3.

P75^{NTR} on neurons and transformed cells

This study revealed that the cellular environment plays a crucial role in determining the characteristics of the interactions between NT3 and p75^{NTR}. Consistent with previous results (Rodríguez-Tébar et al., 1992), high-affinity binding of NT3 to p75^{NTR} was

not obtained with PCNA cells (Figs. 4, 5). Interestingly, the differences in the binding properties of p75^{NTR} between neurons and PCNA cells were observed only with NT3 and not with NGF or BDNF. The high-affinity binding of NT3 to p75^{NTR} is unlikely to be restricted to sympathetic neurons. In a previous study, approximately 10-fold higher numbers of high-affinity NT3 receptors were observed on embryonic chick DRG neurons, compared with BDNF or NGF (Rodríguez-Tébar et al., 1992). We think that p75^{NTR} is likely to be a major contributor of high-affinity NT3 binding on these sensory neurons as well, because they express low levels of trkC (Williams et al., 1993). Also, we observed high-affinity binding of ¹²⁵I-NT3 to suspensions of rat Schwann cells isolated from newborn sciatic nerves (data not shown), suggesting the existence of such p75^{NTR} high-affinity NT3 receptors on various nontransformed cells.

How can the cellular context modify the binding characteristics of p75^{NTR}? The allosteric model for neurotrophin–p75^{NTR} interactions offers an attractive framework (for discussion, see Bothwell, 1995). Indeed, as with other neurotrophins, positive cooperativity has been observed on binding of NT3 to p75^{NTR} (Rodríguez-Tébar et al., 1992), indicating that p75^{NTR} exists in (at least) two conformations with different ligand affinities. Consistent with this model, in addition to high-affinity binding sites for NGF and NT3, sympathetic neurons also express p75^{NTR} low-affinity binding sites for all neurotrophins (Godfrey and Shooter, 1986; Rodríguez-Tébar and Barde, 1988) (our unpublished results). Our results with sympathetic neurons could be explained by a modulation of the ratio between high- and low-affinity forms of p75^{NTR}, driven by hitherto unknown molecules in nontransformed cells. The absence of NGF or BDNF high-affinity binding to p75^{NTR} in our experiments indicates that sympathetic neurons do not equally induce the formation of high-affinity p75^{NTR} binding sites for all neurotrophins. Previous studies with NT3 mutant proteins revealed that several amino acid residues in this neurotrophin contribute to the binding to p75^{NTR}, in particular R114 and K115 (Urfer et al., 1994) as well as Arg31 and His33 (Rydén et al., 1995). Therefore it is conceivable that NT3 contains more than one binding epitope with high and low affinities for p75^{NTR}. Cell type-specific differences in the conformation of p75^{NTR} might determine which of these binding sites is used. Noteworthy are previous observations indicating that the binding properties of p75^{NTR} are sensitive to receptor aggregation. Thus, a shift in the affinity of p75^{NTR} for NGF was observed in the presence of wheat germ agglutinin, NGF antibodies, or the p75^{NTR} monoclonal antibody 192 (Buxser et al., 1983; Grob et al., 1983; Vale and Shooter, 1983; Chandler et al., 1984). Also, aggregation by these agents can stimulate the association of p75^{NTR} to the cytoskeleton (Vale and Shooter, 1982, 1983; Vale et al., 1985). Although the molecular nature of this association is not clear, it is interesting to note that the C terminus of p75^{NTR} contains the critical residues mediating the interaction of various ion channels with the postsynaptic density protein PSD95 (Kornau et al., 1995). Finally, p75^{NTR} molecules carrying a 55 amino acid deletion in the intracellular domain are profoundly affected in cellular sorting, ligand internalization, signaling capacities, and lateral mobility in the membrane (Le Bivic et al., 1991; Dobrowsky et al., 1995; Wolf et al., 1995). The same mutants also have altered binding properties (Hempstead et al., 1990), indicating a role of the intracellular domain in the determination of ligand affinity. In our cross-linking experiments, we did not obtain direct evidence for NT3-driven receptor clustering; the ratio between monomeric and dimeric forms of cross-

linked p75^{NTR} was not overtly changing in correlation to the ligand concentration (data not shown).

At present, the functional consequences of the formation of high-affinity and specific NT3 binding sites are unclear. An efficient removal or ligand neutralization system has already been suggested (Dechant et al., 1993b), but other possibilities exist. The recent and unexpected realization that NGF can uniquely activate p75^{NTR} invites caution in suggesting simplistic functions for the NT3/p75^{NTR} sites on neurons (Carter et al., 1996; Frade et al., 1996).

In conclusion, our data indicate that p75^{NTR} can display remarkable properties of specificity on sympathetic neurons that are not observed when this molecule is expressed in transformed cells. This suggests that molecules profoundly modifying the properties of p75^{NTR} exist that are not ubiquitously expressed. In future studies, it will be interesting to determine the nature of the molecules in sympathetic neurons or other nontransformed cells that modify the properties of p75^{NTR}.

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