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Biochemical and functional interactions between the neurotrophin receptors *trk* and p75^{NTR}

Miriam Bibel[1], Edmund Hoppe[2] and Yves-Alain Barde

Max-Planck Institute of Neurobiology, Department of Neurobiochemistry, 82152 Planegg-Martinsried, Germany

[2]Present address: Hoechst Marion Roussel, Fraunhoferstraße 22, 82152 Martinsried, Germany

[1]Corresponding author
e-mail: miriam@neuro.mpg.de

Neurotrophins bind to two structurally unrelated receptors, the *trk* tyrosine kinases and the neurotrophin receptor p75^{NTR}. Ligand activation of these two types of receptor can lead to opposite actions, in particular the prevention or activation of programmed cell death. Many cells co-express *trk* receptors and p75^{NTR}, and we found that p75^{NTR} was co-precipitated with *trk*A, *trk*B and *trk*C in cells transfected with both receptor types. Co-precipitation of p75^{NTR} was not observed with the epidermal growth factor receptor. Experiments with deletion constructs of *trk*B (the most abundant *trk* receptor in the brain) and p75^{NTR} revealed that both the extracellular and intracellular domains of *trk*B and p75^{NTR} contribute to the interaction. Blocking autophosphorylation of *trk*B substantially reduced the interactions between p75^{NTR} and *trk*B constructs containing the intracellular, but not the extracellular, domains. We also found that co-expression of p75^{NTR} with *trk*B resulted in a clear increase in the specificity of *trk*B activation by brain-derived neurotrophic factor, compared with neurotrophin-3 and neurotrophin-4/5. These results indicate a close proximity of the two neurotrophin receptors within cell membranes, and suggest that the signalling pathways they initiate may interact soon after their activation.

Keywords: BDNF/NT3/NT4/neurotrophins/receptors

Introduction

All known members of the nerve growth factor (NGF) family, designated the neurotrophins, bind to two different types of receptors, the *trk* tyrosine kinases and the neurotrophin receptor p75^{NTR} (Bothwell, 1991; Chao, 1992; Meakin and Shooter, 1992). In mammals, three different *trk* receptors have been identified and are activated by one or more of the four neurotrophins (for review see Barbacid, 1994). Binding of the neurotrophins to the *trk* receptors leads to receptor tyrosine phosphorylation, triggering the activation of pathways leading to the prevention of programmed cell death during development (Kaplan and Miller, 1997). Studies with antibodies and with mouse mutants have established that each neurotrophin and *trk* receptor is required during neural development, the elimination of any one of these components leading to specific deficits in the nervous system (Snider, 1994; Lewin and Barde, 1996).

All neurotrophins also bind to the neurotrophin receptor p75^{NTR}, a member of the tumour necrosis factor (TNF) receptor and FAS/Apo-1/CD95 family. Following the discovery of the *trk* receptors as mediators of the trophic effects of the neurotrophins, the role of p75^{NTR} was mostly discussed as that of an accessory receptor modulating the signalling of the *trk* receptors (Chao and Hempstead, 1995). Recently however, p75^{NTR} has also been shown to mediate cell death in a ligand-dependent fashion (Casaccia-Bonnefil *et al*., 1996; Bamji *et al*., 1998; Davey and Davies, 1998), a function similar to that demonstrated previously with the structural parents of p75^{NTR}, namely the TNF receptor 1 and CD95. Evidence for this new function of p75^{NTR} has also been obtained *in vivo* in the avian retina (Frade *et al*., 1996; Frade and Barde, 1998), in mouse sympathetic ganglia (Bamji *et al*., 1998) and in the developing spinal cord of mice carrying a mutation in the *ngf* or the *p75* gene (Frade and Barde, 1999).

In view of these observations and the fact that many neuronal populations co-express *trk* receptors and p75^{NTR}, we tested to see whether an interaction between both receptor types could be demonstrated by immunoprecipitation in transfected cells. While this question has been addressed in previous studies, work has focused on p75^{NTR} and *trk*A using either cross-linking (Huber and Chao, 1995; Gargano *et al*., 1997) or co-patching techniques (Ross *et al*., 1996). As *trk*B can be activated by three different neurotrophins, namely brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT4/5) and neurotrophin-3 (NT3), we also investigated the extent to which co-expression of p75^{NTR} with *trk*B modulates the ligand specificity of this receptor.

Results

Co-immunoprecipitation of p75^{NTR} with trkA, trkB and trkC

Comparable conditions for immunoprecipitation of the rat receptors *trk*A, *trk*B and *trk*C were established by tagging the three receptors at their N-terminal ends using a nine amino acid hemagglutinin (HA) epitope. The ability of the tagged receptors to bind neurotrophins was determined in cross-linking experiments using radiolabelled neurotrophins and by ligand-induced receptor phosphorylation (data not shown). These three cDNAs were used to transfect A293 cells together with rat p75^{NTR} at a ratio of 1:1. Following cell lysis with 1.0% Triton X-100, the *trk* receptors were precipitated with anti-HA antibodies and the immunoprecipitates analysed by Western blotting with anti-p75 antibodies. These experiments revealed that

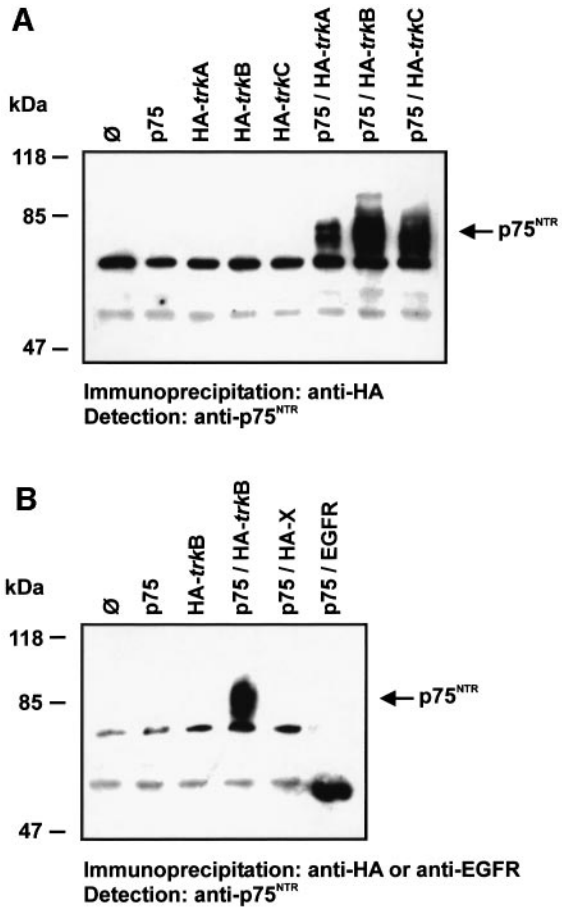

Fig. 1. Co-immunoprecipitation of p75^{NTR} with the *trk* receptors. (**A**) A293 cells were transiently transfected with the various constructs as indicated. Cells were lysed after 2 days and HA-tagged trk receptors were precipitated with an anti-HA mAb. Immunoprecipitates were run on a 7% acrylamide SDS gel followed by Western blotting. Detection was performed with a polyclonal anti-p75 antibody. (**B**) Co-immunoprecipitation was carried out as in (A) except that the EGF receptor (EGFR) was precipitated with a mAb to the EGF receptor. HA-X denotes a HA-tagged phosphodiesterase as a control. The signals observed in all lanes result from antibody cross-reactivities.

p75^{NTR} is co-immunoprecipitated with *trk*A, *trk*B and *trk*C (Figure 1A). Like rat p75^{NTR}, chick p75^{NTR} was also co-immunoprecipitated (data not shown).

When similar experiments were performed with the epidermal growth factor (EGF) receptor using a monoclonal antibody (mAb) to the EGF receptor, no co-immunoprecipitation of p75^{NTR} could be detected (Figure 1B).

To test whether expression of both receptors in the same cells is needed for the *trk*–p75^{NTR} interaction, the *trk* and p75^{NTR} constructs were expressed in separate sets of cells and the immunoprecipitation performed as above after mixing the cell extracts. No co-immunoprecipitation of p75^{NTR} could be detected under these conditions (data not shown).

Although all three *trk* receptors behaved similarly in their ability to associate and co-precipitate p75^{NTR}, subsequent investigations focused on *trk*B, as this is the most abundant of the *trk* receptors expressed in brain tissue. Also, previous studies have established that *trk*B can be activated in fibroblasts by three different neurotrophins (see below).

Mapping trkB and p75^{NTR} interacting domains

We were interested in delineating the receptor domains involved in the interaction and designed various deletion constructs of p75^{NTR} and *trk*B. The *trk*B receptor also exists as a splice-variant form lacking the tyrosine kinase domain, raising the question of whether the truncated form of this receptor would also interact with p75^{NTR}.

To facilitate comparisons between the constructs, the HA epitope was preserved in the *trk*B constructs, as was the p75^{NTR} detection epitope (Figure 2). Deletion mutants were constructed for each receptor lacking either most of the intracellular domain (ΔICD) or the extracellular domain (ΔECD). *trk*B ΔICD essentially corresponds to the naturally occurring *trk*B splice variant designated T1 and lacking the tyrosine kinase domain. p75^{NTR} ΔICD, as well as p75^{NTR} ΔECD, co-immunoprecipitated with full-length *trk*B (Figure 3A). Likewise, *trk*B ΔICD and *trk*B ΔECD co-immunoprecipitated with full-length p75^{NTR}. The weakest interaction was found between p75^{NTR} and *trk*B ΔECD (Figure 3B). Also, p75^{NTR} ΔECD and *trk*B ΔECD, as well as p75^{NTR} ΔICD and *trk*B ΔICD, co-immunoprecipitated, but no detectable interactions could be seen when p75^{NTR} ΔICD was expressed together with *trk*B ΔECD (Figure 3C). In sum, these mapping studies indicate that interaction between p75^{NTR} and *trk*B involves the extracellular, as well as intracellular, domains of both receptors. However, the transmembrane sequence (which is common to all the constructs tested) does not seem to be sufficient for a stable interaction under our experimental conditions.

The trkB–p75^{NTR} interaction is K-252a sensitive

The demonstration by immunoprecipitation of an interaction between *trk* receptors and p75^{NTR} necessitates receptor over-expression. This leads to phosphorylation of the *trk* receptors due to their propensity to dimerize in a ligand-independent fashion. As our results indicate that the intracellular domain of *trk*B interacts with p75^{NTR} (see above), the question arises as to whether the phosphorylation status of *trk*B influences the formation of the *trk*B–p75^{NTR} complex. This possibility was tested using the kinase inhibitor K-252a. A dose-dependent reduction of the *trk*B–p75^{NTR} interaction was observed, with a slight inhibition at 500 nM K-252a and a marked reduction at 1 μM K-252a (Figure 4). To test whether the effects of K-252a result from an action of the alkaloid on the intracellular domain of *trk*B, we used the *trk*B ΔECD construct with p75^{NTR}. The interaction between the two receptors was inhibited almost completely with 500 nM K-252a. In contrast, the interaction of *trk*B T1 with p75^{NTR} was not influenced by K-252a. Therefore these experiments indicate that the K252a-mediated inhibition of receptor interaction is due to its action on the intracellular domain of *trk*B, and that the phosphorylated form of *trk*B is predominantly involved in the interaction with p75^{NTR}.

p75^{NTR} influences the specificity of ligand-dependent phosphorylation of trkB

The close proximity of the two receptors may affect their ability to be activated by different ligands. As *trk*B can

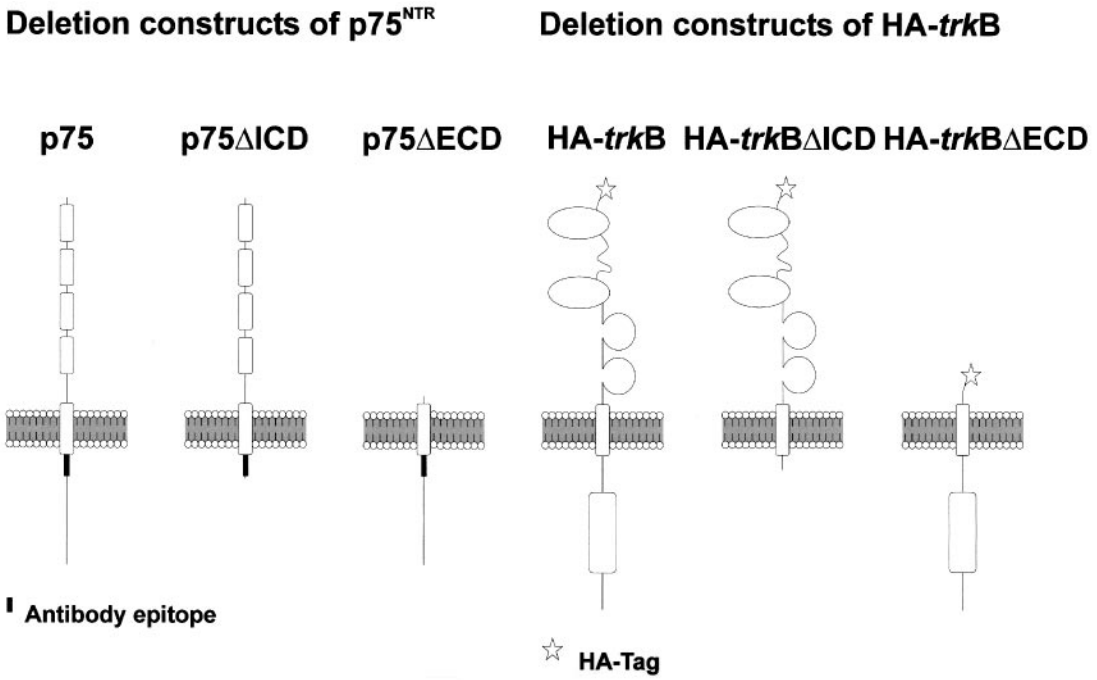

Fig. 2. Deletion constructs of p75^{NTR} and HA-*trk*B.

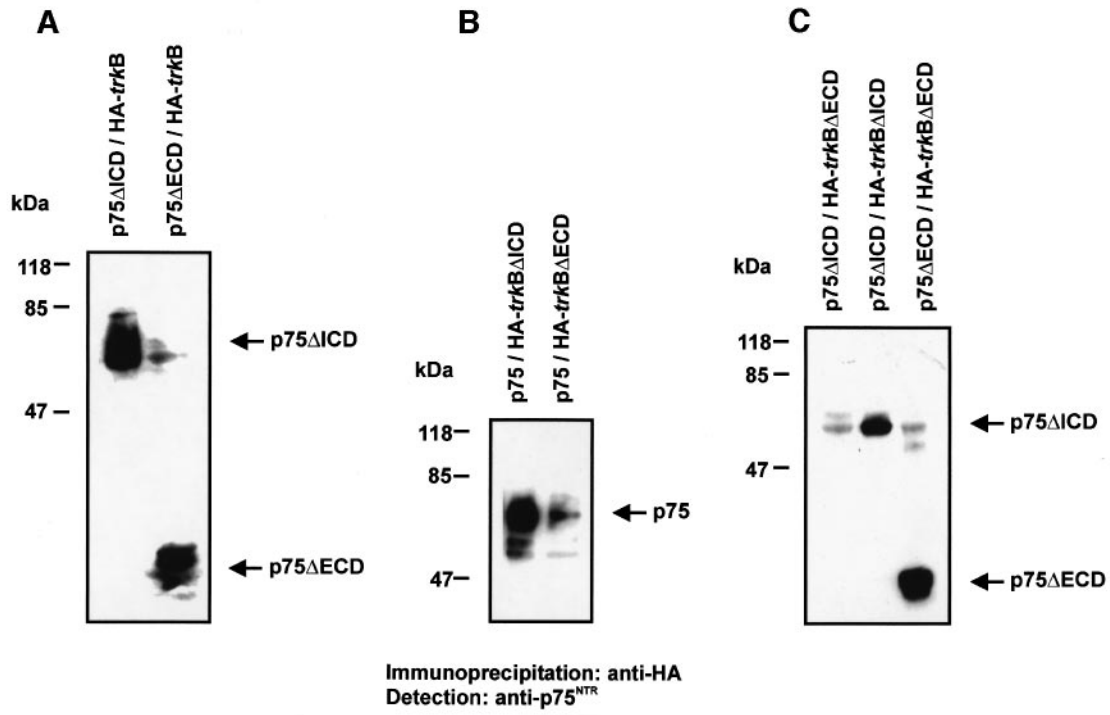

Fig. 3. Co-immunoprecipitation of the deletion constructs of p75^{NTR} and *trk*B. (**A**) Deletion constructs of p75^{NTR} co-immunoprecipitated with full-length HA-*trk*B. (**B**) Full-length p75^{NTR} co-immunoprecipitated with deletion constructs of HA-*trk*B. (**C**) Co-immunoprecipitation of deletion constructs of both receptors. The experiments were performed as described in Figure 1, except that 10% acrylamide SDS gels were used. Note that p75ΔICD and HA-*trk*BΔECD do not interact. The interaction of p75^{NTR} full-length with HA-*trk*BΔECD is significantly weaker.

be activated by BDNF, NT4/5 and NT3, we asked whether p75^{NTR} increases the ligand selectivity of *trk*B. Ligand-dependent *trk*B phosphorylation was investigated in A293 cells stably transfected with a *trk*B construct that can be readily activated by three different neurotrophins (*trk*B-L, Strohmaier *et al*., 1996). These cells were transiently transfected with various p75^{NTR} constructs. Whereas the BDNF-induced phosphorylation of *trk*B remained unchanged with p75^{NTR} co-expression (Figure 5), that induced by NT4/5 and NT3 was clearly reduced. This increased selectivity was dose-dependent, being more prominent at lower neurotrophin concentrations and not readily apparent at 100 ng/ml neurotrophin (data not shown). p75^{NTR} ΔICD also mediated the increased ligand

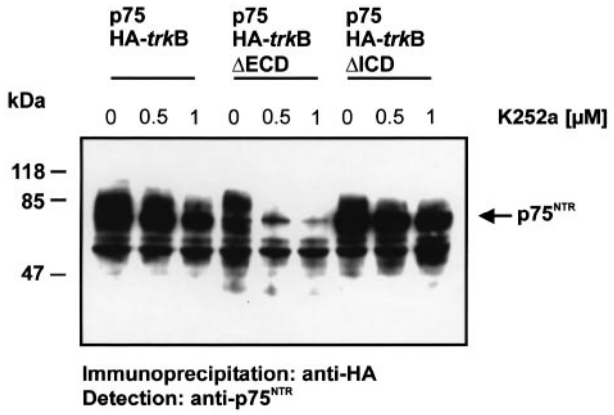

Fig. 4. Inhibition of the p75^{NTR}–HA-*trk*B interaction by K-252a. Transiently transfected A293 cells were treated with K-252a for 24 h before lysis. Co-immunoprecipitation was performed as in Figure 1.

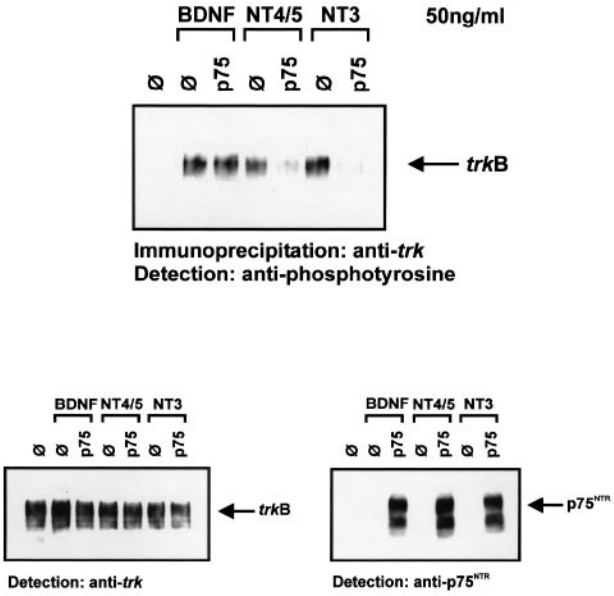

Fig. 5. Influence of p75^{NTR} on ligand-induced autophosphorylation of *trk*B. Following incubation in serum-free medium (see Materials and methods), cells stably transfected with *trk*B were incubated with neurotrophin (50 ng/ml) for 5 min at 37°C. Cell lysates were immunoprecipitated and analysed using a phosphotyrosine antibody. Following stripping, the blots were reprobed with a pantrk antibody to check for receptor expression levels. Cell lysates were also checked for p75^{NTR} expression levels.

specificity of *trk*B (data not shown). From these experiments we conclude that one of the consequences of the *trk*B–p75^{NTR} interaction is a marked increase in the selectivity of *trk*B for BDNF-mediated receptor phosphorylation, and that this effect is more likely to be accounted for by direct receptor interaction than by p75^{NTR}-mediated signalling.

Discussion

The possibility of *trk*–p75^{NTR} receptor association has been raised ever since two different receptors were shown to bind the neurotrophins. This question is all the more pressing since both receptors are now known to signal, and that this signalling may lead to results as different as cell death or cell survival. Our study provides evidence for a direct association between the *trk* receptors and p75^{NTR}, as demonstrated by co-immunoprecipitation. This association is relevant to the *trk* function in intact cells, as it leads to an increase in ligand specificity.

Previous biochemical studies on trk–p75^{NTR} interactions

The question of an association between the *trk* receptors and p75^{NTR} has already been addressed in previous studies with *trk*A. Chemical cross-linking was used, either with radiolabelled NGF (Huber and Chao, 1995), or by reversibly cross-linking receptors expressed in Sf9 insect cells (Gargano *et al*., 1997). Also, co-patching studies with fluorescently labelled receptors (Wolf *et al*., 1995; Ross *et al*., 1996) suggested a co-localization of the receptors in cell membranes using Sf9 cells. Curiously, no such evidence could be obtained in similar experiments using *trk*B instead of *trk*A (Wolf *et al*., 1995; Ross *et al*., 1996). Functionally, co-operation between p75^{NTR} and all three *trk* receptors has been obtained in transfected cells by showing that co-expression increases responsiveness to low neurotrophin concentrations, but in these and other experiments the demonstration of co-immunoprecipitation of p75^{NTR} and *trk* failed (Hantzopoulos *et al*., 1994). The explanation as to why we eventually succeeded in demonstrating a biochemical interaction between p75^{NTR} and all three *trk* receptors may lie in the tagging procedure. It is possible that antibodies used previously to test receptor association may have either interfered with the formation of a *trk*–p75^{NTR} receptor complex or did not recognise the receptor complex. In particular, we note that Gargano and colleagues documented the observation that, whereas antibodies to p75^{NTR} efficiently co-precipitated p75^{NTR} cross-linked to *trk*A, antibodies to *trk*A did not detectably co-precipitated p75^{NTR} (Gargano *et al*., 1997). Also, it is conceivable that the A293 cells used in this study offer a favourable context to study the interactions between p75^{NTR} and *trk* receptors, for example by providing cytoplasmic proteins that stabilise the interaction. However, merely mixing detergent extracts of cells expressing only one receptor type does not lead to receptor association (see Results).

Functional evidence for a trk–p75^{NTR} association

Whereas there is no doubt that the *trk* receptors play a key role in mediating the trophic effects of neurotrophins, it is also clear that the expression of *trk* receptors does not account for all neurotrophin receptor properties on neurons. For example, NGF binds with high affinity to sensory neurons (~10^{-11} M; Sutter *et al*., 1979), and most of the sites formed by *trk*A are of a low-affinity type (Mahadeo *et al*., 1994). Similar observations were made with BDNF and *trk*B [compare for example Rodriguez-Tébar and Barde (1988) with Dechant *et al*. (1993)]. In addition, co-expression of both p75^{NTR} and *trk*A in the same cells leads to the formation of high-affinity receptors (Hempstead *et al*., 1991; Mahadeo *et al*., 1994). That the formation of high-affinity binding sites is of functional significance is well established for NGF. For example, studies with PC12 cells indicate a reduced activation of *trk*A at low NGF concentrations when binding to p75^{NTR} is prevented (Barker and Shooter, 1994; see also Verdi *et al*., 1994). Moreover, in mice carrying a deletion in

the NGF-binding domain of p75^{NTR}, substantial sensory deficits have been observed, and neurons isolated from such mice display a decreased sensitivity to low concentrations of NGF (Lee *et al*., 1992, 1994; Davies *et al*., 1993). In line with this, two recent studies have shown that a mutated form of NGF unable to bind to p75^{NTR}, but capable of activating *trk*A, is less active than wild-type NGF in supporting the survival of p75^{NTR}/trkA expressing neurons at low ligand concentrations (Horton *et al*., 1997; Rydén *et al*., 1997).

Of special relevance to the present study are previous indications that an additional function of the *trk*–p75^{NTR} association may be an increased ligand specificity. Thus, sympathetic neurons isolated from *p75$^{NTR-/-}$* animals are more responsive to NT3 than wild-type neurons (Lee *et al*., 1994) and PC12 cells have an increased responsiveness to NT3 when p75^{NTR} levels are reduced (Benedetti *et al*., 1993).

Finally, there is evidence for a modulation of p75^{NTR}-mediated sphingomyelin hydrolysis by *trk*A (Dobrowsky *et al*., 1995).

Properties of the trk–p75^{NTR} association

Our experiments indicate that the interaction of p75^{NTR} with all three *trk* receptors is stable enough to resist detergent solubilization in a buffer containing 1% Triton X-100. It is thus unlikely that the association results from hydrophobic interactions within the membrane, and co-transfection with constructs only able to interact with their transmembrane domains are in line with this interpretation. In this context, it is interesting to note that a previous study using a reversible cross-linker of *trk*A and p75^{NTR} also concluded that transmembrane interaction is unlikely to participate significantly in receptor interactions (Gargano *et al*., 1997). Our mapping experiments indicate that both the extracellular and intracellular domains of the receptors seem to be sufficient to drive a stable association of the two receptors. This result is in agreement with that of Gargano *et al*. (1997), who used deletion constructs of *trk*A and p75^{NTR}. However, the finding that the association with the intracellular domain of *trk*B is K252a-sensitive raises the possibility that in the absence of receptor phosphorylation, the interaction may be driven mainly by the extracellular domains of the receptor. The possibility therefore exists that the state of phosphorylation of the *trk* receptors modulates the strength of the interactions of the *trk* receptor with p75^{NTR}, and our findings suggest that phosphorylation of *trk* would reinforce the interaction with p75^{NTR}. Clearly not all tyrosine kinase receptors interact with p75^{NTR} as no co-immunoprecipitation could be demonstrated with the EGF receptor.

Beyond co-immunoprecipitation, the most direct indication for an interaction between *trk* receptors and p75^{NTR} is the increase in ligand specificity of *trk*B when p75^{NTR} is co-expressed with *trk*B. The details of the mechanisms by which p75^{NTR} increases ligand specificity are unclear. It is conceivable that the association of p75^{NTR} with the *trk* receptors changes their conformation leading to increased ligand-binding specificity. Such a mechanism could also be responsible for the enhanced binding of NGF to *trk*A in the presence of p75^{NTR} (Barker and Shooter, 1994; Verdi *et al*., 1994). In theory, signalling through p75^{NTR} following ligand binding could also be a mechanism leading to increased ligand specificity. In particular, a recent report indicates that BDNF can activate p75^{NTR} to cause serine phosphorylation of *trk*A (MacPhee and Barker, 1997). But as our results with p75^{NTR} carrying a large deletion in the intracellular domain indicate, this construct is equally effective in increasing ligand specificity, compared with full-length p75^{NTR}, we consider this possibility unlikely. Previously we have reported that a chick *trk*B splice variant lacking exon 9 coding for 11 amino acids in the extracellular juxtamembrane domain of *trk*B also shows an increased selectivity for BDNF, compared with NT4/5 and NT3, even in the absence of expression of p75^{NTR} (Strohmaier *et al*., 1996). A similar variant of *trk*B with increased selectivity for BDNF was also found in human retinal pigmented epithelium (Hackett *et al*., 1998). Taken together, these results indicate that there are at least two different ways by which the selectivity of *trk*B for BDNF can be increased. This is particularly relevant in the context of numerous previous reports indicating that BDNF has trophic activities on a variety of neurons that differ from those of NT4/5, even though both ligands bind to *trk*B.

Conclusion

The results of this study point to a close association of *trk* and p75^{NTR} receptors in cellular membranes and that one outcome of receptor association is an increased ligand specificity. Close association of these two different receptor types suggests that their signalling pathways may interact with each other as soon as they are activated.

Materials and methods

Cell culture and reagents

A293 cells (Graham *et al*., 1977) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum (Boehringer Mannheim) at 37°C and 5% CO_2. The A293 cell line stably expressing chick *trk*B had been established previously and was used as described in Dechant *et al.* (1993). K-252a was from the Alexis Corporation.

Construction of p75^{NTR}- and trk-expression plasmids and deletion mutants

Rat p75^{NTR} full-length and the p75^{NTR} extracellular domain (ΔICD) were cloned in the mammalian expression vector pcDNA3 (Invitrogen). The intracellular domain of p75^{NTR} (ΔECD), rat *trk*B and the *trk*B constructs, as well as *trk*A and *trk*C, were cloned in the pRC/CMV AC7 vector, a derivative of the pRC/CMV vector (Invitrogen), which contains the BM40 signal peptide (Mayer *et al.*, 1994). In all cases expression was driven from the CMV promoter.

Rat p75^{NTR} was isolated from the pGEM1 vector by cloning it with *Eco*RI and *Apa*I in a pcDNA3 expression vector leaving the 5′ and part of the 3′ untranslated region (UTR) intact. Using rat p75^{NTR} subcloned in pcDNA3 as template, a one-step PCR strategy was performed to delete the 118 C-terminal amino acid residues (p75^{NTR}ΔICD). Primers were: 5′-CTGGAATTCCCGGGGATCCG; and the 3′ antisense oligonucleotide with a translation stop codon inserted: 3′-GCCACGGGCCCT-CAGCCACTGTCGCTGTGCAGTT. The resulting cDNA for the construct was subcloned with *Eco*RI and *Apa*I into the pcDNA3 vector and sequenced. The p75^{NTR}ΔECD construct was generated by performing PCR with the primers 5′-GCCCCGCTAGCTCGCGGCACCACCGAC-AACC and 3′-GATCAGTGCGGCCGCTCACACTGGGGATGTGGC-AG, which deletes the 245 N-terminal amino acid residues. The construct was subcloned with *Nhe*I and *Not*I into pRC/CMV AC7 and sequenced. Rat *trk*B with the fused HA tag was cloned in the pRC/CMV AC7 vector and used as a template to generate the construct *trk*BΔECD, in which the 398 N-terminal amino acid residues are deleted. The primers used were 5′-GCCCCGCTAGCTTATCCTTATGACGTGCCTGACT-ATGCCGGGGGACAAACCAATCGGGAGCATCTC and 3′-GATCA-

GTGCGGCCGCCTAGCCTAGGATGTCCAGGTA. *Nhe*I and *Not*I were used for subcloning into pRC/CMV AC7. The T1 isoform of *trk*B was cloned from a pCMV-5 *trk*B.T1 construct using *Hin*dIII and *Xba*I to fuse it to the HA-tagged N-terminus of *trk*B in the vector pB-KS followed by subcloning into pRC/CMV AC7 with *Nhe*I and *Not*I. Rat *trk*A and rat *trk*C were similarly cloned with the HA tag into the vector pRC/CMV AC7. The EGF receptor construct pRK5 was kindly provided by A.Ullrich.

Neurotrophins and antibodies

Recombinant BDNF, NT3 and NT4/5 produced in Chinese hamster ovary cells (CHO) were a gift from Genentech, Inc. In some experiments *Escherichia coli*-derived recombinant neurotrophins were also used (Regeneron Amgen Partners). The antibodies used included anti-human p75^{NTR} pAb (Promega) as well as a rabbit polyclonal antiserum #17 generated against a peptide corresponding to amino acids 248–274 of the cytoplasmic domain of chick p75^{NTR} (kindly provided by A.Rodriguéz-Tébar). Monoclonal anti-HA for immunoprecipitation was used from hybridoma supernatants (a kind gift from S.Werner). Western blotting was performed with anti-HA mouse mAb clone 12CA5 1 µg/µl (Boehringer Mannheim). A rabbit anti-*trk* antiserum (pantrk) recognising all *trk* proteins (raised against a peptide corresponding to the last 14 amino acids of the chick *trk*A sequence) had been established previously (Schröpel *et al.*, 1995). Monoclonal anti-phosphotyrosine (clone 4G10) was purchased from Upstate Biotechnology. Anti-EGF receptor antibodies were #108 (Waterfield *et al.*, 1982) and RK2 (Kris *et al.*, 1985).

Transfection of cell lines

A293 cells were transfected by the calcium phosphate precipitation protocol using the method of Chen and Okayama (1987). Within one experiment, the amount of DNA was kept constant by supplementing samples with pcDNA3 vector DNA. For transient expression, cells were lysed 2 days after transfection. Transfection efficiency was analysed by parallel transfection with a pCMV-GFP vector (pEGFP-N1, Clontech).

Immunoprecipitation

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in 1 ml lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 µg/ml Leupeptin and 1 mM PMSF) followed by centrifugation at 4°C with 10 000 *g* for 10 min. The supernatants were incubated with 200 µl anti-HA antibody for 2–5 h at 4°C followed by precipitation with protein A–Sepharose 6MB beads (Pharmacia Biotech) overnight at 4°C. The EGF receptor was precipitated with 2 µl antibody #108. After washing the beads three times with lysis buffer, containing 0.1% Triton X-100 instead of 1% and no Leupeptin and PMSF, the proteins were eluted by boiling in 30 µl Laemmli loading buffer for 5 min. Samples were subsequently processed by Western blotting.

Receptor phosphorylation studies

A293–c*trk*B-L cells were grown in serum-free medium overnight, incubated in fresh serum-free medium for another 3–5 h and neurotrophins were added for 5 min at 37°C. Immunoprecipitation was performed as described above, except that the lysis buffer was supplemented with 5 mM orthovanadate and 3 mM EDTA. The beads were washed with a buffer containing 1 mM orthovandate. *trk*B was immunoprecipitated with 2 µl pantrk antiserum for 1–2 h at 4 C followed by the addition of protein A–Sepharose beads.

Western blotting

Proteins were separated on 7% or 10% polyacrylamide gels and subsequently transferred onto Immobilon (Millipore) membranes. Following incubation with 2% nonfat milk powder in Tris-buffered saline-Tween (TBST; 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20), the membranes were incubated overnight at 4°C with anti-human p75^{NTR} pAb (Promega), diluted at 1:2000 in blocking buffer or with the p75 antiserum #17, diluted at 1:500 in blocking buffer, followed by incubation for 1 h at room temperature with goat anti-rabbit IgG-POD (Pierce, 1:10 000 in TBST). The EGF receptor was similarly detected with the antibody RK2, diluted at 1:1000.

After blocking with a gelatine solution (0.5% gelatine, 5 mM EDTA in TBST), phosphorylated *trk*B receptors were detected using the anti-phosphotyrosine antibody 4G10 overnight at room temperature, diluted at 1:400 in gelatine blocking buffer, followed by incubation for 1 h at room temperature with goat anti-mouse IgG-POD (Pierce, 1:10 000 in TBST). *trk* receptors were detected following overnight incubation at 4°C with the pantrk antibody, diluted at 1:1000 in TBST/2% milk or with the anti-HA antibody (Boehringer Mannheim), diluted at 1:1000 in TBST/2% milk.

The immune complexes were detected using the ECL detection system (Amersham) and exposure to autoradiographic film (Fuji).

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