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Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor

Alfredo Rodríguez-Tébar[1], Georg Dechant, Rudolf Götz[2] and Yves-Alain Barde

Max-Planck Institute for Psychiatry, Departments of Neurobiochemistry, and [2]Neurochemistry, 8033 Martinsried, Germany and [1]Cajal Institute of Neurobiology, Doctor Arce 37, E-28002 Madrid, Spain

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Neurotrophin-3 (NT-3) has low-affinity ($K_d = 8 \times 10^{-10}$ M), as well as high-affinity receptors ($K_d = 1.8 \times 10^{-11}$ M) on embryonic chick sensory neurons, the latter in surprisingly high numbers. Like the structurally related proteins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), NT-3 also binds to the low-affinity NGF receptor, a molecule that we suggest to designate low-affinity neurotrophin receptor (LANR). NT-3 dissociates from the LANR much more rapidly than BDNF, and more slowly than NGF. The binding of labelled NT-3 to the LANR can be reduced by half using a concentration of BDNF corresponding to the K_d of BDNF to the LANR. In contrast, the binding of NT-3 to its high-affinity neuronal receptors can only be prevented by BDNF or NGF when used at concentrations several thousand-fold higher than those corresponding to their K_d to their high-affinity neuronal receptors. Thus, specific high-affinity NT-3 receptors exist on sensory neurons that can readily discriminate between three structurally related ligands. These findings, including the remarkable property of the LANR to bind three related ligands with similar affinity, but different rate constants, are discussed.

Key words: BDNF/NGF/NT-3/receptors/sensory neurons

Introduction

The survival and maintenance of differentiated function of vertebrate neurons can be dramatically influenced by the availability of specific proteins referred to as neurotrophic factors (for recent reviews, see Purves, 1988; Snider and Johnson, 1989; Barde, 1989). Over a few decades, the protein nerve growth factor (NGF) was the only fully characterized molecule that could be convincingly shown to play such a role (Cohen, 1960; Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). Recently, however, evidence has been provided for the existence of three other neurotrophic factors that are structurally related to NGF: brain-derived neurotrophic factor (BDNF) (Leibrock *et al.*, 1989), neurotrophin-3 (NT-3) (Ernfors *et al.*, 1990; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990) and neurotrophin-4 (NT-4) (Hallböök *et al.*, 1991). Biologically active neutrophins —a name used to refer to the members of this family—consist of essentially the same number of amino acids (~120, after cleavage from their biosynthetic precursors), have a basic isoelectric point (between 9 and 10) and six cysteine residues located at identical positions, almost certainly involved in the formation of three disulfide bridges (for a recent review, see Barde, 1991). Comparative binding studies were performed with NGF and BDNF using chick sensory neurons. These neurons are particularly interesting to study as their survival has been shown to be supported, in various proportions, by all neurotrophins. Both BDNF and NGF have two classes of receptors on embryonic sensory neurons: high-affinity receptors ($K_d \sim 10^{-11}$ M), thought to be involved in the intracellular signalling resulting from ligand binding, and low-affinity receptors ($K_d \sim 10^{-9}$ M) (Sutter *et al.*, 1979; Rodríguez-Tébar and Barde, 1988). These two classes of binding site differ in some important aspects. Of particular relevance to the present study is the observation that the high-affinity sites can readily discriminate between the two ligands BDNF and NGF: a 1000-fold excess of NGF is needed to prevent the binding of BDNF to its high-affinity receptors by 50% (Rodríguez-Tébar *et al.*, 1990). In contrast, the binding of BDNF to its low-affinity sites can be prevented equally well by NGF and BDNF (Rodríguez-Tébar *et al.*, 1990). Evidence has been presented that the low-affinity NGF receptor, a well-defined molecule (Johnson *et al.*, 1986; Radeke *et al.*, 1987), can function as a low-affinity BDNF receptor (Rodríguez-Tébar *et al.*, 1990). These data suggested that the neurotrophins, in addition to their striking relatedness in primary structure, are likely to display similar, but not identical, structural features in solution as revealed by binding experiments. Indeed, even though the affinity constants (at equilibrium) for NGF and BDNF binding to the low-affinity receptor were found to be virtually identical, the surprising observation was made that the binding rate constants of both ligands are very different (Rodríguez-Tébar *et al.*, 1990). In the present study, we find that NT-3 also binds to the same receptor with an affinity close to that previously determined for NGF and BDNF. Interestingly, yet a different rate constant was measured for the dissociation of NT-3 from this receptor. In addition, we determined the binding characteristics of NT-3 on embryonic sensory neurons and examined the extent to which the high-affinity NT-3 neuronal receptors are able to discriminate between NT-3, NGF and BDNF.

Results

NT-3 receptors on sensory neurons

Binding studies were performed using chick sensory neurons, as they can be obtained in large numbers from embryonic dorsal root ganglia. In addition, the survival of some of these neurons is known to be supported *in vitro* by NT-3 (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990). In steady-state binding studies using neurons isolated

from dorsal root ganglia of 8 day old chick embryo (E8), high-affinity, as well as low-affinity binding sites were observed (Figure 1). For the high-affinity sites, we found 11 126 ± 3271 receptors per neurons with a K_d of 1.8 ± 0.7 × 10^{-11} M (Figure 1A), and for the low-affinity sites, 39 190 ± 5047 receptors per neurons with a K_d of 8 ± 2 × 10^{-10} M (Figure 1B). In order to characterize these two sites by criteria other than steady-state binding, we also examined the dissociation rate of NT-3 from these sites (Figure 2). A rate of 4.8 × 10^{-4} s^{-1} was found for the high-affinity receptors (Figure 2A) and of 6.0 × 10^{-3} s^{-1} for the low-affinity receptors (Figure 2B). Thus, the rate of dissociation from the high-affinity receptors is ~12 times slower compared with that measured for the low-affinity receptors.

High-affinity NT-3 binding in the presence of NGF or BDNF

We then wanted to determine the extent to which NGF and BDNF could prevent the binding of NT-3 to its high-affinity receptors. Sensory neurons were incubated with various concentrations of unlabelled NGF or BDNF, followed by the addition of 3 × 10^{-11} M NT-3 for 20 min. It was found that comparatively high concentrations of either NGF, 4 × 10^{-8} M (Figure 3A) or BDNF, 6.3 × 10^{-8} M (Figure 3B) were necessary to prevent NT-3 binding by 50%. 1.3 × 10^{-11} M unlabelled NT-3 reduced binding by 50% from these sites, a figure close to the dissociation constant established using labelled NT-3 (1.8 × 10^{-11} M, see above).

Interaction of NT-3 with the low-affinity NGF receptor

In view of the presence of low-affinity NT-3 receptors on neurons, as well as of a previous study indicating that the low-affinity NGF receptor binds both NGF and BDNF with similar affinities (Rodríguez-Tébar *et al.*, 1990), we next asked the question if NT-3 would also bind to this receptor. To this end, we used PCNA cells, which are fibroblastic cells (L-cells) stably transfected with the NGF receptor gene which express large numbers of this receptor (Radeke *et al.*,

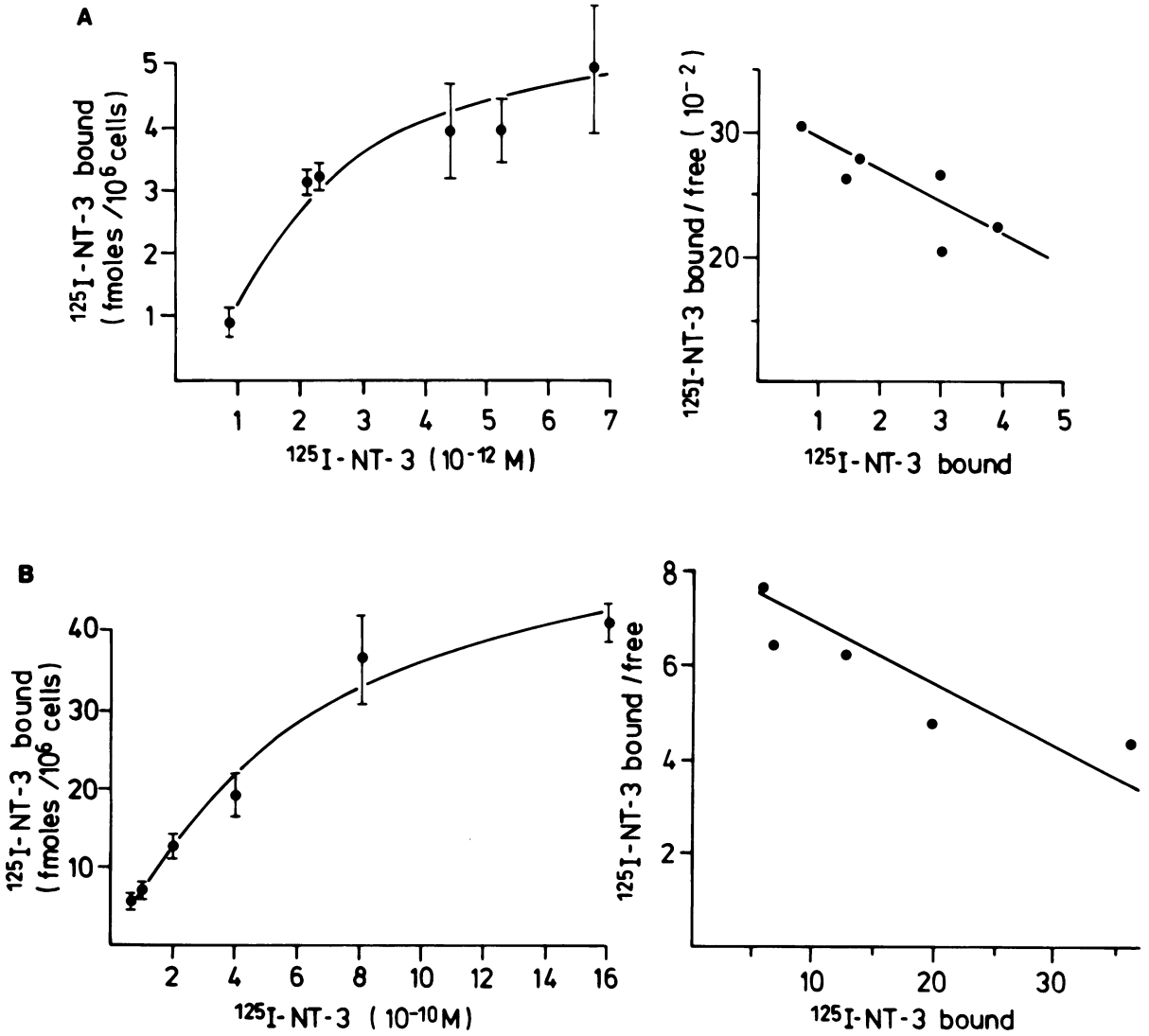

Fig. 1. Binding of labelled NT-3 to sensory neurons. Two ranges of concentrations were used to explore the high-affinity sites (**A**) and the low-affinity sites (**B**). The Scatchard transformations of the data are also shown and the values obtained were: 11 126 ± 3271 high-affinity sites with a K_d of 1.8 (± 0.7) × 10^{-11} M, and 39 190 ± 5047 low-affinity sites with a K_d of 8 (± 2) × 10^{-10} M. The values are the means ± SD of tetraplicate determinations and are representative of three separate experiments.

1987). Steady-state binding experiments indicated that these PCNA cells bind NT-3 (Figure 4A). Half-saturation was obtained with 9.7 (± 1.9) × 10^{-10} M (four experiments), the number of binding sites being ~425 000 per cell. These values are not significantly different from those obtained under identical experimental conditions with either NGF or BDNF as ligands (Rodríguez-Tébar *et al.*, 1990). With NT-3, indications of positive cooperativity were obtained. Though less pronounced than with BDNF, the positive cooperativity is apparent when a Scatchard transformation of the steady-state binding values is attempted (Figure 4B). No binding was observed using the parental L-cells before transfection with the NGF receptor gene (Figure 4). A clear indication that the low-affinity receptor NGF, while binding the three neurotrophins, is also able to distinguish between them was given by examining the rate of dissociation of NT-3 from this receptor (Figure 5). A value of 1.4 × 10^{-2} s^{-1} was obtained. This is 14 times faster than that of BDNF, but distinctly slower than that of NGF, which is so fast that it cannot be measured accurately (Sutter *et al.*, 1979).

In addition to unlabelled NT-3, BDNF was able to prevent the binding of NT-3 to PCNA cells. Fifty per cent inhibition was obtained with BDNF at 3 × 10^{-9} M (50% inhibition of NGF binding on these cells is obtained with 1.3 × 10^{-9} M BDNF, Rodríguez-Tébar *et al.*, 1990).

No NT-3 high-affinity binding was observed using PCNA cells or the cell lines PC12, known to have low-affinity, as well as high-affinity NGF receptors (see Vale and Shooter, 1985), nor NG 115-401L (a neuroblastoma × glioma hybrid cell line that expresses some characteristics of sensory neurons, Hatanaka and Amano, 1981; Jackson *et al.*, 1987).

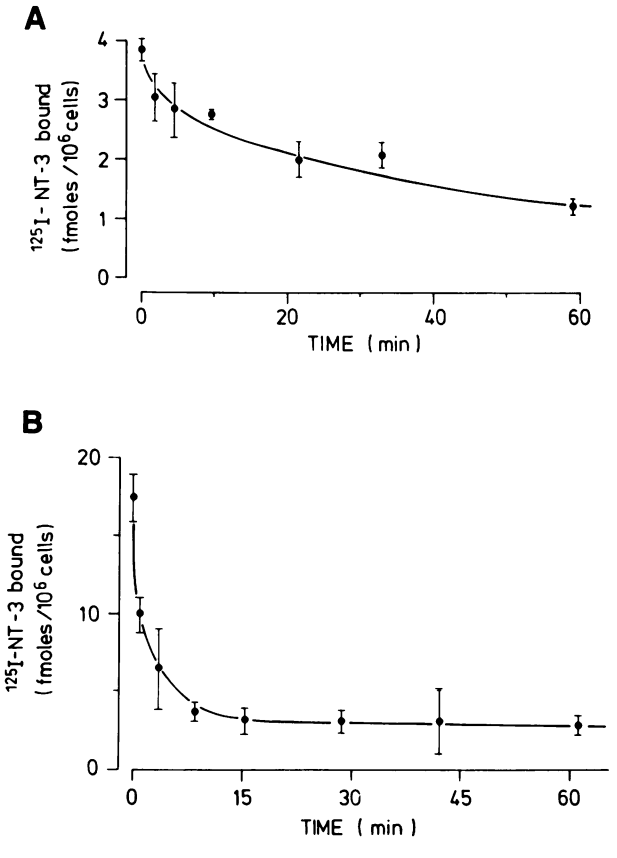

Fig. 2. Dissociation rates of labelled NT-3 from the high-affinity sites (**A**) and from the low-affinity sites (**B**) on sensory neurons. Sensory neurons were mixed with labelled NT-3 and the mixture incubated for 1–2 h, during which equilibrium between bound and free factor was achieved. For the high-affinity sites, [^{125}I]NT-3 was used at 1.5 × 10^{-11} M and for the low-affinity sites, 1 × 10^{-10} M. At the end of the incubation, 100- to 300-fold excess of unlabelled NT-3 was added to the binding mixtures and aliquots were removed at different time intervals, centrifuged and counted. Values of 4.8 × 10^{-4} s^{-1} (**a**) and 6.0 × 10^{-3} s^{-1} (**b**) were obtained (see Materials and methods). The values are the means ± SD of tetraplicate determinations.

Discussion

Low-affinity NT-3 receptors

There are low-affinity binding sites on sensory neurons to which NT-3 binds with a K_d of 8 × 10^{-10} M. This value is close to that determined for NGF (1.7 × 10^{-9} M) and BDNF (1.3 × 10^{-9} M) on the same cells. In view of a previous study showing that the low-affinity NGF receptor also binds BDNF with an affinity similar to that of NGF, but different rate constants (Rodríguez-Tébar *et al.*, 1990),

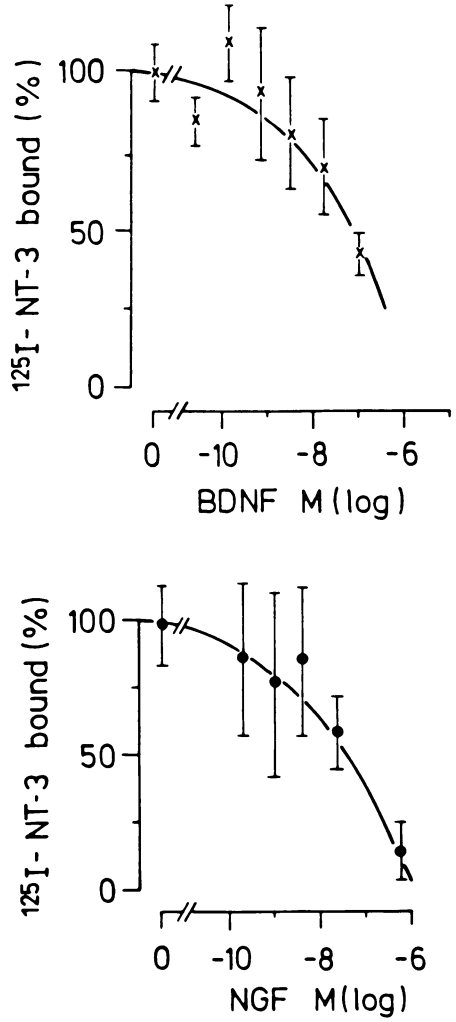

Fig. 3. Binding of labelled NT-3 to its high-affinity receptors on sensory neurons in the presence of various concentrations of unlabelled BDNF (**A**) or NGF (**B**). [^{125}I]NT-3 was used at 3 × 10^{-11} M. Cells were first incubated with various concentrations of unlabelled factor for 60 min, followed by the addition of 3 × 10^{-11} of [^{125}I]NT-3 and incubation for further 20 min. The binding reaction was terminated as described (see Materials and methods). The values are the means ± SD of tetraplicate determinations.

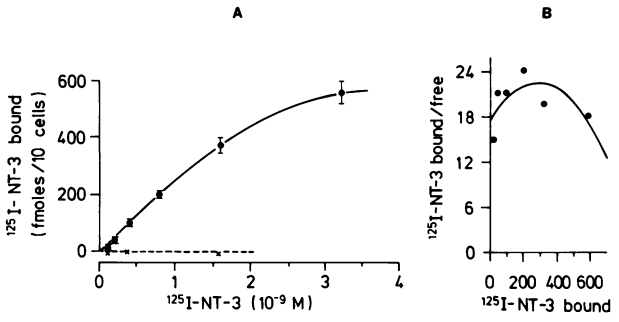

Fig. 4. (**A**) Binding of NT-3 to PCNA cells (solid line) and to the parental L-cells (dashed line). While no binding is observed on L-cells, 425 000 sites with a K_d of 9.7 (± 1.9) × 10^{-10} M are found on PCNA cells. The values are the mean ± SD of tetraplicate determinations. (**B**) Scatchard transformation of the data. The experimental points are fitted using the program Grafit (Erythacus Software, London) and a non-linear regression fit. This transformation illustrates the departure from linearity at low ligand concentrations.

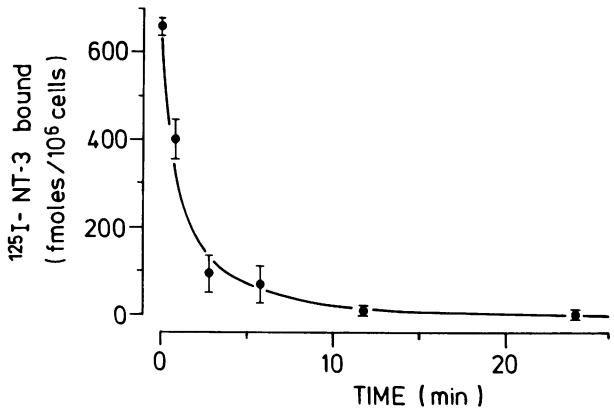

Fig. 5. Dissociation rate of labelled NT-3 from PCNA cells. [^{125}I]NT-3 was used at 1 × 10^{-9} M. A value of 1.4 × 10^{-2} s^{-1} was obtained. The values are the means ± SD of tetraplicate determinations.

and of the primary structure relatedness of NT-3 to both NGF and BDNF, we wanted to determine the binding parameters of NT-3 to the low-affinity NGF receptor. The results of equilibrium binding studies obtained with the PCNA cells (stably transfected with the NGF receptor gene and expressing a very large number of receptors) indicate that this receptor binds NT-3 with an affinity that is similar, if not identical, to that determined for NGF and BDNF. These data are consistent with previous results obtained by others using conditioned medium of COS cells transfected with a NT-3/HDNF cDNA and observing a reduction of [^{125}I[NGF binding to the low-affinity NGF receptor on PC12 cells (Ernfors *et al.*, 1990) and cross-linking experiments using [^{125}I]NT-3 and the low-affinity NGF receptor transiently expressed on COS cells (Squinto *et al.*, 1991). As the low-affinity NGF receptors has now been demonstrated to bind three different neurotrophins, we suggest that this receptor be designated the low-affinity neutrotrophin receptor (LANR). This suggestion also takes into account the results of experiments performed with the recently discovered neurotrophin-4 (NT-4) (Hallböök *et al.*, 1991). Using conditioned medium of COS cells transfected with a NT-4 cDNA, a reduction of [^{125}I]NGF binding to the low-affinity receptor on PC12 cells was observed (Hallböök *et al.*, 1991). Taken together, these data strongly support the idea that the LANR has the property to bind a variety of ligands (Rodríguez-Tébar *et al.*, 1990). In view of the similar affinity constants determined for NGF, BDNF and NT-3, it is surprising that the rate constants for the binding to the LANR are distinct for each of the three ligands: the dissociation rates are too fast to be measured accurately with NGF, 1.4 × 10^{-2} s^{-1} for NT-3 (this study) and 5.8 × 10^{-4} s^{-1} for BDNF (Rodríguez-Tébar *et al.*, 1990). It thus appears that the LANR, while binding three neurotrophins, also recognizes differences between them. At present, the neurotrophin sequences that participate in binding to the LANR are not known. Our results indicate that these sequences are unlikely to correspond to segments consisting of identical amino acids. Based on the observed rate constants, the hypothesis can be put forward that NT-3 is likely to display a binding site more similar to that of NGF than to that of BDNF. This might be of significance if the LANR contributes to the formation of a receptor complex with high-affinity binding characteristics (see below).

The LANR on PCNA cells binds NT-3 with characteristics that are close to those observed for the low-affinity NT-3 receptor on sensory neurons. However, a contribution to low-affinity NT-3 binding resulting from the presence of other components expressed on the surface of sensory neurons cannot be ruled out at present. For example, *trk*B (expressed on NIH3T3) has been shown to bind NT-3 with an affinity indistinguishable from that determined here for the LANR (Soppet *et al.*, 1991). But for two reasons, we find it likely that the LANR represents the major NT-3 low-affinity receptor on sensory neurons.

First, in the case of the low-affinity sites, the rates of dissociation of NT-3 from sensory neurons and PCNA cells are quite similar, the slightly slower rate observed with sensory neurons being most likely due to the contribution of the high-affinity receptors, from which NT-3 dissociates considerably more slowly. Second, the number of NT-3 low-affinity sites (~39 000 per neuron) is close to that determined with NGF on the same neurons (between 23 000 and 45 000 depending on the technique used, Sutter *et al.*, 1979).

High-affinity NT-3 receptors

As with NGF and BDNF, high-affinity NT-3 receptors were also found on neurons. The value obtained for the dissociation constant using sensory neurons (1.8 × 10^{-11} M) is close to that obtained for NGF (2.3 × 10^{-11} M) and BDNF (1.7 × 10^{-11} M). In terms of neuronal survival, the observed biological activity of NT-3 is high, half-maximal values being obtained with ~ 2 × 10^{-12} M (Götz *et al.*, 1992). As noted before with NGF (Sutter *et al.*, 1979), it seems that with NT-3, as well as with BDNF (Rodríguez-Tébar and Barde, 1988) neuronal survival is induced below full occupancy of the high-affinity receptors. When studying these sites, only very small concentrations of ligands were used (see, for example, Figures 1 and 3), in order to avoid significant occupancy of the low-affinity sites. This approach was facilitated by the positive cooperativity of NT-3 binding to the low-affinity receptor, which reduces the occupancy of the low-affinity receptor to negligible values at low ligand concentrations (10^{-12} to 10^{-11} M). For example, in this range of concentrations, the PC12 cells used in this study

do not show any specific binding, in spite of the presence of 50 000 low-affinity sites on these cells (Vale and Shooter, 1985). In addition, the high-affinity receptors can also be distinguished from the low-affinity ones on the basis of the considerably slower rates of dissociation.

It is interesting to note that the high-affinity NT-3 receptors are also characterized by their remarkable ability to discriminate between the three neurotrophins. In fact, even higher concentrations (~5 000-fold) of either NGF or BDNF are necessary to prevent by 50% the binding of NT-3 to its high-affinity receptor (Figure 3A and B), compared with either NGF or BDNF in the case of the high-affinity BDNF or NGF receptors (~1000-fold, Rodríguez-Tébar *et al.*, 1990). There are reasons to think that the selectivity of binding seen so far in our studies using sensory neurons is of relevance, simply because there are neurons that respond to one particular neurotrophin and not to the others (see Barde, 1991 for a recent review). This also is the case with NT-3, even though the description of its biological effects is still at a preliminary stage. For example, basal forebrain cholinergic neurons have been reported to respond to NGF and BDNF, but not to NT-3 (Knüsel *et al.*, 1991). Also, BDNF, used at saturating concentrations, is able to support the survival of only ~50% of the neurons isolated from E8 chick nodose ganglion. But in combination with NT-3, which on its own supports fewer neurons than BDNF, >80% of the neurons can be rescued (Hohn *et al.*, 1990; Götz *et al.*, 1992).

Our experimental approach does not allow us to decide whether or not the inhibition of NT-3 high-affinity binding seen with NGF and BDNF is competitive. However, the results of preliminary experiments in which various concentrations of [^{125}I]NT-3 ($0.5-2.0 \times 10^{-11}$ M) were added to the neurons in the presence or in the absence of BDNF (5×10^{-8} M) indicate that both ligands compete for the same binding sites.

In the present study, we find one characteristic of the high-affinity NT-3 binding to sensory neurons to be surprising: the number of high-affinity receptors is substantially higher (~15 times) than that found for either NGF or BDNF on the same neurons, under the same experimental conditions. This is all the more astonishing that it is with NT-3 that we find the lowest percentage of neuronal survival, 28% at E8 (Hohn *et al.*, 1990; Götz *et al.*, 1992), compared with ~35 and 50% with BDNF and NGF, which have ~500–1000 high-affinity sites per neuron (Sutter *et al.*, 1979; Rodríguez-Tébar and Barde, 1988). The possibility exists that there are high-affinity NT-3 receptors that are not linked with the transduction of any particular biological function, and/or that there are other biological functions for NT-3, the promotion of *in vitro* neuronal survival being only one of them. Concerning the first possibility, it is of interest to note that as with other receptors, including those of various interleukins, truncated forms of *trk*B have been described (Klein *et al.*, 1990; Middlemas *et al.*, 1991).

At present, our results do not allow conclusions to be drawn as to the molecular nature of the specific high-affinity NT-3 receptors on sensory neurons. One component might be the most recently identified member of the *trk* family, *trk*C (Lamballe *et al.*, 1991). While it is not known whether or not this gene is expressed in sensory neurons, experiments carried with NIH3T3 cells expressing this gene indicate that such cells can indeed bind NT-3. Furthermore, neither NGF nor BDNF can readily prevent the binding of NT-3 to *trk*C, suggesting a substantial degree of ligand specificity (Lamballe *et al.*, 1991). While it is premature to conclude that *trk*C represents the NT-3 high-affinity neuronal binding sites described here, this is an interesting possibility to test. At present however, binding results obtained with transfected fibroblasts expressing various members of the *trk* family cannot readily be extrapolated to neurons. For example, NT-3 has been reported to bind not only to *trk*C (see above), but also to *trk*B, thought to be part of a high-affinity BDNF receptor (Klein *et al.*, 1991; Squinto *et al.*, 1991; Soppet *et al.*, 1991) and *trk* (Cordon-Cardo *et al.*, 1991), almost certainly part of the high-affinity NGF receptor (see Bothwell, 1991 for review). Thus, the results obtained with cell lines transfected with the *trk* family members do not consistently show one typical feature of the neurotrophins when their binding characteristics are assessed using sensory neurons: high-affinity binding for any of the three neurotrophins is accompanied by high selectivity. One obvious candidate known to be present in sensory neurons that could contribute to increase the binding specificities of the *trk* family members is the LANR. As discussed above, the observation that NGF, BDNF and NT-3 bind, though differently to the LANR, invites the speculation that it is the co-operation between the various *trk*s and the LANR that results in increased ligand discrimination.

Materials and methods

Materials

Recombinant NT-3 was produced using vaccinia virus infection of cultured rabbit kidney cells (Götz *et al.*, 1992). Briefly, the entire coding sequence corresponding to mouse NT-3 was introduced into the vaccinia virus genome, and the viruses used to infect cultured rabbit kidney cells. The conditioned medium was adsorbed over a glass bead column and the adsorbed proteins eluted with 0.1 M NaCl and 0.1 M acetic acid containing 30% acetonitrile. The eluted material was concentrated by evaporation and applied onto a C8 microbore reverse phase column. Elution was performed with a linear gradient of acetonitrile in 0.1% TFA at 0.4 ml/min. NT-3 was eluted as a major peak with ~30% acetonitrile. This fraction was evaporated and contained only one protein band as revealed by SDS–PAGE, migrating exactly at the position of BDNF. Amino-terminal sequencing was performed for 14 cycles and revealed one sequence, corresponding to that predicted by the cDNA sequence. Half-maximal activity was obtained with 20–30 pg/ml and maximal activity with 200 pg/ml NT-3 using the survival of chick dorsal root ganglion neurons as an assay system (Götz *et al.*, 1992). NGF was prepared from adult male mouse submandibular glands (Suda *et al.*, 1978) and BDNF from pig brain (Hofer and Barde, 1988) or as a recombinant protein using the vaccinia virus system described above.

Radiolabelling

NGF was labelled as described (Rohrer and Barde, 1982), and NT-3 according to the following procedure: ~2.5 μg NT-3 were mixed in a total volume of 30 μl M NaP_i buffer (pH 7.2), with 0.2 μg of lactoperoxidase (Sigma), 400 μCi of [^{125}I]Na (2000 Ci/mmol, New England Nuclear) and H_2O_2 at a final concentration of 170 μM. This reaction mixture was incubated for 30 min at 0°C and subsequently diluted with the NaP_i buffer to 250 μl. After 2–3 h at 0°C, 2–3 mg bovine serum albumin was added to the reaction mixture. Labelled NT-3 was found to have a mean specific activity of 160 c.p.m./pg protein (corresponding approximately to one radioatom per molecule of NT-3, taking into consideration a counting efficiency of 50%). [^{125}I]NT-3 was used within 10 days after the reaction. Under these conditions, >90% of the radioactivity could be precipitated with trichloroacetic acid (TCA), 90% being already precipitable 30 min after starting the reaction. Gel filtration on a P30 (Bio-Rad) column was initially used to separate bound from free radioactivity, but led to considerable losses (up to 90%) of [^{125}I]NT-3, without significant increase in TCA-precipitable material. Also, no measurable differences were found in binding parameters using PCNA cells with or without gel filtration. These results led to the omission of the gel filtration step in the standard iodination procedure. In a bioassay using embryonic dorsal root sensory neurons, radiolabelled NT-3 was found to have the same specific biological activity as unlabelled NT-3.

Cell preparation

LTK$^-$ L-cells and PCNA cells were grown and harvested as described (Rodríguez-Tébar *et al.*, 1990). PC12 cells were cultured in DMEM containing 5% fetal calf serum and 5% horse serum, and NG 115-401 L in DMEM with 10% fetal calf serum.

For binding experiments, dorsal root ganglia (E8) were prepared, the ganglia dissociated, and the neurons obtained as described in Sutter *et al.* (1979) and Rodríguez-Tébar and Barde (1988) with the modifications indicated below. About 500–750 ganglia were collected in ice-cold F-14 medium containing 10% heat-inactivated horse serum. The ganglia were briefly centrifuged, resuspended in 5 ml $Ca2^+/Mg2^+$-free Gey's buffer and incubated for 10 min at 37°C. Trypsin (Worthington) was added at a final concentration of 0.12 mg/ml and the incubation continued for further 10 min. Trypsinization was terminated by the addition of soybean trypsin inhibitor (Sigma) at 0.15 mg/ml (final concentration) and 10% horse serum. The tube was placed on ice and the ganglia dissociated mechanically by passing them through a wide-bore 5 ml pipette using six to eight gentle strokes. Non-dissociated ganglia were allowed to settle and the supernatant removed and saved. The remaining ganglia were resuspended in 3 ml Gey's buffer containing 10% horse serum and mechanical dissociation was performed as before. This step was repeated twice and the combined supernatants were filtered through a nylon net (50 μ pore size) and centrifuged for 10 min at 120 *g*. The cell pellet was resuspended in F-14 medium containing 10% heat-inactivated horse serum and pre-plated onto four to six 10 cm tissue culture dishes that were incubated for 3 h at 37°C in a water-saturated atmosphere containing 3% CO_2. Non-adhering cells were gently resuspended and collected by centrifugation for 10 min at 120 *g*. This pre-plating procedure has the double purpose to enrich for neurons by eliminating more adherent non-neuronal cells, and to let sufficient time to elapse for the cells to recover from the trypsin digestion step. The cell pellet was resuspended in Krebs–Ringer HEPES buffer, pH 7.35 (Herrup and Thoenen, 1979), containing 5 mg/ml bovine serum albumin (Sigma) and 0.1 mg/ml horse heart cytochrome C (Sigma) (=binding buffer).

Binding assays

All binding studies were performed at 4°C, in a total volume of 425 or 525 μl. Incubation of the cells (150 000–500 000 cells/ml) with labelled NT-3 was typically for 2 h, allowing for equilibrium to be reached at the lowest concentration of ligand used. Non-specific binding was determined by pre-incubating the cells for 45 min with a 100- to 1000-fold excess of unlabelled factor and was subtracted from total binding to obtain specific binding. It was found to account for maximally 10–20% of the total binding. All values reported in this study refer to specific binding and result from tetraplicate determinations.

For high-affinity binding studies, binding was terminated by centrifugation of aliquots of cell suspensions (100 or 125 μl) through to two-step sucrose gradient, as previously described (Sutter *et al.*, 1979; Rodríguez-Tébar and Barde, 1988). Immediately after centrifugation, the tubes were frozen in an ethanol–dry ice bath and the bottoms of the tubes cut and counted (bound radioactivity), as well as the remaining upper part of the tubes (free radioactivity). Most of the experiments reported here on high-affinity binding were performed using very low concentrations of [^{125}I]NT-3, 1.8×10^{-11} M or less. At such low concentrations, the occupancy of the low-affinity receptor (see below) is negligible. For example, using PC12 cells which have ~50 000 low-affinity receptors per cell (Vale and Shooter, 1985), no specific high-affinity binding could be detected.

For the study of low-affinity binding, the reaction was terminated by centrifuging 100 μl aliquots in 500 μl tubes as described in Rodríguez-Tébar *et al.* (1990). Supernatants were carefully removed by aspiration and the bottoms of the tubes cut and counted. Thus, this methodology differs from that used for measuring high-affinity binding which was found to be inadequate owing to the fast dissociation rate of [^{125}I]NT-3 from its low-affinity receptors.

Treatment of kinetic data

In the results presented in Figure 1, both the dissociation constants and the number of binding sites were obtained from linearization of the saturation curves by Scatchard transformation of the saturation curves and the experimental points fitted by a linear regression programme. Dissociation rates of [^{125}I]NT-3 from its high- and low-affinity receptors were calculated assuming a single exponential decay $k_{-1} = \ln 2/t_{1/2}$, whose $t_{1/2}$ is the time taken for 50% of the bound ligand to dissociate from its receptor.

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