High Affinity Not in the Vicinity?

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Functional interactions between the p75 neurotrophin receptor (p75NTR) and the Trk receptors were demonstrated several years ago, but their mechanistic basis remains uncertain. In this issue of *Neuron*, Wehrman et al. provide a three-dimensional structure of the full TrkA ectodomain complexed to NGF and examine the possibility of a ternary p75NTR-NGF-TrkA complex.

The p75NTR has been getting a lot of attention over the last few years for its role as an apoptotic receptor and for its participation in neuronal growth inhibition. However, the function originally identified for p75NTR concerned its function as an accessory protein that modulates responses of the Trk tyrosine kinase receptors. In vivo and in vitro data clearly indicate that p75NTR and Trk receptors functionally interact, but the precise means by which this occurs has remained unresolved. In this issue of *Neuron*, Wehrman et al. (2007) provide a three-dimensional structure of the entire TrkA extracellular domain (ECD) in complex with NGF; with this data in hand, the authors examine the possibility that a tripartite p75NTR-NGF-TrkA assembly facilitates their functional collaboration, with some intriguing results.

p75NTR and Trk Receptors Collaborate to Sharpen Neurotrophin Responses

Functional interactions between p75NTR and TrkA can be broken into two main categories. The first is derived from studies showing that p75NTR enhances the response of Trk to neurotrophins. This work showed that antibodies directed against p75NTR reduced NGF-mediated TrkA phosphorylation in PC12 cells and primary neurons and demonstrated that coexpression of p75NTR with TrkA in heterologous expression systems enhanced NGF-induced TrkA phosphorylation (reviewed in Roux and Barker, 2002). The second group of studies showed that p75NTR increases the specificity of the Trk receptors for particular ligands. For example, work by Bibel et al. (1999) showed that TrkB is readily activated by BDNF, NT3, and NT4 in the absence of p75NTR, but only BDNF can efficiently activate the receptor when p75NTR is coexpressed. Other studies have shown that function-perturbing antibodies to p75NTR enhance the response of TrkA to NT3, consistent with the notion that p75NTR acts to suppress TrkA responses to this ligand (Clary and Reichardt, 1994). Taken together, these and other related studies indicate that p75NTR enhances Trk responses to preferred ligands (e.g., NGF for TrkA, BDNF for TrkB) while attenuating responses to nonpreferred ligands (e.g., NT3 for TrkA).

There is ample evidence showing that these functional collaborations have physiological relevance. Primary dorsal root sensory neurons and sympathetic neurons derived from p75NTR null animals show an ~3-fold decrease in survival responses to NGF. This deficit may seem modest but can have serious consequences for a neuron that must respond to the low quantities of neurotrophin present in target tissues. Indeed, this reduction in responsiveness likely accounts for the defects in cutaneous sensory innervation originally described in p75NTR null mice (Lee et al., 1994). In separate studies, analyses of p75NTR nulls and neurons derived from them showed that p75NTR reduces TrkA responses to NT3 in sympathetic neurons, sharpening dependence on the target-derived factor, NGF (Brennan et al., 1999; Kuruvilla et al., 2004).

Layered on top of these functional interactions is a rich history of work examining the biochemical and kinetic features of neurotrophin binding. Early studies indicated that NGF receptors on neurons have two kinetic forms, a high-affinity complex with a Kd of approximately 10^{-11} and a low-affinity receptor with a Kd in the nM range. Neither TrkA nor p75NTR form high-affinity binding sites when expressed alone, but coexpression of the two receptors in heterologous cells results in formation of high-affinity NGF binding sites (Hempstead et al., 1991). Thus, the notion that p75NTR and TrkA combine to form receptor complexes that give rise to high-affinity NGF binding sites has become well entrenched in the field. However, this view is challenged by the studies from the Garcia group reported in this issue of *Neuron*.

Neurotrophin and Neurotrophin Receptor Structures

The TrkA ECD contains a canonical LLR consisting of three leucine-rich repeats capped at either end with cysteine-rich domains, followed by an Ig-C1 and then an Ig-C2 domain. By solving the three-dimensional structure of the entire TrkA extracellular domain (ECD) in complex with NGF, Wehrman et al. (2007) reveal that the TrkA ECD is a surprisingly rigid structure that is constrained by several interdomain contacts. They show that the LRR is an integrated domain that has extensive contacts with the Ig-C1 structure and reveal that receptor rigidity is reinforced by an interdomain disulfide bond between the LRR and

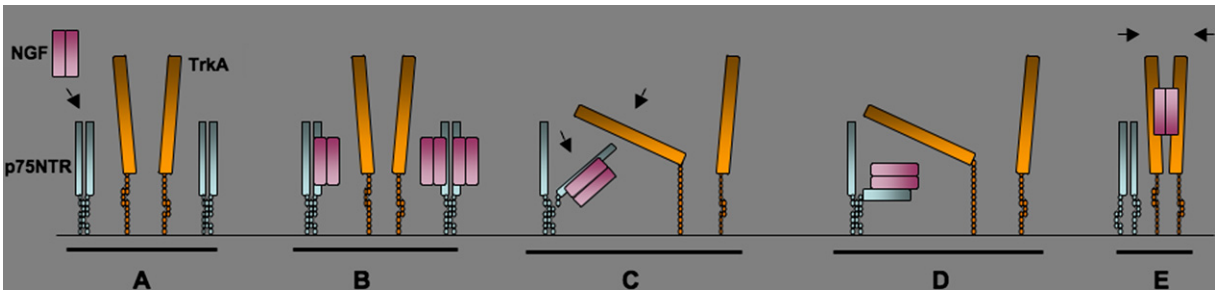

Figure 1. A Ligand-Passing Model for p75NTR and TrkA
(A) In the absence of neurotrophin, p75NTR exists as preformed dimers, but TrkA chains do not form homo- or heterocomplexes.
(B) NGF binds p75NTR with a fast association rate; both configurations shown are possible since NGF binding to the p75NTR does not disrupt the p75NTR dimer (Wehrman et al. 2007). Note, however, that two p75NTR chains cannot simultaneously bind a single NGF dimer (He and Garcia, 2004).
(C) NGF bound to p75NTR has a binding interface available for interaction with TrkA. p75NTR and TrkA bind NGF in different orientations, and therefore an "NGF sandwich" that accommodates this requirement is indicated.
(D) In this orientation, NGF is presented to TrkA in a conformation that favors rapid association with the receptor tyrosine kinase. This complex is likely to dissociate very rapidly when the ligand is NGF but more slowly when the ligand is NT3.
(E) The NGF dimer binds a second chain of TrkA to allow the receptor kinase to form an active homodimer.

Ig-C1 region. The Ig-C1 and Ig-C2 domains have extensive interdomain contacts that also contribute to overall stiffness of the receptor. Earlier work had established that NGF binds TrkA through the Ig-C2 domain of TrkA, and the present study confirms this and demonstrates that the Ig-C2 domain is the only region within the TrkA ectodomain that contacts NGF (Wehrman et al., 2007).

In another recent study, Garcia and colleagues presented a three-dimensional structure of NGF bound to the p75NTR ECD (He and Garcia, 2004). These results were surprising because they showed that the interaction of a single p75NTR ECD with an NGF dimer resulted in conformational changes in NGF that prevented a second molecule of p75NTR from binding the complex. The conformational change induced in NGF by binding p75NTR does not alter its TrkA binding surface, raising the possibility that NGF bound to p75NTR might simultaneously bind to a TrkA ectodomain. By combining structural solutions for the TrkA-NGF and p75NTR-NGF complexes, Wehrman et al. (2007) were able to model putative p75NTR-NGF-TrkA ternary complexes. They show that p75NTR and TrkA can, at least theoretically, bind to NGF in a 1:2:1 stoichiometry without steric clashes, provided that the distinct receptor ECDs bind to opposite sides of an NGF dimer. Creation of a ternary complex between membrane-tethered p75NTR and TrkA would require that the receptors' ECDs are arranged to create an overlapping NGF sandwich in which the receptors are arranged in opposite orientations (Figure 1).

Taken together, one can envision an appealing scenario in which NGF initially binds to a single chain of p75NTR but, because of allosteric changes in the NGF dimer, is prevented from binding a second p75NTR molecule. This would leave a binding surface available for interaction with TrkA, and thus a ternary complex could be formed. But does this actually occur? To address this, Wehrman and colleagues fused α and ω fragments of β-galactosidase to the tail of p75NTR and to the tail of a truncated form of TrkA (to prevent endocytosis) and then asked whether NGF binding to these receptors reconstitutes β-galactosidase enzymatic activity. When TrkA-α and TrkA-ω were coexpressed, NGF treatment caused a marked increase in β-galactosidase enzymatic activity. However, when cells coexpressed p75NTR-α with TrkA-ω, NGF exposure led to a slight decrease in β-galactosidase activity, indicating that a tripartite structure does not form. In cells transfected with p75NTR-α and p75NTR-ω, NGF treatment had no effect. Taken together, these results indicate that TrkA fusions are capable of NGF-induced homodimerization, as expected, but that NGF exposure does not facilitate formation of p75NTR homodimers or p75NTR-TrkA heterodimers.

Garcia's group then went on to perform I^{125}-NGF Scatchard analyses on PC12 cells and on HEK293 cells transfected with p75NTR and trkA. In contrast to previous results from others, high-affinity NGF binding sites were not observed. Experiments in Wehrman et al. (2007) were first performed using correction factors for each Scatchard data point (by subtracting counts present in wells exposed to 1000× excess of unlabelled NGF); however, when the experiments were performed without background correction, high-affinity NGF binding sites were observed, leading Wehrman and colleagues to conclude that high-affinity binding sites resulting from p75NTR and TrkA coexpression may result from systematic errors inherent in Scatchard analyses.

Is Finding Binding Blinding?

We are left with an interesting conundrum. The structural data suggest the possibility of a ternary complex, yet the biochemical data in Wehrman et al. (2007) indicate that a p75NTR:NGF:TrkA ternary complex does not form. How then, does p75NTR enhance TrkA tyrosine phosphorylation in response to NGF, and what is actually happening at the cell surface? An important clue comes from studies that

show that the simple presence of p75NTR is not enough to enhance NGF-induced TrkA activation; the ligand must actually bind p75NTR for enhancement of Trk tyrosine phosphorylation to occur. In fact, blocking the association of NGF with p75NTR actually decreases the amount of NGF that ultimately binds TrkA. Another important element is that NGF associates with p75NTR very rapidly, essentially at the limit of diffusion, whereas the NGF association rate with TrkA is much slower. Finally, although the extracellular domains of p75NTR and TrkA do not associate directly (Wehrman et al., 2007), p75NTR and Trk receptors can be coimmunoprecipitated (e.g., (Bibel et al., 1999) and are almost certainly in close proximity to one another.

Much of the data on p75NTR-TrkA interactions can be explained with a ligand-passing model in which NGF rapidly associates with p75NTR and then is presented to TrkA in a favored conformation that lowers the energy barrier for association with this receptor. We and others have previously provided biochemical findings that were consistent with a ligand-passing model but concluded that available kinetic evidence argued against this type of receptor collaboration (Barker and Shooter, 1994). In Wehrman et al. (2007), cutting-edge structural and modeling approaches indicate that a ligand-passing model is feasible, yet kinetic and cell-based assays did not generate evidence favoring this mechanism. Of course, absence of evidence is not evidence of absence, and so before consigning this hypothesis to the dust-heap, lets turn this problem around and consider findings that favor the notion that ligand-passing is required for functional interactions between p75NTR and Trk receptors.

If It Walks Like a Duck and Talks Like a Duck...

Lets assume that NGF initially becomes bound to p75NTR and is presented to TrkA in a conformation that lowers its TrkA association rate. For this to be true, a structure that can accommodate this model must be obtained (it has), blocking NGF binding to p75NTR should attenuate NGF binding to TrkA and NGF-induced TrkA activation (it does), and the propensity of p75NTR to enhance or attenuate TrkA activation by specific neurotrophins should be related to their p75NTR association and dissociation kinetics (they are). Regarding this last point, Dechant et al. (1997) have shown that the dissociation rate of NT3 with p75NTR is considerably slower than that for NGF; from the perspective of a ligand-passing model, decreasing the dissociation rate of neurotrophin from p75NTR would actually inhibit Trk activation, precisely what is observed with regard to NT3-induced TrkA activation. Finally, it would be nice if kinetic analyses indicated that the association rate of NGF with TrkA was enhanced in the presence of p75NTR (it has, in Mahadeo et al., 1994).

When presented in this light, the main unresolved question concerning functional interactions between p75NTR-Trk is not whether the ligand-passing model reflects reality, but rather why it cannot be detected kinetically. Here, the kinetic properties that provide a solution to the biological problem may make life miserable for the experimentalist. There are two inherent problems. First, a ternary complex involved in ligand passing has to be transient to meet its functional goal. Second, the passing receptor must be one that readily dissociates its ligand, in order to mediate transfer to the recipient receptor. The fact that NGF dissociates from p75NTR very rapidly, even at 4°C, raises a problem in I^{125}-NGF binding experiments because a substantial amount of I^{125}-NGF dissociates from p75NTR during the process of separating free I^{125}-NGF from bound material (typically by rapid cooling and centrifugation). It is certainly possible that the low numbers of high-affinity I^{125}-NGF binding sites identified by others do actually represent a ternary complex, but the very nature of this receptor system suggests that it will remain difficult to definitively resolve this experimentally using standard binding assays.

The real issue raised by Wehrman et al. (2007) is whether neurotrophins actually form ternary complexes with the two receptors. A prediction of the ligand-passing model is that a p75NTR-NT3-TrkA ternary complex may be more stable, and therefore easier to identify, than one consisting of p75NTR-NGF-TrkA. It is noteworthy that p75NTR is required to produce the abundant high-affinity NT3 binding sites that are present on developing sympathetic neurons (Dechant et al., 1997). In this regard, the β-galactosidase complementation assay designed by Wehrman et al. (2007) will be a useful addition to the experimental toolbox—although this system may lack the temporal sensitivity to demonstrate a p75NTR-NGF-TrkA complex in real time, it will be very useful in other formats. For example, it would be interesting to determine how NGF- or NT3-induced TrkA-α and TrkA-ω complementation is altered by p75NTR overexpression.

What other mechanisms could account for the complex crosstalk between p75NTR and the Trks? One possibility is that signaling mechanisms activated independently by these receptors may converge to activate survival or differentiation pathways. An interesting example of this used a chimeric receptor containing the extracellular domain of the epidermal growth factor receptor coupled to the TrkA transmembrane and intracellular domain (ET-R). When expressed in $PC12^{nnr5}$ cells that do not express TrkA, EGF activated the ET-R kinase and induced partial differentiation. Addition of NGF to activate p75NTR greatly enhanced differentiation of these cells through a mechanism that seemed to involve Akt activation (Lachyankar et al., 2003). Other studies have used p75NTR and Trk agonist antibodies or used Trk- and p75NTR-selective ligands to show that p75NTR and TrkA can activate distinct yet converging signaling cascades (e.g., Ivanisevic et al., 2003).

Trk signals are induced at the cell surface but are maintained in signaling endosomes that travel from distal neuronal tips to the cell body. Our understanding of the spatial and temporal regulation of signaling has become increasingly sophisticated, and

it is now clear that signaling events, such as Erk and PI3K activation, are induced from distinct cellular compartments with different latencies. Events that impinge on receptor trafficking and degradation are therefore key junction points for understanding the physiological consequences of receptor activation. TrkA endocytosis and transport has been well studied, and the concept of a signaling endosome that functions as a retrograde platform that supports TrkA survival signaling is established in the field. Ubiquitination of cell-surface receptors has recently emerged as a key regulatory event important for internalization, signaling, and receptor degradation. Recent studies have not only demonstrated that Trk receptors become ubiquitinated but that this is regulated by p75NTR (Geetha et al., 2005; Makkerh et al., 2005). It therefore seems likely that regulated ubiquitination of p75NTR and TrkA will prove to be an important intersection point that will also facilitate cross-regulation between these receptors.

The work of Wehrman et al. (2007) provides key insights into the structural and kinetic issues concerning p75NTR and Trk interactions. With this structural information, improving technical tools, and an increased focus on the cell-biological events that underlie receptor activation and signaling, the future is bright, and the precise mechanisms that regulate the p75NTR-TrkA regulatory network are certain to emerge.

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The *blu* Blur: Mutation of a Vesicular Glutamate Transporter Reduces the Resolution of Zebrafish Vision

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Vesicular transporters mediate the packaging of neurotransmitters into synaptic vesicles and can therefore control the amount of neurotransmitter released into the synaptic cleft. In this issue of *Neuron*, Smear et al. demonstrate that mutation of a vesicular glutamate transporter (Vglut) found in the retinal ganglion cells (RGCs) of zebrafish alters both the synaptic transmission and connectivity between RGCs and their targets, limiting the transfer of visually evoked activity from RGCs and degrading behaviors that depend on high-acuity vision.

Discovered in an anatomical screen for zebrafish retinotectal projection defects, the *blumenkohl* (or *blu*) mutant was characterized by enlarged termination zones and defasciculation of RGC axons in a way reminiscent of the shape of its namesake, the cauliflower (Baier et al., 1996; Neuhauss et al., 1999). Reported in this issue of *Neuron*, Smear et al. (2007) began their investigation by assaying the mutant's vision using the optomotor response, an innate behavior where zebrafish swim in the same direction as a drifting grating stimulus presented at the