On Trk for Retrograde Signaling

Minireview

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Target-derived neurotrophins like nerve growth factor (NGF) mediate biological effects by binding to and activating Trk neurotrophin receptors at nerve terminals. The activated Trk receptors then stimulate local effects at nerve terminals, and retrograde effects at neuronal cell bodies that often reside at considerable distances from the terminals. However, the nature of the retrograde signal has been mysterious. Recent experiments suggest that the major retrograde signal required for survival and gene expression consists of activated Trk itself. Remarkably, signaling by Trk may differ at the terminal versus the neuronal cell body as a consequence of the retrograde transport mechanism, thereby allowing NGF to not only promote growth locally, but to specifically support survival and gene expression retrogradely.

One of the classic concepts in neurobiology is the neurotrophic factor hypothesis, which states that the biology of peripheral neurons is dependent upon limiting concentrations of neurotrophic factors present in their peripheral target tissues. These target-derived growth factors bind to receptors on the nerve terminal and then generate a signal that somehow travels the entire length of the axon to promote survival in the neuronal cell body. The past several years have seen a flurry of research attempting to elucidate this long-distance signaling mechanism. While many questions still remain, this review will discuss recent work with the neurotrophin family of growth factors that has shed significant insight on this fascinating biological question.

Nerve growth factor (NGF) is the prototypic target-derived trophic factor and is best known for its essential role during development of peripheral sensory and sympathetic neurons. During development, target-derived NGF signals retrogradely to the cell body to support neuronal survival, and signals locally at axon terminals in the target tissue to promote sprouting and target innervation. In addition to this developmental role, target-derived NGF also regulates the phenotype of peripheral neurons throughout the life of the animal. In particular, NGF derived from the terminals of mature peripheral neurons retrogradely regulates cell body and dendrite size, gene expression, and neurotransmitter phenotype, and locally ensures appropriate innervation as the size of the animal alters.

It is now clear that the positive local and retrograde effects of NGF are largely mediated via the TrkA tyrosine

kinase receptor (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001), which is localized over the entirety of the neuron's surface. A second neurotrophin receptor, the p75 neurotrophin receptor, also modulates and/or antagonizes TrkA functions in peripheral neurons, and can retrogradely transport neurotrophins itself (Neet and Campenot, 2001). However, we will not focus on the p75 receptor here since it is not essential for retrograde transport of NGF in peripheral neurons (Neet and Campenot, 2001), and its biological role and interactions with Trk have been recently reviewed (Kaplan and Miller, 2000). Instead, we will focus upon one key unsolved question in this area; how does NGF binding to receptors on axon terminals retrogradely signal back to the cell body to regulate the survival and phenotype of peripheral neurons?

Activated Trk as a Retrograde Signal

Much of what we know about how Trk signals neuronal survival and growth comes from studies on mass cultures of neurons where it is impossible to distinguish local versus retrograde signals (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). In recent years, however, a number of groups have utilized compartmentalized cultures of peripheral neurons to attempt to distinguish local versus retrograde signaling mechanisms. These cultures, called Campenot chambers (Campenot, 1994), segregate the distal axons from cell bodies and proximal axons by a distance of approximately 1 mm (Figure 1). It was early work using these chambers that conclusively demonstrated (1) that axons will grow locally only in response to a local source of NGF, and (2) that NGF derived from distal axons alone is sufficient to retrogradely support neuronal survival (reviewed in Campenot, 1994). Molecular insights into how NGF regulates local growth and retrograde responses have come from recent studies using this same system. With regard to local growth, a recent report demonstrates that axonal sprouting in response to local axonal NGF is due to TrkA-mediated activation of two major downstream effector pathways, the MEK1-ERK1/2 and PI3-kinase pathways (Atwal et al., 2000). As for how NGF applied to axons retrogradely regulates responses in the neuronal cell body, work performed in this system has led to a number of conclusions, some predictable and some surprising.

A priori, one can envisage three distinct types of retrograde signals that could derive from NGF applied to axon terminals. First, NGF itself might be retrogradely transported, and bind to an intracellular receptor upon its arrival in the cell body. However, in spite of the fact that NGF, and the other neurotrophins, are retrogradely transported intact to neuronal cell bodies both in vivo and in vitro, this possibility was ruled out by early studies demonstrating that injection of NGF directly into neuronal cell bodies could not support survival (reviewed in Campenot, 1994). Second, NGF might bind and activate TrkA, and then the activated TrkA, either in a complex with NGF or on its own, might provide the retrograde signal. A third possibility is that NGF activates TrkA on nerve terminals, TrkA then activates downstream substrates, and it is these substrates that provide a retrograde signal. These latter two

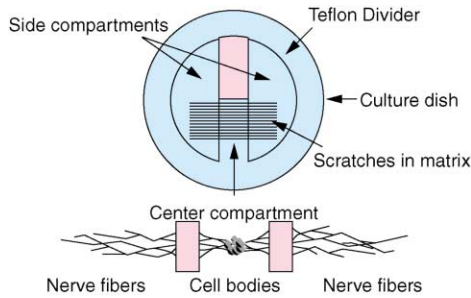[1] Correspondence: freda.miller@mcgill.ca (F.M.), david.kaplan@mcgill.ca (D.K.)

Figure 1. Schematic Representation of a Campenot Chamber

Cell bodies are plated in and restricted to the central chamber while axons grow into side chambers containing neurotrophins such as NGF. Since the fluid environment of the central chamber is isolated from that of the side chambers, this system can be used to study how neurotrophins added to distal axons signal retrogradely to regulate gene expression and survival in cell bodies.

Figure 2. Three Models of Trk Retrograde Signaling Supported by Recent Experimental Studies

possibilities are not mutually exclusive, and there may be multiple retrograde signals.

Do the recent studies in Campenot chambers distinguish these possibilities? All the studies strongly support the notion that a major retrograde signal consists of activated Trk itself. In particular, three different groups have confirmed that activated Trk is rapidly observed in the cell body compartment following neurotrophin application to axons (Senger and Campenot, 1997; Riccio et al., 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999, 2001; see below for discussion of the nature of this activated Trk). A second series of experiments took advantage of the pharmacological Trk inhibitor K252a to inhibit Trk activity in cell bodies when NGF was applied to distal axons, and demonstrated that activated Trk must be transported to the cell bodies to induce a signaling cascade that culminates in CREB phosphorylation and c-*fos* induction (Riccio et al., 1997; Watson et al., 1999).

While it is thus clear that activated Trk is a key retrograde signal, a second question is whether or not this activated Trk is transported bound to its neurotrophin ligand. Crosslinking and coimmunoprecipitation studies indicate that at least some activated Trk is retrogradely transported bound to neurotrophin, even at short time points following receptor activation (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). However, these studies provide no information on the percentage of activated Trk associated with neurotrophin. This quantitative issue becomes important in light of work on sympathetic neurons (Ure and Campenot, 1997; Senger and Campenot, 1997), showing that when NGF is washed out and 125I-NGF is added back to distal axons, virtually no 125I-NGF is retrogradely transported to the cell body within the first hour. Since, in the same experiments, the activated Trk signal peaks by 20 min, this argues that a significant amount of the activated Trk observed in the first hour is not bound to neurotrophin, although it is difficult to estimate the precise proportion due to potential differences in sensitivity of the assays. Arguing against the conclusion that Trk is retrogradely transported without NGF, Riccio et al. (1997) crosslinked NGF to beads and showed that when these beads were applied to distal axons, they were unable to retrogradely induce CREB phosphorylation, presumably because the retrograde signal required internalization of NGF. The
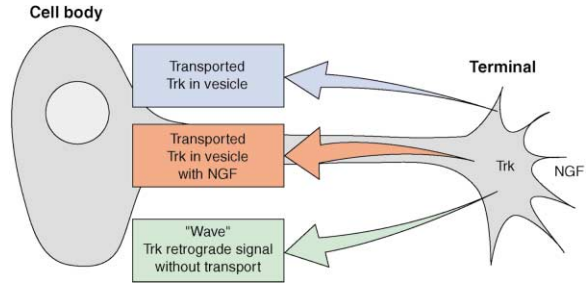
beads were, however, capable of inducing Trk activation in cell bodies of PC12 cells (although activation of Trk on distal neurites was not explicitly tested), suggesting that internalization of ligand was required for propagation of the retrograde signal. Together these studies show clearly that some activated Trk is retrogradely transported bound to neurotrophin, but also leave open the possibility that a biologically active retrograde Trk signal may not require cotransport of the ligand.

Is there precedent for Trk activation in the absence of neurotrophin? In fact, one of the unusual properties of TrkA is its propensity to dimerize and auto-activate when its concentration in the plasma membrane reaches high enough levels. For example, overexpression of TrkA in PC12 cells (Hempstead et al., 1992) led to TrkA activation in the absence of NGF. A second unique property of TrkA is its sustained activity following NGF binding. For comparison, receptor tyrosine kinases such as the EGF receptor are rapidly internalized and "turned-off" following EGF binding. These unique properties thus make TrkA ideally suited to be a retrograde signal carrier. One can imagine a scenario where NGF binds and activates TrkA, the dimerized receptors become clustered on the terminal axonal membrane, and subsequent internalization into a vesicle does not downregulate TrkA, but instead maintains a high local TrkA concentration, resulting in an active retrograde signaling vesicle, even if NGF dissociates during the process (Figure 2). Such vesicle internalization may also sequester TrkA away from phosphatases that normally attenuate receptor tyrosine kinase signaling, thereby fulfilling a dual function.

The Mechanism of Retrograde Signaling

How then does activated Trk traverse the length of the axon to get back to the cell body? Any model(s) must account for at least two temporal components that have been associated with retrograde NGF signaling. Estimates of the rate of the bulk of activated Trk retrograde transport in peripheral neurons varies between 2 and 20 mm/hr, consistent with the estimated speed of vesicular transport, and leading to the first, and prevalent, model that activated Trk is retrogradely transported in a signaling vesicle (Figure 2). While data strongly support this idea (as discussed below), a number of lines of evidence also support the existence of a second, faster but weaker Trk signaling mechanism. In particular, Senger and Campenot (1997) demonstrated that Trk was activated in the cell body compartment of Campenot chambers within 1 min of NGF addition to distal axons. Moreover, Watson et al. (1999) showed that some level of activation of CREB in the cell

body could be observed as early as 5 min after NGF was added to distal axons, with maximal activation occurring at 20 min, suggesting that NGF added to axons can cause an almost immediate activation of CREB in at least some neurons. How can phosphorylated Trk appear in the cell body this rapidly? While various mechanisms have been proposed (Neet and Campenot, 2001), an intriguing possibility is that Trk uses a similar mechanism to that used by the EGF receptor ErbB1. EGF addition to a subpopulation of ErbB1 receptors resulted in a rapid ligand-independent lateral signaling "wave" that activated all receptors at the plasma membrane (Verveer et al., 2000). The wave was propagated when the EGF-activated receptor locally overcame receptor-associated phosphatase activity, causing a spreading of unliganded receptor activity over the cell. NGF addition to distal axons might cause a similar rapid "wave" of Trk phosphorylation across the axonal membrane that could explain the rapid appearance of activated Trk in the cell body compartment 1 mm away (Figure 2).

While the precise nature of the faster, weaker Trk "wave" is still largely speculative, all of the studies agree that the majority of activated Trk from distal axons arrives back at the cell body with a time course consistent with retrograde vesicular transport (Riccio et al., 1997; Watson et al., 1999; Senger and Campenot, 1997). Strong evidence that activated Trk is present in vesicles also comes from studies on signaling endosomes largely performed in PC12 cells, and just recently confirmed in sensory neurons (Howe et al., 2001 [this issue of Neuron] and references therein). These studies show that Trk bound to NGF undergoes clathrin-mediated endocytosis into a "signaling" endosome that also contains substrates of TrkA known to regulate survival, growth, and gene expression (Howe et al., 2001). In support of this model, NGF induced an increase of clathrin at plasma membranes that associated with Trk. Dynamin, required for Trk internalization (Zhang et al., 2000), caused the clathrin-coated vesicles to pinch from the membrane, and then to uncoat into a "transport" vesicle containing Trk oriented with its cytoplasmic tail outside of the vesicle bound to its substrates such as PLC-γ1 and signaling proteins such as Ras, Rap1, Erk1/2, and PI3-kinase (Howe et al., 2001 and references therein). Trk in such vesicles may then subsequently associate directly with the dynein motor machinery by binding to the dynein light chain (Yano et al., 2001), which can initiate retrograde transport. Further support for vesicular retrograde transport of activated Trk comes from in vivo studies showing accumulation of phosphorylated Trk in the sciatic nerve distal to a nerve ligation (Ehlers et al., 1995; Bhattacharyya et al., 1997), and studies in cultured neurons showing that the microtubule-disrupting agent colchicine inhibited transport of activated Trk to cell bodies in Campenot chambers (Watson et al., 1999). However, it should be kept in mind when interpreting these studies that neurotrophins are known to be locally induced in the nerve distal to a nerve injury such as ligation (Neet and Campenot, 2001), and colchicine also efficiently blocked Trk signaling when NGF was applied to the cell bodies themselves (Watson et al., 1999).

Consideration of all of the above studies suggests that the rapid, nonvesicular and the slower, vesicle-associated mechanisms for transport of activated Trk likely both take place. Moreover, as discussed above, vesicles may well transport biologically active Trk both with and without its neurotrophin ligand. These findings therefore lead to a model where NGF binding to Trk on nerve terminals elicits a rapid, but weak, nonvesicle-mediated Trk signal to the cell body, followed by a more robust Trk vesicle-associated signal (Figure 2). One of the challenges for the future will be to determine the biological relevance of each of these components of the retrograde signaling mechanism.

Regulation of Signaling by Retrograde Transport

Are the signals mediated by activated Trk the same in the local axonal environment as they are distally in cell bodies? Recent evidence suggests that the retrograde transport machinery may in fact regulate the selectivity of Trk signaling. A dramatic example of spatial segregation of neurotrophin-mediated signal transduction was recently reported by Watson et al. (2001), who showed that neurotrophin addition to distal axons of sensory neurons promoted local axonal stimulation of Erk1 and 2 activity, previously shown to be required for local axonal growth (Atwal et al., 2000). However, the same axonal stimulus did not activate Erk1 or 2 in the cell body compartment, but instead led to cell body activation of another MAP kinase Erk5. This retrograde stimulation of Erk5 activity was responsible for cell body CREB phosphorylation and for a portion of neurotrophin-regulated retrograde survival responses (Figure 3). Thus, the signaling substrates "seen" and activated by Trk differed as a function of local versus retrograde signaling.

Why would the signals transduced by activated Trk differ locally versus retrogradely? One possibility is that endocytosis and retrograde transport direct Trk signaling complexes to specific targets in the cell body, and thereby act to stimulate or attenuate specific aspects of Trk signal transduction. In this regard, evidence suggests that endocytosis into transport vesicles is a necessary step in receptor-mediated activation of Erk1/2 (Howe et al., 2001 and references therein), and may provide a means to attenuate PLC-γ1 signaling by sequestering this enzyme from its lipid substrates (Matsuda et al., 2001). Moreover, the recent demonstration that the EGF receptor can translocate to the nucleus and function as a transcription factor in nonneuronal cells (Lin et al., 2001) suggests that transported Trks could even conceivably regulate gene transcription on their own if directed to the nuclear compartment.

Is there any evidence for similar spatial regulation of other Trk signaling effectors? In contrast to the Erks, PI3-kinase is apparently activated both locally in distal axons and retrogradely in sympathetic neuron cell bodies in response to NGF on axons (Kuruvilla et al., 2000). This activation of PI3-kinase in sympathetic neuron cell bodies is essential for survival in response to NGF applied either locally on cell bodies (Kaplan and Miller, 2000) or distally on axons (Kuruvilla et al., 2000). PI3-kinase also plays a number of important roles in the axonal Trk signaling response, including regulation of the initiation of NGF retrograde transport (Kuruvilla et al., 2000 and references therein), and of local axonal growth (Atwal et al., 2000). It would, however, be interesting to determine whether the targets of PI3-kinase differ as a consequence of whether TrkA is activated on cell bodies or whether it is transported retrogradely from axons.

What is the biological relevance of such spatially segregated signaling? One interesting possibility is that it might provide a means to selectively direct Trk to the subset of
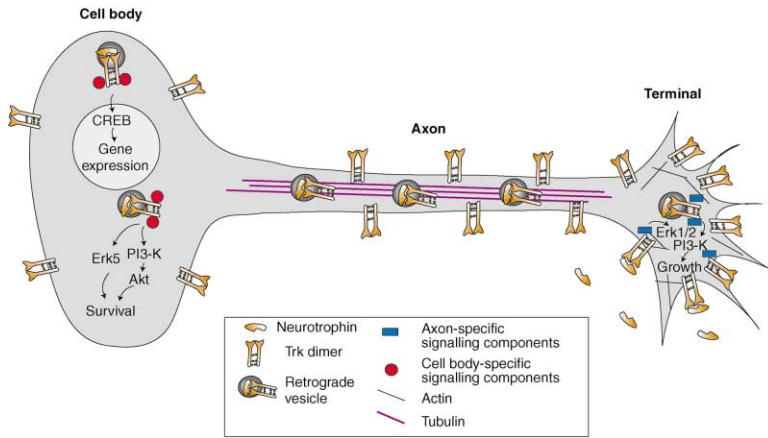
Figure 3. Activation of Trk on Nerve Terminals Signals Locally via One Set of Signaling Pathways to Promote Axonal Growth, and Retrogradely via a Second Set of Signaling Pathways to Promote Cell Body Gene Expression and Survival

substrates specifically involved in growth versus survival, thereby maintaining a highly localized growth response at axon terminals while at the same time mediating an essential retrograde survival and gene expression signal (Figure 3). A second possibility is suggested by a study showing that the magnitude of NGF-induced increases in neuronal gene expression were significantly lower when the same amount of NGF was applied to distal axons versus cell bodies (Toma et al., 1997), indicating that at least some Trk signals are attenuated, potentially by retrograde transport. Since sympathetic neuron phenotype is determined by multiple influences, including NGF and neuronal activity, then such selective attenuation of neurotrophin signaling from axon terminals would allow them to still respond to these other signals even in the presence of saturating concentrations of NGF at their terminals.

The past few years have seen significant advances in our understanding of how target-derived NGF can bind to receptors on nerve terminals, and then generate a signal that traverses the entire axon to regulate neuronal survival, phenotype, and gene expression. This important biological event appears to involve, perhaps not surprisingly, multiple ways of getting activated Trk receptors back to the cell body, including a fast, but relatively weak, "wave" of Trk signaling followed by a robust and sustained retrograde transport of signaling vesicles that contain activated Trk and at least some NGF and downstream Trk substrates. Remarkably, but again perhaps not surprisingly, the retrograde transport mechanism also regulates the selectivity of Trk signaling, with the signaling substrates "seen" and activated by Trk differing as a function of local versus retrograde signaling. Such spatially segregated signaling in response to target-derived NGF would provide a cellular mechanism for maintaining a highly localized growth response at axon terminals while at the same time mediating essential survival and gene expression signals back at the cell body. However, in spite of this progress, many important questions remain to be answered: what are the signals in different cellular compartments that mediate the various responses of neurons to neurotrophin? What is the biological role of the fast wave of retrograde signaling? How does the retrograde signaling system decide whether Trk signals locally or to the cell body? How do retrogradely transported vesicles communicate with signaling proteins once they reach the cell body? Elucidating the answers to these questions will help unravel one of the fundamental mysteries of neurobiology.

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