FUNCTIONS AND MECHANISMS OF RETROGRADE NEUROTROPHIN SIGNALLING

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Abstract | Neuronal connections are established and refined through a series of developmental programs that involve axon and dendrite specification, process growth, target innervation, cell death and synaptogenesis. Many of these developmental events are regulated by target-derived neurotrophins and their receptors, which signal retrogradely over long distances from distal-most axons to neuronal cell bodies. Recent work has established many of the cellular and molecular events that underlie retrograde signalling and the importance of these events for both development and maintenance of proper neural connectivity.

PRONEUROTROPHINS
Uncleaved forms of the neurotrophins that bind with high affinity to $p75^{NTR}$.

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The correct establishment of neuronal connections in the central and peripheral nervous systems during development is essential for the proper function of the nervous system. Such connections are generated through a developmental program that involves axon and dendrite specification, process growth, target innervation, cell death, synaptogenesis and synaptic refinement. Many target-derived factors are crucial for this developmental program. Of these instructive cues, the neurotrophins are perhaps the best described. However, although there is considerable evidence for their roles in development, the precise molecular mechanisms that underlie retrograde neurotrophin signal transduction from the tip of the axon to the soma remain unresolved.

The neurotrophins are a family of low-molecular-weight proteins that includes the prototypical neurotrophin nerve growth factor (NGF) as well as brain-derived neurotrophic factor (BDNF) and neurotrophins 3 and 4/5 (NT3 and NT4/5). Each neurotrophin binds with high affinity to receptor tyrosine kinases known as Trk receptors; NGF binds to TrkA, BDNF and NT4/5 to TrkB, and NT3 to TrkC[1,2]. NT3 also binds TrkA and TrkB. As in other families of receptor tyrosine kinases, ligand binding induces dimerization of Trk receptors and their autophosphorylation at specific tyrosine residues in the cytoplasmic domain. This, in turn, leads to the recruitment of various downstream effectors and the activation of numerous signal transduction cascades that support growth and survival[3]. Both mature neurotrophins and PRONEUROTROPHINS also bind to a structurally distinct neurotrophin receptor, p75 ($p75^{NTR}$), which also mediates neuronal development through local and retrograde signalling[4].

Early observations that neurotrophins are retrogradely transported from the target towards the cell body have led to decades of inquiry regarding not only the underlying mechanism of this developmental phenomenon but also the physiological significance of retrograde growth factor signalling. In this review, we summarize the functions of retrograde neurotrophin signalling during development and analyse the mechanisms through which this signalling occurs.

Functions of retrograde signalling

Regulation of neuronal survival. The importance of target fields for the survival of developing neurons was first disseminated through a series of elegant and classical experiments in the chick. Ablation of the chick wing bud was found to result in hypoplasia and death of brachial spinal motor neurons. Conversely, transplantation of supernumery wing buds results in increased numbers of neurons[5–7]. It is now known that the establishment of the proper complement of neurons for a given target field is governed by a developmental

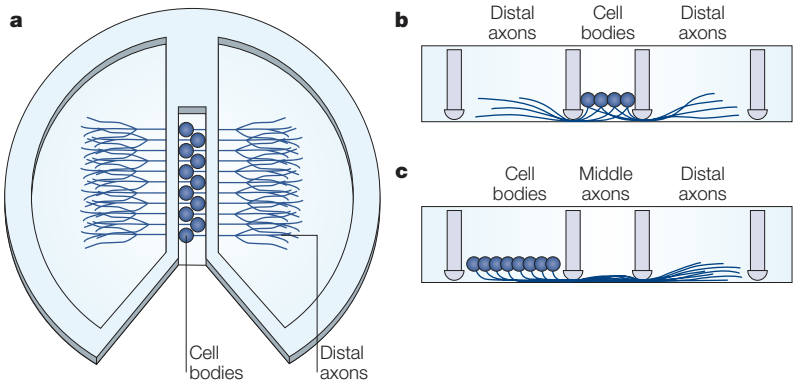

Figure 1 | **Compartmentalized neuronal cell culture system.** This *in vitro* model system is used to study the retrograde transport of neurotrophins and their receptors, as well as downstream events in retrograde neurotrophin signalling. Compartmented cultures of sympathetic and sensory neurons are established by placing Teflon dividers onto a thin layer of vacuum grease, which allows diffusion-limited compartmentalization of neuronal cell bodies and distal axons[11,108]. **a** | Schematic top view of three-compartment culture chamber (Campenot chamber). **b** | Side view of compartmentalized culture depicting cell body and distal axon compartments. **c** | Side view of three-compartment chamber system used to study spatial requirements of tyrosine receptor kinase (Trk) activity, and downstream pathways such as phosphatidylinositol 3-kinase (PI3K)–Akt (v-akt murine thymoma viral oncogene homologue, also known as protein kinase B) signalling. Unlike conventional three-compartment chambers, cells are plated in one of the two side compartments, which allows axonal projections to be divided into a middle axon compartment and a distal axon compartment. Development of the compartmentalized cell culture system has revolutionized the study of retrograde neurotrophin signalling.

sequence in which neurons are overproduced and then compete for target-derived survival factors[8]. The control of this developmental process is best understood for PNS neurons. Several target-derived trophic factors, typified by NGF and other neurotrophins, have been identified and found to be expressed in many peripheral and central neuronal targets[9]. Antigenic neutralization and genetic ablation of these secreted factors and their cognate receptors, as well as intraperitoneal injection and transgenic overexpression of neurotrophins, have revealed that these target-derived signals are essential for the growth and survival of select populations of developing PNS neurons[7,9].

How do target-derived signals, such as the neurotrophins, influence neuronal survival? Disruption of neuron–target interactions through axonal transection has shown that axonal transport of neurotrophic factors, or their downstream effectors, is necessary for their function[10]. Further insight into the nature of retrograde neurotrophin signalling has been gained through the use of compartmentalized cell culture systems (FIG. 1), which allow neuronal cell bodies or distal axons to be stimulated separately. In this system, sympathetic neurons survive even when NGF is applied exclusively to their distal axons[11]. These *in vivo* and *in vitro* findings support the idea that limiting amounts of target-derived neurotrophic factors signal retrogradely to support neuronal survival, thereby sculpting connectivity.

Much of what we know about the neurotrophin signalling events that promote neuronal survival has been gleaned from *in vitro* studies of trophic factor deprivation. When neurotrophic factors are withdrawn, neurons follow a stereotyped temporal progression of apoptotic signalling events, which include the translocation of BAX to mitochondria, the release of cytochrome *c*, activation of the APOPTOSOME and caspase-mediated cell death[12–15]. Retrograde neurotrophin signalling impinges on these apoptotic pathways (FIG. 2a), in part by regulating transcriptional events, such as those mediated by the transcription factor cyclic AMP responsive element-binding protein (CREB)[14,16–18]. In addition, several signalling cascades, such as the phosphatidylinositol 3-kinase (PI3K)–Akt (v-akt murine thymoma viral oncogene homologue, also known as protein kinase B) and Ras–Raf–MEK (MAPK (mitogen activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase)–ERK pathways, mediate retrograde neurotrophin-dependent survival[19–21]. However, much remains to be learned about how pro-survival and pro-apoptotic pathways intersect in developing neurons. Which downstream targets of PI3K and MITOGEN ACTIVATED PROTEIN KINASE SIGNALLING are essential for neurotrophin-dependent survival? Which transcriptional targets downstream of retrograde neurotrophin signals contribute to survival? To address these questions, it will be helpful to merge recent advances in our understanding of apoptosis and mitochondrial physiology with improved spatial and temporal resolution of neurotrophin signalling pathways in compartmentalized cultures. *In vivo* analysis of transcriptional targets of retrograde neurotrophin signalling, using, for example, *Ngf/Bax* and *Nt3/Bax* double mutants, in which deletion of the pro-apoptotic B-cell leukaemia/lymphoma 2 (*Bcl2*) family member *Bax* prevents neuronal apoptosis in the absence of neurotrophins[22–25], will also prove beneficial. These types of investigation promise a better understanding of how and where apoptotic machinery and pro-survival signalling intersect to regulate retrograde neuronal survival.

Regulation of axonal growth. As developing axons traverse long distances towards their final targets, they respond to growth and guidance cues that are derived from both intermediate and final target fields. In compartmentalized sympathetic neuronal cultures, the direct application of NGF to distal axons, but not to cell bodies, supports the extension of these axons. Importantly, if NGF is removed from the distal axons but left in the medium bathing the cell bodies, the distal axons retract and degenerate, but the neurons survive[11]. Therefore, neurotrophin signalling initiated at distal axons is both necessary and sufficient to support distal axon extension, whereas NGF signalling in cell bodies alone cannot support distal axon extension.

Retrograde signalling from distal axons to the nucleus also contributes to axonal extension. Downstream of neurotrophin-receptor activation, several pathways signal to transcription factors, including CREB[26], that contribute not only to survival but also to axon growth and target innervation[16]. Interestingly, other transcription factors, such as nuclear factor of activated T cells (NFAT), seem to be dedicated exclusively to axon growth[27].

BAX
Pro-apoptotic BCL2 family member. BAX translocation from the cytosol to the mitochondria facilitates cytochrome *c* release.

APOPTOSOME
Heteromeric protein complex containing cytochrome *c*, APAF-1 and procaspase-9. Triggers a cascade of caspase activation and proteolysis.

Ras, Rap
Small GTPases that are involved in growth, differentiation and cellular signalling. They require the binding of GTP to enter into their active state.

MITOGEN-ACTIVATED PROTEIN KINASE SIGNALLING
A signalling cascade that relays signals from the plasma membrane to the nucleus. Mitogen-activated protein kinases (MAPKs), which represent the last step in the pathway, are activated by a wide range of proliferation- or differentiation-inducing signals. ERKs are among the best-characterized MAPKs.

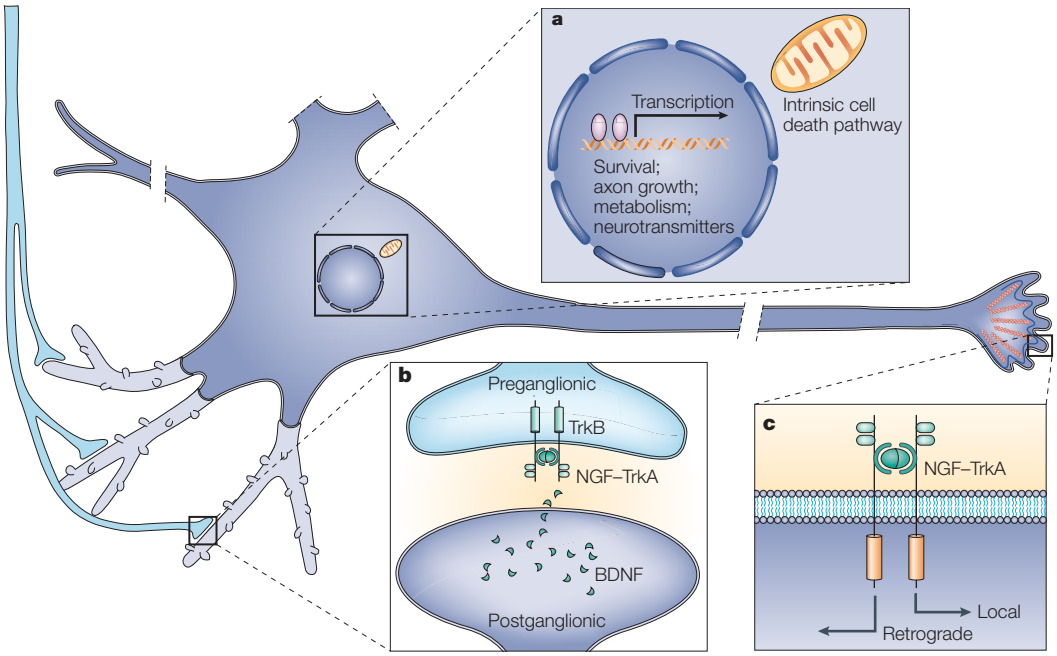

Figure 2 | **Retrograde neurotrophin signalling instructs various developmental programs. a** | Neurotrophin signalling events initiated in developing axons signal retrogradely to neuronal cell bodies to inhibit intrinsic cell death pathways. These signalling events also influence transcriptional programs that are involved in cell survival, axon growth, synaptogenesis, metabolism and the establishment of neurotransmitter and neuropeptide phenotypes. **b** | The establishment of pre- and postganglionic synaptic contacts in sympathetic neurons is influenced by retrograde nerve growth factor (NGF)–TrkA signalling. A potential synaptogenic signal downstream of retrograde NGF–TrkA signalling is the neurotrophin brain-derived neurotrophic factor (BDNF). BDNF regulates the formation and maintenance of presynaptic contacts by signalling trans-synaptically to TrkB receptors on preganglionic sympathetic neurons. **c** | Neurotrophin-dependent axon growth is supported by both local and retrograde signalling through the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signalling pathways. Retrograde neurotrophin signalling also regulates axon growth and target innervation through the activation of transcriptional programs.

In addition to regulating resident factors, such as CREB and NFAT, retrograde signalling also controls *de novo* expression of transcription factors that are required for axon growth. For example, retrograde NT3–TrkC signalling promotes the expression of the ETS transcription factor ER81 in proprioceptive dorsal root ganglion (DRG) neurons[25]. Genetic ablation of either NT3 or ER81 results in the formation of defective proprioceptive afferents that terminate abnormally in the intermediate region of the spinal cord rather than establishing proper connections with motor neurons in the ventral cord[25]. Similarly, in a subset of brachial motor neurons, target-derived GDNF (glial cell line-derived neurotrophic factor) induces the expression of another ETS transcription factor, PEA3, and the absence of either GDNF or PEA3 leads to failure of these neurons to innervate target muscles[28]. These findings indicate that target-derived neurotrophic factors influence axonal growth at several levels, from local signalling in axons to the retrograde modulation of transcription factors such as CREB and NFAT, and the regulated expression of transcription factors. An important challenge is the identification and characterization of the transcriptional targets of CREB, NFAT, ER81, PEA3 and other transcription factors that work with local neurotrophin signals to support axonal growth.

Regulation of dendrite growth. For most neurons, the elaboration of dendritic arbors is a prerequisite for the establishment of functional connections with presynaptic partners. In most cases, dendrites begin to extend before axons have innervated their target fields, but a number of studies indicate that target-derived factors control the growth and maintenance of dendrites in the PNS. In sympathetic neurons, for example, the size of the target field influences the size and complexity of dendritic arbors[29,30], and neurons that project to different targets adopt distinct dendritic morphologies[30], perhaps hinting that target-specific cues specify dendrite growth and synaptic differentiation.

Target-derived NGF is necessary for sympathetic dendrite growth *in vivo* in both neonatal and adult mice, and post-ganglionic axotomy results in retraction of post-ganglionic dendrites[31–33]. However, *in vitro* studies indicate that, alone, NGF is insufficient to support dendrite growth[34], which suggests either that other target-derived signals, such as bone morphogenic proteins (BMPs), are required, or that signals derived from pre-ganglionic partners act cooperatively with target-derived NGF to support this developmental process (FIG. 2b). Consistent with the latter hypothesis, axotomy or disruption of axonal transport in postganglionic sympathetic neurons leads to the withdrawal of

presynaptic contacts[35–37]. Conversely, the regeneration of postganglionic axons in the periphery, mediated in part by an NGF-dependent mechanism, restores these contacts[38].

These findings indicate that the effects of target-derived NGF on pre- and postsynaptic growth might be mediated by retrograde induction of a 'synaptogenic signal'. A potential downstream target of NGF and candidate synaptogenic signal is BDNF, which is synthesized in postganglionic sympathetic neurons[39,40]. In addition, BDNF's cognate receptor, TrkB, is expressed in preganglionic neurons[41,42]. Moreover, overexpression of BDNF in sympathetic neurons results in an increase in the density of preganglionic synapses in the superior cervical ganglion (SCG), whereas deletion of BDNF results in a decrease in both synapse density and the number of preganglionic axons[39]. Intriguingly, TrkB and NT4/5, but not BDNF, are required for the survival of preganglionic sympathetic neurons[41,43], which indicates that local and/or retrograde BDNF signalling alone might modulate synapse number. Whether the synthesis of BDNF in postganglionic sympathetic neurons is regulated by retrograde NGF signalling is not known. Nevertheless, these findings point towards an interesting model in which a retrograde neurotrophin signalling cascade, acting across two neuronal populations, sculpts neuronal connectivity.

Regulation of neuronal specification. Retrograde signalling by target-derived neurotrophins influences not only early developmental events in peripheral neurons, but also later steps in the differentiation of neurons, such as the acquisition of signature neurotransmitter phenotypes. Examples of the influence of neurotrophins on neuronal specification have been provided by studies in which NGF was injected into neonatal and adult rats, and from genetic models. NGF increases the expression of genes that establish a neurotransmitter phenotype in sympathetic neurons[44,45]. In BAX$^{-/-}$;TrkA$^{-/-}$ double-mutant mice, the absence of NGF–TrkA signalling in nociceptive neurons prevents the expression of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (REF. 24). In addition, an unidentified retrograde signal controls the ability of a subset of sympathetic neurons that innervate rat sweat glands to undergo a switch from a noradrenergic to a cholinergic phenotype during development[46]. A number of target-derived factors, including ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), NT3 and GDNF have been proposed as potential candidates for this cholinergic differentiation factor[46]. Therefore, retrograde signals from the periphery might act with cell-intrinsic programs to control the neurotransmitter and peptidergic phenotype of neurons, which thereby defines neuronal identity.

Mechanisms of retrograde signalling

Early observations that neurotrophins and their receptors are internalized and retrogradely transported from distal axons to neuronal cell bodies indicated that an active ligand–receptor signalling complex might transmit target-derived neurotrophin signals to the soma to mediate target-dependent survival, growth and gene expression[47–51]. Biochemical and ultrastructural analyses of these retrograde neurotrophin signalling complexes indicate that they form a unique organelle, the signalling endosome. Although the exact nature of the signalling endosome remains unresolved, there is evidence that it is a specialized constituent of the endocytic pathway. Substantial evidence from several laboratories supports the 'signalling endosome model', but there might also be alternative modes of retrograde signalling. These include retrograde propagation of signalling effectors; retrograde waves of Trk receptor activation along the plasma membrane; and retrograde calcium waves emanating from activated Trk receptors[52]. However, whether these distinct modes of retrograde signalling amalgamate to support growth and survival, or the extent to which these mechanisms contribute to the target-dependence of neuronal development, is not clear.

Is the propagation of internalized ligand–receptor signalling complexes necessary or sufficient for the growth and survival of developing neurons? As mentioned previously, studies using compartmentalized neuronal cultures have shown that the addition of neurotrophins exclusively to distal axons can support survival[11,20,21,53]. In addition, inhibition of Trk kinase activity in either the distal axons or the cell body and proximal axons attenuates or eliminates retrograde accumulation of tyrosine kinase signalling events[54], signalling to the transcription factor CREB[14,18], and cell survival[53,55]. Disruption of neurotrophin endocytosis[53,55–57] and pharmacological or molecular disruption of dynein-dependent microtubule transport *in vitro*[55] or *in vivo*[58] also lead to neuronal apoptosis. These findings, taken together, provide compelling support for the idea that ligand–receptor complexes are essential carriers of retrograde NGF signalling. However, although several reports indicate that sustained retrograde TrkA activity in cell bodies is required to mediate neurotrophin-dependent cell survival signalling[20,53,55], others report that TrkA activity in cell bodies is not required for retrograde survival[59]. These disparate findings underscore the importance of determining the catalytic requirements of Trk receptors using more specific and stable inhibitors of Trk kinase activity and *in vivo* paradigms.

Some studies also indicate that ligand internalization might not be crucial for retrograde neurotrophin–receptor signalling. These alternative assertions are based on experiments in which neurotrophins are covalently coupled to large beads, which allow Trk receptors to be activated without the ligand being internalized. In these experiments, bead-mediated immobilization of neurotrophin greatly reduces, but does not completely abrogate, the retrograde accumulation of activated Trk receptors[55], and does not prevent the survival of compartmentalized neurons[55,60]. Paradoxically, the presentation of ligand in

this manner greatly attenuates the rapid retrograde activation of CREB[17], which contributes to neuronal survival both *in vitro* and *in vivo*[14,16]. This reported support of survival by non-internalized neurotrophin could be explained if small quantities of soluble ligand are liberated from bead complexes, which are notoriously 'leaky'. It has been argued that leakage might be insufficient to support survival, because the effective concentration of free neurotrophin that is required for survival is higher than the detected concentration of leaked neurotrophin in the medium from neurons treated with neurotrophin-bead complexes[60]. Nonetheless, if we consider the high local concentration of ligand coupled to beads then it would seem possible to achieve maximal receptor activation locally, and this might allow small quantities of free 'leaked' ligand to be internalized with the receptors and to form competent signalling complexes.

It is reasonable to speculate that both ligand-dependent and ligand-independent modes of receptor transport exist and work cooperatively to support neuronal survival. Consistent with this idea, peptide-mediated delivery of NGF-neutralizing antibodies into cell bodies attenuates, but does not eliminate, retrograde survival[53]. In addition, there is a marked increase in the rate of retrograde accumulation of Trk in cell bodies when soluble ligand is applied rather than bead-conjugated ligand[55], which indicates that although Trk receptors and downstream effectors might be transported in the absence of ligand, both the rate and magnitude of transport are augmented by the presence of internalized ligand. It is therefore likely that the primary mode of retrograde signalling occurs through the transport of a ligand–receptor complex; however, ligand-independent transport of Trk receptors might contribute to some portion of retrograde survival signalling.

Given the physiological significance of retrograde neurotrophin signalling, it is important to determine the mode of retrograde ligand–receptor transport. Biochemical and ultrastructural analyses indicate that internalized ligand–receptor complexes are localized to small membrane-bound organelles and are retrogradely transported in a dynein-dependent manner along a microtubule network towards neuronal cell bodies, where their signals are disseminated. In sensory neurons, these NGF–TrkA-containing organelles have characteristics of early endosomes and are associated with signalling components of the MAPK, PI3K and phospholipase C-γ (PLCγ) signalling pathways, which provides further support for the idea of a specialized signalling vesicle, or signalling endosome[61,62].

Although there is considerable evidence from many laboratories for the signalling endosome mechanism, there might also be alternative modes of retrograde signalling. One conceivable alternative involves the retrograde propagation of downstream signalling effectors. When Trk receptors are activated by their ligand, specific effectors are recruited that link active receptors to downstream signalling pathways, such as the Ras or Rap1–Raf–MEK–ERK and PI3K–Akt pathways, as well as directly to PLCγ[3]. Activation of these signalling pathways is linked to receptor internalization, local control of axon growth and signalling within cell bodies to support survival and cellular metabolism. Furthermore, many of these signalling proteins undergo retrograde axonal transport themselves[61,63]. Interestingly, blockade of neurotrophin receptor internalization and neutralization of neurotrophins at intracellular locations prevents the retrograde accumulation of many of these signalling effectors, as well as the survival of compartmentalized cultures[53,55]. These and other findings indicate that activation of effector proteins alone in distal axons is not sufficient for retrograde survival signalling.

Kinase signalling cascades might not be the only pathways that can mediate retrograde signalling. For example, the transcription factor ATF2 is localized to axons of nociceptive DRG neurons, and neutralization of NGF results in accumulation of phosphorylated ATF2 distal to a rat sciatic nerve ligature[64]. Given that neurotrophin neutralization results in retrograde transcription factor activation, it seems plausible that other transcription factors are transported retrogradely in response to active neurotrophin signalling. Consistent with this idea, the activation of calcineurin in response to neurotrophin stimulation leads to the nuclear translocation of the transcription factor NFAT, which mediates axon growth[27]. In this case, however, it is unclear whether the signal is propagated by Trk-containing endosomes, calcineurin or NFAT itself.

So, although target-dependent neuronal survival can be mediated by the retrograde transport of actively-signalling ligand–receptor complexes, other modes of signalling might also contribute to a lesser extent. Moreover, signalling endosomes might not be sufficient to support all neurotrophin-dependent processes. Therefore, it will be important to establish the contribution of alternate retrograde neurotrophin signalling mechanisms to developmental processes, including axon growth, acquisition of neurotransmitter phenotype, dendrite growth and presynaptic differentiation, as well as their mode of propagation.

The signalling endosome

Retrograde endosomal signalling in neurons is a multi-step process that includes the internalization of ligand–receptor complexes in axon terminals, the sorting of complexes into active signalling vesicles, physical translocation of these endosomes along the axonal microtubule network to cell bodies, endosomal signalling and the dismantling of the retrograde endosomal signalling complex. The molecular machinery that underlies these events is largely unknown. It is also not clear whether the retrograde transport of neurotrophins and their receptors in neurons differs in specific respects from the endocytosis and trafficking of other receptor tyrosine kinases in non-polarized cells.

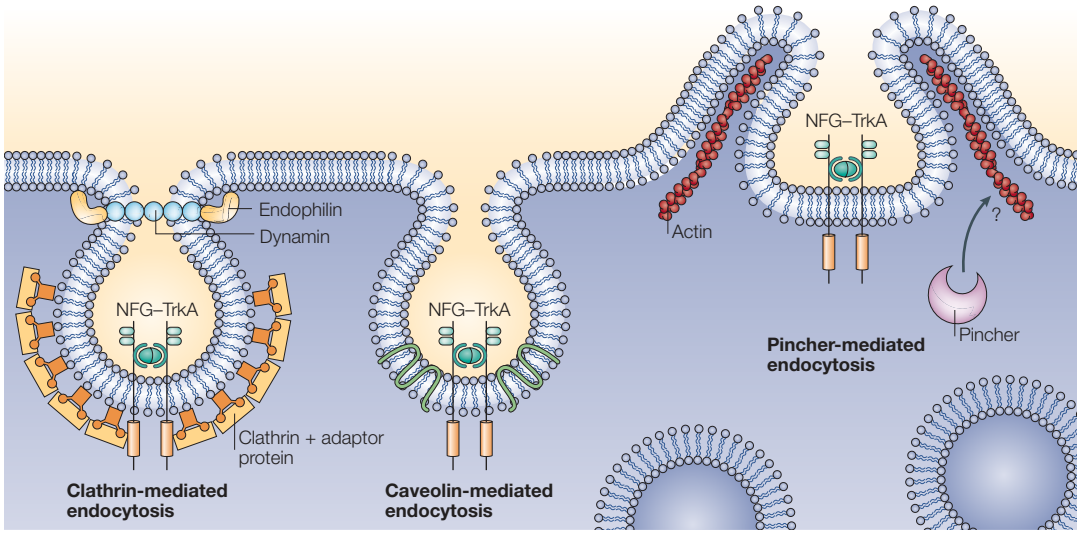

Figure 3 | **Neurotrophins and their receptors use various modes of internalization.** The archetypal neurotrophin–receptor complex, nerve growth factor (NGF)–TrkA, uses three distinct internalization pathways. Although there is considerable evidence that NGF–TrkA complexes are internalized through a clathrin/dynamin-dependent process, TrkA has been localized to caveolin-like domains in PC12 cell lines, and disruption of Pincher-mediated pinocytosis prevents intracellular accumulation of NGF–TrkA complexes in both PC12 cell lines and primary sympathetic cultures. Whether Trk activity is required for internalization and how Trk receptor signalling modulates these pathways remain unresolved.

NGF–TrkA internalization. The main endocytic routes for membrane receptors include at least four mechanistically diverse and highly regulated pathways: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis. The best studied endocytic pathway is clathrin-mediated endocytosis, and most of the information on trafficking of receptor tyrosine kinases has emerged from the study of the archetypal receptor tyrosine kinase epidermal growth factor (EGF) receptor (EGFR). On binding EGF, EGFRs undergo dimerization, recruitment to clathrin-coated pits, invagination and fission of clathrin-coated vesicles into the cytosol, followed by their uncoating to form endosomes[65].

At present, there is some debate as to which endocytic route is the dominant mechanism for the uptake of neurotrophins and Trk receptors, and there is evidence to support both clathrin-dependent and -independent mechanisms (FIG. 3). NGF increases the association of clathrin with membranes in both PC12 cells and DRG neurons, and induces the formation of complexes that contain activated TrkA, the clathrin heavy chain and the clathrin adaptor protein AP2 (REFS 66,67). In addition, clathrin-coated vesicles isolated from NGF-treated PC12 cells contain not only NGF–TrkA complexes, but also activated signalling effectors of the MAPK pathway[67]. Trk receptor internalization also depends on dynamin, a GTPase that is involved in the scission of clathrin-coated vesicles from the plasma membrane, which indicates that a clathrin-mediated mechanism might be the preferred mode of Trk endocytosis[53,57]. However, dynamin also participates in caveolin-dependent internalization[68] and other membrane scission events, so dynamin inhibition alone cannot implicate the involvement of clathrin-coated pits.

Alternate modes of internalization, including clathrin- and caveolin-independent mechanisms, also contribute to Trk receptor trafficking. The recent identification of Pincher as a membrane trafficking protein that mediates endocytosis and the trafficking of NGF–TrkA complexes by clathrin-independent macropinocytosis illustrates the diverse and complex nature of neurotrophin-receptor internalization[69]. Overexpression of Pincher in PC12 cells and sympathetic neurons enhances the ligand-dependent endocytosis of TrkA at plasma membrane ruffles where pinocytic structures are formed[56,69]. Interestingly, Pincher is not localized to clathrin-coated invaginations, and expression of a dominant-negative form of Pincher blocks TrkA internalization without affecting clathrin-mediated endocytosis of the transferrin receptor[69].

The multiplicity of pathways by which receptors can be internalized indicates that the mechanism of ligand–receptor internalization might depend on the type of receptor, the cellular context in which the ligand is presented, and/or the magnitude of the signal generated by ligand stimulation. Indeed, ligand concentrations can profoundly affect the endocytic fate of the EGFR: low EGF concentrations favour clathrin-dependent endocytosis, whereas high — but still physiologically relevant — concentrations of EGF promote primarily caveolin-dependent internalization[70,71]. One exciting possibility is that during neuronal development axons that project into target fields encounter a concentration gradient of a particular neurotrophin, which could establish different routes of Trk receptor internalization, and form distinct signalling complexes.

Although *in vitro* analyses support the existence of different modes of neurotrophin receptor endocytosis, studies that use overexpressed receptors might reveal mechanisms that are fundamentally different from those used in physiological scenarios. Recent advances in long-term *in vivo* imaging in both cells and animals, the use of siRNA-based methods to deplete key endocytic molecules, and the use of conditional mutant mouse models in which components of the endocytic machinery can be spatially and temporally inactivated will provide essential insights into how neurotrophins and their receptors undergo endocytosis *in vivo*.

Role of Trk kinase activity. The role of Trk kinase activity in receptor internalization is still unclear. Pharmacological inhibition of Trk kinase activity and ectopic expression of mutant Trk receptors that lack kinase activity blocks ligand–receptor endocytosis and subsequent retrograde transport in sympathetic and sensory neurons (REFS 53,55,72; R. K. and D. D. G., unpublished observations). Inhibition of TrkA kinase activity in distal axons, but not in middle axons, of compartmentalized cultures of sympathetic neurons blocks retrograde transport of radiolabelled NGF, which indicates that Trk activity is required for internalization, sorting or both, but is not required for ligand–receptor transport in axons[53]. In addition, neuronal activity and Ca^{2+} influx facilitate the internalization of TrkB by enhancing its kinase activity in hippocampal neurons[73]. However, the results of other studies argue that TrkA kinase activity is not required for the initial steps of receptor endocytosis. Inhibition of TrkA kinase activity with K252a (a cell-permeable protein kinase inhibitor) and the expression of mutant receptors that lack the intracellular kinase domain of TrkA do not affect the intracellular accumulation of radiolabelled NGF in PC12 cells[74–76]. However, $p75^{NTR}$ also mediates NGF internalization in PC12 cells[77], and might, therefore, contribute to measurements of ligand internalization. Furthermore, differences in Trk kinase requirements could reflect cell-intrinsic properties, as studies in PC12 cells have generally found that the kinase activity is not required, whereas studies that use primary neuronal cultures have found that it is.

Discrepancies in kinase requirements notwithstanding, putative endocytic motifs in Trk receptors have been identified. For example, the tyrosine-based signals FxNPxY (where x is any amino acid) and Yxxϕ (where ϕ is a large hydrophobic residue) in the juxtamembrane and cytoplasmic domains might be crucial for the internalization of Trk receptors[78]. Consistent with this idea, deletion of the putative trafficking motifs ^{531}ECYNLL and ^{496}NPQY in the TrkA cytoplasmic domain greatly attenuates receptor internalization[74]. Phosphorylation at these tyrosine-based motifs could recruit endocytic adaptors such as Src homology 2 domain-containing transforming protein C (Shc) or fibroblast growth factor (FGF) receptor substrate 2 (FRS2) through their SH2 or PTB DOMAINS, respectively. Alternatively, Trk kinase activity could regulate receptor internalization by enhancing the recruitment of unique endocytic adaptor proteins or by stabilizing nascent clathrin-coated pits. Consistent with this idea, the endocytosis of EGFR is facilitated by the tyrosine-phosphorylation of the endocytic coat protein EPS15 and the clathrin heavy chain[79,80]. Determining the exact role of Trk kinase activity in receptor internalization and sorting will prove useful in the identification of downstream targets of Trk signalling that are required for these processes. Genetic models, such as Trk-knockin mice, which allow specific, potent, and reversible inhibition of kinase activity[81], will be useful for investigating the kinase requirements of Trk receptor trafficking.

Sorting of Trk receptors in axons. It is well established that ligand stimulation of distal axons results in the transport of active Trk receptors to cell bodies. Intriguingly, only a fraction of the ^{125}I-NGF that is internalized at distal axons of sympathetic neurons is transported to cell bodies, which indicates that only a small amount of activated TrkA in distal axonal processes is retrogradely transported[82,83]. These findings indicate that most internalized axonal Trk receptors undergo recycling and/or proteolysis in axons; however, it is not known how those receptors that are bound for retrograde transport are sorted from those that are not, and the vesicular destination of receptors remains elusive.

NGF and TrkA co-localize in different types of vesicle with distinct morphological characteristics, which indicates that internalized Trk receptor signalling complexes might use various modes of retrograde transport. Ultrastructural analyses of sympathetic nerves *in vivo* indicate that gold-labelled NGF is concentrated in multivesicular bodies[84,85]. By contrast, immunoelectron microscopy studies in sciatic nerves show that phosphorylated TrkA is found in both coated and uncoated 50–200-nm vesicles, as well as in multivesicular structures[86]. To determine which of these vesicular compartments retrogradely transports activated Trk receptors, Delcroix *et al.*[61] used chamber preparations of intact rat sciatic nerve, which allow proteins undergoing retrograde or anterograde transport to be isolated and characterized. They found that retrogradely transported vesicles that contain ^{125}I-NGF also contain not only activated TrkA but also molecular markers that are characteristic of early endosomes, such as the small G-protein Rab5 and its effector, early endosome antigen 1 (EEA1), as well as constituents of the MAPK and PI3K pathways[61]. These elegant biochemical analyses indicate that early endosomes mediate retrograde neurotrophin signalling in sensory neurons. Determining the contribution of other vesicular compartments, such as multivesicular bodies, to the sorting and retrograde transport of Trk receptors will shed light on the itinerary of internalized neurotrophin–receptor signalling complexes.

With coated and uncoated vesicles, early endosomes, and multivesicular bodies potentially serving as transport shuttles for retrograde neurotrophin signals, it is important to determine the molecular machinery that sorts and targets internalized Trk receptors for

SH2 AND PTB DOMAINS
Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains bind directly to canonical sites of tyrosine phosphorylation (pYXN and NPXpY, respectively, where p represents phosphorylation) that are found in many tyrosine kinases. These domains are commonly found in adaptor proteins such as Shc and FRS2. For the Trk receptors, Shc and FRS2 bind to Y-490.

retrograde transport. PI3K might be a candidate for regulating the endocytic itinerary of internalized Trk receptors. Constituents of the PI3K pathway associate with Trk-containing vesicles, and inhibition of PI3K activity attenuates NGF internalization and retrograde transport[20,61,87]. Moreover, inhibition of PI3K at distal axons — but not middle axons — of compartmentalized sympathetic neurons attenuates the retrograde transport of ^{125}I-NGF (REF. 20). Although these findings strongly implicate PI3K signalling in the regulation of Trk receptor trafficking, the downstream targets of this signalling cascade and how it regulates sorting remain unclear. Intriguingly, many endocytic proteins contain phosphoinositide binding modules (FYVE, ENTH, FERM, PH and PX domains) that are necessary for the proper fusion and sorting of vesicles[88]. One such protein, the early endosomal marker EEA1, is localized to retrogradely transported NGF–TrkA complexes[61] and binds to phosphatidylinositol-3-phosphate through a carboxy (C)-terminal FYVE domain[89]. EEA1 also interacts directly with the endocytic protein Rab5, and this interaction is necessary for the sorting of internalized proteins to early endosomes[90]. It will be interesting to determine whether Trk receptor signalling through PI3K is directly linked to endosomal effectors such as EEA1 and Rab5, and whether these proteins are involved in Trk receptor sorting and/or trafficking.

In addition to internalization, sorting, and transport of neurotrophin signals, the signalling endosome must also avoid proteolysis as it travels from axons to cell bodies. It is widely believed that late endosomes and lysosomes are restricted to the soma and dendrites, but ultrastructural analyses and immunostaining experiments point to their existence in axons[91,92]. ^{125}I-NGF is retrogradely transported in sympathetic axons with little or no degradation or release en route to the cell bodies[83], which indicates that signalling endosomes evade degradation in axons. Moreover, studies of the internalization and fate of ^{125}I-NGF have revealed that NGF is poorly targeted to lysosomes and remains tightly associated with its receptor at a relatively acidic pH[93], which further indicates that NGF–TrkA signalling endosomes might be specialized vesicles that have evolved to propagate long-distance signalling in axons by subverting proteolytic degradation. Avoidance of proteolytic degradation might involve the macropinocytic protein Pincher. Overexpression of Pincher in cultured sympathetic neurons prevents activated Trk receptors from targeting to lysosomes[56]; however the exact mechanism by which Pincher prevents lysosomal degradation of internalized Trk receptors remains unresolved. It would be interesting to investigate whether other proteins that are involved in the avoidance of axonal degradation are also linked to axonal transport of Trk signalling complexes.

Axonal transport of Trk receptors. An important question is how target-derived neurotrophins and their receptors efficiently travel along axons, which are up to one metre long. There is considerable evidence that they use a microtubule-based motor system in which neurotrophins and Trk receptors are retrogradely transported by the microtubule minus-end-directed motor dynein. Phosphorylated TrkA co-localizes with dynein and components of the dynein complex, such as dynamitin in axons of the sciatic nerve, and the distal region of the TrkA juxtamembrane domain interacts with the 14-kDa dynein light chain in both heterologous cells and brain lysates[86,94]. Moreover, molecular and chemical disruption of microtubules[95,96] or the dynein–dynactin complex[55] attenuates retrograde transport of neurotrophins and their receptors both *in vitro* and *in vivo*, and dynein-dependent transport is required for the survival of PNS neurons *in vivo*[58] and *in vitro*[55]. However, whether the neuronal degeneration in dynein mutant mice is due to the disruption of transport of target-derived neurotrophins, or of some other cargo that is necessary for survival, remains to be seen. These findings indicate that interactions between TrkA and components of the dynein machinery might underlie the recruitment and loading of TrkA-containing vesicles onto retrograde microtubule motors and the retrograde movement of these vesicles. Using new genetic models and advanced imaging techniques, such as two-photon microscopy, it might soon be possible to visualize ligand–receptor trafficking in real-time, both *in vitro* and *in vivo*. These techniques will allow more thorough investigations into the requirements and mechanisms of dynein-dependent retrograde transport, and will help us to identify new molecular components and cellular pathways that are required for retrograde neurotrophin signalling.

Signalling from the endosome. How do target-derived signals, which emanate from remote axonal locations, impart their messages to neuronal cell bodies? As mentioned above, there is considerable evidence that neurotrophin–Trk receptor complexes are associated with the early endosome, and possibly other organelles, and that these vesicles are a necessary and sufficient platform from which various signalling pathways are initiated[61,97,98]. Indeed, the early endosome presents several potential advantages as a platform for Trk signalling by providing access to unique effectors that are not localized at the cell surface (FIG. 4). For example, sustained MAPK signalling seems to be maintained exclusively from endosomal locations through activation of Rap1 (REF. 99). Consistent with this idea, NGF induces the activation of Rap1 at endosomal membranes, which, in turn, leads to the recruitment and activation of B-Raf[62,99] (a serine/threonine protein kinase). Moreover, NGF and TrkA, along with various constituents of the MAPK transduction cascade, including Rap1, B-Raf, ERK1 and ERK2, are also localized and retrogradely transported with early endosomes[61]. Although it is not clear whether Rap1 is recruited to this complex from the cytosol or is constitutively associated with the early endosomes to which Trk receptors are sorted, the Rap1 signalling pathway seems to be important for the signalling function of the endosome. It has also been found that members of the ERK kinase family of signalling effectors can be activated at discrete cellular locations. Retrograde accumulation of ERK5

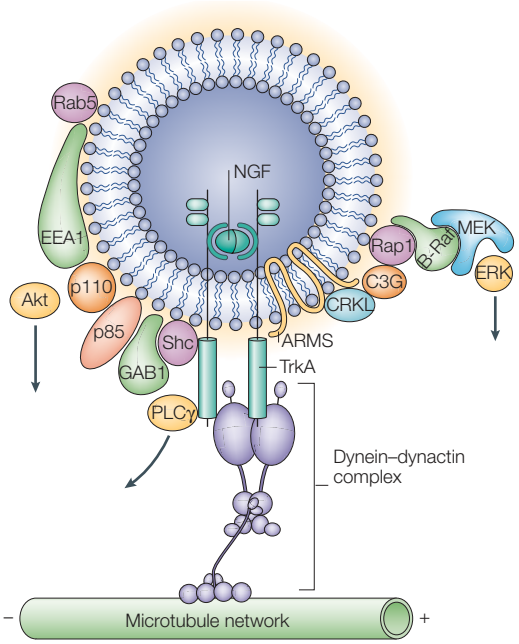

Figure 4 | **The signalling endosome.** The main mode of retrograde neurotrophin signalling is through the retrograde transport of a membrane-enveloped ligand–receptor complex with characteristics of early endosomes. These signalling endosomes are platforms for unique signalling effectors such as the small G-proteins Rap1 and Rab5, which are essential for activation of downstream signalling pathways and transport of retrograde neurotrophin signals. Various signalling cascades (arrows) that are crucial for neurotrophin-dependent survival and axon growth are associated with nerve growth factor (NGF)–TrkA-containing endosomes. These include constituents of the phospholipase C-γ (PLCγ), Raf (a serine/threonine protein kinase)–MEK (MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase)–ERK and phosphatidylinositol 3-kinase (PI3K) signalling pathways. The mechanisms responsible for the nucleation of these complexes remain unresolved. Akt, v-akt murine thymoma viral oncogene homologue, also known as protein kinase B; ARMS, ankyrin-rich membrane-spanning protein; B-Raf, v-raf murine sarcoma viral oncogene homologue B1; CRKL, v-crk sarcoma virus CT10 oncogene homologue; C3G, Rap guanine nucleotide exchange factor (GEF) 1; EEA1, early endosome antigen 1; GAB1, GRB2 (growth factor receptor-bound protein 2)-associated binding protein 1; p85, regulatory subunit of phosphatidylinositol 3-kinase; p110, catalytic subunit of phosphatidylinositol 3-kinase; Shc, Src homology 2 domain-containing transforming protein C.

but not ERK1 or ERK2 in response to neurotrophin stimulation of distal axons of compartmentalized DRG neurons indicates that retrograde Trk signalling complexes might use distinct effector pathways depending on the cellular location of neurotrophin stimulation. However, sustained retrograde activation of ERK1 and 2 has been observed in compartmentalized cultures of sympathetic neurons[23,53], and activated ERK1 and 2 are localized to retrogradely-transported Trk-containing endosomes from sciatic nerve preparations as well as to the cell bodies of DRG neurons *in vivo*[61]. Discrepancies such as these highlight the importance of purifying and characterizing retrograde Trk signalling complexes from various cell types.

Other endosomal proteins that are retrogradely transported with TrkA might also provide signalling functions for neurotrophins. For example, Rab5 has been functionally linked to signalling molecules through direct and indirect associations with its effectors Rabaptin 5, EEA1, Rabenosyn 5, and the phosphatidylinositol 3-kinases p-110β, p85α and p150 (REFS 61,89,90,100–102). However, although TrkA localizes to Rab5- and EEA1-positive endosomes, it is not clear whether TrkA activity modulates Rab5 activity, or whether Rab5 itself is required for endosomal activation of Trk effectors. Future experiments designed to purify and characterize the proteins associated with Trk-containing endosomes will provide insight into the mechanisms by which neurotrophin signals are maintained during retrograde transport and disseminated on arrival at neuronal cell bodies.

Concluding remarks

Target-derived trophic factors such as the neurotrophins are crucial for the establishment of proper neuronal connectivity during development. Target instruction is imparted through both local and retrograde activation of various signalling cascades by neurotrophins. These signalling pathways coordinate several developmental processes, including cell survival, axonal and dendritic growth, synaptogenesis, and the acquisition of neurotransmitter phenotype. When neurotrophins bind to Trk receptors, these complexes recruit the cellular machinery that is responsible for endocytosis, trafficking to transport organelles, and, ultimately, their retrograde transport and signalling in cell bodies.

Although the function of signalling endosomes is becoming clear, many questions regarding retrograde signalling remain to be addressed. What is the molecular composition of signalling endosomes and how do they avoid lysosomal degradation in axons? Do alternative modes of retrograde signalling contribute to distinct neurotrophin-dependent processes? Are different modes of ligand–receptor internalization required for spatially distinct cellular processes such as axon growth and cell survival? The increasing sensitivity of mass spectrometry and improved methods for purifying retrogradely-transported vesicles will undoubtedly provide a clearer picture of the molecular fingerprint of retrograde signalling complexes. In addition, coupling advanced imaging techniques to high-throughput molecular screens should prove invaluable in determining the molecular mechanisms that are important for the internalization and sorting of ligand–receptor complexes that are destined for retrograde transport. Finally, as there is growing evidence that aberrant retrograde neurotrophic factor signalling is involved in the aetiology of neurological diseases such as Alzheimer's disease[103–106], Huntington's disease[107] and amyotrophic lateral sclerosis[96], it is essential that we determine the modes of retrograde target influence, not only during development but also in adulthood.

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Competing interests statement

The authors declare no competing financial interests.

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