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The Coming of Age of Axonal Neurotrophin Signaling Endosomes

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Abstract

Neurons of both the central and the peripheral nervous system are critically dependent on neurotrophic signals for their survival and differentiation. The trophic signal is originated at the axonal terminals that innervate the target(s). It has been well established that the signal must be retrogradely transported back to the cell body to exert its trophic effect. Among the many forms of transmitted signals, the signaling endosome serves as a primary means to ensure that the retrograde signal is delivered to the cell body with sufficient fidelity and specificity. Recent evidence suggests that disruption of axonal transport of neurotrophin signals may contribute to neurodegenerative diseases such as Alzheimer's disease and Down syndrome. However, the identity of the endocytic vesicular carrier(s), and the mechanisms involved in retrogradely transporting the signaling complexes remains a matter of debate. In this review, we summarize current insights that are mainly based on classical hypothesis-driven research, and we emphasize the urgent needs to carry out proteomics to resolve the controversies in the field.

Keywords

neurotrophin; NGF; Trk; endosomal signaling; scaffold; axonal traffic; retrograde; neurodegeneration; quantum dots; 2-D DIGE

I. Retrograde signaling and neuronal survival

Neurotrophins are a family of small protein factors that regulate many aspects of neuronal functions including neuronal survival, differentiation, migration and synaptic plasticity [1–7]. These trophic factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4 (NT3 and NT4) [8–10]. While all these neurotrophic factors each bind to the p75 neurotrophin receptor (p75), each binds to and acts additionally through a specific member(s) of the surface tyrosine receptor kinases (Trks): NGF to TrkA, BDNF and NT4 to TrkB, NT3 to TrkC and to TrkA, to exert its trophic effects [5, 10].

The downstream signaling pathways activated by neurotrophin/Trk are, in many ways, very similar to other receptor tyrosine kinase-mediated cascades. Chief among these cascades are:

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the mitogen-activated kinase (MAPK) pathway; the phosphoinositol-3-kinase (PI3K) pathway and the phospholipase C-gamma (PLC γ) pathway, each of these signaling pathways contributes to modulating neuronal functions [3–5,11,12]. However, due to the unique architecture of neurons, intracellular transmission of the neurotrophin/Trk signal represents a daunting challenge for many types of neurons. Unlike other cells, all neurons possess elaborate and long processes called dendrites and axons (Figure 1A). As such, active communications e.g. neurotrophin signaling between these processes and the soma are paramount for neuronal functions. Neurotrophins released from the target must bind to and activate Trks at the axonal terminal. The signal must be then transmitted to the soma *via* the long axon insulated by myelin (Figure 1A). In the case of human lumbar motoneurons, the average size of the soma falls within 30–50 μ m in diameter, while the length of the axons often exceeds 1 meter [13]. Therefore, the mechanism(s) that underlies the extraordinarily long-distance retrograde transmission of trophic signal has been one of the most intriguing enigmas in modern neurobiology and this issue has attracted extensive attention from many investigators in the field [13–16].

The discovery of NGF by Levi-Montalcini and Hamburger provided an important clue to the mechanism: a soluble “nerve growth factor” produced in the target dramatically influenced specific populations of neurons [17]. It is now well established that NGF, produced in and released from target tissues, activates specifically TrkA at the distal axons of innervating neurons. The signals thus elicited are subsequently retrogradely transported to the cell bodies of these neurons to regulate nuclear and cytosolic events known to be important for the survival and maintenance of these neurons. The generation, intracellular sorting, and maintenance of these signals have been the subject of intensive investigations.

II. Retrograde Neurotrophic Signaling: Proposed Mechanisms

Over recent years, a number of models have been put forward to explain retrograde neurotrophin (e.g. NGF) signaling. We will describe each of these models in detail.

The Wave Model

The “wave model” suggests that NGF binds to and activates surface TrkA at distal axonal terminals. Activated surface TrkA and/or activated signaling molecules, downstream of activated surface TrkA receptors, are subsequently transported in a “wave” fashion to the cell body. Endocytosis of NGF, as mediated by its receptors, is not envisioned as necessary in this model. This is contrary to large amount of experimental evidence revealing that NGF/p75/TrkA are indeed internalized following binding of NGF to its receptors at the cell surface. Ligand-receptor internalization is apparently necessary for survival of neurons supported by NGF on distal axons [18]. Although there is no direct evidence supporting the “wave model” in the field of neurotrophin signaling, recent work with the epidermal growth factor receptor (ErbB1) has demonstrated that focal stimulation of cells with beads crosslinked to epidermal growth factor (EGF) induced a rapid lateral propagation of ligand-independent receptor activation in the plasma membrane leading to full activation of all EGF receptors [19].

The Signaling Effector Model

The “signaling effector model” postulates that second messengers such as ionic flux (e.g. cytosolic Ca²⁺) or a downstream kinase, instead of the NGF/pTrkA signaling complex, are retrogradely transmitted as the primary source of neurotrophic signal. A major deficiency in this model is that ionic fluxes and second messengers have a limited radius of diffusion, thus compromising the ability to traffic the signal down the long axon to reach the soma. Additionally, many types of stimuli other than neurotrophins can trigger ionic fluxes and it is difficult to conceive how signaling specificity would be achieved under this model.

Interestingly, a recent study with cultured cerebellar granule cells showed that application of a Slit-2 gradient in front of the leading process caused a retrogradely propagating Ca^{2+} wave from the growth cone to the soma leading to the collapse of the growth cone and the reversal of neuronal migration [20]. However, application of immobilized NGF to the distal axons of cultured rat sympathetic neurons was shown to cause retrograde transport of pTrkA [21] or its downstream signaling proteins [22], which appears to be necessary and sufficient to support neuronal survival [21,22]. It is not clear whether or not a retrogradely propagating Ca^{2+} wave was also resulted in and whether or not the Ca^{2+} wave alone was sufficient to propagate the retrograde neurotrophic signal under these experimental conditions. On the other hand, the wave mechanism and the signaling effector model may both exist to compensate other mechanism(s).

The Signaling Endosome Hypothesis

This signaling endosome hypothesis is built on the observation that NGF binds to and activates TrkA receptors at the axonal terminal to elicit the formation of signaling protein complexes. Following receptor-mediated endocytosis, the NGF/activated TrkA signaling complexes are sorted into a subpopulation of endosomes to give rise to the signaling endosomes [2,18,23–29]. In addition to causing down-regulation of the signaling complexes, the signaling endosome serves as a retrograde vesicular carrier to maintain signal fidelity and to sustain the signaling of the NGF/activated TrkA complex during their transit from the terminal to the soma *via* the axon. The hypothesis points to signaling endosomes as an important source of retrogradely transmitted signals. It is consistent with the emerging view that cellular signaling pathways are highly organized and compartmentalized, features that are used to confer specificity and sustainability of signal transduction [14,18,30–32].

The signaling endosome hypothesis is supported by an increasing body of experimental evidence from a number of laboratories. NGF induced endocytosis of TrkA and remained bound to pTrkA in endosomes in PC12 cells [23]. The NGF-pTrkA complexes were found in clathrin-coated vesicles (CCVs) together with activated components of the Ras/Erk1/2 pathway as well as PI3K and PLC γ [33]. In an *in vitro* kinase assay, the NGF signaling complex present in CCVs was capable of phosphorylating Elk-1, a downstream target of Erk1/2 [33]; When the receptor-mediated endocytosis was inhibited by overexpression of a dominant-negative dynamin mutant, neuronal differentiation promoted by catalytically active Trks within endosomes was abolished in PC12 cells [12]. In what appears to be the next step in the maturation of the signaling endosome within the endosomal pathway, NGF induced the formation of complexes containing pTrkA and activated components of the Rap1/Erk1/2 pathway in early endosomes [34,35]. Retrograde NGF signaling [36] and the generation of this long-lasting NGF signaling complex required active microtubule-based transport [34,35]. Moreover, NGF at axon terminals must be internalized and retrogradely transported to neuron cell bodies for the NGF signal to induce phosphorylation of CREB (cAMP response element-binding protein) and to increase survival of immature neurons [37–40]; NGF-induced activation of TrkA in axon terminals was also required for survival [41–43]. Furthermore, inhibition of axonal retrograde transport of the Erk5 survival signal [44] and the NGF/pTrkA signaling complex [35] by impairing the dynein activity through overexpression of dynamitin (the 50 kD subunit of the dynein/dynactin complex) leads to neuronal cell death.

The signaling endosome hypothesis is also supported by some *in vivo* and *ex vivo* studies. Following hippocampal injection *in vivo*, ^{125}I -NGF is co-immunoprecipitated with TrkA, and NGF was localized within early endosomes in cholinergic terminals [45]. Early endosomes containing NGF-pTrkA, together with activated components of the Rap1/Erk1/2 and PI3K pathways, were retrogradely transported in isolated sciatic nerve [46]. Increasing NGF levels at the target of innervations increased retrograde transport of these membranes and increased

pErk1/2 in the cell bodies of DRG neurons [46]. Conversely, sequestering NGF at the target of innervations using specific NGF antibodies decreased the retrograde transport of endosomes containing pTrkA and pErk1/2 [46].

The importance of endosomal signaling, first proposed to explain long-distance retrograde signaling of neurotrophic factors, has also been appreciated for transmitting intracellular signals of other receptor-tyrosine kinases (RTK) [47–49]. Until recently, endosomal trafficking of activated RTK signaling cascades was believed to merely serve as a means for controlling signaling by down-regulating surface receptors. A series of elegant studies with the epidermal growth factor receptor (EGFR) have demonstrated that endosomal trafficking of activated EGFR is critical for signaling specificity. Through the identification of a highly conserved adaptor protein, p14, that is peripherally associated with the cytoplasmic face of late endosomes/lysosomes, Huber and colleagues found that p14 was required to anchor the Erk1/2 cascade onto endosomes mediated by the scaffold protein MP1 (MEK1 partner) [50]. Reduction of MP1 or p14 protein levels by siRNA disrupted EGF-induced endosomal signaling thus resulting in defective signal transduction [51]. When endosomal trafficking was disrupted through the overexpression of dynamin, prolonged EGFR activation was retained in late endosomes at the cell periphery. The consequence was slowed EGFR degradation and sustained activation of the Erk1/2 and the p38 signaling cascades leading to hyperactivation of nuclear targets, such as Elk-1 [52]. Taken together, these findings suggest that appropriate endosomal trafficking of the activated RTKs ensures the spatial and temporal regulation of signaling pathways [50–52]. Mutations in p14 that modified these processes may contribute to a previously unknown human primary immunodeficiency disorder [53].

The hypothesis that spatial distribution of signaling proteins, such as those within subcellular organelles, contributes to signaling specificity has been recently investigated in zebrafish [31]. APPL1, one of the Rab5 effectors [49], displays a different subcellular localization pattern with respect to two of the Akt substrates: GSK3 β and tuberous sclerosis protein 2 (TSC2), a key player regulating cell growth through the mammalian target of rapamycin complex (mTOR) pathway. APPL1 did not colocalize with TSC2 but showed a significant overlap with GSK3 β in endosomes. Stimulation with growth factor caused a transient recruitment of Akt to the APPL1-positive endosomes and rapid dissociation of GSK-3 β from these compartments. Furthermore, GSK3 β , but not TSC2 was recruited to the APPL1-containing enlarged endosomes in cells overexpressing the constitutively active Rab5 mutant (Rab5Q79L). As a result, suppression of APPL1 expression selectively decreased phosphorylation of GSK3 β leading to apoptosis [31]. These apoptotic effect were only reversed by endosomally localized APPL1, but not by either cytosolic or nuclear APPL1. Therefore, the survival effect of the Akt signaling pathway appears to be critically dependent on signaling events occurring on endosomes [31].

NGF-independent Retrograde Signaling

There is also evidence that NGF retrograde signaling may not always be coupled to retrograde transport of NGF. Senger and Campenot found that it took at least 30 min for ^{125}I -NGF to arrive at the cell body/proximal chamber at a detectable level following addition of ^{125}I -NGF to the distal chamber in the compartmentalized culture of sympathetic neurons [54]. TrkA and other signaling molecules were more rapidly activated in the cell body/proximal axons [54]. Utilizing NGF that was covalently cross-linked to beads, thus rendering it incapable of internalization, Campenot's group further demonstrated that this source of NGF was able to trigger activation and axonal transport of signaling proteins including pTrkA, PI3K and pAkt at levels that were adequate to support survival of sympathetic neurons for up to 30 hrs [21]. These findings suggested that signaling events initiated by NGF at the axonal terminals can be transported retrogradely without NGF. More recently, Campenot and colleagues used either

K252a [42] or Go6976 [22], both potent inhibitors for the TrkA kinase, to block TrkA phosphorylation in the cell body/proximal axons of compartmentalized sympathetic neurons. They discovered that addition of the inhibitors to locally block TrkA phosphorylation in the distal axons impeded local axonal growth and induced apoptosis. However, when the cell body/proximal axons were pretreated with the inhibitors to block TrkA activity locally, NGF added to distal axons still resulted in activation of survival signaling pathways (e.g. pAkt, pCREB) and supported survival of these neurons [22,42]. These investigators concluded that retrograde transport of signaling molecules downstream of phosphorylated TrkA, but not phosphorylated TrkA itself, were necessary to support neuronal survival.

Due to the long-distance nature of retrograde axonal signaling, the argument for the requirement of NGF lies in the fact that the presence of NGF sustains TrkA activation and thus its down-stream signaling. Without binding to NGF, activated TrkA can be quickly inactivated by phosphatase(s) that will make it difficult for the signal to reach its final destiny. Nevertheless, the finding that retrograde transport of neither NGF nor phosphorylated TrkA is required to convey a survival signal is intriguing. These mechanisms may exist to complement the NGF/TrkA signaling endosome hypothesis. However, it remains to be seen how the signal is sustained and transmitted under these paradigms.

III. Defining the endocytic vesicular carriers for the retrograde NGF signal

Although the signaling endosome hypothesis is considered to be one of the most plausible mechanisms for explaining retrograde axonal trafficking of the NGF signal, no consensus has been reached thus far as to what endocytic vesicle(s) transports the retrograde trophic signal in axons. Similar to most other receptor-mediated endocytic pathways, neurotrophins bind to and activate Trk receptors at the surface, which, in turn, triggers its internalization into early endosomes. The signal is then sorted either into recycling endosomes to return back to the cell surface, or progresses from the early endosomes to late endosomes, then into multivesicular bodies (MVBs) and finally to lysosomes. Therefore, each of these different subcellular organelles has been found to contain internalized NGF using different approaches and experimental systems. Based on data derived from classical immuno-chemical and molecular biological research, these organelles include but are not restricted to early endosomes, late endosomes, lysosome/MVBs and macroendosomes (Figure 1B).

The early endosomes

Experimental evidence, both *in vitro* and *in vivo*, strongly points to a subpopulation of early endosomes may serve as an axonal transport carrier for the neurotrophin signaling endosome. Following NGF treatment of PC12 cells, a long lasting signaling complex was formed that contained pTrkA, Gab2, C3G, Rap1, B-Raf, MEK and Erk1/2, a pathway that is essential for NGF-induced differentiation in PC12 cells. Furthermore, following treatment of PC12 cells with NGF for 30 min, activated Rap1, pTrkA and pErk1/2 were all recovered from the same endosomal fraction that was enriched with Rab5, EEA1, both markers for early endosomes [34,55]. In addition, generation of the long-lasting Rap1 signaling complex was critically dependent on an intact cellular microtubule network and required functional dynein motor activity. Depolymerization of the microtubule by nocodazole, or inhibition of dynein motor activity by overexpression of dynamitin, selectively suppressed the activity of the Rap1 signaling pathway and blocked retrograde transport of the signaling complex [35].

In a study also using PC12 cells, Liu and colleagues showed that NGF treatment activated rapidly a Rab5 GTPase-activating protein (Rab5GAP) to suppress the cellular levels of active Rab5 i.e. the GTP bound form [56]. The consequence of a decreased level of Rab5GTP is not only to block homotypic fusions of Rab5-positive early endosomes [57] but also to prevent subsequent conversion of early endosomes into late endosomes (i.e. Rab5 to Rab7) as

demonstrated by Rink et al [58]. Consistent with this notion, expression of a dominant-negative Rab5 mutant sustained TrkA activation in early endosomes and enhanced NGF-mediated neurite outgrowth, whereas expression of a constitutively active Rab5 mutant or Rabex-5 inhibited this process [56]. Furthermore, suppression of Rab5GAP by RNA interference increased the level of Rab5GTP and blocked NGF-mediated neurite outgrowth [56]. Additionally, a recent study has demonstrated that endophilin B1 interacted with both TrkA and early endosome marker EEA1, the early endosome antigen-1. Knockdown of endophilin B1 resulted in enlarged EEA1-positive vesicles in NGF-treated PC12 cells [59]. Therefore, the implication of these elegant studies is that the NGF/TrkA signaling endosomes somehow manage to remain positive for Rab5 by blocking further maturation to a Rab7-positive endosome.

Signaling endosomes, with the characteristics of early endosomes, were also characterized using isolated rat sciatic nerves [46]. Retrogradely transported endosomes were found to contain NGF, activated TrkA, and other signaling proteins of the Rap1/Erk1/2, p38MAPK, and PI3K/Akt pathways. Retrograde transport of p-Erk1/2, p-p38, and pAkt in these vesicles was enhanced by NGF injection. Injection of an NGF antibody in the peripheral target of DRG neurons diminished retrograde transport of these signals [46]. These signaling molecules appeared to be harbored on early endosomes by virtue of their co-localization with Rab5 by immunostaining and co-fractionation with Rab5 on a step-density gradient system [46].

Further supporting evidence that the Rab5 positive early endosomes may serve as the vesicular carrier for neurotrophin signaling has come from recent demonstration that APPL proteins play an important role in TrkA intracellular trafficking and signaling [60,61]. The APPL proteins, APPL1 and APPL2, are two Rab5 effectors that reside on a subpopulation of Rab5 positive early endosomes [49]. Both APPL1 and APPL2 were essential for HeLa cell proliferation stimulated by EGF and their function required Rab5 binding. An endosomal compartment bearing Rab5 and APPL proteins was shown to be an intermediate in EGF signaling between the plasma membrane and the nucleus [49]. Studies from two separate groups have recently demonstrated in sympathetic neurons as well as in a PC12 cell line that NGF treatment resulted in the recruitment of APPL to activated TrkA either directly through the PTB domain of APPL [61] or indirectly via the PDZ domain of GIPC1 [60,61]. GIPC1 is a PDZ protein located on peripheral endosomes that binds to the juxtamembrane region of the TrkA receptor [62]. APPL1, GIPC1, and phosphorylated TrkA were enriched in the same endosomal fractions by high-resolution centrifugation. Reduction of APPL1 protein levels suppressed NGF-induced activation of MEK, Erk1/2 and Akt kinases and inhibited neurite outgrowth in PC12 cells [60,61].

More interestingly, APPL was recruited to and remained associated with the TrkA-containing peripheral endosomes prior to or during their transit from the cell periphery to the juxtanuclear region, where they acquired EEA1 [60]. Together, these results point again to the view that at least one population of early endosomes bearing APPL-Rab5 play a role in transmitting intracellular NGF signals [60,61]. The intriguing questions are how many such populations exist and the signaling pathways they carry. Furthermore, how these early endosomes are prevented from entering the late/lysosomal degradation pathway prior to conveying their signals.

Single-molecule methods have been recently developed to study NGF trafficking and signaling. By applying Cy3-NGF onto chick dorsal root ganglion growth cones, Tani and colleagues found that most of Cy3-NGF molecules were internalized as a single NGF dimer [63]. Quantum dot-labeled NGF (QD-NGF) was also used to track the movement of NGF in real time in compartmentalized culture of rat DRG neurons [64]. A physiological level of QD-NGF was added to the distal axons and the real time movement of QD-NGF was captured

within the proximal axons leading to the cell body. Quantitative analysis has revealed that the majority of retrogradely transported NGF-containing endosomes carry only a single NGF dimer. Again, retrogradely transported QD-NGF within the proximal axons was shown to co-localize with Rab5 [64]. Electron microscopic analysis of axonal vesicles carrying QD-NGF confirmed this finding. In addition, the majority of QD-NGF was found to localize in vesicles 50–150 nm in diameter with a single lumen and no visible intraluminal membranous components. This result is consistent with a previous report showing that activated Trks together with dynein within rat sciatic nerve axons were preferentially localized to simple-membrane vesicles with a diameter of ~ 60 nm [65].

The late endosomes

Late endosomes marked by the small Rab7 GTPase have also been implicated in regulating TrkA signaling and axonal transport of neurotrophin signals [66]. Kruttgen and colleagues found that following NGF treatment, TrkA co-immunoprecipitated with Rab7 in PC12 cells [67]. Expression of a dominant-negative Rab7 mutant resulted in endosomal accumulation of TrkA and pronounced enhancement of TrkA signaling in response to limited stimulations with NGF. In addition, activation of Erk1/2 (extracellular signal-regulated kinase 1/2) was increased, and neurite outgrowth was enhanced in these cells [67]. Using a different approach, Rab7 was identified as a functional marker of a specific pool of axonal retrograde carriers for a fragment of the tetanus neurotoxin, Tent H(C)[66]. This pool of Rab7-positive axonal carriers was also found to transport BDNF and the TrkB receptors in mouse motoneurons that were microinjected with a TrkB-GFP construct. These investigators reported that Rab5-positive early endosomes appeared to be involved only in an early sorting step but were not responsible for axonal transport of vesicles. Based on these findings, the Rab7-late endosomes was stated to serve as an important retrograde transport carrier for axonal neurotrophin signals [66,67].

The Lysosomes and multivesicular bodies (MVBs)

Using electron microscope autoradiography, Campenot and colleagues localized the ^{125}I -NGF in the cell body of cultured sympathetic neurons following a 1 hr exposure to ^{125}I -NGF [68]. They found that the majority of silver grains were associated with lysosomal organelles, including secondary lysosomes, residual bodies, and MVBs. MVBs contained most of the ^{125}I -NGF signal with a labeling density (L.D.) of 21; the lysosomes had a L.D. of 3.1 [68]. To study retrograde transport of ^{125}I -NGF, these investigators cultured sympathetic neurons in compartmentalized culture dishes and incubated distal processes with ^{125}I -NGF. They discovered that the radiolabel was transported to the cell bodies at the rate of approximately 3 mm/hr. After 8 hr of transport, the radioactivity in cell bodies reached a steady state. Most of the label was again localized primarily to MVBs (L.D. = 16.8) and lysosomes (L.D. = 3.8)[68].

Studies from the Hendry laboratory showed that ^{125}I -NGF transported from the eye to the sympathetic and sensory ganglia was also concentrated in MVBs [25]. They examined the transport of ^{125}I -labelled neurotrophins from the eye to sympathetic and sensory ganglia as well as in cultures of dissociated sympathetic ganglia to which rhodamine-labeled NGF was added. Most of the label was present within large vesicles at the growth cone and in axons. The retrogradely transported NGF signal was sporadically located in large structures in axons of the sympathetic nerve trunk *in vivo*. Ultrastructural examination of the sympathetic nerve trunk after transport of gold-labeled NGF showed that gold particles were concentrated in relatively few large organelles that consisted of accumulated MVBs with a diameter >1 μm . These findings led to the proposal that MVBs are major vesicular carriers for retrograde NT signaling within axons [25,69]. The attractiveness of this model is that the entire signaling complex along with its vesicle is encapsulated in a secondary membrane system prior to axonal transport. As such, the signaling machinery is protected from degradation by cytosolic factors

during axonal transport. One challenge to this model is that the relatively large size of MVBs ($>1\ \mu\text{m}$) often exceeds the diameter of many axons ($0.2\text{--}1.0\ \mu\text{m}$). The possibility that MVBs might be too large to effectively transport the neurotrophin signals needs to be addressed. More concerning is the fact that, once MVBs reach the cell body, the signaling complex encapsulated within the MVBs would need to be released from the MVBs to fulfill its signaling function. To our knowledge, no experimental evidence supports this postulated process.

The macroendosomes

Halegoua and colleagues have identified another population of endosomes that could serve as a vesicular carrier for retrograde neurotrophin signaling complex [70–72]. Through the identification of a pinocytic chaperone, Pincher, these investigators have studied the involvement of macroendosomes in neurotrophin signaling and trafficking. In PC12 cells, overexpression of Pincher enhanced TrkA internalization induced by NGF via a clathrin-independent macropinocytosis process. Overexpression of Pincher resulted in the formation of extended tubular structures within the cytoplasm. Following internalization TrkA receptor accumulated in these Pincher-containing tubules. A dominant inhibitory mutant form of Pincher (G68E) suppressed NGF-induced endocytosis of TrkA, and selectively blocked TrkA-mediated cytoplasmic signaling of Erk5, but not Erk1/2, kinases [70]. Similar observations were made in primary cultured sympathetic and hippocampal neurons. Overexpression of Pincher led to the formation of macroendosomes containing Trk receptors in the soma, axons, and dendrites of these neurons. Trk-containing macroendosomes were derived from plasma membrane ruffles and were subsequently trafficked to MVBs. In compartmentalized sympathetic neuronal cultures, suppression of Pincher by the Pincher mutant (G68E) prevented the formation of Trk macroendosomes and eliminated retrograde neuronal survival signaling [71].

Mechanistically, formation of the Pincher-containing Trk signaling endosomes uniquely required internalization of Trk at the ruffle site(s) mediated by Rac, an Rho-GTPase [72]. These Trk-containing macroendosomes appeared to have the characteristics of immature MVBs that retained Rab5 but not Rab7 during axonal transit to the cell body. Combined with results of a recent study in PC12 cells [56], these findings appear to indicate that, unlike what characterizes EGFR signaling, Trk signaling endosomes, once generated, manage to avoid rapid progression from a Rab5-positive to a Rab7-positive compartment, thereby possibly avoiding prompt degradation in lysosomes [72]. Because the formation of Trk-containing macroendosomes apparently requires overexpression of Pincher, it would be important to see if these organelles also exist within the axons of neurons under physiological conditions. It is conceivable that the macroendocytic pathway may play a prominent role in transmitting neurotrophin signals in some neurons or under some conditions that are not entirely clear at present. Another important question is the relative contribution of Pincher-mediated macroendocytosis versus that of clathrin-, lipid raft-mediated endocytosis to the formation of signaling endosomes in untransfected neurons. Clearly, a detailed quantitative analysis is required to resolve this issue.

IV. Proteomic analysis of the neurotrophin signal endosomes

Ultimately, the goal of studying endosomal signaling and trafficking of neurotrophins is not only to elucidate a fundamental cellular process but also to pin-point possible defect(s) under neurodegenerative conditions. Such efforts could potentially lead to the discovery of novel targets for treatments. To this end, compilation of a complete and unbiased map of endosomal signaling molecules i.e. functional signaling endosome proteomics (FSEP) under various physiological conditions and with spatial and temporal resolution becomes an absolute necessity. Unfortunately, despite tremendous advance has been made in the field of endosomal signaling and trafficking of neurotrophins, our current knowledge in this regard is lacking. This

is in part due to the fact that many different types of signaling endosomes may exist and play different roles in retrograde signaling. There is no consensus as to whether or not a specific class of endosomes plays a specific role in signaling. Nor is it clear whether or not a specific type of endosomes can signal through a single pathway. Importantly, because of the diversity of these endocytic compartments, it has been difficult to purify these subcellular organelles to the extent needed for proteomic analysis [73].

We have been attempting to carry out FSEP analysis of NGF signaling endosomes. We took advantage of a number of recent observations and technological advances: 1) the addition of Quantum dot labeled-NGF (QD-NGF) to live neurons greatly helps to visualize the intracellular trafficking pathways of NGF-containing endosomes [64]; 2) QD-NGF co-localizes with Rab5 within axons of DRG neurons [64] and Rab5-positive early endosomes may serve as an important source for the signaling endosomes as outlined in the previous section; 3) a number of Rab5 mutants, such as the constitutively active mutant Rab5Q79L, are available to manipulate NGF/TrkA containing compartments [56]. When overexpressed, the Rab5Q79L mutant promotes homotypic fusion of Rab5-positive early endosomes thus causes the enlargement of Rab5-positive early endosomes within the cell [74]. It would be of great interest to isolate these organelles and to profile the protein composition of these NGF-containing organelles by functional organelle proteomics, as successfully applied to identify endosomal EGF receptor signaling target [75].

Dr Huber and colleagues have developed the strategy of functional signaling endosome proteomics and they have made tremendous progress in identifying endosomal targets for the EGF receptor signaling pathway [75]. Employing subcellular fractionation, two-dimensional differential gel electrophoresis (2-D DIGE), fluorescence labeling of phosphoproteins, and MALDI-TOF/TOF mass spectrometry techniques, Dr Huber and colleagues have discovered that 23 phosphoproteins were regulated by EGF and were differentially associated with endosomal fractions. Certain protein molecules include Alix, myosin-9, myosin regulatory light chain, Trap1, moesin, cytokeratin 8, septins 2/11, R-Ras and CapZbeta that all are known to be involved in endosomal trafficking and cytoskeleton rearrangement [75].

V. Concluding remarks and future directions

Retrograde neurotrophic signaling plays a critical role in regulating survival and differentiation of specific populations of neurons in both the central nervous system and the peripheral nervous system. Increasing evidence suggests that signaling endosomes serve as an important source of retrograde signals. Many types of endocytic compartments have been proposed to fulfill the requirement of retrograde signaling, but identities and molecular components of these vesicles are far from clear. Significant efforts are needed to further characterize the trafficking and signaling properties of endosomes.

Until now, most of our knowledge about the composition of NGF signaling endosomes has been gathered by immunoblotting/immunostaining of subcellular compartment(s) with specific antibodies, thus is a labor-intensive and cumbersome process. In contrast, proteomic approaches offer a much more efficient and less biased means to gain a global view of signaling endosomes. However, several issues need to be addressed before such an approach is taken. One lies in the technical difficulties in generating endosomal fractions with sufficient purity that is suitable for proteomic analysis. A potential solution is to utilize fluorescence activated organelle sorting (FAOS) pioneered by Dr Huber and his colleagues [76]. In the case of NGF, PC12 cells can be transfected with GFP-Rab5 constructs followed by binding/internalization of QD605-NGF. Conventional density gradient centrifugation can be used to generate a crude endosomal fraction that is enriched for the GFP-Rab5 construct. The endosomal fraction is then subjected to high speed FAOS in a flow cytometer in sequentially. GFP-Rab5 labeled

endosomes would be first separated from non-labeled vesicles. Due to the extreme brightness and resistance to photobleaching of quantum dots, the purified GFP-Rab5 endosomes would be further sorted using the QD605 channel to collect the subpopulation of GFP-Rab5 endosomes that are also positive for QD605-NGF. Protein profiling GFP-Rab5/QD605-NGF endosomes could be then analyzed using standard proteomic techniques. Similar approaches can be applied to analyze other putative type of signaling endosomes. We can mark late endosomes with a GFP-Rab7 construct, lysosomes with LAMP1/LAMP2-GFP, macroendosomes with Pincher-GFP in PC12 cells. Organelles, positive for each of these constructs and for QD605-NGF, can be purified by two rounds of high speed FAOS for further analysis with proteomic techniques. It would be interesting to identify changes, if any, that takes place as the signaling endosomes mature within a cell. In addition, pretreatment of cells with specific inhibitors such as K252a [42] or Go6976 [22] for TrkA can be employed to decipher what role that specific target(s) play in the signaling endosomes. These studies will potentially facilitate identification of novel neurotrophin signaling molecules and pathways.

Protein phosphorylation by kinases is a critical regulatory mechanism of intracellular signaling in neurons [5]. Changes in the phosphorylation status of some kinase targets are associated with neurodegenerative disorders. For instance, Tau, the microtubule-binding protein, is hyperphosphorylated and present in neurofibrillary tangles (NFTs) that were found in the neurons of patients of Alzheimer's disease [77,78]. The kinases that phosphorylate Tau proteins include: glycogen synthase kinase, cyclin-dependant kinase-5, MEK, Erk-1/2, c-Jun NH(2)-terminal kinases, casein kinase, calcium calmodulin-dependant kinase II, microtubule affinity regulating kinase and protein kinase A (PKA/cAMP-dependant protein kinase) among others [77,78]. Therefore, proteomic analysis of all phosphoproteins i.e. phosphoproteome profiling [79], on signaling endosomes might be informative in directing efforts to define neurodegenerative disease mechanisms.

Other strategies include the enrichment of phosphorylated proteins or peptides by subcellular fractionation, immunoprecipitation or chromatography prior to 2-D DIGE analysis. In addition, several gel-free methods combining chromatography with highly sensitive MS have been successfully applied for the analysis of complex phosphoproteomes. Recently developed approaches like KESTREL or 'chemical genetics' and protein micro-arrays offer new possibilities for the identification of specific kinase targets. All these methodologies and other strategies are summarized in an excellent recent review [80]. With new tools in hand, "mapping" the molecule networks on signaling endosomes is now possible.

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A: Structure of a nerve cell

B: Different types of NGF signaling endosomes

Figure 1.

A. A schematic representation of a nerve cell (adapted from learn.genetics.utah.edu/.../neuron_labeled.jpg). A typical neuron possesses an extensive network of dendrites and axons. The axon is often insulated with the myelin sheaths that are interrupted by the nodes of Ranvier. The axon terminal innervates the target. The neurotrophic signal, released from the target, must be retrogradely transported to the cell body (soma). B: The different types of signaling endosomes. Neurotrophins such as NGF, are released from the target and bind to its Trk receptor at the axonal terminals. The ligand and receptor complex is internalized and is shuttled via the endocytic pathways. Endocytic compartments such as the early endosomes, MVBs and late endosomes have all been proposed to traffic the neurotrophic signal to the cell body.

Through a different internalization pathway, macroendosomes may also be formed to carry the neurotrophin signals retrogradely.