A specialized endosomal population mediates Trk, but not EGFR, retrograde signaling.

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Abstract of the Dissertation

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During development, target-derived neurotrophins promote the survival and phenotype of innervating neurons. Neurotrophins exert their effects by initiating tropomyocin receptor kinase (Trk) signaling cascades at nerve terminals that transmit gene expression changes to the soma. According to the widely accepted signaling endosome model, ligand-bound and activated Trk receptors are endocytosed and retrogradely transported to signal at the soma. However, sustained signaling endosomes are counterintuitive to the classic role of endocytosis in receptor down-regulation and so the mechanisms that dictate their formation and processing have been poorly understood and controversial.

I have developed a novel, multi-faceted approach toward elucidating mechanisms of Trk endosomal signaling. In this approach, Quantum dot-conjugated EGF bound to an EGFR/TrkB chimera or EGF-receptor is tracked by confocal and electron microscopy, along sympathetic axons grown in compartmentalized neuronal cultures that separate axons from soma.

Using this approach, I define the retrogradely transported axonal endosomes of Trk, but not EGFR, as immature multivesicular bodies that avoid lysosomal degradation.
These properties of Trk endosomes are dictated by unique molecular mechanisms that govern their formation and processing and which depend principally on the membrane trafficking protein Pincher. Trk, but not EGFR endosomes thereby mediate sustained Erk signaling to gene induction in soma. I further identified the Trk-binding adaptor protein Adaptor protein containing PH domain, PTB domain and Leucine zipper motif (APPL) as the critical component that links Trk to Pincher, and showed that disruption of the APPL/Pincher interaction causes rapid lysosomal degradation and eliminates long-term Trk endosomal signaling. I find that the early endosome processor, Rab5, and the Pincher-binding Rab5-effectors, Rabankyrin and Rabenosyn, are also involved in Trk endosomal processing, which is likely to involve a highly specific protein network mediating the unique features of Trk endosomes.

I further show the generality of Trk endosomal signaling in dendrites of hippocampal neurons. Given the essential role of the neurotrophin BDNF in hippocampal plasticity, Pincher-mediated Trk macroendocytosis may thus provide an underlying mechanism for neurotrophin-mediated synaptic plasticity in the central nervous system. An exciting possibility emanating from these studies is that a specialized Trk endosomal population provides the major signaling platform for a broad spectrum of neurotrophin-induced phenotypes.
DEDICATION

To my parents, Costas and Irene Philippides

And to my sister, Anna
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Chapter I

General Introduction

Neurotrophins (NTs) are a family of target-derived peptide growth factors initially identified as critical mediators of developing neuron survival in the peripheral nervous system (Levi-Montalcini and Hamburger, 1951). Since their discovery, NTs have been implicated in multiple functions including regulation of axonal and dendritic growth, axonal guidance, synapse formation, cell proliferation, cell migration and synaptic plasticity, both in the peripheral and central nervous system (for review see Zweifel et al., 2005). The neurotrophin family consists of Nerve Growth Factor (NGF), Brain Derived Neurotrophic factor (BDNF), Neurotrophin-3 (NT3) and Neurotrophin 4/5 (NT4/5). NTs exert their biological functions by binding to two types of transmembrane receptor, tropomyosin receptor kinase (Trk) and p75NTR. p75NTR indiscriminately binds all NTs with low affinity. Additionally, neurotrophins selectively bind Trk receptors with high affinity: NGF to TrkA, BDNF and NT4 to TrkB and NT3 to TrkC, although NT3 also binds the other Trk isoforms with lower affinity (Ryden and Ibanez, 1996).

Neurotrophin Signaling:

Despite the fact that p75NTR, a member of the tumor necrosis factor receptor superfamily, was the first NT receptor to be discovered (Johnson et al., 1986), its role in NT signaling is still a matter of debate. Described as a cell-death receptor, p75NTR mediates ligand induced apoptosis through activation of the JNK pathway, ceramide production and association with a number of cell-death inducing proteins such as NADE and N-RAGE (Miller and Kaplan, 2001). A recent study has suggested that p75NTR-mediated death eliminates a population of neurons during development, as part of a neuronal competition for survival mechanism (Deppmann et al., 2008). The proapoptotic effects of p75NTR manifest in the absence of Trk receptors while, paradoxically, in the presence of Trk the two receptors cooperatively promote cell survival (Hamanoue et al., 1999). This reflects the major complexity in deciphering p75NTR functions; its signaling
outcome is dramatically altered upon Trk receptor activation. P75NTR interaction with Trk results in the formation of a high affinity binding receptor complex (Hempstead et al., 1991) and p75NTR also interacts with Trk to alter Trk ligand specificity (Brennan et al., 1999; Davies et al., 1993). Based on the regulatory effects of p75NTR on Trk signaling, p75NTR was originally thought to act exclusively as a Trk modulator. Although Trk-independent roles of p75NTR, such as pro-neurotrophin binding and regulation of axonal growth, are beginning to emerge (Arevalo and Wu, 2006), Trk receptors are still thought to mediate the majority of NT classical biological actions.

Trk receptors are prototypical receptor tyrosine kinases, consisting of an extracellular domain containing two immunoglobulin-like domains that mediate ligand binding, a single transmembrane domain and a cytoplasmic region with a kinase domain (Kaplan et al., 1991). Ligand binding results in dimerization and trans-phosphorylation of the receptor, recruitment of a number of adaptors and effectors and activation of signaling cascades. Assembly of signaling complexes on Trk centers around two key tyrosine-containing motifs: the NPXY motif in the juxtamembrane region (corresponds to Y490 in TrkA) and the YLDIG motif in the carboxy terminus (corresponds to Y785 in TrkA) (Loeb et al., 1994). Phosphorylated Y490 interacts with shc (Stephens et al., 1994) or SNT (suc-associated neurotrophic factor-induced tyrosine phosphorylated target), also known as fibroblast growth receptor substrate 2 (FRS2) (Rabin et al., 1993), which lead to the activation of ras, rap and PI3 kinase (PI3K) while phosphorylation of Y785 creates a binding site for phospholipase Cγ (PLCγ) (Loeb et al., 1994). The signaling cascades emanating from these interactions are considered to be the classical Trk signaling pathways, however recent work has identified a number of Trk effectors (discussed below) that are likely to have important roles in mediating NT biological effects.

Binding of shc to phosphorylated Y490 leads to activation of the ras/MAPK signaling cascade that has been implicated in neuronal survival and differentiation. Briefly, shc recruits the adaptor Grb2 complexed with the ras exchange factor sos. Activated ras then leads to raf-mediated activation of MEK1/2 and phosphorylation of ERK1/2 (Patapoutian and Reichardt, 2001). Ras activation also triggers a raf-independent signaling cascade that results in activation of ERK5 through sequential activation of MEKK3 and MEK5 (Watson et al., 2001). ERK1/2 activation through the ras pathway
has been suggested to be transient. A distinct signaling cascade initiated by FRS2 binding to phosphorylated Y490 mediates sustained ERK1/2 activation in response to NT. In one proposed scenario, FRS2 provides a docking site for crk, which activates the guanyl nucleotide exchange factor C3G for rap1. Rap-1-GTP then stimulates B-raf leading to ERK1/2 prolonged activation (Meakin et al., 1999; York et al., 1998). Activation of ERK1/2 and ERK5 results in transcriptional responses via activation of multiple transcription factors, including ribosomal s6 kinase (rsk)-mediated activation of cAMP response element binding protein (CREB). Specificity of transcriptional responses is regulated qualitatively by activation of distinct transcription factors by ERK1/2 vs ERK5, temporally by the amount of time ERKs remain activated and also appears to be spatially regulated since the ERK5 and rap1 pathways are thought to be preferentially activated from endosomal membranes (Zhang et al., 2000).

Activation of ras also leads to direct activation of the PI3 kinase pathway, a major survival pathway induced by NT treatment. Additionally, the PI3 kinase pathway can be activated independently of ras, through Grb2 recruitment of the adaptor protein Gab1 (Holgado-Madruga et al., 1997). Phosphorylated Gab proteins recruit and facilitate the activation of class I PI3-kinases. PI3 kinase activity results in the production of P3-phosphorylated phosphoinositides and the activation of phosphatidylinositol-dependent protein kinase (PDK-1) that synergistically activate the protein kinase Akt. Akt mediates prosurvival effects by phosphorylation of substrates involved in cell survival pathways, such as NFκB-dependent transcription (Datta et al., 1999).

Binding of PLCγ to phosphorylated Y785 results in its activation and the subsequent hydrolysis of PtdIns(4, 5)P₂ to generate inositol tris-phosphate (IP₃) and diacylglycerol (DAG). DAG stimulates DAG-regulated protein kinase C isoforms while IP₃ promotes release of Ca²⁺ from internal stores resulting in activation of kinases, including Ca²⁺-regulated isoforms of protein kinase C and Ca²⁺-calmodulin-regulated protein kinases (Patapoutian and Reichardt, 2001). Activation of the PLCγ pathway selectively mediates the induction of the late genes peripherin and the peripheral nerve sodium channel Nav1.7 (also known as SCN9A or PN1) and has been implicated in neurite outgrowth (Choi et al., 2001, see fig.1 for a summary of these signaling cascades).
Although the above signaling cascades are the best studied and understood in regards to Trk signaling, they are by no means the only ones. A plethora of novel Trk interactors have been identified over the past years and their important roles have begun to emerge. A number of these effectors regulate NT-induced neurite outgrowth through either cytoskeletal rearrangements or crosstalk with the MAPK/ERK pathways. Among those that influence MAPK signaling are the adaptors rAPS, SH2-B (Qian and Ginty, 2001) and the DnaJ domain-containing protein tumorous imaginal disc 1 (TID1) (Liu et al., 2005) that bind to the kinase activation loop of Trk receptors, the csk homologous kinase (chk) that binds Y785 (Yamashita et al., 1999) and Fas apoptosis inhibitory molecule (FAIM) (Sole et al., 2004). Ras guanine-releasing factor 1 (rasGrf1), a guanine nucleotide exchange factor for members of the ras and rho family that binds the Trk HIKE domain (a candidate binding site for PH domain-containing proteins present in all three Trk receptors) (Robinson et al., 2005), the non-receptor tyrosine kinase abl that binds the Trk juxtamembrane region (Yano et al., 2000) and the Rho GTPase-activating protein (Grit) for Rho/Rac/Cdc42 GTPases that binds the C-terminus of Trk (Nakamura et al., 2002) all mediate neurite outgrowth in PC12 cells via cytoskeletal rearrangements during neuronal differentiation. Other direct Trk effectors include calmodulin, which binds to the C-terminal domain of TrkA in a Ca2+-dependent manner and regulates Trk intracellular processing (Llovera et al., 2004), SLAM-associated protein (SAP), which binds to the kinase activation loop of Trk receptors and may serve as a negative regulator of Trk receptor activation (Lo et al., 2005), the rhoGEF kalirin that increases Trk catalytic activity and activates Rac (Chakrabarti et al., 2005) and the nucleotide exchange factor dbs that is directly phosphorylated by TrkC and enhances schwann cell migration (Yamauchi et al., 2005). TrkB also interacts with ephrinA to regulate retinal axon branching and synaptogenesis (Marler et al., 2008).

Trk interacting partners that have been implicated in Trk endosomal signaling, recruitment to the endosomes or Trk trafficking are of particular interest. These include an ankyrin-rich membrane spanning protein (ARMS), the adaptors GAIP-interacting protein, C terminus (GIPC) and Adaptor protein containing PH domain, PTB domain and Leucine zipper motif (APPL), the atypical protein kinase C-interacting protein p62, the
dynein light chain, caveolin and the ubiquitin-ligase nedd4-2. The role of this particular group of proteins will be addressed in detail in a later section.

The multiplicity of signals emanating from Trk receptors is only the first layer of complexity of Trk signaling. Additional signaling branch points exist downstream of Trk, that contribute to the diversity of neurotrophin actions (D'Arcangelo and Halegoua, 1993). Perhaps more intriguing are the temporal and spatial regulation that have to be superimposed on these signaling cascades for the correct manifestation of neurotrophin-induced phenotypes. In vivo, target-secreted neurotrophins must elicit survival responses of innervating neurons that may have an axon up to 1m long. Therefore the fundamental question in NT biology has been uncovering the mechanisms that dictate long distance, retrograde NT signaling.

**Neurotrophin Retrograde Signaling:**

The neuronal architecture presents a challenge for the transmission of NT signals and makes apparent the need for a mechanism to transmit NT signals from the nerve terminal to the cell body. Many theories have been put forth to explain NT retrograde signaling, including (but not limited to) the wave model, the signaling effector model and the signaling endosome hypothesis.

Both the wave and the signaling effector models have been based on evidence that NGF covalently crosslinked to beads (thus unable to internalize) retrogradely supports neuronal survival of neurons grown in compartmentalized chamber cultures (MacInnis and Campenot, 2002). This raises the possibility that retrograde signals are transmitted independently of NGF/Trk transport to the cell body. According to the wave model NGF binds to and activates surface Trk at distal axonal terminals and Trk activation is then transmitted to the cell body in a “wave” fashion. Support for this model is derived from the observation that surface activation of the epidermal growth factor receptor (EGFR) induces a rapid lateral propagation of ligand-independent receptor activation in the plasma membrane (Verveer et al., 2000). This type of receptor activation has not, however, been extended to Trk receptors. The signaling effector model postulates that activation of Trk at the nerve terminals generates activated signaling complexes that are then transported to the cell body independently of Trk or ligand transport. In support of
this model, it was demonstrated that inhibiting Trk kinase activity at the cell body of neurons grown in compartmentalized chamber cultures by application of K252a does not block retrograde survival signaling (MacInnis et al., 2003). While both these models can be used to explain NT transport-independent propagation of retrograde signals they appear to be mutually exclusive, as the support for a Trk kinase-independent model contradicts the wave model. Furthermore, a different study demonstrated that Trk kinase activity in the cell body is required for retrograde survival of sympathetic neurons and that blocking Trk endocytosis with a dominant negative dynamin eliminates retrograde-signaled neuronal survival (Ye et al., 2003). This study points against both aforementioned models and strongly supports the signaling endosome hypothesis.

The signaling endosome hypothesis (Halegoua et al., 1991) has by far been the most verified model of retrograde NT signal propagation. According to this model, target-derived neurotrophins bind their respective Trk receptors at axon terminals, the ligand/receptor complexes are then internalized and transported to the cell body via microtubule-dependent axonal transport. Support for this model has been overwhelming. Early work in the field demonstrated that NGF itself is retrogradely transported in sympathetic and sensory neurons, both in vivo and in vitro. Injection of \(^{125}\text{I}-\text{NGF}\) into the anterior eye chamber led to accumulation of radioactivity in the cell bodies of postganglionic superior cervical ganglia (Hendry et al., 1974). Subsequent autoradiogram studies demonstrated that the neurotrophins BDNF and NT3 are also retrogradely transported along neuronal axons. When injected into a target field, \(^{125}\text{I}-\text{NGF}\), BDNF and NT3 are retrogradely transported by distinct innervating neuronal populations (DiStefano et al., 1992). Similarly, the retrograde transport of Trk receptors has also been demonstrated. Following ligation of the rat sciatic nerve, phosphorylated TrkA accumulates distal to the ligation site; this accumulation is enhanced by NGF injection in the footpad and abolished by anti-NGF administration (Ehlers et al., 1995). Downstream effectors of Trk signaling such as PI3K, ERK and MEK also accumulate distal to the ligation site (Johanson et al., 1995). More recently, coprecipitation studies showed that TrkA and TrkB receptors are retrogradely cotransported with their respective ligands (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999).
Further support for the signaling endosome model comes from experimental evidence linking the microtubule network/transport machinery to NT/Trk retrograde transport and signaling. Colchicine treatment, which blocks microtubule-assisted transport, reduced NGF immunoreactivity of basal forebrain cholinergic neurons (Conner and Varon, 1992). The light chain of cytoplasmic dynein binds TrkA, B and C (Yano et al., 2001) and inhibition of dynein-based transport resulted in block of NT dependent retrogradely signaled survival (Heerssen et al., 2004).

Furthermore, endocytosis is required for NT retrograde signaling. In one set of experiments, using neurons grown in compartmentalized chambers, NGF was crosslinked to beads that are unable to internalize and applied at distal axons. Unlike soluble NGF, this did not induce CREB phosphorylation at the cell body (Riccio et al., 1997). In a second set of experiments, expression of a dominant negative form of dynamin prevented ERK5 phosphorylation in response to NGF (Watson et al., 2001) and retrogradely-signaled neuronal survival (Ye et al., 2003). Despite the amount of evidence supporting the signaling endosome model, the molecular processes involved in the generation and processing of signaling endosomes are largely unknown. Fundamental questions that have not yet been conclusively answered involve the mode of endocytosis for NGF/Trk complexes, the interactions that recruit Trk to endosomal populations, the nature of the Trk endosome and the factors that influence Trk endosomal processing and lifetime. The generation of long-lasting signaling endosomes is counterintuitive to the classical role of receptor endocytosis as a means of receptor downregulation and signal attenuation. Therefore, specialized mechanisms must operate to generate these unconventional endosomes.

**Modes of receptor endocytosis:**

In neuronal (or the neuronal-like cell line PC12) cells, three major modes of endocytosis have been implicated in NT/Trk endocytosis:

*Clathrin-mediated endocytosis* is the classic mode of receptor tyrosine kinase endocytosis as exemplified by the epidermal growth factor receptor, EGFR (Beguinot et al., 1984). Clathrin-mediated endocytosis involves the concentration of receptor/ligand complexes in clathrin coated pits at the plasma membrane. Multiple adaptor proteins,
such as AP-2 and AP180, coordinate clathrin nucleation and subsequent clathrin polymerization into curved lattices at the plasma membrane. These events lead to deformation of the attached membrane that acquires increasing curvature until a deeply invaginated coated pit is formed. The large GTPase dynamin mediates the fission of these pits to release clathrin-coated vesicles (CCVs), which are about 100nm in diameter, into the cell interior. CCVs are then rapidly uncoated by auxilin and hsc70; uncoated vesicles are trafficked through early endosomes, where they are sorted and processed either for degradation or recycling through a series of events that involve, among others, the early endosomal protein Rab5 and its effectors EEA1 and Rabenosyn, the late endosomal protein Rab7 or the recycling protein Rab11 (reviewed in Doherty and McMahon, 2009; Le Roy and Wrana, 2005; Takei and Haucke, 2001). A complex protein network controls clathrin-mediated endocytosis and regulates cargo recruitment and sorting.

**Caveolae-mediated endocytosis** involves the protein caveolin and it occurs at plasma membrane microdomains, rich in cholesterol and sphingolipids. Caveolae are flask-shaped invaginations from which vesicles of about 50-100nm in diameter originate. They have been involved in cholesterol homeostasis and transport and have been implicated in the endocytosis of some GPI-anchored proteins (Kurzchalia and Parton, 1999). Caveolae are highly enriched in signaling molecules, hinting for a role of these organelles as signaling platforms.

**Macropinocytosis** involves massive membrane ruffling and actin reorganization at the plasma membrane and it leads to the formation of large, dynamic, heterogeneous vesicular structures, called macropinosomes. Growth factors have been shown to induce macropinocytosis and the small GTPase Rac is required for this process (Ridley et al., 1992). Traditionally, macropinocytosis has been associated with the non-specific uptake of extracellular solutes and plasma membrane (Swanson and Watts, 1995). Recently, however, a significant percentage of EGFRs in fibroblasts was shown to internalize via clathrin-independent, actin- and dynamin-dependent circular dorsal ruffles (waves). Endocytosis of EGFR in waves was ligand-induced and specific, since other receptors were not internalized in these structures (Orth et al., 2006). These findings indicate that macropinocytic-like mechanisms could mediate regulated and specific receptor endocytosis and be more complex than originally thought.
Which of these processes mediates the internalization of NT/Trk complexes is currently a subject of debate. Due to its role in the internalization of other tyrosine kinase receptors, such as EGFR, clathrin-mediated endocytosis was originally thought to be the best-suited for NT/Trk internalization. Immunocytochemical assays showed that NGF treatment of PC12 cells increases clathrin at the plasma membrane and enhances clathrin-mediated membrane trafficking (Beattie et al., 2000). It has also been shown that clathrin-coated vesicles isolated by subcellular fractionation contain TrkA/NGF complexes (Grimes et al., 1996), while other biochemical studies have pointed to Trk/NGF endosomes as being small vesicles distinct from clathrin-coated vesicles (Grimes et al., 1997). Treatment of dorsal root ganglia (DRG) axon terminals with monodansylcadaverine (MDC), a transglutaminase inhibitor thought to specifically block clathrin-mediated endocytosis, prevented BDNF-induced TrkB internalization (Watson et al., 2001; Zheng et al., 2008). However, transglutaminases are cross-linking enzymes that are involved in other processes as well, such as cell adhesion (Greenberg et al., 1991; Menter et al., 1991). MDC also interacts with autophagic vacuole membrane lipids and labels autophagosomes in vivo (Niemann et al., 2000). Therefore, transglutaminase inhibition with MDC is hardly a specific inhibition of clathrin-mediated endocytosis. Another manipulation that has been used in support of a clathrin-dependent mechanism for Trk endocytosis has been the blocking of endocytosis by dominant negative forms of dynamin (Zheng et al., 2008). However, it has recently become clear that dynamin is involved in clathrin-independent endocytosis as well. It appears that the evidence in support of clathrin-mediated Trk endocytosis is outdated; while many experimental manipulations were once perceived as specifically disrupting clathrin-mediated endocytosis, an increased understanding of clathrin-independent mechanisms demonstrates that, in fact, that is not the case (Sandvig et al., 2008). Given the fact that clathrin-mediated endocytosis of receptors results in rapid degradation or recycling, it would not appear to be a suitable process for generating long lasting, retrogradely transported signaling endosomes.

Other lines of evidence indicate that Trk/NGF complexes are internalized through a caveolin-mediated mechanism. In support of this, TrkA and caveolin colocalize in caveolae microdomains, isolated caveolae fractions contain active TrkA associated with
she and PLCγ (Huang et al., 1999; Peiro et al., 2000) and TrkA coimmunoprecipitates with caveolin (Bilderback et al., 1999). The above studies were performed in PC12 cells but the role of caveolin in neurons is unclear. Whether caveolin is expressed in the nervous system is currently a subject of debate. Some studies were unable to detect caveolin mRNA in the brain (Oka et al., 1997), while in others caveolin was found to be expressed in isolated hippocampal nerve terminals (synaptosomes) (Braun and Madison, 2000), brain astrocytes (Cameron et al., 1997) and in Dorsal Root Ganglion neurons (DRGs) (Galbiati et al., 1998). The unambiguous expression of caveolin in neuronal populations must be established before assigning a role to this mechanism in NT retrograde signaling. Intriguingly, it has been proposed that the vast majority of caveolae remain surface-bound and tethered to the membrane (Sandvig et al., 2008). If this is in fact the case, caveolae-mediated generation of signaling endosomes is highly unlikely. Instead, caveolae could serve as a cell surface platform to augment signaling from the cell-surface or as a nucleating membrain domain to concentrate cargo for other endocytic routes.

Support for a role of macropinocytosis in Trk internalization comes from early observations that NT treatment induces membrane ruffling, both in PC12 cells and in sympathetic neurons (Connolly et al., 1981; Connolly et al., 1979). Inhibition of PI3 kinase activity, an upstream activator of Rac, by wortmannin blocks Trk internalization in PC12 cells and neurons (York et al., 2000). Also, GFP-TrkA is found in large, retrogradely moving, globular vesicles together with dextran, a marker for macropinosomes, in the axons of DRG neurons (Nakata et al., 1998). Recent evidence from our lab has implicated a novel, micropinocytic-like mechanism, in the endocytosis and retrograde transport of NT/Trk complexes. We have termed this process macroendocytosis because, while it shows mechanistic similarities to macropinocytosis such as membrane ruffling and actin reorganization, it is specific and receptor-mediated. This process is dependent on the function of the trafficking protein Pincher (Shao et al., 2002; Valdez et al., 2005), a member of the eps15 homology domain (EHD) protein family.
**EHD proteins:**

Mammalian cells express four highly homologous EHD isoforms. Despite their high similarity, the four members have distinct functions and subcellular distributions (George et al., 2007). EHD1 and EHD3 have the highest degree of similarity and play a role in the recycling of a number of receptors, including transferrin, major histocompatibility complex class I and GLUT4. EHD2, the least similar paralog, plays a role both in clathrin-mediated endocytosis of GLUT4 and transferrin and a similar role to EHD1 and EHD3 as a regulator of recycling (Grant and Caplan, 2008). Our own data (discussed in the next section) strongly support that Pincher exhibits the most divergent function of the group by mediating a specialized pathway involved in NT-induced Trk endocytosis. A recent study has implicated Pincher in early endosomal transport by demonstrating colocalization of Pincher with the Rab5 binding partner early endosomal antigen 1 (EEA1) and demonstrating the enlargement of GTP-Rab5 containing early endosomes upon Pincher depletion by RNAi (Sharma et al., 2008). However, a different study did not find any colocalization of Pincher with EEA1. Interestingly, the same study showed that out of the four family members, Pincher is the only one that does not colocalize with Rab11 or transferrin, both markers of recycling endosomes, supporting a distinct role for Pincher from that of the other family members (Blume et al., 2007).

EHD proteins consist of a nucleotide-binding region at the N-terminus, followed by a coiled-coil region and the EH domain at the C-terminus (Naslavsky and Caplan, 2005) (Fig. 2A). While the nucleotide-binding region closely resembles the G-domain of dynamin, it binds to adenine nucleotides, rendering EHD proteins ATPases (Daumke et al., 2007; Lee et al., 2005). The structure of EHD2 bound to a non-hydrolysable ATP analogue was recently solved, providing new insights to EHD function (Fig. 2B). EHD proteins were shown to share many features with the dynamin superfamily, such as low affinity for nucleotides, the ability to tubulate liposomes in vitro, oligomerization around lipid tubules in ring-like structures and lipid binding-induced nucleotide hydrolysis (Daumke et al., 2007). This analysis reveals new potentials for EHD proteins. Their ability for membrane scission could mediate their involvement in dynamin-independent endocytosis. In accordance to this, Pincher is involved in dynamin-independent endocytosis of Nogo (A. Hatkic, personal communication).
EHD proteins oligomerize through their G domain and bind other proteins through their EH domain. EH domains bind to proteins containing the tripeptide NPF and so far all EHD protein binding partners identified contain at least one NPF motif. It has been suggested that the electrostatic surface potential at EH domains may regulate binding specificity to different NPF-containing proteins, depending on the residues surrounding the NPF motif (Grant and Caplan, 2008). This hypothesis can explain specificity of protein binding to EHD proteins as opposed to eps15 but cannot differentiate between EHD family members. Additional work needs to be done to address binding specificity within the EHD protein family. The vast majority of EHD binding proteins identified, such as syndapin, Rab11-FIP2 and Rabenosyn-5 are involved, not surprisingly, in endocytic trafficking and recycling. A yeast two-hybrid assay carried out in our lab identified a number of Pincher-binding proteins. Among them, Rabenosyn-5 and Rabankyrin-5 are also Rab5-binding partners (G. Valdez and S. Halegoua, unpublished). These dual interactors may serve to coordinate Pincher and the Rab5 endocytic machinery on Trk signaling endosomes and Rab5 may also play a role in Pincher-dependent Trk endocytosis.

**Pincher-dependent Trk macroendocytosis and retrograde transport:**

Recent research from our lab implicates Pincher in NT endocytosis and retrograde transport. Pincher was originally identified as an NGF-inducible protein in PC12 cells. Overexpression of Pincher enhances NGF-induced TrkA endocytosis. A single amino acid mutation in the ATP-binding P-loop results in a dominant-negative form of Pincher (pinG68E) that blocks TrkA internalization (Fig. 2C). Pincher associates with extensive ruffles at the cell surface that are internalized to form macroendosomes. In neurons, Pincher also mediates Trk endocytosis and expression of pinG68E eliminates retrogradely-signaled neuronal survival (Shao et al., 2002; Valdez et al., 2005).

This data strongly support a role for Pincher in retrograde endosomal signaling. However, the exact role of Pincher is not clear. While Pincher activity is required for the generation of Trk endosomes, it is not clear if it is needed for subsequent endocytic processing. An interesting observation is that in PC12 cells, Pincher recycles from endocytosed Trk and returns to the cell surface, while it remains associated with Trk.
endosomes along axonal projections in sympathetic neurons. This suggests an important role for Pincher in subsequent endosomal processing events along the axons. How Pincher is recruited to Trk endosomes and how it affects endosomal processing are currently unresolved questions and will be addressed in this thesis.

**Endosomal processing:**

Receptor downstream endocytic processing for clathrin-mediated endocytosis has been extensively studied. Following endocytosis, ligand-bound receptors are directed to two major destinations: the plasma membrane through recycling pathways, as exemplified by transferrin receptors or the lysosome where they are degraded, as seen for EGFR. Delivery of endocytosed material into their target compartment requires the formation of mobile vesicles, their tethering to target membranes and their subsequent fusion with the target organelle. The early endosome serves as the sorting station for cells; from there receptors are sorted either to recycling or to late endosomes. Receptor sorting to different intracellular compartments is dictated by trafficking motifs and post-translational modifications such as ubiquitination and phosphorylation. The addition of ubiquitin molecules marks receptors for entry into the late endosomal/lysosomal pathway. The presence of amino acid sequences, such as the NPxY motif, targets receptors to recycling endosomes (Seaman, 2008).

Endosomal processing is mediated by the small Rab GTPases and their binding effectors. Rab GTPases cycle between a GTP-bound active state (controlled by guanine nucleotide exchange factors-GEFs) and a GDP-bound inactive state (regulated by GTPase activating proteins-GAPs). When bound to GTP, Rab proteins are inserted into endosomal membranes and can bind their effectors. Different Rab proteins bind membranes of endosomes at different stages. Rab5 acts at the early endosome to recruit effectors such as EEA1 and Rabenosyn that subsequently regulate different aspects of endosomal processing, such as vesicle tethering and membrane fusion. Rab4 and Rab11 are found on the membrane of recycling tubulovesicular structures and Rab7 and Rab9 mediate late endosomal processing at the multivesicular body stage (Grosshans et al., 2006).
Ubiquitin is the major signal for directing receptors to the multivesicular body/lysosome pathway. It recruits the *endosomal sorting complexes required for transport* (ESCRT) proteins that organize the progression of ubiquitinated cargo into the intralumenal vesicles (ILVs) of multivesicular bodies. In mammalian cells there are four ESCRT protein complexes (0-III) that act sequentially. Initially ESCRT 0, consisting of hrs and STAM, binds ubiquitinated receptors, PtdIns3P—a lipid abundant on early endosomes—and clathrin to localize cargo destined for degradation in limiting membrane microdomains. ESCRT III, the final component in the pathway, mediates deubiquitination of receptors before their incorporation into ILVs. Disrupting the ESCRT pathway by deleting or mutating proteins of these complexes results in defects in receptor degradation, as seen for EGFR (Williams and Urbe, 2007). It is interesting that clathrin acts as an organizer of the ESCRT pathway. Receptors undergoing clathrin-independent endocytosis may interact with the ESCRT pathway in a different manner. One possibility would be that without clathrin, endosomal membranes might have less affinity for ESCRT complexes, ESCRT assembly would be delayed and, as a result, so would receptor degradation. Also, Rab5 acts through its effectors to increase PtdIns3P at early endosomes, which are necessary for ESCRT 0 recruitment. Receptor downregulation cannot be completed without recruitment of de-ubiquitinating enzymes by the ESCRT III complex, which does not bind PtdIns3P but PtdIns(3,5)P2, suggesting that cargo degradation, ESCRT recruitment and Rab protein membrane specificity are tightly coupled. Changing the temporal profile of Rab membrane association (for example delaying the Rab5 to Rab7 conversion on late endosomes) could potentially delay the progression of receptors to different ESCRT complexes and, subsequently, receptor degradation. It appears that changing the temporal dynamics of both ESCRT and Rab progression would result in a delay in receptor degradation.

**Determinants of Trk trafficking and processing:**

How Trk endocytosis and processing are regulated is poorly understood. Besides the fact that surface receptors are internalized upon ligand stimulation, there hasn’t been a consensus about much else. Conceptually, in order for signaling endosomes to be retrogradely trafficked, they must avoid recycling and degradation pathways and be
trafficked in a way that sustains their signaling potential. Nevertheless, there have been
reports of Trk trafficking to both the recycling (Chen et al., 2005) and proteosomal
(Geetha and Wooten, 2008) pathways. And while a number of reports agree that Trk
receptors eventually end up in lysosomes, there are discrepancies in the reported lifetimes
of the receptors, from 35 min (Jullien et al., 2002) to more than two hours (Zapf-Colby
and Olefsky, 1998).

The Trk residues and modifications that regulate endocytic trafficking and
processing are a matter of debate. The kinase activity of the receptor was shown to be
required for retrograde endosomal transport (Heerssen et al., 2004). To the contrary, a
different study showed that truncated mutants lacking kinase activity were still able to
internalize in cells lacking endogenous Trk receptors, in fact more efficiently than wild
type Trk. This same study showed that deletion mutants lacking almost the entirety of
their intracellular domain, excluding the juxtamembrane region and an adjacent region
(amino acids 477-565), show no defect in endocytosis and proposed that receptor
dimerization, without kinase activity, is sufficient for inducing internalization (Jullien et
al., 2003). Recently, a point mutant at Tyr701 was shown to slow the internalization rate
of Trk, presumably through disruption of the endocytic motif YRKF (de Pablo et al.,
2008).

The role of ubiquitination in Trk trafficking has been very controversial. One
study suggests that TrkA (but not TrkB) is multi-monoubiquitinated by the ubiquitin
ligase nedd4-2 and this ubiquitination event requires both the kinase activity of the
receptor and Tyr785. Nedd4-2 binds TrkA directly and constitutively at the PPXY785
motif and is phosphorylated upon NGF treatment. This group found that disrupting Trk
ubiquitination by knocking down nedd4-2 with RNAi has no effect on endocytosis but
decreases Trk degradation, indicating that ubiquitination plays a role in later events such
as lysosomal targeting but not early events such as endosome generation (Arevalo et al.,
2006b). A different study found that Trk is polyubiquitinated at Lys485 and that mutation
of that site blocks receptor endocytosis, implicating ubiquitin at early endocytic steps. In
this scenario, ubiquitination is mediated by the TRAF-6 ubiquitin ligase, complexed with
p75NTR and the Trk interactor p62 and p75NTR enhances ubiquitination (Geetha et al.,
2005). Contrary to this, p75NTR was found to reduce Trk ubiquitination and delay Trk
endocytosis and degradation (Makkerh et al., 2005). More work needs to be done to address the above conflicting results.

Several adaptor proteins have also been reported to play a role in Trk endocytosis, endocytic signaling or trafficking:

Ankyrin Rich Membrane Spanning protein (ARMS): ARMS binds the Trk transmembrane region and provides a docking site for the crkL-C3G-rap1 pathway that leads to sustained ERK activation. ARMS colocalizes with TrkA at early endosomes and mediates Rap1 activation but it is not clear if this function depends on its endosomal association or whether ARMS can affect endosome biogenesis or processing (Arevalo et al., 2006a; Arevalo et al., 2004).

Endophilin B1: Endophilin B1 interacts with TrkA and the early endosomal marker EEA1. Endophilin B1 knockdown by RNAi results in enlargement of early endosomes and increased Trk degradation, implicating endophilin in the Trk trafficking pathway. The mechanisms of endophilin action still remain to be elucidated (Wan et al., 2008).

GIPC and APPL: The adaptor proteins GIPC and APPL both interact with the Trk juxtamembrane region and each other and are localized on Trk endosomes. APPL also interacts with Rab5 and has been proposed to couple trafficking and signaling to the nucleus in the case of EGFR (Miaczynska et al., 2004). Trk, APPL and GIPC are enriched in endosomal fractions and knockdown of either GIPC or APPL attenuates NGF-induced MAPK signaling and neurite outgrowth (Lin et al., 2006; Lou et al., 2002; Varsano et al., 2006). In this thesis, I have examined the role of APPL in Pincher-mediated macroendocytosis (chapter IV).

Although phosphorylation and ubiquitination of Trk receptors are very likely to play a role in their endosomal localization and signaling, a consensus has yet to be reached about the critical residues/proteins in these processes. A network of proteins that may mediate endocytosis and trafficking has also begun to emerge but the underlying mechanisms are still unclear. While Trk endocytosis may be mediated by a unique pathway in order to generate long lasting signaling endosomes there also exists an interplay with common endocytic machinery. For example both the early endosomal marker Rab5 and the late endosomal marker Rab7 have been implicated in Trk
endocytosis, endocytic signaling and trafficking (Deinhardt et al., 2006; Liu et al., 2007; Saxena et al., 2005). While the presence of these markers on Trk endosomes may indicate that Trk receptors follow canonical endocytic processing from early endosomes to late endosomes and then lysosomes, the specialized role of these endosomes seems to indicate otherwise. An appealing possibility is that while Trk receptors utilize the common endocytic machinery nucleated around Rab5 and Rab7, they can uniquely modify these pathways to generate distinct signaling endosomes. Interestingly, Pincher interacts with a number of Rab5 binding partners, raising the possibility that it can interfere with the regular Rab5 protein network and alter the properties of the Trk endosomes (see chapter III).

Trk signaling endosomes provide a unique platform for mediating NT long-distance and long-term signals. In this thesis, I demonstrate that Pincher-mediated macroendocytosis is indispensable for generating long lasting Trk signaling endosomes. The properties of these endosomes compared to EGFR endosomes were examined as well as the mechanism of recruiting Pincher to Trk endosomes. Finally I examined the nature and function of retrogradely Trk signaling endosomes in sympathetic neurons grown in compartmentalized cultures and also demonstrated that these endosomes can be found in dendrites of hippocampal neurons as well, pointing to a more global role of the Trk signaling endosome.
Figure 1. Schematic representation of signaling cascades activated by neurotrophins through Trk receptors.
Upon ligand binding, Trk receptors homodimerize and autophosphorylate at multiple tyrosine residues to activate signaling cascades. The two major phosphorylation sites are Tyr490 and Tyr785, which recruit signaling effectors such as shc, FRS2 and PLC\(\gamma\) to initiate signaling cascades and elicit NT-dependent phenotypes.
Figure 2. Pincher, a member of the EHD family, is involved in Trk macroendocytosis.

(A) Domain structure of Pincher. Pincher, like the other EHDs, contains an ATP binding site and an EH domain. A single-point mutation of Pincher in the ATP binding loop (G68E), results in a dominant-negative protein that stays bound to the cell surface (Shao et al., 2002).

(B) Structure of the Pincher-family member EHD2 (reproduced from Daumke et al., 2007).

(C) Pincher involvement in various stages of Trk macroendocytosis in PC12 cells. Initially, Pincher and Trk co-localize at the cell surface, are endocytosed together and eventually Pincher recycles back to the cell surface (reproduced from Shao et al., 2002).
Chapter II

Materials and Methods

Cell Culture

Superior Cervical Ganglia (SCG) and hippocampi were dissected from postnatal day 1 (P1) and embryonic day 18 (E18) Sprague Dawley rats, respectively. SCG neurons were dissociated, plated onto collagen-coated tissue culture dishes and maintained in high glucose Dulbecco’s Modified Eagles (DME) medium supplemented with 10% fetal bovine serum and 100ng/ml NGF, as previously described (Geetha and Wooten, 2003; Mains and Patterson, 1973; Ye et al., 2003). Compartmentalized Campenot chambers (CAMP 10-Tyler Research Corporation) were set up and maintained as previously described (Campenot, 1977). Hippocampi were dissociated, plated onto poly-d-lysine-coated ACLAR pieces (Ted Pella) and maintained in Neurobasal medium supplemented with B27, glutamax and glutamate (for the first four days in culture) as previously described (Matsuoka et al., 1998). PC12 cells (Greene and Tischler, 1976), TrkA-PC12 (Hempstead et al., 1992), EGFR-PC12 (Yoon et al., 1997) and dynaminG273D-PC12 (Zhang et al., 2000) cell lines were grown on tissue culture dishes or poly-L-lysine- and laminin-coated glass coverslips in Dulbecco’s Modified Eagle’s (DME) medium supplemented with 5% fetal bovine serum (FBS) and 10% horse serum (HS). HEK293 cells were grown on tissue culture dishes and maintained in DME supplemented with 10% FBS.

Growth factors and growth factor-fluorescent conjugates

Recombinant human EGF, EGF-biotin, streptavidin-conjugated Quantum dot 525 and streptavidin-conjugated Quantum dot 605 were purchased from Invitrogen. Streptavidin-conjugated fluoronanogold was from Nanoprobes. EGF biotin was coupled to either streptavidin- conjugated Quantum dot or fluoronanogold as previously described (Lidke et al., 2004). Briefly, biotinylated EGF and streptavidin quantum dot/ fluoronanogold were mixed at 6:1 molar ratio for at least 30 min at 4°C and then passed through a Biospin 30 Tris column (Biorad) that had been prewashed with 1 % BSA in
PBS. The resulting complex was used at 80ng/ml (active EGF concentration) for
treatment at distal axons and 20ng/ml for treatment at cell bodies. NGF, EGF and BDNF
were used at 50ng/ml unless otherwise stated.

**Recombinant cDNA Constructs**

The following cDNA constructs were used as previously described: Pincher-HA,
PincherΔEH-HA and PincherG68E-HA (Shao et al., 2002), Rat TrkA, EGFR/TrkAJM (a
chimeric receptor consisting of the EGFR extracellular and intracellular domains and the
TrkA juxtamembrane domain (JM chimera)(from M. Chao, New York University),
EGFR-GFP (from A. Sorkin, University of Colorado at Denver), EGFR, EGFR/TrkB, a
chimera containing the EGFR extracellular domain and TrkA juxtamembrane and
intracellular regions (E/TrkB), E/TrkBΔBY15A and E/TrkBΔKFG (from M. Goldfarb,
Hunter College), Rab7-GFP (from B. van Deurs, University of Copenhagen), GFP-PH
(from S. Grinstein, University of Toronto), Rac1(V12)-GFP (from M. A. Schwartz,
University of Virginia), eps15Δ(95–295)-GFP (from P. P. Di Fiore, Instituto Firc di
Oncologia Molecolare, Milan, Italy), EEA1-GFP and Rab11-GFP (from S. Corvera,
University of Massachusetts Medical School), dynamin2(K44A)-GFP (from M. A.
McNiven, Mayo Clinic, Rochester, NY), Rab5(S34N)-GFP (from P. Stahl, Washington
University, St. Louis, MO), NcoR-Flag (from G. Mandel-Vollum Institute), Rabenosyn-
Flag (from S. Caplan, University of Nebraska Medical Center), pGEX-EH4 (from J.
McGlade, Hospital for Sick Children, Toronto), APPL-YFP (from D. Kaplan, Hospital
for Sick Children, Toronto) and Rac1-T7, Rac1(N17)-T7 and Rab5-GFP (from D. Bar-
Sagi, New York University). We generated the following constructs:

(1) pCMV-myc-Rabenosyn (encoding myc-Rabenosyn). PC12 cells mRNA were
reversed transcribed and the cDNA was PCR amplified with Rabenosyn-specific primers
(Forward Primer = ATCGAATTCCATGGCATCTTTGGATGACG and Reverse Primer =
AGTTTCTGCCGCTGTCCGAGTNTAGG) to generate full-length Rabenosyn. Rabenosyn
was then cloned into the EcoR I/Sal I sites of the pCMV-myc vector in frame with the
myc-epitope.

(2) pEGFP-C1/ Ankhzn/Rabankyrin (encoding Ankhzn/Rabankyrin -GFP). Mouse
ankhzn (Kuriyama et al., 2000) was PCR amplified (Forward Primer =
GGGAATTCCATGGCGGAAGAGGAGGTG and Reverse Primer = GGCCCCCGGGAGAGACTCCACCCAGAGT) and cloned into the EcoRI/XmaI sites of the pEGFP/C1 vector in frame with GFP.

(3) pShuttle/T7-RacN17 (encoding T7-Rac N17). Human T7-Rac N17 was PCR amplified (Forward primer= GCGTATGTCGACTAGGATGGCATCGATG and Reverse Primer= CAATCAAGCGGCCGCAAACCTCACCC ) and cloned into the SalI and NotI sites of the pShuttle vector.

(4) pCGTVIP16DC-eps15Δ95-295 (encoding T7-eps15Δ95-295, missing the EH domains). Human eps15Δ95-295 (Benmerah et al., 1999) was PCR amplified (Forward Primer = GCTCTAGAATGGCTGCGGCAGCCCAG and Reverse Primer = CGGGATCCTCTAGCTTCTGAGATATCAG) and cloned into the Xba/BamHI sites of the pCGTVIP16DC vector in frame with the T7-epitope tag.

(5) pShuttle/T7-eps15Δ95-295 (encoding T7-Eps15Δ95-295). T7-eps15Δ95-295 was PCR amplified (Forward primer= GCGTATGTCGACTAGGATGGCATCGATG and Reverse Primer: CAATCAAGCGGCCGCAAACCTCACCC) and cloned into the SalI and NotI sites of the pShuttle vector.

(6) pEYFP-N1/TrkA (encoding TrkA-YFP). Base pairs 1-2200 of rat TrkA-CMV5 were excised from the CMV5 vector with EcoRI and KpnI and ligated to the corresponding sites of pEYFP/N1. Base pairs 1978-2400 were PCR amplified (Forward Primer = GGTATGTTGTCATCCATAGCCAGCCC and Reverse Primer = CATCCCGGGAGCAGGAACTCCAGGTA) and cloned into the SpeI site in the TrkA coding region and the Xma site of the pEYFP/N1 vector.

(7) pShuttle/EGFR-GFP (encoding EGFR-GFP). Human GFP-EGFR was PCR amplified (Forward Primer=GGGTCGACATGCGACCCCTCAGGCAGCG and Reverse Primer = CCTCTACAAATGTGTTGATGGCTTG) and cloned into the SalI/NotI sites of the pShuttle vector.

(8) pShuttle/EGFR (encoding EGFR): Human EGFR was PCR amplified (Forward Primer= GGATCGACATGCGACCCCTCAGGCAGCG and Reverse Primer= GCTCTAGATCATGCTCCAATATCC) and cloned into the SalI/XbaI sites of the pShuttle vector.

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(9) pShuttle/Rab5-GFP. Human Rab5-GFP was cloned into the NotI site of the pShuttle vector.

(10) pShuttle/Rab7-GFP. Human Rab7-GFP was cloned into the NotI site of the pShuttle vector.

(11) pShuttle/RacV12-GFP. RacV12-GFP was isolated from the pEGFP-C1 vector with NheI and SmaI and ligated into the XbaI/EcoRV sites of the pShuttle vector.

(12) APPLΔNPF-YFP. The internal APPL fragment containing the NPF site was excised with XbaI/ApaI and replaced with the corresponding sequence of APPLΔNPF.

(13) PshuttleAPPLΔNPF-YFP. APPLΔNPF-YFP was cloned into the KpnI site of pShuttle.

(14) PshuttleAPPLWT-YFP. APPL-YFP was cloned into the XbaI site of pShuttle.

(15) PshuttleAPPLΔNPF-myc. APPLΔNPF-myc was cloned into the NotI and XbaI sites of pShuttle.

(16) pHSVPrPUC PinG68E (encoding PincherG68E-HA). PincherG68E-HA was subcloned into the XbaI/KpnI sites of pHSVPrPUC.

**Recombinant Adenoviruses**

Defective recombinant adenoviruses containing T7-RacN17, T7-eps15Δ95-295, EGFR, E/TrkB, EGFR-GFP, Rab5-GFP, Rab7-GFP, RacV12-GFP, APPL-YFP, APPLΔNPF-YFP, APPLΔNPF-myc were created using the Ad-Easy system (Stratagene). Recombinant adenoviruses containing GFP (Vaillant et al., 1999), TrkB-GFP (Watson et al., 1999), dynaminK44A (Ye et al., 2003) and HA-Pincher and HA- PincherG68E (Valdez et al., 2005) have been described.

Herpes Simplex virus containing PincherG68E was packaged into helper virus-free amplicon particles by the W. Bowers lab (University of Rochester) as previously described (Halterman et al., 2006).

**Transfections and Viral Infections**

Transient transfection with Lipofectamine 2000 (Lifetechnology) was used to introduce constructs into PC12 cells, HEK293 cells and hippocampal neurons. 24 hours later, PC12 cells were replated to poly-L-lysine- and laminin- coated coverslips. Prior to
treatments, PC12 cells were starved in 1% horse media for at least 12 hours. Hippocampal neurons grown on poly-D-lysine-coated ACLAR were transfected after 7 days in culture. For biochemical analyses by Western blot, the adenoviruses were titered and cultures were infected to obtain virally encoded gene expression in 70-80% of the cells. For immunofluorescence studies, 30-40% of the neurons were infected.

**Western Blot Analysis**

Proteins were extracted from PC12 cells using RIPA buffer (150mM NaCl, 10mM NaPhos, 2mM EDTA, 50mM NaF, 10mM Na pyrophosphate, 1mM Na3VO4, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate), diluted in 2x sample buffer (2% SDS, 80mM Tris-HCl pH 6.8, 10% glycerol, 10% β-mercaptoethanol, 2ug/ml bromophenol blue) and analyzed by western blot. The following antibodies were used to probe western blots: rabbit anti-TrkA (1:500; Chemicon), mouse anti-Flag-M5 (1:1000; Sigma-Aldrich), mouse anti-GFP (1: 400; Molecular Probes), rabbit anti-phospho-ERK1/2 specific antibody (1:500 Cell Signaling). Anti-mouse or anti-rabbit horseradish peroxidase linked secondary antibodies (1:2000, Amersham) were used and blots were developed using a chemilumminesence reagent (Perkin Elmer) and a Konica SRX-101A developer. Alternatively, anti-rabbit or anti-mouse Alexa680 secondary antibody was used (1:5000, Invitrogen) and blots were developed using an Odyssey Infrared Imaging System (Version 2.1, Licor). Films were analyzed using a Super CoolScan9000ED film scanner (Nikon) and quantitation of scanned images was done using ImageJ (NIH).

**In vitro binding assays**

BL21 bacterial cells were transformed with pGEX-EH$_4$ (a construct containing the EH domain of Pincher fused to glutathione-S-transferase (GST) or His-APPL WT or His-APPL-ΔNPF and inoculated in Terrific Broth (1.2% tryptone, 2.4% yeast extract and 0.4% glycerol) until reaching an OD600 of 0.5. Cells were then induced with 1mM Isopropyl-β-D-thio-galactoside (IPTG) for 4hrs and then centrifuged at low speed. Bacterial pellets were then resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 0.1M NaF, 1% Triton X-100, 10% glycerol, 1mM EGTA, 2 µg/ml aprotinin and 1 µg/ml leupeptin), sonicated and added to
pre-equilibrated glutathione-sepharose beads (Amersham Pharmacia) or a pre-equilibrated nickel nitriloacetic acid (Ni-NTA) agarose resin (Novagen) for 90 min at 4°C. The beads were then washed with lysis buffer three times and resin-bound protein concentration was determined by comparison to BSA standards on a Coomassie gel. 1-5ug of resin-bound protein was incubated with 100ug of HEK293 lysates transfected with the corresponding constructs for each experiment (see text and figure legends) for 2hrs at 4°C. The beads were then washed three times with lysis buffer, resuspended in 2x sample buffer, spun down and the supernatant was resolved with SDS-PAGE and analyzed by Western Blot.

**Coimmunoprecipitation assays**

HEK293 cells were co-transfected with Pincher-HA and either APPL-YFP or EEA1-GFP or co-transfected with PincherΔEH-HA (Pincher lacking the EH domain) and APPL-YFP. 48 hrs post-transfection cells were lysed with RIPA buffer and Pincher was immunoprecipitated from 500ug of cell lysate for 2hrs at 4°C using 5ug of anti-HA antibody coupled to proteinA beads. The beads were then washed three times with lysis buffer, resuspended in 2x sample buffer, spun down and the supernatant was resolved with SDS-PAGE and analyzed by Western Blot.

**Receptor Internalization and Degradation Assays**

Internalization of cell surface TrkA was assessed using surface receptor biotinylation. PC12 cells grown on poly-L-lysine coated dishes were either infected with a GFP control virus or increasing concentrations of RacN17-T7 virus. 72 hrs later cells were cooled to 4°C and biotinylated on ice (Pierce; 1.5 mg/mL EZ-Link NHS-LC-Biotin in PBS) for 20 minutes to detect total TrkA surface levels before NGF treatment. Unreacted biotin was removed in the cold using PBS containing 50mg/ml glycine. To measure surface TrkA remaining after NGF-induced internalization, the cells were NGF-treated for 30 minutes prior to cell surface biotinylation. Cell surface biotin-TrkA was measured as described below.

For receptor degradation measurements, PC12 cells were serum starved for 24 hours. The cells were biotinylated as described above and, then, shifted to 37°C for the
indicated time points (see figure legends) in the presence of NGF (50 ng/mL) or EGF (50 ng/mL) to allow endocytosis and degradation of TrkA and EGFR respectively. Proteins were extracted using RIPA buffer. For the APPL degradation experiments in chapter IV, cells were infected with either a GFP control virus or APPL-WT-GFP virus or APPL\_NPF-GFP 60 hrs prior to biotinylation. Biotinylated receptors were immunoprecipitated for 2 hours with neutravidin-coupled beads using equal amounts of protein from different samples. After immunoprecipitation, the beads were washed 3 times with RIPA buffer and 2x sample buffer was added to resuspend biotinylated receptors. All samples were analyzed by SDS electrophoresis and western blot detection. Biotinylated TrkA and biotinylated EGFR were detected using rabbit anti-TrkA (1:500; Chemicon) and rat anti-EGFR (1:75; Agazie and Hayman, 2003), respectively.

**Surface Receptor Signaling**

To assess surface receptor signaling, PC12 cells were infected with a defective recombinant adenovirus containing a GFP control or the dominant negative dynaminK44A to block internalization. 48 hrs after infection medium was changed to DMEM+1% horse serum overnight and cells then treated with 50 ng/mL EGF. Alternatively, PC12 cells expressing a temperature-sensitive dynaminG273D and kept overnight in DMEM + 1% horse serum, were preincubated either at the permissive (33ºC) or the non-permissive temperature (39ºC) for 20 min before EGF (50 ng/mL) treatment. Proteins were extracted using RIPA buffer. ERK1/2 activation was analyzed by western blot using an anti-phospho-ERK1/2 specific antibody (1:500 Cell Signaling).

**Receptor Endosomal Signaling**

PC12 cells, kept overnight in DMEM + 1% horse serum, were used to assess receptor signaling from within endosomes. The cells were treated with 10uM of the specific MAPK1/2 inhibitor U0126 for 15 min. The cells were then stimulated with ligand (50 ng/mL NGF or 50 ng/mL EGF) for 5 minutes in the presence of U0126. Following treatment and except for control experiments, the cells were washed 3 times with DMEM to remove inhibitors and placed at 37° C, in DMEM containing anti-NGF (1:10,000, Sigma) for the indicated times in the figures. Proteins were extracted using
RIPA buffer. Endosomal signaling was analyzed by western blot using an anti-phospho-ERK1/2 specific antibody (1:500 Cell Signaling).

Immunofluorescence Staining and Confocal Microscopy

To monitor events in the cell bodies of sympathetic neurons, in the neurites of hippocampal neurons and in PC12 cells, cells were cultured as described above and either transfected or infected with the corresponding constructs/adenoviruses as described in the text and figure legends. 48-72hrs post transfection/infection and before growth factor treatments, sympathetic neurons and PC12 cells were neurotrophin and serum starved in high glucose DME medium containing 1%FBS and low glucose DME medium containing 1%HS respectively, for 12 hours. In all cases, after treatments as indicated in the text, cells were washed with cold PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature (RT). After fixation, the cells were washed 3 times with cold PBS and incubated in blocking solution (5.5% goat serum, 0.5% Triton/PBS) for 1 hour at RT. After exposing the cells to primary antibodies diluted in blocking solution overnight at 4°C, the cells were washed 3 times with PBS, then probed with fluorescently-labeled secondary antibodies for 30 min at RT, washed 3 times and mounted on slides. Primary antibodies used were: rabbit anti-P-ERK5 (1:500; Biosource), mouse-anti-HA (1:200; Santa Cruz Biotechnology), rabbit anti-P-Trk (1:100; Cell Signaling), rabbit anti-Pincher (1:1000; Shao et al., 2002), mouse anti-T7 (1:400, Novagen ), mouse anti-myc (1:250, Zymed ), mouse anti-Flag M5 (1:1000, Sigma ) mouse anti-EGFR (1:100, Oncogene) , goat anti-cathepsin (1:100, Santa Cruz Biotechnology), rabbit anti-VGF (1:750, from S. Salton, (Levi et al., 2004), mouse anti-EEA1 (1:200; BD Transduction laboratories), rabbit-anti-Rabenosyn (1:500, from M. Zerial; Nielsen et al., 2000), rabbit-anti-Rabankyrin (1:500, from M. Zerial; Schnatwinkel et al., 2004) and mouse anti-dynamin (1:250; Transduction Laboratories). The fluorophore-conjugated secondary antibodies used in this study were: donkey anti–mouse Alexa 488/Alexa 546 or donkey anti–rabbit-Alexa 488/Alexa 546 (MolecularProbes), goat anti–rabbit-Cy5 or goat anti-mouse-Cy5 (Jackson Labs).

To simultaneously detect actin, phospho-Trk, HA-Pincher and T7-Rac, cells were fixed with 3.7% formamide + 0.12 M sucrose in PBS for 15 minutes at room temperature
and washed two times with PBS. After labeling with secondary antibodies as described above, cells were incubated with Alexa488-tagged phalloidin (1:40; Molecular Probes) at room temperature for 20 minutes and washed 3 times.

Alexa 647-tagged dextran (Molecular Probes) was used to visualize macropinosomes. Hippocampal neurons infected with E/TrkB adenovirus were incubated with EGF-Alexa555 and Alexa647-tagged dextran for 15 minutes. The cells were washed 5 times with cold PBS to remove excess dextran, fixed with 4% formaldehyde for 15 minutes at room temperature and washed two times with PBS.

For cathepsin staining, cells were blocked in 3% FBS (instead of 5.5% goat serum) and the procedure described above was followed.

Coverslips were mounted on slides and images obtained using a LSM 510 laser scanning confocal microscope (Zeiss). To avoid bleed-through, fluorophores were activated and signal captured sequentially using the following filters: 505-530/GFP/Alexa488, 560-610/Alexa546 and LP 650/Cy5.

**Block of retrograde transport assays**

Sympathetic neurons were cultured in compartmentalized chambers as described (Tsui-Pierchala and Ginty, 1999). 8-10 days after plating, the cells were infected with either E/TrkB or EGFR-GFP containing adenoviruses together with PincherG68E-containing adenovirus and treated with carboxylate modified microspheres (fluospheres) (1:500, Invitrogen) at the distal axons for 48hrs. Cells were then starved for 12 hrs and the next day treated with EGF-Qdot605 for 2hrs at the distal axons, fixed and stained as described above. Alternatively, sympathetic neurons grown in compartmentalized cultures were infected with an E/TrkB adenovirus for 48hrs, starved for 12 hrs and then treated with EGF-Qdot525 for 6hrs at the distal axons. After treatment, the distal axon compartments were washed thoroughly and cells were infected with an adenovirus containing either RacN17-T7 or PincherG68E-HA. 48hrs after infection, cells were starved for 12 hrs and treated with EGF-Qdot605 for 2hrs at the distal axons, fixed and stained as described.
Retrograde VGF induction

Sympathetic neurons in compartmentalized chamber cultures were starved for 60 hrs in the presence of anti-NGF (1:10,000-Sigma), then treated with 100 ng/ml EGF or NGF for 10 hrs and stained for VGF as described above. Depending on the experiment, at the time of starvation cells were either also infected with an adenovirus expressing EGFR-GFP or a herpes simplex virus (applied at distal axons) containing HA-PincherG68E (see figure legends for individual experiments).

Live imaging

Hippocampal neurons were grown on polylysine-coated bottom glass dishes for 10 days, then infected with E/TrkB virus for 48hrs and treated with EGF-Qdot for 15 min. Cells were then washed and imaged in a temperature-controlled chamber kept at 37°C with a Nikon Eclipse Ti microscope. Images were captured with a high-speed camera (38.6 frames/s).

Electron Microscopy and Immunogold Labeling

PC12 cells and hippocampal neurons were plated on poly-D-lysine coated ACLAR (Ted Pella) and cultured as described above. SCGs were plated in compartmentalized Campenot chambers, on collagen-coated tissue culture dishes and maintained as described. Immunogold electron microscopy was carried out as described (Shao et al., 2002), except that cells were microwave fixed for 30 sec. at 650 watts using a Pelco3451 microwave (TedPella) in a cold mixture of 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH7.4. To detect TrkB-GFP or EGFR-GFP and Pincher, we used rabbit anti-GFP (1:20,000, Molecular Probes or 1:6,000, Eusera) and rabbit anti-Pincher (1:50,000), respectively, on ultrathin sections of Durcapan-embedded cells.

For silver enhanced Qdot experiments, cultured neurons were fixed for 15 minutes in a mixture of cold 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (PB), pH 7.4. After several washes in PB and then dH2O, fixed cells were silver enhanced for 1 minute at RT with a silver enhancing kit (British Bio Cell, Ted Pella). Cells were then extensively washed in dH2O, post-fixed with 1% osmium tetroxide.
for 15 minutes, en bloc stained with aqueous 1% uranyl acetate for 30 minutes, dehydrated through an ascending series of ethanols, and embedded in Durcupan (Fluka, Electron Microscopy Sciences) for 48 hours at 60°C. Ultrathin sections (60-90nm) were cut and then stained with 1% methanolic uranyl acetate and 0.3% aqueous lead citrate, and observed with a JEOL 1200EX transmission electron microscope.

For gold-enhanced fluoronanogold experiments and anti-GFP double labeling, cultured neurons were microwave fixed for 30 seconds with a mixture of cold 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB), pH 7.4. After several washes in PB and then dH2O, fixed cells were gold enhanced for 5-7 minutes at RT with a gold enhancing kit (Nanoprobes). Cells were then extensively washed in dH2O, post-fixed with 1% osmium tetroxide for 30 minutes, en bloc stained with aqueous 1% uranyl acetate for 30 minutes, dehydrated through an ascending series of ethanols, and embedded in Durcupan (Fluka; Milwaukee, WI). Serial ultrathin sections (60-90nm) were cut on a Reichert Ultracut E ultramicrotome and picked up on formvar-coated nickel slot grids. Sections were post embedding immunogold labeled for GFP within 24 hours of sectioning using a modification of a previously described protocol (Phend et al., 1995 in addition to a postscript to this paper and a personal conversation with the authors.) Briefly, grids were rinsed in Tris-buffered saline containing 0.005% Tergitol NP-10 (Sigma; St. Louis, MO), pH 7.6 (hereafter referred to as Tris-tergitol pH 7.6), incubated in saturated (10%) sodium meta-periodate for 5 seconds, rinsed, incubated in 1% sodium borohydride for 1 minute, rinsed, and incubated in primary antibody (anti-GFP, 1: 5000, Eusera) overnight at room temperature. The next day, grids were rinsed in Tris-tergitol pH 7.6, followed by Tris-tergitol pH 8.2, incubated in secondary antibody (goat anti-rabbit IgG conjugated to 15nm gold particles) (Amersham; Arlington Heights, IL) 1:25 in Tris-tergitol pH 8.2 for 1 hour and rinsed. Grids were then stained with 1% methanolic uranyl acetate and 0.3% aqueous lead citrate, and observed with a JEOL 1200EX transmission electron microscope.

Image Processing and Quantitative Analysis

All fluorescence images were digitally analyzed using Aim 3.2 software (Carl Zeiss) and processed using Photoshop (Adobe). Weighted colocalization coefficients
were calculated using the Aim 3.2 colocalization Macro (Manders et al., 1993), which gives a higher value to brighter pixels.

Unless otherwise indicated, statistical analyses on quantified experiments were carried out using the Mantel-Haenszal (M-H) Test for equal rates (odds ratio of 1.0), a statistical test that compares two groups on a binary response.
Chapter III

**Trk signaling endosomes are generated by Rac-mediated macroendocytosis.**

**Introduction**

Neurotrophin (NT) control of neuronal survival and phenotype requires the persistent activation of signaling intermediates over hours and days, a result of which is long-term alterations in gene expression (Halegoua et al., 1991). The molecular basis for long-term NT signaling, exemplified by the PC12 cell model, is persistent signaling by NGF through TrkA receptor tyrosine kinase effectors, including ras, rap and the ERK/MAP kinases (Qiu and Green, 1991; Thomas et al., 1992; York et al., 1998). Interestingly, and unlike the case for TrkA, the EGF receptor tyrosine kinase (EGFR), which signals through the same ERK/MAP kinases in PC12 cells, mediates transient rather than sustained signaling. Indeed, transient signaling through ERK/MAP kinases has been invoked to explain the inability of EGFR to mediate alterations of neuronal phenotype and survival (see Marshall, 1995). The mechanisms underlying sustained vs. transient ERK/MAP kinase signaling, however, have not been elucidated.

That sustained ERK signaling by Trk is dictated directly by the receptor is suggested by the preferential binding of signaling adaptors to TrkA, such as SNT/FRS2 and ARMS, and the stimulation of a rap1-based signaling pathway (Arevalo et al., 2006a). Consistent with this, long-term signaling by TrkA receptors has been hypothesized to be specifically mediated through endosomes associated with rap1 (Wu et al., 2001; York et al., 1998; Zhang et al., 2000). However, another characteristic of Trk endosomal signaling is long-lived endosomes, as exemplified by Trk endosomal signaling that persists from the nerve terminal to the soma (Halegoua et al., 1991; Ye et al., 2003). These endosomes are apparently refractory to degradation during and after retrograde transport, and can signal through ERK5 (Watson et al., 2001) and ERK1/2 kinases (Delcroix et al., 2003). The Trk signaling endosome responsible for retrograde...
signaling in neurons is formed by an unusual endocytic mechanism mediated by the membrane trafficking protein, Pincher (Shao et al., 2002; Valdez et al., 2005). Trk is specifically concentrated and endocytosed at complex plasma membrane ruffles by a process termed “macroendocytosis”. Pincher confers on Trk endosomes the ability to avoid lysosomal processing (Valdez et al., 2005) although the underlying mechanism is not known.

In contrast to Trk, EGFR endosomes are short-lived and the signaling of ERK kinases is transient. These properties are consistent with EGFR’s established clathrin-based endocytotic pathway wherein signaling is terminated by rapid lysosomal processing (Sorkin, 2004). Thus, the differences between EGFR and Trk raise the possibility that sustained signaling is a function of a long endosome lifetime (Yamada et al., 1997). The extent to which transient or sustained signaling is an intrinsic property of Trk receptors and/or a function of the endosomal process has yet to be determined.

An understanding of Trk endosomal processing would yield insights into the selective ability of Trk to mediate sustained retrograde signaling. However, with the exception of dynamin (Zhang et al., 2000) and Pincher, the molecular entities that mediate the formation, processing and fate of specialized Trk signaling endosomes are relatively unexplored. Here, we identify molecular components associated with the subcellular structures that distinguish the endocytic processes between Trk and EGFR. Some of these components are recruited directly by Pincher and participate in Trk macroendosome formation and processing. The resulting Trk multivesicular bodies tenaciously retain molecular characteristics of early endosomes, allowing for long-term Trk endosomal signaling.

Results

**Rac mediates Trk internalization at macropinocytic plasma membrane ruffles.**

Trk internalization in PC12 cells and neurons was found to initiate at complex ruffle-like structures at the plasma membrane (Shao et al., 2002; Valdez et al., 2005). To molecularly characterize these cell surface structures we first determined whether they were associated with actin filaments. In NGF-treated TrkA-PC12 cells we examined the
distributions of active P-TrkA, actin and, when present, Pincher-HA, using anti-phospho-Y490-TrkA, Alexa488-tagged phalloidin, and anti-HA antibodies, respectively. In the absence of Pincher-HA overexpression, actin and P-TrkA receptors were concentrated at plasma membrane ruffles, seen in z-sections by confocal microscopy (Fig. 3A).

Similarly, in Pincher-HA expressing cells, Pincher-HA, P-TrkA and actin were all concentrated at large peripheral membrane ruffles. Confocal z-sections illustrating the dorsal surface of Pincher-HA-expressing cells revealed large (5 µm) circular ruffles (Fig. 3B), similar to the ruffles at which growth factor-induced macropinocytosis occurs (Bar-Sagi and Feramisco, 1985; Lanzetti et al., 2004).

PI(4,5)P$_2$ (PIP$_2$) participates in actin remodeling at sites where macropinocytic ruffles are formed (Tall et al., 2000). To determine if PIP$_2$ was also a component of Pincher and TrkA-positive ruffles, we examined PIP$_2$ localization in Pincher-HA-expressing PC12 cells by co-expressing a GFP-tagged PH-domain of PLC$_\delta$ that binds PIP$_2$. After 5 min of NGF treatment, PIP$_2$ was found co-localized with P-TrkA at dorsal cell surface ruffles (co-localization frequency for PIP$_2$ = 87%, P-TrkA = 70%; see fig. 3C, top panel). After 10 min NGF treatment the localizations changed; PIP$_2$ and P-TrkA became co-localized at accumulations of cytoplasmic endosome-like structures (co-localization frequency for PIP$_2$ = 85%, P-TrkA = 80%; fig. 3C, bottom panel).

Because the small GTPase, Rac, drives the formation of actin- and PIP$_2$-rich circular membrane ruffles that distinguish macropinocytosis, we tested for a role for Rac in the formation of Pincher and Trk membrane ruffles. In TrkA-PC12 cells co-transfected with T7-tagged Rac and Pincher-HA constructs, T7-Rac overexpression enhanced the formation and size of Rac-T7-labeled ruffles at the cell surface that were marked by actin staining (Fig. 4A). In these ruffles, Rac was concentrated together with P-TrkA (Fig. 4A) and with Pincher (Fig. 4B). Expression of Rac-T7 also enhanced Trk internalization. In three independent experiments, after NGF treatment, the number of cells displaying cytoplasmic accumulation of P-TrkA (76%; n=48) was enhanced in Rac-T7 expressing cells compared to control cells (27%; n=51). T7-Rac appeared to dissociate from Trk endosomal membranes, as it was associated poorly with the cytoplasmic accumulations of internalized P-Trk (Fig. 4C). That the internalized Trk endosome is derived from the Rac-mediated macropinocytic ruffles is further indicated by its association with an
activated mutant form RacV12, which does not dissociate from internalized membranes (Fig. 4D).

To assess the requirements for Rac function in Trk endocytosis, surface Trk receptors were biotinylated before and after a 30 min NGF treatment, in the presence or the absence of T7-tagged, mutant RacN17 (RacN17-T7), which blocks Rac-mediated ruffling and macropinocytosis. In control cells, NGF treatment caused an internalization of receptor, resulting in a decrease of surface biotinylated receptor. On the contrary, in cells overexpressing RacN17, similar amounts of biotinylated receptor were observed before and after NGF treatment, indicating that RacN17 blocks NGF-induced endocytosis of TrkA (Fig. 5). The effects of RacN17 on Trk internalization were further tested by expression of RacN17-T7 in TrkA-PC12 cells that resulted in the co-accumulation of RacN17-T7, P-TrkA and Pincher-HA at clusters along the plasma membrane, which lacked ruffles (Fig. 6A, top panel). Additionally, the expression of RacN17-T7 caused a dramatic reduction in the percentage of cells containing Pincher-HA-mediated cytoplasmic accumulations of P-TrkA (from 73%, n=51 to 27%, n=223 from 3 independent experiments, \( P<0.0001 \)).

Overexpression of either Pincher or Rac results in enhancement of Trk internalization, and expression of dominant-negative RacN17 (shown herein) or a dominant inhibitory mutant PincherG68E (Shao et al., 2002; Valdez et al., 2005) blocks TrkA internalization in PC12 cells and neurons. Because RacN17 inhibits Pincher-stimulated TrkA internalization, we asked whether Rac-mediated Trk internalization was conversely dependent on Pincher function. This was assessed by examining P-TrkA localization in TrkA-PC12 cells co-expressing wild type Rac-T7 and HA-tagged, dominant-negative mutant HA-PincherG68E. In these cells, regardless of NGF treatment, both P-TrkA and Rac-T7 were limited to peripheral membranes lacking ruffles (Fig. 6A, bottom panel) indicating that the functions of Rac and Pincher are each necessary for Trk internalization.

Dynamin plays an essential role in endosome formation and is crucial to Trk endocytosis and retrograde signaling (Ye et al., 2003; Zhang et al., 2000). To determine if dynamin and Pincher mediate Trk internalization through the same or different endocytic pathways, the requirement for dynamin in Pincher-mediated Trk endocytosis was tested.
by expressing a dominant-negative mutant dynaminK44A together with Pincher-HA. Expression of dynaminK44A in TrkA-PC12 cells resulted in the accumulation of both P-TrkA and Pincher-HA at the plasma membrane (Fig. 6B). The cells had aberrantly rough surface morphologies, which did not show normal ruffling. Additionally, the expression of dynaminK44A caused a dramatic reduction in the percentage of cells containing Pincher-mediated cytoplasmic accumulations of P-TrkA (from 78%, n=50 to 25%, n=51 from 2 independent experiments, P<0.001). Unlike the effects of RacN17, expression of dynaminK44A did not cause a co-clustering of Trk with Pincher, and did not co-localize with either protein (Fig. 6B).

**Trk and EGFR are internalized by distinct modes of receptor-mediated endocytosis.**

The above results indicate that the Trk receptor tyrosine kinase is macro-endocytosed via Rac-regulated macroendocytic ruffles. This stands in contrast to the clathrin-based endocytic mechanism described for the EGF receptor tyrosine kinase. To begin to elucidate the basis for this distinction, we tested the ability of RacN17 to inhibit the internalization of GFP-tagged EGFR (EGFR-GFP). PC12 cells expressing either EGFR-GFP alone or EGFR-GFP and RacN17-T7 were treated with EGF for 30 min. and examined for cytoplasmic EGFR-GFP accumulations. Unlike the case for Trk, internalization of EGFR was not prevented by expression of RacN17-T7 (Fig. 6C). No significant difference in the levels of EGFR-GFP internalization was seen after EGF treatment in RacN17-T7 expressing cells compared to the control cells (86%; n=108, compared to 88%; n=146, from 3 independent experiments; P>0.2), indicating that Rac is required for Trk but not EGFR internalization.

We assessed the contribution of clathrin-mediated endocytosis to the internalization of both EGFR and Trk in PC12 cells using either a T7- or GFP-tagged dominant inhibitory form eps15Δ(95-295), which blocks clathrin coat assembly. PC12 cells expressing EGFR-GFP alone or together with eps15Δ(95-295) were treated with EGF for 30 min., and assessed for cytoplasmic EGFR-GFP accumulations. As expected, EGFR-GFP internalization was lower in cells expressing eps15Δ(95-295) compared to cells expressing EGFR-GFP alone (12%; n= 46, vs 66%; n=127, from 3 independent experiments; P<0.0001). After treatment with EGF for 30 min., EGFR-GFP was found
highly concentrated at the surface of cells expressing eps15Δ(95-295) but was internalized in cells expressing EGFR-GFP alone (see Fig. 6D, top panel). In contrast, the redistribution of P-TrkA from the plasma membrane to cytoplasmic accumulations, caused by NGF treatment, was not significantly affected (P>0.2) by expression of eps15Δ(95-295) (see Fig. 6D, bottom panel). In three independent experiments, 80% (n=129) of cells expressing Pincher-HA alone contained large cytoplasmic P-Trk accumulations; similarly, large cytoplasmic P-Trk accumulations were observed in 85% (n=148) of cells expressing eps15Δ(95-295) and Pincher-HA.

To further compare the endocytic processes for Trk and EGFR, we examined the ultrastructural localization of GFP-tagged receptors using immunogold-electron microscopy of hippocampal neurons, which normally express TrkB and EGFR. In neurons expressing TrkB-GFP, immunogold-labeling for TrkB-GFP could be seen sporadically all along the plasma membrane, and associated with membrane loops (Valdez et al., 2005). Most often, complex ruffles at the plasma membrane and internalized macroendosomal structures were seen (15 complex ruffles and 241 macroendosomes seen in 31 cells), all of which were gold-labeled for TrkB-GFP (Fig. 7A, top panel). Although clathrin-coated pits and vesicles were observed in the gold-labeled TrkB-GFP expressing neurons (6 CCPs and 12 CCVs in 31 cells), none of these structures were immunogold-labeled for TrkB-GFP (Fig. 7A, top right). Indeed, overexpression of TrkB-GFP caused a two-fold reduction in the number of typical clathrin-coated structures seen per neuron (from 2.31/cell in control GFP expressing neurons, n=29, to 0.58/cell in TrkB-GFP expressing neurons, n=31; P<0.0001). Similar results were seen for the distribution of Pincher in Pincher-HA overexpressing neurons. All complex ruffles and macroendosomes were immunogold-labeled for Pincher (n=77 from 67 cells), but clathrin-coated pits and vesicles in the same cells were never gold-labeled (n=35), and overexpression of Pincher-HA reduced the number of clathrin-coated structures (from 2.31/cell in control GFP expressing neurons, n=29, to 0.52/cell in Pincher expressing neurons, n=67; P<0.0001). These findings are in stark contrast to cells overexpressing EGFR-GFP, in which complex ruffling structures were never observed. In contrast, immuno-gold labeling for EGFR-GFP was observed in clathrin-coated pits
and clathrin-coated vesicles (41.2% of CCPs plus CCVs, n=68 from 25 cells; see Fig. 7A, bottom panel) as well as in classic early endosomes throughout the cytoplasm.

A possible role for Pincher in EGFR endocytosis in PC12 cells was examined by expressing the dominant-inhibitory mutant HA-PincherG68E. While expression of the HA-PincherG68E blocked NGF-stimulated Trk internalization (Shao et al., 2002; Valdez et al., 2005; see fig. 7B, top panel), EGFR-GFP internalization was unaffected after EGF treatment (% of EGFR internalized in control cells=92.0%, n=25; in PincherG68E expressing cells=92.6%, n=27; P>0.95; fig. 7B, bottom panel), indicating that EGFR internalization is independent of Pincher function.

Identification of molecular components for Trk macroendosomal processing.

To gain insights into the Pincher-mediated macroendocytic process for Trk, we determined whether the endocytic mediator Rab5, was involved. First, the distributions of GFP-tagged Rab5 (Rab5-GFP), Pincher-HA, and P-TrkA, co-expressed in Trk-PC12 cells, were examined. At early time points Rab5-GFP, P-TrkA and Pincher-HA were localized together at plasma membrane ruffles and at cytoplasmic foci. After 10 min of NGF-treatment Rab5-GFP became co-localized with P-TrkA in large cytoplasmic accumulations surrounded by Pincher-HA (Fig. 8A, top panel). Thus, unlike Pincher, Rab5-GFP was not sequestered or recycled away from accumulated P-TrkA endosomes, even after one hour of NGF treatment. To assess the role of Rab5 in the accumulation of TrkA endosomes, the internalization of TrkA was examined in PC12 cells expressing the dominant inhibitory mutant form of Rab5, Rab5(S34N)-GFP. Although large P-TrkA foci were formed on the cytoplasmic side of the plasma membrane in Rab5(S34N)-GFP expressing cells, these foci appeared to be tethered or proximal to the plasma membrane (see Fig. 8A, bottom panel). Large cytoplasmic P-TrkA accumulations, often perinuclear, in Rab5-GFP expressing cells, were never seen in cells expressing Rab5(S34N)-GFP.

Previous work in our lab identified additional components of the Pincher endocytic machinery by yeast two-hybrid screens, using full-length Pincher or the EH-domain of Pincher as bait. Interestingly, two Rab5 binding proteins, Rabankyrin/Ankhzn and Rabenosyn, were identified as Pincher-binding partners using either bait suggesting
that Rabankyrin and Rabenosyn interact with Pincher through its EH domain. Moreover, neither Rabankyrin nor Rabenosyn interacted with the EH domain mutant PincherW488A, indicating that the EH domain specified the interactions (Valdez G. and Halegoua S., unpublished). To further corroborate these results, interaction of both Rabankyrin and Rabenosyn with Pincher was tested in GST-pulldown assays using a Pincher EH domain GST fusion protein. In this assay, both Rabenosyn and Rabankyrin, but not a non-specific control protein (NcoR), were retained with the EH domain GST fusion (Fig. 8B). Interaction with Pincher was further corroborated by immunofluorescence confocal microscopy analysis of PC12 cells co-transfected with Pincher-HA and Rabankyrin-GFP or Rabenosyn-myc (see below).

Rabankyrin, through its binding to Rab5, has been implicated in both clathrin-mediated endocytosis and macropinocytosis (Schnatwinkel et al., 2004). Having established that Rabankyrin is a Pincher-binding partner, but that the Pincher pathway is clathrin-independent, we asked whether Rabankyrin was localized to Pincher-mediated Trk macroendocytic structures. Several distributions of Rabankyrin were observed in NGF-treated TrkA-PC12 cells expressing both Rabankyrin-GFP and Pincher-HA. Rabankyrin-GFP was together with Pincher-HA and P-TrkA at plasma membrane ruffles (Fig. 9A, top panel) and within cytoplasmic accumulations (Fig. 9A, middle panel) prior to Pincher-TrkA sorting. However, as with Rab5, Rabankyrin-GFP remained mostly localized with P-TrkA in the cytoplasm and was associated poorly with the surrounding Pincher-HA putative recycling tubules (Fig. 9A, bottom panel). Even after one hour of NGF treatment, when most of Pincher-HA had recycled to the peripheral membrane, Rabankyrin remained associated with P-TrkA in the cytoplasm. These data indicate that Rabankyrin is involved at all stages of Trk macroendocytosis. The interaction between Pincher, Rabankyrin and Trk was further tested by the expression of PincherG68E. Similar to its effects on TrkA, expression of PincherG68E caused Rabankyrin to become re-localized to PincherG68E clusters at the plasma membrane (Fig. 9B; surface co-clustering with PincherG68E = 56.25%; n=48 and Pincher = 4.6%; n=65; P<0.0001).

Rabenosyn and EEA1 are Rab5 effectors involved in the trafficking and processing of clathrin-derived early endosomes (Nielsen et al., 2000). In agreement with this, in PC12 cells transfected with Rabenosyn-myc or co-transfected together with
EGFR-GFP, Rabenosyn-myc and endogenous EEA1 were each co-localized with EGFR-GFP at small cytoplasmic foci, presumably clathrin-derived endosomes (Fig. 10B). As expected, because Rabenosyn was identified as a Pincher-binding protein, Rabenosyn was co-localized with Pincher in cytoplasmic foci of TrkA-PC12 cells at early times of NGF-treatment (Fig. 10A). Rabenosyn-myc was also localized with P-TrkA in large cytoplasmic accumulations in NGF-treated PC12 cells expressing both Pincher-HA and TrkA (Fig. 10C, top panel). Like Rabankyrin, Rabenosyn did not associate well with putative Pincher recycling tubules surrounding cytoplasmic accumulations of P-TrkA. In contrast to the findings with Rabenosyn, EEA1-GFP was rarely localized with Pincher-HA and P-TrkA structures in TrkA-PC12 cells expressing EEA1-GFP and Pincher-HA (Fig. 10C, bottom panel). The distribution of EEA1-GFP in Pincher-HA expressing cells was similar to that in cells expressing EEA1-GFP alone. In both situations, EEA1 was associated predominantly with scattered cytoplasmic foci. Together with the yeast two-hybrid results, these results suggest that Pincher preferentially recruits Rabenosyn over EEA1 to Trk macroendosomes.

To better visualize the Pincher-mediated endosomal structures with which early endosome markers were associated, we carried out immunogold EM analyses for Rab5-GFP or Rabankyrin-GFP expressed together with Pincher-HA in PC12 cells. In serial thin sections of PC12 cells, immunogold labeling demonstrated that Rab5-GFP and Pincher-HA (Fig. 11A), or Rabankyrin-GFP and Pincher-HA (Fig. 11B), were localized to the same cell surface complex ruffles at the cell surface, and to the same macroendosomes throughout the cytoplasm. As with Pincher (Valdez et al., 2005), both Rab5-GFP and Rabankyrin-GFP were mostly limited to the peripheral membrane of multivesicular bodies (Fig. 11), which contain Trk in both their lumen and peripheral membrane (Valdez et al., 2005), indicating that the Pincher macroendosome-derived multivesicular bodies retain early endosome markers.

Trk-containing structures, which accumulate via Pincher, appear to continually associate with early endosome markers. To determine whether these endosomal structures are destined for recycling to the plasma membrane, we examined Trk association with the recycling endosome marker, Rab11. Rab11-GFP and Pincher-HA were similarly localized after expression in TrkA-PC12 cells. After NGF-treatment of
these cells, tubule-like Rab11-GFP structures were found encircling large cytoplasmic accumulations of P-TrkA (Fig. 12A); this localization coincided with the putative Pincher recycling tubules. Thus Pincher, but not TrkA was associated with Rab11 recycling tubules. To assess whether internalized P-Trk receptor endosomes were targeted for lysosomal degradation, we examined whether the late endosomal marker Rab7, was co-localized with P-TrkA. TrkA-PC12 cells were transfected with a cDNA for GFP-tagged Rab7 (Rab7-GFP) and Pincher-HA. In these cells, P-Trk and Rab7-GFP were rarely co-localized in cytoplasmic structures (weighted co-localization coefficient for Rab7-GFP = 0.16 ± 0.14; P-TrkA=0.11 ± 0.19; n=17) even after 1 hour of NGF treatment (Fig. 12B, top panel). In contrast, EGFR was associated robustly with Rab7-GFP cytoplasmic foci after one hour of EGF treatment (Fig. 12B, bottom panel; weighted co-localization coefficient for Rab7-GFP = 0.6 ± 0.18; EGFR=0.6 ± 0.1; n=14). These results suggest that, unlike clathrin-derived EGFR endosomes, Pincher-derived Trk receptor endosomes remained associated stably with early endosome markers, but not recycling or late-endosomal markers.

Sustained signaling through ERK kinases is mediated differentially by compartmentalized Trk and EGFR receptors.

The above results indicate that Trk and EGFR endosomes differed significantly in their fate, with all types of Trk endosomes associated persistently with early endosome markers and EGFR endosomes targeted for lysosomal degradation. The association of Trk and EGFR with early and late endosomal structures, respectively, could explain their differential abilities to mediate sustained signaling, by, for example, the ERK kinases. If transient activation of ERK kinases was due to removal of activated EGFR through endocytosis, then preventing endocytosis, by maintaining EGFR on the cell surface, should extend the time of ERK activation by EGF. To test this, a dominant-negative dynaminK44A mutant was expressed in PC12 cells to block EGFR endocytosis (Vieira et al., 1996). This resulted in an enhanced activation of ERK1/2 kinases with 5 min of EGF treatment (Fig. 13A), consistent with the accumulation of EGFRs on the cell surface. Furthermore EGF treatment caused a more sustained activation of the ERK1/2 kinases over a 60 min time course (Fig. 13A). To verify that the sustained ERK activation was
not simply due to the “overexpression” of EGFRs on the cell surface, we examined EGF stimulated ERK activation in PC12 cells stably expressing a temperature-sensitive mutant, dynamin G273D, in which dynamin function is attenuated acutely by temperature shift from 33°C to 39°C (Zhang et al., 2000). As expected, at the permissive temperature of 33°C, EGF stimulated ERK signaling was transient (Fig. 13B, top panel). However, after the shift to the non-permissive temperature (39°C), ERK signaling by EGF was now sustained for at least two hours (Fig. 13B, bottom panel).

To examine whether Trk and EGFR differentially activated ERKs in their associated endosomes, PC12 cells were preincubated with either NGF or EGF respectively, to allow receptor-mediated internalization. This was performed in the presence of the MEK inhibitor, U0126, to prevent ERK signaling. Subsequently, the external ligand and MEK inhibitor were removed so that only internalized receptors were ligand-bound and activated. As demonstrated in fig. 13C, internalized NGF/Trk signaling led to sustained ERK1/2 activation over a one-hour time course. In contrast, internalized EGF/EGFR signaling was transient, such that the signal after five minutes was greatly decreased by 10 minutes and nearly gone after 30 minutes of EGF treatment. Thus, surface but not endosomal EGFRs are capable of sustained ERK activation.

To determine whether receptor downregulation by lysosomal degradation was responsible for the differential attenuation of signaling by Trk and EGFR we compared the lifetimes of the two receptors after ligand treatment. Surface receptors were biotinylated and cells were treated with either NGF or EGF. No significant degradation was seen for Trk receptors, even 1 hour after ligand treatment (76% of initial levels remain) while EGF receptors were rapidly degraded after 1 hour of EGF treatment (20% of initial remaining, fig. 13D). This is consistent with the known fate of rapid degradation for EGFR after clathrin-mediated endocytosis. It also supports the idea that Trk endosomes are resistant to lysosomal degradation.

**Discussion**

The Trk neurotrophin receptors use endosomes to transmit signals retrogradely over long axonal distances, in order to elicit long-term changes within the neuronal soma.
Trk receptor endocytosis is mediated through a unique process of Pincher-mediated internalization of complex ruffles en mass, the formation of macroendosomes, and their subsequent processing to multivesicular bodies (Shao et al., 2002; Valdez et al., 2005). The macroendocytosis step is key. We show that the molecular events underlying Trk macroendocytosis differ fundamentally from the clathrin-based pathway for EGF receptor-mediated endocytosis. As a result, macroendocytosis endows Trk endosomes with the ability to sustain ERK kinase activation, and likely other downstream signaling events as well, events required for neurotrophin-specific control of neuronal survival and phenotypic traits.

**Trk Macroendocytosis at membrane ruffles**

In both PC12 cells and neurons, NT-induced Trk activation rapidly initiates plasma membrane ruffling where Trk receptors are concentrated (Shao et al., 2002; Valdez et al., 2005). We show here that these ruffles are actin- and PIP2-rich circular plasma membrane ruffles, the typical sites of macropinocytosis. Trk macroendocytosis is distinct from classical macropinocytosis, however, because it mediates the specific internalization of Trk receptors rather than non-specific bulk internalization of plasma membrane constituents. Additionally, unlike typical macropinocytic ruffles, Trk macroendocytic ruffles demonstrate an unusual complexity of tubulovesicular structures, whose formation are governed at least in part by the amount of Pincher and Trk (Shao et al., 2002; Valdez et al., 2005).

We found that Rac, a known mediator of membrane ruffling and macropinocytosis (Ridley et al., 1992), also played a crucial role in the specific internalization of Trk. Overexpression of wild type or activated RacV12 facilitated Trk internalization at ruffles, and conversely, expression of dominant-negative RacN17 blocked this internalization. Overexpression of Rac and Pincher each increased the density, basal activation, and internalization of Trk at plasma membrane ruffles. Furthermore, expression of RacN17 or of PincherG68E inhibited membrane ruffling and caused a change in the localizations of Trk, Pincher and Rac into coincident clusters at the plasma membrane. Thus, Trk internalization likely involves a coordination of the activities and distributions of Rac, Pincher and the actin cytoskeleton at membrane
ruffles. This coordination also involves the dynamin2 GTPase, which is an essential component of the endocytic process for Trk (Zhang et al., 2000). We found that expression of dominant-negative dynamin2-K44A interfered with membrane ruffling and caused co-accumulation of Trk and Pincher at the plasma membrane, thus preventing Pincher-mediated Trk endocytosis. This result was consistent with the necessity of dynamin for Rac localization and function in directing growth factor-induced macropinocytosis (Schlunck et al., 2004), and indicates that dynamin and Pincher mediate Trk endocytosis through a common endocytic pathway.

**Macroendosome Processing**

Once internalized, Trk macroendosomes are processed in an unusual manner, with the complex tubulovesicular arrays directly giving rise to multivesicular bodies (Shao et al., 2002; Valdez et al., 2005). We found that some of the underlying molecular components were shared with other endocytic processes. A primary example is the small G-protein, Rab5, which mediates both clathrin-based (Bucci et al., 1992) and macropinocytic (Lanzetti et al., 2004) pathways. Consistent with the association of Rab5 with Trk endosomes in axons in vivo (Delcroix et al., 2003), we found that Rab5 was localized to and appeared to be required for processing of Trk macroendosomes.

Interestingly, two established Rab5 effectors, Rabenosyn and Rabankyrin, were directly recruited by the Pincher EH-domain presumably by binding to their NPF motifs (Naslavsky et al., 2004). On the other hand, the Rab5 effector, EEA1, lacks NPF motifs and does not bind Pincher. EEA1 associated poorly with Trk macroendosomes, suggesting that Pincher is the primary determinant in recruiting the Rab5 effectors for macroendosomal processing. Rabenosyn, like EEA1, is involved in directing the fusion of clathrin-derived vesicles to early endosomes that lead to the formation of multivesicular bodies (Nielsen et al., 2000). Rabenosyn may play an analogous role in Trk macroendosomes, in directing the fusion of tubulovesicular structures, leading to the formation of multivesicular bodies. Rabankyrin /Ankhzn (Ito et al., 1999), has been shown recently to be a Rab5-binding partner associated with both macropinocytic and clathrin-derived EGFR endosomes and is required for macropinocytosis (Schnatwinkel et al., 2004). Rabankyrin, together with Rab5, are present with Pincher from the earliest
stage of Trk macroendocytic ruffles to the latest stage of multivesicular body formation, and are likely to be involved in multiple stages of Trk macroendosome formation and processing.

Trk versus EGFR endocytosis

Trk macroendocytosis is unlike other pathways for receptor-mediated endocytosis, including that mediating endocytosis of EGFR. The primary mode for EGFR internalization is by clathrin-dependent endocytosis (Carpenter, 1987). Although previous biochemical studies have suggested that Trk is associated with clathrin-containing vesicles (Howe et al., 2001), our immunogold EM analyses failed to reveal any significant association of Trk with clathrin coated pits or vesicles. Moreover, no inhibition of Trk internalization was observed in cells in which clathrin-dependent EGFR endocytosis was prevented by expression of dominant-negative eps15\(\Delta\)(95-295) (Benmerah et al., 1999). If Trk is internalized by a clathrin-based mechanism under certain conditions, it is either a relatively minor route of internalization or perhaps a recycling pathway (Chen et al., 2005) that does not allow an accumulation of Trk endosomes. A fraction of EGFR was internalized via a macropinocytic-like pathway (Schnatwinkel et al., 2004; Yamazaki et al., 2002), and we did occasionally see large macropinosome-like EGFR-containing vesicles in PC12 cells. However, the EGFRs were not concentrated at macropinocytic sites, and the predominant mode of EGFR internalization in PC12 cells and neurons was clathrin-mediated and Pincher-independent.

Trk and EGFR endosomal signaling

While EGFR mediates only transient activation of the ERK kinases, Trk mediates sustained activation that underlies its selective ability to mediate long-term changes in neuronal phenotype and survival (Cowley et al., 1994; Qui and Green, 1992) as well as retrograde axonal signaling (Ginty and Segal, 2002). We demonstrated that the transient signaling by EGFR is due at least in part to the mechanism of endocytosis. Consistent with previous studies (Lloyd et al., 2002), we found that EGFR endosomes lost their signaling capacity as they were processed from early into late, acidified endosomes.
headed for lysosomal degradation. We showed that the half-life of Trk endosomal signaling is much longer than that for EGFR. This may in part be explained because unlike EGFR, Trk activation persists in acidified endosomes (Zapf-Colby and Olefsky, 1998). Furthermore, the maturation of EGFR early endosomes to late endosome/multivesicular bodies is accompanied by the exchange of Rab5 for Rab7 (Rink et al., 2005). Our findings indicate that this event does not readily occur for Trk endosomes, due at least in part to the direct recruitment and maintenance of early endosome components by Pincher. This may contribute to the accumulation and maintenance of “immature” Trk signaling endosomes, which surprisingly includes multivesicular bodies. Our findings are consistent with previous studies suggesting that sustained signaling by Trk is endosome-based (Wu et al., 2001; York et al., 1998; Zhang et al., 2000). The macroendocytic internalization and processing of active Trk signaling complexes may provide an important mechanism by which long-term and long-range endosomal signaling occurs in neurons.
Figure 3. P-TrkA and Pincher are co-concentrated at actin-rich, macroendocytic, circular plasma membrane ruffles.
PC12 cells were transfected with TrkA alone (A) or together with Pincher-HA (B). TrkA-PC12 cells were transfected with PH-GFP (to detect PIP_2) and Pincher-HA (C). Cells were treated with NGF for the indicated times, fixed, stained and confocal microscopy carried out as described in materials and methods. Confocal z-sections are at dorsal cell surface (B, C, upper panel) or at the middle of cell (A, C, bottom panel). Anti-phospho-TrkA (P-TrkA, red, A-C), phalloidin (actin, green, A, B), anti-HA (Pincher-HA, cyan in B), and PH-GFP (PIP_2, green in C). Scale bar = 2µm.
Figure 4. Rac mediates the formation of plasma membrane ruffles from which activated TrkA is internalized.

PC12 cells were co-transfected with TrkA and either Rac-T7 (C) or RacV12-GFP (D). TrkA-PC12 cells were co-transfected with Rac-T7 and Pincher-HA (A, B). Cells were treated with NGF for the indicated times, fixed and stained. P-Trk (red, A, C, D), phalloidin (actin, green, A, B), anti-T7 (Rac-T7, cyan in A, B, green in C), RacV12-GFP (green in D), anti-Pincher (red in B). Scale bar = 2µm.
Figure 5. Active Rac is required for TrkA internalization.
TrkA-PC12 cells were infected with an adenovirus expressing GFP (control) or two different concentrations of RacN17 virus (x1-low, x2-high). 48hrs after infection cells were either left untreated or treated with NGF for 30 min and then cell surface proteins were biotinylated and biotinylated proteins were immunoprecipitated with streptavidin beads and resolved with SDS-PAGE, as described in materials and methods. Biotinylated TrkA was identified by western blot using an anti-TrkA antibody.
Figure 6. TrkA and EGFR are differentially internalized via Rac-dependent versus eps15-dependent mechanisms.

PC12 cells were triple-transfected with TrkA-YFP, Pincher-HA and RacN17-T7 (A, upper panel); TrkA-YFP, PincherG68E-HA and Rac-T7 (A, lower panel); or co-transfected with EGFR-GFP and RacN17-T7 (C); EGFR-GFP and eps15Δ(95−295)−T7 (eps15DN-T7, D, upper panel); or transfected with TrkA-YFP (A-left panels) or EGFR-GFP (left panels in C and upper D). TrkA-PC12 cells were co-transfected with Pincher-HA and dynaminK44A-GFP (B), Pincher-HA and eps15Δ(95−295)−GFP (eps15DN-GFP, D, lower panel), or transfected with Pincher-HA alone (B, Control). Cells were treated with NGF for 10min (A, B) or 30min (D, lower panel), or treated with EGF for 30min (C, D upper panel), then fixed and stained. TrkA-YFP (green, A), anti-P-Trk (P-TrkA, green, B and D, lower panel), EGFR-GFP (green, C and D, upper panel), anti-T7 (RacN17-T7, red, A upper panel, and C), anti-HA (Pincher-HA, red, B), anti-T7 (Rac-T7, red, A lower panel), anti-T7 (eps15DN-T7, red, D, upper panel), eps15DN-GFP (red, D, lower panel), anti-Pincher (cyan, A), dynaminK44A-GFP (cyan, B). Scale bar = 2µm.
Figure 7. Internalization of EGFR, but not Trk is mediated by clathrin-coated pits and clathrin-coated vesicles in a Pincher-independent manner.
(A) Cultured hippocampal neurons were infected with adenoviruses expressing TrkB-GFP and treated with BDNF for 15 min (upper panel) or EGFR-GFP and treated with EGF for 15 min (lower panel), and immunogold electron microscopy using antibodies against GFP carried out as described in materials and methods. Immunogold labeled TrkB-GFP (upper panel) was associated with plasma membrane ruffles (left side) and macroendosomes (right side) but not coated pits (indicated by arrowhead, right side), while EGFR-GFP (lower panel) was gold-labeled at coated pits (arrowhead, left side) and coated vesicles (arrowhead, right side). (B) PC12 cells were co-transfected with PincherG68E-HA and either TrkA-YFP (upper panel) or EGFR-GFP (lower panel). Cells were treated with NGF for 20 min (upper panel) or EGF for 30 min (lower panel), fixed and stained. TrkA-YFP (green, upper panel), EGFR-GFP (green, lower panel), anti-HA (HA-pinG68E, red). Scale bars = 200 nm in A, 2µm in B.
Figure 8. Pincher-mediated P-TrkA macroendocytosis requires Rab5 and Pincher binds the Rab5-binding partners Rabenosyn and Rabankyrin.

(A) TrkA-PC12 cells were co-transfected with Pincher-HA and either Rab5-GFP (upper panel) or Rab5 (S34N)-GFP (lower panel). Cells were treated with NGF for 30 min (upper panel) or 10 min (lower panel), fixed and stained. Anti-P-Trk (P-TrkA, red), Rab5-GFP (green, upper panel), Rab5(S34N)-GFP (green, bottom panel), anti-HA (Pincher, cyan). Scale bar = 2µm.

(B) Lysates of HEK293 cells transfected with Rabenosyn-Flag, NcoR-Flag or Rabankyrin-GFP were incubated with a GST fusion of the EH domain of Pincher coupled to glutathione sepharose beads, as described in materials and methods. Whole cell lysates (top) and GST-EH pulldowns (GST-EH pulldown, bottom) were resolved by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a mouse anti-Flag antibody (left panel) or with a mouse anti-GFP antibody (right panel), followed by an anti-mouse-HRP conjugated antibody.
A

P-Trk Rab5 Pincher

P-Trk Rab5(S34N) Pincher

B

NcoR-Flag Rabenosyn-Flag Rabankyrin-GFP

Whole Cell Lysates

-130kDa

-80kDa

GST-EH Pulldown

Anti-Flag Anti-GFP

-130kDa

-80kDa
Figure 9. TrkA endosomes associate with the Pincher- and Rab5-binding protein, Rabankyrin.
TrkA-PC12 cells were co-transfected with Pincher-HA and Rabankyrin-GFP (A, B-upper panel) or Rabankyrin-GFP and PincherG68E-HA (B-lower panel). Cells were treated with NGF for 30 min, fixed and stained as described in materials and methods. Anti-P-Trk (P-TrkA, red A), Rabankyrin-GFP (green, A and B), anti-HA (Pincher, cyan, A and red, B-upper panel and PincherG68E, red, B-lower panel). Scale bar = 2µm.
Figure 10. Pincher-mediated P-TrkA endosomes preferentially associate with the Pincher- and Rab5-binding protein, Rabenosyn, over EEA1.

TrkA-PC12 cells were co-transfected with Pincher-HA and Rabenosyn-myc (A, C-upper panel) or Pincher-HA and EEA1-GFP (C-lower panel). PC12 cells were co-transfected with EGFR-GFP and Rabenosyn-myc (B-upper panel) or transfected with EGFR-GFP alone (B-lower panel). Cells were treated with NGF for 10 min (A) or 30 min (C), or with EGF for 15 min (B), and then fixed and stained. Anti-Pincher (green, A), EGFR-GFP (green, B), P-TrkA (green, C), anti-myc (Rabenosyn-myc, red, A, B-upper panel, C-upper panel), anti-EEA1 (red, B-lower panel), EEA1-GFP (red, C-lower panel). Scale bar = 2µm.
Figure 11. Pincher is co-localized with both Rab5 and Rabankyrin at complex plasma membrane ruffles, macroendosomes, and multivesicular bodies. PC12 cells were co-transfected with Pincher-HA and either Rab5-GFP (A) or Rabankyrin-GFP (B), treated with NGF and immunogold-EM analyzed using antibodies against GFP or Pincher as described in materials and methods. Ultrathin serial sections (or sections from different cells in the same grid in B, bottom panel) were alternately labeled with anti-GFP antibody for Rab5-GFP (A) and for Rabankyrin-GFP (B) or with anti-Pincher antibody for Pincher-HA as indicated. Rab5-GFP and Rabankyrin-GFP were each associated with Pincher within the same plasma membrane ruffles (top panels), macroendosomes (middle panels), and peripheral membranes of multivesicular bodies (bottom panels). Scale bar=200nm.
Figure 12. P-TrkA endosomes avoid the recycling and late endosome effectors Rab11 and Rab7.
TrkA-PC12 cells were co-transfected with Rab11-GFP and Pincher-HA, and treated with NGF for 10 min (A). PC12 cells were triple-transfected with TrkA, Pincher-HA and Rab7-GFP and treated with NGF for 60 min (B, upper panel), or co-transfected with EGFR and Rab7-GFP and treated with EGF for 60 min (B, lower panel). Cells were fixed and stained as in figure 1. Anti-P-TrkA (green, A, B upper panel), anti-EGFR (green, B lower panel), Rab11-GFP (red, A), Rab7-GFP (red, B). Scale bar = 2μm.
Figure 13. Endosomal TrkA, but not EGFR, resist degradation and mediate sustained ERK activation.

(A) PC12 cells were infected with an adenovirus expressing a dominant negative form of dynamin (dynK44A) (bottom panel) or an adenovirus expressing GFP (top panel). 48 hrs after infection, cells were starved overnight and then treated with EGF for the indicated times.

(B) PC12 cells stably expressing a temperature-sensitive mutant form of dynamin, dynG273D, were starved overnight and pre-incubated either at 33°C (permissive temperature, top panel) or 39°C (non-permissive temperature, bottom panel) for 20 min. Cells were then treated with EGF for the indicated times.

(C) Endosomal signaling was assessed in TrkA-PC12 cells (upper panel) or EGFR-PC12 cells (lower panel) pre-treated with the MEK inhibitor U0126 for 15 min as indicated, and then also treated with NGF or EGF as indicated for 5 min. The ligands and inhibitor were removed and the cells washed and reincubated for the indicated times, all as described in materials and methods. In all experiments, whole cell lysates were blotted and probed with an anti-P-ERK1/2 antibody.

(D) PC12 cells were surface biotinylated and treated with either NGF (top panel) or EGF (bottom panel) for the times indicated. Biotinylated were immunoprecipitated, resolved by SDS-PAGE and analyzed by western blot, as described in materials and methods. The top blot was probed with an anti-Trk antibody (Chemicon) while the bottom one was probed with an anti-EGFR antibody (gift from M. Hayman).
Chapter IV

The adaptor protein APPL provides the link between Trk receptors and Pincher-mediated macroendocytosis.

Introduction

In the developing nervous system, growth factors mediate an array of phenotypes, including survival and differentiation. Growth factor actions are determined to some extent by the duration of the signaling cascades elicited. For example, in the PC12 cell model system, EGF-induced transient activation of ERK1/2 results in cell proliferation while NGF-induced sustained ERK1/2 activation leads to differentiation. Termination of receptor-emanating signals is accomplished by receptor downregulation via endocytosis and subsequent lysosomal degradation. While endocytosis serves in part to attenuate receptor signaling, increasing evidence supports a role for endocytosis in signal augmentation and specificity. Endosomal signaling can mediate distinct phenotypes from cell surface signaling; for example cell surface Trk receptors mediate neuronal survival while internalized receptors mediate neuronal differentiation (Zhang et al., 2000).

Sustained signaling by Trk receptors has been proposed to be mediated specifically from endosomal structures. After endocytosis, a signaling complex involving the small GTRase rap1 nucleates around the receptor and mediates sustained endosomal signaling through MAPK (York et al., 1998). Rap1 activation has been proposed to succeed ras activation, which predominantly occurs at the cell surface, on endosomes (York et al., 2000). Rap1 is activated by the guanyl nucleotide exchange factor C3G, which is activated by crk. The Ankyrin-rich membrane-spanning protein (ARMS) and the signaling adaptor SNT/FRS2 have been proposed to provide docking sites for crk by binding to Trk (Arevalo et al., 2004; Meakin et al., 1999). SNT/FRS2 competes with shc for binding to phosphorylated Tyr490 in the juxtamembrane region of Trk and can reconstitute the ability of mutant Trk receptors to mediate differentiation (Meakin et al., 1999).
The juxtamembrane region of Trk plays a key role in NGF-induced PC12 differentiation as mutations in this region abolish NGF-induced neurite outgrowth (Meakin and MacDonald, 1998). Multiple Trk signaling effectors that bind the juxtamembrane region, including GIPC and APPL have also been implicated in mediating the neuritogenic effects of NGF. APPL also interacts with Rab5 and has been proposed to couple trafficking and signaling to the nucleus in the case of EGFR (Miaczynska et al., 2004). GIPC and APPL associate with Trk on endosomes and disrupting the Trk interaction with these effectors or reducing their levels by RNAi results in decreased neurite outgrowth in response to NGF (Lin et al., 2006; Lou et al., 2001; Varsano et al., 2006). Interestingly, replacing about 80 amino acids of the EGFR juxtamembrane region with the corresponding region of Trk results in neurite outgrowth in response to EGF (Yoon et al., 1997). Despite the evidence that both endocytosis and the juxtamembrane region of Trk play a role in sustained ERK1/2 signaling and neuronal differentiation, the molecular mechanisms underlying this connection are unclear.

We have previously shown that the selective capacity of Trk to induce sustained ERK/MAPK signaling lies in its ability to form long lived signaling endosomes via a Pincher-mediated mechanism (Valdez et al., 2007). Since not all endocytic routes can generate long-lived endosomes, the mode of receptor endocytosis is tightly linked to its signaling potential. We have shown that Pincher-mediated endocytosis, unlike clathrin mediated endocytosis, can generate long-lived, degradation resistant endosomes (Valdez et al., 2007). However, it was unclear how Trk, but not EGFR, is able to recruit Pincher and undergo Rac-mediated macroendocytosis.

Here we show that the juxtamembrane region of Trk is sufficient for Pincher mediated endocytosis and identify the Rab5-binding protein APPL as the link between Trk and the Pincher endocytic machinery. APPL has been previously shown to interact with Trk and we now demonstrate its interaction with Pincher. We show that disrupting the APPL-Pincher interaction by mutating the NPF motif of APPL results in faster Trk degradation and attenuation of ERK signaling. These results indicate that APPL links receptor signaling and endocytosis in a receptor specific manner.
Results

The juxtamembrane region of Trk is sufficient for Pincher recruitment.

In order to define the Trk domain required for the recruitment of the Pincher machinery we utilized chimeric receptors containing different domains of EGFR and Trk. Trk has been shown to internalize via Pincher-mediated endocytosis, while EGFR does not (Valdez et al., 2007). We examined the colocalization of each chimeric receptor with Pincher upon internalization and the ability of dominant negative Pincher, Pincher G68E to block endocytosis of the receptor in order to determine the contribution of individual Trk domains to Pincher-mediated endocytosis.

In order to determine whether the ligand binding domain of Trk is required for Pincher-mediated endocytosis, we first examined the endocytosis of the E/TrkB chimeric receptor, a receptor consisting of the extracellular domain of EGFR and the intracellular and transmembrane domains of TrkB (Wang et al., 1996). When the receptor was expressed alone in PC12 cells, treatment with EGF for 20 min induced its internalization (Fig. 14A-left panels). When wild type Pincher was co-expressed in these cells it was seen colocalized with E/TrkB in internalized structures after EGF treatment (weighted colocalization coefficient for E/Trk=0.73±0.11, n=24 cells, fig. 14A-upper right panels). Pincher was also localized in tubular structures surrounding the receptor (Fig. 14A-lower right panels), as demonstrated for Trk (Shao et al., 2002).

Coexpression of PincherG68E with E/TrkB resulted in a block of E/TrkB endocytosis. Cells were considered to have undergone receptor endocytosis when the ratio of cell surface: intracellular fluorescence for the E/TrkB channel was less than 1. In cells expressing receptor alone 85% of cells showed E/TrkB endocytosis after 30 min of EGF treatment (n=20) as opposed to only 10% of cells co-expressing PincherG68E (n=10). In these cells E/TrkB co-accumulated with PincherG68E at surface clusters (Fig. 14B) as previously seen for Trk but not EGFR (Valdez et al., 2007). The above results indicate that the extracellular domain of Trk is not necessary for Pincher recruitment.

Since Pincher recruitment is independent of the extracellular Trk domain and ligand binding, it must depend on signals generated by an intracellular Trk domain. We hypothesized that the domain responsible for Pincher recruitment on Trk would confer
Pincher-recruiting abilities to EGFR. Therefore we followed a domain swap approach between Trk and EGFR to identify the region responsible for Pincher recruitment. The juxtamembrane region of Trk mediates rap1 recruitment through FRS2 and rap1 is thought to mediate sustained signaling from Trk endosomes. When the EGFR juxtamembrane region was replaced with the corresponding region of Trk, EGF receptors were able to mediate sustained ERK signaling (Yoon et al., 1997). Based on the above, the juxtamembrane region of Trk seems a likely candidate for Pincher recruitment; therefore we decided to examine the ability of a chimera in which the juxtamembrane region of EGFR is replaced by the juxtamembrane region (amino acid residues 434-509) of TrkA (JM chimera; Yoon et al., 1997) to undergo Pincher mediated endocytosis. The JM chimera contains the extracellular and intracellular domains of EGFR and is endocytosed in response to EGF treatment (Fig. 15A and B-left panels) and colocalizes with Pincher in endocytic structures in cells co-expressing Pincher (weighted colocalization coefficient for JM=0.77±0.09, n=13 cells, fig. 15A-right panels).

PincherG68E sequesters JM to the cell surface (Fig.15B) and dramatically reduces receptor endocytosis from 70% in control cells (n=10) to 17% in cells expressing PincherG68E (n=18). This indicates that the juxtamembrane region of Trk is sufficient for Pincher recruitment.

**Tyr515 and the KFG sequence within the juxtamembrane region are not essential for Pincher recruitment.**

Since the juxtamembrane region of Trk is sufficient for Pincher-mediated endocytosis we looked for the specific sites required for Pincher recruitment. For this purpose we used the E/TrkB receptor chimera that was mutated at two key sites within the juxtamembrane region. The first receptor had a point mutation, Y515A. Y515 is phosphorylated upon ligand binding to Trk and is responsible for recruiting and phosphorylating shc, the first step in a signaling cascade leading to ERK activation and gene expression changes and FRS2. The second receptor was a deletion mutant lacking the KFG residues responsible for SNT/FRS2 phosphorylation. Both receptors appeared to retain the ability to interact with Pincher and they both co-clustered at the cell surface
with PincherG68E (Fig. 16). Therefore, it appears that neither Tyr515 nor the KFG motif is required for Pincher recruitment by Trk.

**APPL is the link between Trk and Pincher.**

Since Pincher and Trk do not interact directly with each other (Shao Y., Rosenbaum J. and Halegoua S., unpublished) we looked for an adaptor protein that would recruit Pincher to Trk receptors. This adaptor protein must be able to interact both with Trk and Pincher. Since the juxtamembrane region of Trk is sufficient for Pincher-mediated endocytosis, we searched for proteins that specifically bind to that region of Trk. Pincher-binding partners must contain either a NPF or a DPF motif, both of which can bind the EH domain of Pincher. APPL is a Rab5-binding partner that interacts with Trk both directly and indirectly through its phosphotyrosine-binding (PTB) domain (Lin et al., 2006; Varsano et al., 2006). Both sites of Trk-APPL interaction are in the Trk juxtamembrane region. APPL also contains an NPF motif that can presumably interact with the EH domain of Pincher (Fig. 17A).

In order to test whether APPL interacts with Pincher we performed a protein pulldown assay using the EH domain of Pincher bound to GST as bait. GST-EH fusion protein was immobilized on glutathione sepharose beads and incubated with 293 whole cell lysates transfected to express either APPL-YFP or EEA1-GFP, a Rab5-binding partner that does not contain a NPF motif. The Pincher EH domain pulled down APPL but not EEA1 (Fig. 17B).

To test whether APPL and Pincher interact in cells we performed coimmunoprecipitation (co-IP) assays. 293 cells were transfected with Pincher-HA and either APPL-YFP or EEA1-GFP or with PincherΔEH and APPL-YFP, as indicated for each lane in fig. 17C. Whole cells lysates were immunoprecipitated with an agarose bound HA antibody, proteins resolved by SDS electrophoresis, transferred to a nitrocellulose membrane and blotted with an anti-GFP antibody. Pincher-HA pulled down APPL-YFP (Fig. 17C-right lane). This interaction is specific because Pincher-HA did not pull down EEA1-GFP (Fig. 17C-left lane) and requires the EH domain of Pincher because a deletion mutant of Pincher lacking the EH domain failed to co-IP with APPL-YFP.

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To determine whether APPL is a component of Trk endosomes, we looked at the relative localization of Trk, APPL and Pincher within cells. We found APPL to co-localize with Trk and Pincher at various stages of Trk endocytosis (Fig. 18A). Phosphorylated TrkA, APPL and Pincher are all found together at membrane ruffles at the initial stage of Trk endocytosis (Fig. 18A-top panels). Once the majority of TrkA is internalized and Pincher starts to recycle away from the receptor, APPL can be found either associated with phospho-Trk (Fig. 18A-middle panels) or Pincher (Fig. 18A-bottom panels). PincherG68E sequesters Trk signaling endosomal complexes to the cell surface (Shao et al., 2002). To determine whether APPL is part of that complex, we examined the ability of PincherG68E to sequester APPL to the cell surface. We found that APPL can either colocalize at the cell surface with PincherG68E (37.5% of cells examined, n=48, fig. 18B) or it can be found localized in enlarged internal structures independent of Pincher function (62.5% of cells, n=48, fig. 18B). It is not clear why PincherG68E is not always able to restrict APPL to the cell surface but one possibility is that APPL is differentially localized depending on its expression levels.

**The NPF motif of APPL is required for the APPL-Pincher interaction but not for Trk endocytosis.**

Since the EH domain of Pincher is required for the APPL-Pincher interaction we examined whether this interaction also requires the NPF motif of APPL. We performed in vitro binding assays using either His-APPL-WT or His-APPL-ΔNPF (a mutant APPL construct in which the NPF residues were replaced with AAA) as bait and whole cell lysates of 293 cells transfected to express HA-Pincher as prey. Only WT-APPL but not APPLΔNPF pulled down HA-Pincher (Fig. 19A). When Pincher and APPLΔNPF were coexpressed in PC12 cells they were no longer colocalized (Fig. 19B).

Given that mutating the NPF motif of APPL disrupts the APPL-Pincher interaction we expected that there would no longer exist a link between Trk and Pincher, therefore Trk endocytosis would be blocked in the presence of APPLΔNPF. We examined Trk endocytosis in cells expressing APPLΔNPF and Pincher but we found no significant reduction in the levels of Trk endocytosis after NGF treatment as compared to cells expressing APPL WT and Pincher (82.2% of control cells show endocytosis, n=45
and 80% of cells expressing APPLΔNPF, n=15, fig. 19C). It appears that the APPL-Pincher interaction is not required for Trk endocytosis.

**APPLΔNPF expression results in accelerated Trk degradation and early termination of Trk endosomal signaling.**

Disrupting the APPL-Pincher interaction does not block Trk endocytosis but significantly decreases the affinity of the receptor for Pincher. To investigate the affects of this decreased affinity on the lifetime of the resulting endosomes, we performed Trk biotinylation assays. PC12 cells were infected with either a control GFP virus, APPL WT virus or an APPLΔNPF virus. Surface proteins were biotinylated and the cells were then treated with NGF for 45 min, lysed and the cell lysates were immunoprecipitated with streptavidin beads. We found that in the presence of APPLΔNPF the majority of the receptor was degraded (Fig. 20A). After 45 min of NGF treatment, Trk levels in GFP-infected control cells were at 83±14% of Trk levels before NGF treatments. APPL-WT infected cells showed a small non-significant increase at Trk levels after NGF treatment (104%±41% of original levels), while APPLΔNPF-expressing cells showed a significant reduction of Trk levels after NGF treatment to 46.3±12.5% of original levels (averaged over 3 independent experiments), indicating that APPLΔNPF expression accelerates Trk degradation.

The increased rate of Trk degradation prompted us to examine whether signaling by the receptor is also attenuated in the presence of APPLΔNPF. Therefore we performed an endosomal signaling assay as previously described (Valdez et al., 2007), also see materials and methods) to examine the lifetime of Trk signaling. We found that in the presence of APPLΔNPF overall signaling from ERK1/2 is decreased at 5 min of NGF treatment. When we looked specifically at endosomal signaling we found that there is significantly less activated ERK 1/2 even after 5min of endocytosis and that levels of activated ERK1 and ERK2 rapidly decrease over time (Fig. 20B). While activated ERK1/2 levels remain constant between 15 and 30 min in GFP-control infected cells (ratio of 30 min to 15 min signal=1.01±.06), they decrease significantly in APPLΔNPF-expressing cells (ratio of 30 min to 15 min signal=0.52±.12, averaged over 3 independent
experiments). These results show that disrupting the Trk-Pincher interaction decreases the signaling potential of Trk receptors.

**Discussion**

The mechanisms that connect Trk signaling to endocytosis have been elusive. Here we identify the adaptor protein APPL as one molecular link between Trk and the Pincher endocytic machinery. We demonstrate that the juxtamembrane region of Trk is sufficient to recruit the Pincher endocytic machinery, presumably through binding of APPL. The APPL-Pincher interaction is mediated by the EH domain of Pincher and the NPF motif of APPL. Disruption of the APPL-Pincher interaction results in a decrease in the duration of NGF-induced ERK endosomal signaling and an increase in the degradation rate of Trk. This is a demonstration of the defining connection between Trk endocytosis and signaling and provides evidence that the nature of the Trk signaling endosome dictates its signaling potential.

We demonstrate that the juxtamembrane region of Trk is sufficient for Pincher recruitment and that this recruitment is independent of Tyr490 phosphorylation, which is required for shc and SNT/FRS2 binding and the KFG residues required for SNT/FRS2 recruitment. Consistent with this, APPL interacts with Trk at its juxtamembrane region, both directly through its PTB domain and indirectly through GIPC, and both interactions are independent of Trk phosphorylation at Tyr490 (Lin et al., 2006; Varsano et al., 2006). Interestingly the juxtamembrane region of Trk is sufficient for neuritogenesis in PC12 cells (Yoon et al., 1997) and knockdown of APPL with siRNA has been shown to significantly decrease neurite outgrowth (Lin et al., 2006; Varsano et al., 2006). APPL recruitment by the Trk juxtamembrane region and establishment of a signaling platform on endosomes may be the major signaling event required for NGF-induced differentiation.

APPL interacts with various receptors, such as adiponectin receptors (Mao et al., 2006) and NMDA receptors (Husi et al., 2000) through its PTB-binding domain and with Rab5 through its BAR and PH domains (Miaczynska et al., 2004; Zhu et al., 2007). Due to its interaction with both receptors and Rab5, APPL has been thought to bridge receptor
signaling and trafficking. Here we demonstrate a novel interaction of APPL with Pincher. This interaction is mediated by binding of the NPF motif of APPL to the EH domain of Pincher, thus providing another mechanism by which APPL can be linked to trafficking, via interaction with EH proteins. It remains to be determined whether APPL specifically interacts with Pincher or it can also bind other EHD family members in different contexts.

APPL colocalizes with Trk and Pincher at membrane ruffles and endocytic structures, indicating that it is part of the Trk endosomal complex through various stages. This is consistent with a previous study, which found APPL colocalized with Trk endosomes at different time points after internalization (Varsano et al., 2006). Surprisingly, PincherG68E did not always sequester APPL to the cell surface. It is possible that the APPL interaction with Rab5 is strong enough to localize APPL endosomally even in the presence of PincherG68E. When endosomal fractions were isolated in NGF-treated PC12 cells, APPL was found in two fractions: one that contained Trk and GIPC as well and one that contained only APPL (Lin et al., 2006). Presumably PincherG68E only blocks the formation of the Trk endosomal population but not the second population of APPL endosomes. Therefore APPL can still be localized on that second population even in the presence of PincherG68E. Whether APPL is sequestered to the cell surface or not may depend on its expression level as well as the expression levels of its different interacting proteins.

Mutation of the NPF motif of APPL disrupts its interaction with Pincher but does not prevent Trk endocytosis. Although puzzling, this is consistent with previous studies in which siRNA knockdown of APPL did not inhibit Trk endocytosis (Varsano et al., 2006). This raises the possibility that while APPLΔNPF significantly weakens the interaction between Trk and Pincher some residual interaction still remains that allows for Pincher-mediated Trk endocytosis or Pincher is recruited to Trk via an alternate route, independent of APPL. Both of these scenarios predict the formation of a Trk signaling endosome that has less affinity for Pincher, in which endocytosis is unaffected but partial uncoupling from the Pincher machinery affects further processing of these endosomes. Alternatively, Trk could be internalized via a different mechanism, such as clathrin-
mediated endocytosis, that generates endosomes that are rapidly targeted for lysosomal degradation.

We have previously shown that Pincher- and Rac- mediated endocytosis of Trk results in long-lived signaling endosomes that avoid lysosomal degradation. These endosomes are unique in their ability to retain Rab5, unlike clathrin derived EGFR endosomes. Since APPL is a Rab5-binding partner, it is possible that Trk, Pincher and APPL form a complex that retains Rab5 on the membrane through its interaction with APPL. APPL only binds the GTP-bound form of Rab5; its presence on endosomes should coincide with the presence of activated Rab5, which would prevent the association of these membranes with Rab7 and targeting to lysosomes. Consistent with this idea, APPLΔNPF overexpression results in an increased rate of Trk degradation and an attenuation of NGF induced endosomal signaling through phosphorylated ERK1 and 2. We propose that the disruption of the APPL-Pincher interaction alters the composition of Trk signaling endosomes and leads to their accelerated processing in lysosomes.

APPL exists on a population of EGFR endosomes as well. However, no complexing of APPL with EGFR has been demonstrated to date. Trk on the other hand interacts with APPL both directly and through the adaptor GIPC (Lin et al., 2006; Varsano et al., 2006). Therefore APPL is recruited to Trk and EGFR endosomes via two distinct mechanisms; to Trk endosomes via direct interaction with the receptor and to EGFR endosomes via Rab5. The mode of APPL recruitment may play an important role in the function of APPL on these endosomes. The Pincher machinery is not recruited to EGFR endosomes despite APPL presence on these endosomes, suggesting that a direct receptor-APPL interaction might be required for Pincher-mediated endocytic processing. In the case of EGFR, APPL dissociates from endosomes and translocates to the nucleus to associate with a histone deacetylase complex required for cell proliferation. Such nuclear translocation has not been observed in response to NGF. APPL release from membranes and nuclear translocation is dependent on Rab5-GTP hydrolysis. If nuclear translocation is a tightly regulated temporal event and presence of Pincher on Trk endosomes delays Rab5-GTP hydrolysis, Pincher could act to prevent APPL nuclear translocation in response to NGF. APPL acts in the nucleus to promote proliferative effects that are known to occur in response to EGF. NGF on the other hand mediates cell
differentiation and not cell proliferation. APPL may therefore play multiple roles depending on the type of receptor endosome it is associated with. In the case of EGFR its most fundamental role is in the nucleus while in the case of Trk it is on the endosomes, where it serves to retain Rab5 and delay lysosomal degradation. We propose that the presence of Pincher on these endosomes may dictate the function of APPL.

The subcellular localization of APPL plays a major role in its signaling outcome. Endosomal, but not cytosolic or nuclear, APPL can mediate Akt dependent cell survival (Schenck et al., 2008). Rab5 alone is not sufficient to retain APPL on endosomes because the APPL BAR domain that detects membrane curvature is indispensable for its endosomal localization. Pincher shares many features of the dynamin superfamily and has the ability to create membrane curvature (Daumke et al., 2007). It is possible that the coincident existence of Pincher and Rab5 on endosomes retains APPL on these endosomes. Retrogradely transported Trk endosomes mediate neuronal survival in sympathetic and sensory neurons. APPL presence on these endosomes may be crucial in mediating the transmission of Akt-mediated survival signals. Therefore it appears that Pincher, APPL and Rab5 are the backbone of a highly specific Trk endosomal signaling platform that transmits survival signals to the cell bodies of peripheral neurons.
Figure 14. The extracellular domain of Trk is not required for Pincher-mediated endocytosis.

PC12 cells were transfected with a construct expressing a chimeric receptor consisting of the EGFR extracellular domain and the TrkB intracellular and transmembrane domains (E/TrkB, A and B-left panels) or co-tranfected with E/TrkB and either Pincher-HA (A-right panels) or PincherG68E-HA (B-right panels). Cells were treated with EGF for 20 min, fixed and stained. E/TrkB (anti-EGFR, green, A and B), Pincher-HA (anti-Pincher, red, A) and PincherG68E (anti-Pincher, red, B) were visualized by confocal microscopy.
Figure 15. The juxtamembrane region of Trk is sufficient for Pincher-mediated endocytosis.

PC12 cells were transfected with a construct expressing a chimeric receptor consisting of the EGFR extracellular and intracellular domains and the TrkA juxtamembrane domain (JM chimera, A and B-left panels) or co-transfected with JM chimera and either Pincher-HA (A-right panels) or PincherG68E-HA (B-right panels). Cells were treated with EGF for 20 min, fixed and stained. JM chimera (anti-EGFR, green, A and B), Pincher-HA (anti-Pincher, red, A) and PincherG68E (anti-Pincher, red, B) were visualized by confocal microscopy.
Figure 16. The E/TrkBY515A and the E/TrkBΔKFG mutants are internalized via Pincher-mediated endocytosis.

PC12 cells were transfected with constructs containing E/TrkBY515A and either Pincher-HA (A-upper panel) or PincherG68E(A-lower panel) or with constructs containing E/TrkBΔKFG and either Pincher-HA (B-upper panel) or PincherG68E (B-lower panel). E/TrkBY515A (anti-EGFR, green, A), E/TrkBΔKFG (anti-EGFR, green, B), Pincher (anti-Pincher, red A and B-upper panels) and PincherG68E (anti-Pincher, red, A and B-lower panels) were visualized by confocal microscopy.
**Figure 17. The adaptor protein APPL1 interacts with the EH domain of Pincher.**

(A) Domain structure of APPL1 protein.

(B) In vitro binding assays were performed using the EH domain of Pincher bound to GST as bait. The EH domain pulled down APPL-YFP (lane 1), but not another Rab5-binding partner, EEA1-GFP (lane 2).

(C) HEK 293 cells were transfected with the constructs indicated on each lane. Immunoprecipitation was performed using an anti-HA antibody. Pincher-HA coimmunoprecipitated with APPL-YFP (lane 3), but not EEA1-GFP (lane 1). APPL-YFP did not coimmunoprecipitate with a deletion mutant of Pincher lacking the EH domain (PincherΔEH, lane 2).
### Table 1

<table>
<thead>
<tr>
<th>BAR domain</th>
<th>PH domain</th>
<th>NPF</th>
<th>PTB domain</th>
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BAR (Bin–Amphiphysin–Rvs) domain = Membrane curvature binding domain.

PH (Pleckstrin homology) domain = Phosphatidylinositol lipid binding domain.

NPF motif = Asn-Pro-Phe, binds to EH (Eps15-homology) domain.

PTB domain = Phospho-tyrosine binding domain, site of Trk interaction.

### Figure B

- Whole Cell Lysates
- GST-EH pulldown
- APPL-YFP
- EEA1-GFP
- anti-GFP

### Figure C

- Pincher-HA
- PincherEH-HA
- Pincher-HA+APPL-YFP
- IP anti-HA
- anti-GFP
Figure 18. APPL participates at various stages of Pincher-mediated Trk endocytosis.
Trk-PC 12 cells were co-transfected with constructs expressing APPL-YFP and either Pincher-HA (A) or PincherG68E (B). 48hrs after transfection cells were starved and the next day treated with NGF for 30min, fixed and stained. Phospho-TrkA (anti-P-Trk, cyan, A), APPL-YFP (YFP, green, A and B), Pincher (anti-HA, red, A) and PincherG68E (anti-HA, red, B) were visualized by confocal microscopy.
Figure 19. The APPL NPF motif is required for the APPL-Pincher interaction but not for Trk endocytosis.

(A) In vitro binding assays were performed using His-APPL (middle lane) or His-APPL NPF→AAA (right lane) as bait and HEK293 transfected with Pincher-HA whole cell lysate as prey. Samples were resolved with SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-HA. The band representing HA-Pincher is shown with an arrow. Other bands are non-specific, due to the HA antibody.

(B, C) Trk-PC12 cells were co-transfected with APPLΔNPF-YFP and Pincher-HA for 2 days, starved overnight, treated with NGF for 15 min, fixed and stained. APPLΔNPF (YFP, green) and Pincher (anti-HA, red) were localized at distinct cytoplasmic locations. The endocytosis of phosphorylated TrkA (anti-P-Trk, cyan, C) was not impaired.
A

Whole cell lysates  His-APPL WT  His-APPL ΔNPF

anti-HA

B

APPLΔNPF  Pincher  Merge

C

APPLΔNPF  Pincher  Phospho-TrkA
Figure 20. APPLΔNPF overexpression results in an increased Trk degradation rate and disruption of sustained Trk endosomal signaling.

(A) PC12 cells were infected with adenoviruses expressing GFP, WT-APPL-YFP or APPLΔNPF-YFP. 48 hrs after infection, surface proteins were biotinylated. Biotin was then removed, cells were treated with NGF for 45 min, lysed and lysates were immunoprecipitated (IP) with neutravidin beads. IPs were then run on a polyacrylamide gel, transferred to a nitrocellulose membrane and blotted with an anti-Trk antibody.

(B) Endosomal signaling was assessed in PC12 cells infected with a GFP virus (upper panel) or infected with a APPLΔNPF virus (lower panel) pre-treated with the MEK inhibitor U0126 for 15 min as indicated, and then also treated with NGF as indicated for 5 min. The ligands and inhibitor were removed and the cells washed and reincubated for the indicated times. In all experiments, whole cell lysates were blotted and probed with an anti-P-ERK1/2 antibody.
A

Control  APPL-WT  APPLΔNPF
-    +    -    +    -    +    +

Trk

B

Inhibitor  -  -  +  +  +  +  +
Ligand    -  +  +  +  +  +  +
Wash-Incubate  -  -  -  15  30  45

GFP virus

APPLΔNPF virus

Anti-P-Erk1/2
A specialized endosomal population mediates Trk, but not EGFR, retrograde axonal signaling.

Introduction

During development, target-derived neurotrophins promote the survival and phenotype of innervating neurons. Neurotrophins exert their effects by initiating Trk signaling cascades at nerve terminals that result in gene expression changes at the soma. According to the widely accepted signaling endosome model, ligand-bound and activated Trk receptors are endocytosed and retrogradely transported to signal at the soma. However, sustained signaling endosomes are counter to the classic role of endocytosis in receptor down-regulation, and so the mechanisms that dictate their formation and processing have been poorly understood and controversial. Given that the classical mode of clathrin-mediated receptor endocytosis generates endosomes that are rapidly targeted for recycling or lysosomal degradation, it would not appear to be suitable for generating retrogradely transported signaling endosomes. Thus, alternative mechanisms must form long-lasting Trk endosomes at neuronal terminals.

A role for macropinocytosis in NT/Trk internalization was first demonstrated by Shao et. al. with the discovery of the protein Pincher (Shao et al., 2002). Pincher functions as a pinocytic chaperone for vesicles containing NGF and TrkA via a novel macroendocytic process. Macroendocytosis is defined as a receptor-mediated, specific mode of endocytosis with characteristics of macropinocytosis, such as membrane ruffling and actin remodeling at the cell surface. Trk/Pincher endosomes recruit ERK5 and persistently signal for up to 24 hours. Overexpression of a dominant negative form of Pincher leads to accumulation of activated TrkA and ERK5 on distinctive membrane regions and block of internalization (Shao et al., 2002; Valdez et al., 2007). Additional support for a role of macroendocytosis in Trk internalization comes from early observations that NT treatment induces membrane ruffling, both in PC12 cells and in
sympathetic neurons (Connolly et al., 1981; Connolly et al., 1979). Also, GFP-TrkA is found in large, retrogradely moving, vesicular structures together with dextran, a marker for macropinosomes, in the axons of DRG neurons (Nakata et al., 1998). Recently, Valdez et al. showed that Pincher also mediates endocytosis and retrograde transport in sympathetic neurons, and that expression of dominant negative Pincher in sympathetic neurons, cultured in compartmentalized chambers, eliminates retrogradely signaled neuronal survival (Valdez et al., 2007).

The structure of the retrogradely transported endosome has also been a subject of debate. Early studies involving treatment of neuronal terminals with $^{125}$I-NGF found the majority of the retrograde label located in multivesicular bodies (MVBs) with some labeling of lysosomes as well (Claude et al., 1982a). MVBs containing NGF have been observed both in vivo (Schwab, 1977) and in vitro (Claude et al., 1982b). However a recent study by Cui et al (Cui et al., 2007) using NGF bound to quantum dot proposed that the Trk retrograde signal carrier in dorsal root ganglia (DRG) is an early endosome, containing as few as a single ligand-receptor complex. The authors also found that retrogradely transported NGF-Qdot is associated with Rab5 and this was taken as further evidence that the ligand is transported in early endosomes. Rab5, but not Rab7 was also found to be retrogradely co-transported with NGF-biotin after sciatic nerve ligation (Kaasinen et al., 2008). A different study, however, suggested that Rab7 and not Rab5 is found in axonally transported BDNF vesicles in motor neurons (Deinhardt et al., 2006). Subcellular fractionation of Trk endosomes did not reveal the presence of either Rab5 or Rab7 on these membranes (Yano and Chao, 2005).

A major caveat in the aforementioned studies is the use of NGF or BDNF to track retrogradely transported endosomes. Besides Trk, NGF and BDNF also bind to their low affinity receptor, p75NTR. Therefore, the endosomal population being followed is not a pure Trk endosomal population but also includes p75NTR endosomes that have quite distinct properties (Bronfman et al., 2003). Here, we have developed a system utilizing Campenot chambers, a chimeric EGFR/Trk receptor and EGF bound to quantum dot, that allows for the selective tracking and manipulation of Trk endosomes, independently of ligand binding to p75. Using this system we demonstrate that Trk retrograde transport
depends on a macropinocytic mechanism that generates long-lived Rab5-containing multivesicular bodies that mediate sustained retrograde signaling.

**Results**

**Developing a system for tracking retrogradely transported Trk endosomes.**

In order to define the nature and the molecular characteristics of retrogradely transported Trk endosomes, I first had to devise a system that allows for the selective visualization and manipulation of nerve terminal-derived Trk signaling endosomes (Fig. 21A). Compartmentalized Campenot chambers have been used as a tool to isolate the environment of distal axons from that of cell bodies (Campenot, 1977). To identify endosomes originating from neuronal terminals, labeled NGF can be applied to the distal axon compartment and followed to the cell body. This however poses the problem that, since labeled NGF can also bind the p75NTR receptor, the endosomal population being followed does not only consist of Trk endosomes but is a mix of Trk and p75NTR endosomes, two endosomal populations that have been shown to have quite distinct properties (Bronfman et al., 2003). To circumvent that problem I used an adenovirus containing an EGFR/TrkB chimera (E/Trk), consisting of the extracellular domain of EGFR and the intracellular and transmembrane domains of TrkB. The chimeric receptor has been shown to retain all Trk signaling (Wang et al., 1996) but is activated in response to EGF binding. Therefore I can follow these receptors to the cell body by treating the distal axon compartment with EGF coupled to quantum dot (EGF-Qdot605). No significant expression of EGFR in peripheral neurons has been reported and uninfected neurons do not show an uptake of EGF-Qdot605 (not shown), demonstrating that any appearance of EGF-Qdot605 at the cell body corresponds to retrogradely transported E/Trk endosomes. Cultured sympathetic ganglion neurons (SCGs) grown in Campenot chambers were infected with the E/Trk adenovirus, starved overnight 48hrs after infection and treated with EGF-Qdot605 at the distal axon compartment for 2hrs. EGF-Qdot605 was found to be retrogradely transported together with E/Trk along proximal axons (Fig. 21B) and to reach the cell body also together with the receptor (Fig. 21C).
Trk is retrogradely transported in multivesicular bodies.

In order to identify the ultrastructure of the Trk retrograde endosomes, we performed EM of SCGs grown in Campenot chambers, infected with the E/Trk adenovirus. Cells were treated with EGF-Qdot605 at the distal axons for 2hrs and then processed for EM. Quantum dots are electron-dense and can be directly visualized by EM but are very small. To make their visualization easier we used a silver enhancing method (see materials and methods). Using this approach we were able to see retrogradely transported quantum dot accumulate in partially filled multivesicular bodies in the cell bodies of infected neurons (Fig. 22A) and along the axons (Fig. 22B).

Trk retrograde transport is Pincher- and Rac-dependent.

To begin to uncover the molecular mechanisms responsible for Trk retrograde transport, I looked at the requirements for this process. Since Trk endocytosis requires Pincher and Rac, it is likely that these two proteins also play a role in Trk retrograde transport. To test that I slightly modified the chamber paradigm previously used (Fig. 23A). In this modified paradigm SCGs were grown in Campenot chambers for two weeks and then infected with the E/Trk adenovirus. Two days later they were treated with EGF-Qdot525 (EGF bound to a quantum dot of a different emission wavelength that the one previously used) for 6hrs at the distal axon compartment. The distal axons were then washed and the neurons were infected with either dominant-negative Pincher (PincherG68E) or dominant-negative Rac (RacN17). Three days later the distal axons were treated with EGF-Qdot605 for two hours and the cells were fixed and stained. Cells were found to either contain EGF-Qdot525 alone or both EGF-Qdot525/605 in their cell bodies. Appearance of both quantum dot-bound ligands in the cell body indicates that cells were capable of retrograde transport at both times tested. Appearance of EGF-Qdot525 but not EGF-Qdot605 means that cells that could retrogradely transport ligand before infection with a dominant negative construct were unable to do so after. In control cells, infected only with E/Trk, 65.7% of cells containing EGF-Qdot525 also contained EGF-Qdot605 (n=872 cells in 44 chambers). The reason this number is not closer to 100% is that over time receptor expression increases and neurons with very high receptor numbers show a deficiency in retrograde transport (P. Philippidou, unpublished...
observation). When PincherG68E or RacN17 were expressed only 10.2% or 32% respectively of EGF-Qdot525 containing cells also contained EGF-Qdot605 (n=88 cells in 13 chambers for PincherG68E and n=72 cells in 16 chambers for RacN17) indicating that both Rac and Pincher are required for Trk retrograde transport (Fig. 23B).

Since Rac and Pincher are critical for the formation of Trk endosomes, it is possible that they will be associated with the endosomes as they reach the cell body. Wild type Rac dissociates from endosomes after mediating their formation, but a constitutively active form of Rac, RacV12, remains associated with the membranes of endosomes derived from Rac-mediated macroendocytosis (Valdez et al., 2007). If Trk endosomes are generated by a Rac-mediated process, RacV12 will be associated with them in the cell body. To investigate that, SCGs grown in Campenot chambers were infected with E/Trk and RacV12-GFP containing adenoviruses and treated with EGF-Qdot605 at the distal axons for two hours. It was found that 52.6% of E/Trk/EGF-Qdot605 complexes were in fact associated with RacV12 (n=211 EGF-Qdot605 particles in 49 cells, Fig. 24A). Pincher has been shown to colocalize with Trk along sympathetic axons (Valdez et al., 2005). To see if it is also found with Trk retrograde endosomes in sympathetic neuron cell bodies, SCGs in chambers were co-infected with E/Trk- and Pincher-expressing adenoviruses and treated with EGF-Qdot605 for 2hrs at the distal axons. 53.8% of E/Trk/EGF-Qdot605 complexes were found to also contain Pincher (n=91 EGF-Qdot605 particles in 61 cells, Fig. 24B).

**EGFR is also retrogradely transported in MVBs in SCGs.**

Trk retrograde signaling is critical for peripheral neuron survival and the development of a neuronal phenotype. We hypothesized that perhaps only receptors that have an indispensable role in neuronal function can be retrogradely transported. To test this hypothesis I used an EGFR-expressing adenovirus in place of E/Trk and treated the distal axon compartments of Campenot chambers with EGF-Qdot605. Surprisingly, EGFR also has the ability to retrogradely transport in SCGs (Fig. 25A). Analysis of the ultrastructure of the retrogradely transported EGF-Qdot605 endosomes revealed that these endosomes are also multivesicular bodies (Fig. 25B).
Trk and EGFR employ different mechanisms for retrograde transport.

Since EGFR is capable of retrograde transport, I asked whether it utilizes the same molecular mechanisms as Trk. Previous findings in PC12 cells (Valdez et al., 2007) demonstrated that the endocytic pathways of Trk and EGFR are quite distinct. Pincher is required for Trk endocytosis but EGFR endocytosis is Pincher-independent. To test whether Pincher plays a role in EGFR retrograde transport I developed another paradigm utilizing Campenot chambers. SCGs grown in chambers were infected with either E/Trk or EGFR-GFP virus alone or together with PincherG68E. At the same time the distal axons were treated with fluospheres, carboxylate-modified microspheres that are non-specifically taken up by the cells, in order to mark projecting neurons. Three days later the distal axons were treated with EGF-Qdot605 for 2hrs (Fig. 26A). Retrograde transport was measured as the percentage of fluosphere positive cells that showed appearance of EGF-Qdot605 at the cell bodies. 78.3% of cells expressing E/Trk alone (n=152 cells in 9 chambers) and 88.8% of EGFR expressing cells (n=116 cells in 3 chambers) exhibited retrograde transport. When PincherG68E was co-expressed with the receptors, only 13.3% of E/Trk expressing cells (n=45 cells in 3 chambers) as opposed to 83.3% (n=114 cells in 2 chambers) of EGFR expressing cells exhibited retrograde transport (Fig. 26B), indicating that Pincher is required for Trk, but not EGFR, retrograde transport.

E/Trk and EGFR retrograde endosomes are differentially processed.

The differential modes of retrograde transport for E/Trk and EGFR may affect the processing of axon derived endosomes that reach the cell body. To test that idea I looked at the colocalization of these endosomes with the early endosomal marker Rab5 and the late lysosomal marker Rab7. SCGs grown in Campenot chambers were infected with adenoviruses containing either E/Trk or EGFR and either Rab5-GFP or Rab7-GFP. Two days after infection the distal axons were treated with EGF-Qdot605 for 2hrs, fixed and stained. In the case of E/Trk, 59.3% of receptor/ligand complexes (n=273 EGF-Qdot particles in 100 cells) were associated with Rab5 and 34.8% (n=264 EGF-Qdot complexes in 47 cells) were associated with Rab7. In EGFR expressing cells, 34.2% (n=222 EGF-Qdot particles in 51 cells) of receptor/ligand complexes were found
colocalized with Rab5 while 50.9% (n=266 EGF-Qdot particles in 93 cells) were colocalized with Rab7 (Fig. 27).

Since E/Trk endosomes are preferentially associated with Rab5 while EGFR ones are preferentially associated with Rab7, it appears that EGFR processing is more rapid. Once endosomes acquire Rab7, they are targeted for lysosomal degradation shortly thereafter. I examined the association of both E/Trk and EGFR endosomes with the lysosomal marker cathepsin to determine whether EGFR endosomes are indeed degraded at a faster rate than E/Trk ones. When EGF-Qdot605 is continuously applied at the distal axons new endosomes are constantly generated, creating a heterogeneous endosomal population in regards to the time of appearance at the cell body. Therefore instead of treating the distal axons with EGF-Qdot continuously I applied a pulse chase protocol. I incubated the distal axons with EGF-Qdot at 4°C for 30min and after washing the axons multiple times I moved the cells to 37°C for various time points. After 4hrs at 37°C in cells expressing E/Trk, 29.5% of EGF-Qdot (n=88 EGF-Qdot particles in 15 cells) was associated with cathepsin. In cells expressing EGFR-GFP, 41.2% of EGF-Qdot (n=284 EGF-Qdot particles in 19 cells) was found colocalized with cathepsin (Fig. 28). This indicates that EGFR retrograde endosomes are targeted for degradation at a greater rate than E/Trk ones.

The association of different markers with receptor endosomes must correspond to the nature of these structures. The selective association of EGFR endosomes with Rab7 and cathepsin indicates a fast progression of the receptor to late endosomes/lysosomes. To examine the ultrastructure of EGF-Qdot containing endosomes EM analysis was carried out in cells expressing E/TrkB or EGFR-GFP. Infected SCGs in Campenot chambers were incubated with EGF-Qdot605 for 2hrs. Detailed analysis of quantum dot containing structures of E/Trk infected cells revealed that both in the proximal axons and in the cell bodies the vast majority of endosomes are multivesicular bodies (MVBs) (Fig. 29A-left panels). Out of those, 62% are partially filled “light” MVBs and 38% are electron dense (n=42). However, the majority of EGFR-containing MVBs appear to be more filled and electron dense than the E/Trk ones (63% electron dense, n=35, fig. 29B-right panels). Interestingly, proximal axons of EGFR-GFP infected neurons show a high density of lysosomes (Fig. 29B-top panel), contrary to E/TrkB infected neurons that are
essentially devoid of lysosomes (Fig. 29B-bottom panel). Cell bodies of both neuronal populations contain comparable numbers of lysosomes (P. Philippidou, W. Akmentin, S. Halegoua, unpublished observations). This indicates that lysosomal degradation of EGFR complexes may begin in the proximal axon, while Trk receptors avoid degradation until reaching the cell body.

Contrary to EGFR, E/Trk does not rapidly progress to lysosomes/dark MVBs. It was intriguing that after 2hrs of treatment with EGF-Qdot605 more than 50% of the receptor/ligand complexes are associated with Rab5, yet the only structures that appear at the cell body are MVBs. This is surprising given the current view that Rab5 is displaced for Rab7 once early endosomes become MVBs (Rink et al., 2005). To address this apparent contradiction, we examined Rab5 localization in relation to EGF coupled to fluoronanogold (FNG) by using double-labeling EM. We used EGF-FNG for EM double-labeling instead of EGF-Qdot605 because EGF-FNG can be gold enhanced instead of silver enhanced. To test whether EGF ligand and Rab5-GFP can be seen in the same structures we had to post-embed label the cells for GFP. This process results into breaking down of silver but not gold, therefore silver enhanced quantum dot would be damaged while gold enhanced FNG stays intact.

We found that a number of Trk-containing MVBs are in fact positive for Rab5 (Fig. 30), which could explain their relative resistance to degradation when compared to EGFR.

**Retrogradely transported Trk, but not EGFR, is capable of sustained ERK signaling and gene induction.**

Differential processing of axon derived Trk and EGFR complexes might result in differences in their signaling potential. To test that idea, I looked at the association of the two receptor/ligand complexes with activated ERK5 (phospho-ERK5). SCGs grown in Campenot chambers were infected with either the E/Trk or EGFR-GFP virus, treated with EGF-Qdot605 for two hours and then stained with an antibody against phospho-ERK5. I found that 53.7% (n=160 EGF-Qdot605 particles in 52 cells) of E/Trk/EGF-Qdot605 complexes were associated with phospho-ERK5 as opposed to 32.6% (n=178 EGF-Qdot605 particles in 30 cells) of EGFR/EGF-Qdot605 complexes (Fig. 31).
The fact that Trk shows an increased association with signaling effectors could be indicatory of its ability to induce downstream trophic factor effects more efficiently than EGFR. To test that hypothesis I asked whether retrograde Trk and EGFR signaling are each capable of gene induction. VGF is a neuropeptide that has been shown to be upregulated in PC12 cells in response to NGF, through the ras-ERK pathway. To see whether retrogradely transported Trk or EGFR signaling endosomes would induce VGF expression I used SCGs grown in chamber cultures that were either uninfected or infected with EGFR-GFP. Receptor overexpression has been shown to cause autoactivation of the receptor at high numbers and bypass the need for a ligand. Therefore I used a low amount of EGFR-GFP virus that did not result in receptor autoactivation and only collected data from cells with low receptor numbers. SCGs were starved for 60hrs in the presence of anti-NGF and then treated for 10 hrs with either NGF+fluospheres (to mark projecting cells) for uninfected cells or EGF-Qdot605 for EGFR infected cells. I found that only NGF treatment caused an upregulation of VGF in these cells (Fig. 32A). To test whether VGF upregulation by NGF requires the retrograde transport machinery, I infected the cells with a herpes simplex virus expressing PincherG68E. Hsvs can infect sympathetic neurons retrogradely so I applied the virus in the distal axon compartment of Campenot chambers so as to also mark the projecting neurons. Cells were again starved for 60 hrs after infection and treated with NGF and fluospheres at distal axons for 10hrs. Expression of PincherG68E prevented VGF induction by NGF (Fig. 32B), demonstrating that the ability of Trk for retrograde gene induction requires Pincher-dependent retrograde transport of the receptor.

**Discussion**

Direct visualization of ligand/receptor complexes provides a powerful quantitative tool for dissecting the mechanisms of receptor retrograde transport. NGF binds to a high affinity receptor, Trk, and a low affinity receptor, p75NTR. I have developed a novel method for distinguishing and tracking retrogradely transported Trk endosomes. This technique allows for the selective visualization and manipulation of Trk endosomes, as opposed to previous methods that could not differentiate between Trk and p75NTR
endosomes (Cui et al., 2007). This is especially important given the fact that Trk and p75NTR signaling endosomes are spatially and temporally distinct (Bronfman et al., 2003) and depend on different mechanisms. Retrograde transport of p75NTR in sympathetic neurons occurs via clathrin-dependent and lipid-raft dependent mechanisms (Hibbert et al., 2006) while we have previously shown that macroendocytosis plays a major role in Trk retrograde transport and signaling (Valdez et al., 2005).

The E/Trk chimera is crucial for this system. This chimeric receptor has the same signaling potential as Trk (Obermeier et al., 1993; Wang et al., 1996). I have shown that this chimera follows the same endocytic mode as Trk in PC12 cells (chapter IV). The cytoplasmic and transmembrane domains of Trk and p75NTR may regulate NGF high affinity binding to Trk, independent of ligand binding to p75NTR (Esposito et al., 2001). Therefore, in regards to receptor signaling, endocytosis and ligand binding, E/Trk can be considered equivalent to Trk.

By using this method I was able to demonstrate that the Trk retrograde carrier is a multivesicular body (MVB) that contains multiple receptor/ligand complexes. This is consistent with previous in vivo and in vitro studies (Claude et al., 1982a; Hendry et al., 1974; Schwab, 1977) that also identified MVBs as the primary axonally-derived NGF containing structures at the cell bodies of sympathetic neurons. It has been proposed that the double membrane of MVBs serves a protective role for the enclosed signaling complexes (Weible and Hendry, 2004). Other studies (Delcroix et al., 2003; Howe and Mobley, 2004), as well as a recent study utilizing NGF-Qdot (Cui et al., 2007) suggested that early endosomes are the primary NGF retrograde vehicles. We do not see any evidence of early endosomes reaching the cell bodies in our cultures—in fact we only see MVBs even along the axon. Early endosomes are short lived and are either targeted to recycling pathways or degradation pathways soon after their generation—approximately within 10-15 min (Rink et al., 2005). It is hard to imagine how early endosomes would avoid these pathways along sympathetic axons, with the average time of travel to reach the cell body being 45 min (Cui et al., 2007). As the authors of this study only looked at the EM structure of vesicles at proximal axons, it is possible that they captured newly generated early endosomes along the axon. It was not demonstrated whether these vesicles actually reached the cell body.
This study also demonstrates that Trk retrograde transport is dependent on a macropinocytic mechanism that requires Pincher and Rac function. We have previously demonstrated that Trk endocytosis is Rac- and Pincher- dependent in PC12 cells (Valdez et al., 2007) and that Pincher is required for Trk retrograde signaling in sympathetic neurons (Valdez et al., 2005). By using a direct visualization approach we see a dramatic reduction in the receptor/ligand complexes that reach the cell bodies in the presence of dominant negative Pincher and Rac. We also see more than 50% of these complexes colocalize with Pincher and RacV12. Pincher and Rac may dissociate from a number of these endosomes after a period of being at the cell body. RacN17 does not block Trk retrograde transport to the extent that PincherG68E does. It is possible that the RacN17 is less efficient as a dominant negative than PincherG68E. We think it is unlikely that a Pincher-dependent, Rac-independent mechanism for retrograde transport exists since we have previously shown a co-dependence of these two proteins for each other’s endocytosis, as well as Trk endocytosis (Valdez et al., 2007).

The retrograde transport of EGFR came as a surprise to us. Since EGFR uses a clathrin-dependent endocytic pathway (Vieira et al., 1996) we hypothesized that early endosomes would be rapidly processed at neuronal terminals or along axons and would never reach the cell body. This was not the case as EGFR/EGF complexes were retrogradely transported in sympathetic neurons, indicating that clathrin-derived endosomes can be retrogradely transported, as shown for p75NTR (Hibbert et al., 2006). As expected, EGFR retrograde transport was Pincher-independent, just like EGFR endocytosis in PC12 cells (Valdez et al., 2007). EGFR was also transported in MVBs suggesting that MVBs might be the only endocytic structure stable enough to transverse the axon. EGFR MVBs appeared to be darker than Trk ones upon reaching the cell body, demonstrating that endosomal processing can begin in the axons. In fact we can see lysosomes along the axons of sympathetic neurons, which suggests that retrograde transport of receptor/ligand is not sufficient for retrograde signaling, since it is possible for this complex to reach the cell body in a structure already destined for degradation.

Since Trk and EGFR are retrogradely transported via distinct mechanisms we sought to examine their processing state upon reaching the cell body. EGFR receptor complexes appeared to be further along their processing since their majority associated
with Rab7 and cathepsin as opposed to Trk receptors that were mainly associated with Rab5. Association of retrograde Trk with Rab5 is consistent with other studies (Cui et al., 2007; Kaasinen et al., 2008). A recent study showed that Rab7, and not Rab5, is associated with Trk retrogradely transported endosomes. This study used fluorescent tetanus neurotoxin (TeNT) to monitor these endosomes after demonstrating that 96% of these endosomes also contain TrkB and p75NTR (Deinhardt et al., 2006). In other cases, however, a much lower colocalization has been seen for p75NTR and Trk (Kaasinen et al., 2008). It is possible that TeNT redirects Trk to the p75NTR trafficking pathway or that the presence of TeNT in those endosomes alters their properties. In our case, we looked exclusively at Trk/ligand endosomes and found their majority to be associated with Rab5.

The association of Rab5 with MVBs also came as a surprise given the current notion that Rab7 displaces Rab5 from endosomes once they become MVBs (Rink et al., 2005). Since Pincher and Rab5 have common binding partners, we propose that Pincher presence on Trk retrograde endosomes serves to retain Rab5 there, even as they become MVBs. It has been proposed that Rab5 activity serves to recruit Rab7 on membranes and Rab7 then acts to decrease Rab5 activity, leading to its membrane dissociation. Constitutively active Rab5 does not dissociate from these membranes (Del Conte-Zerial et al., 2008). It is possible that Pincher acts to increase Rab5 activity through prolonging its association with effectors such as Rabankyrin, Rabenosyn and APPL, essentially turning it into a constitutively active form. Presence of Rab5 on these MVBs may serve to protect them from lysosomal degradation, explaining the difference in the amount of processing seen between Trk and EGFR retrogradely transported MVBs.

Another difference between Trk and EGFR retrogradely transported endosomes is that a higher percentage of Trk endosomes are associated with activated ERK5. As endosomes move further along lysosomal processing, signaling molecules are inactivated by acidification and degradation. Increased association of Trk with phospho-ERK5 is consistent with less lysosomal processing. Induction of VGF by Trk but not EGFR retrograde signaling, demonstrates that only Trk endosomes have the properties required for long-term signaling that leads to late-gene induction. The fact that VGF induction by...
NGF is blocked by PincherG68E shows that the transport of retrograde signaling endosomes is required for gene induction.

We have demonstrated that Trk is retrogradely transported in Rab5-positive multivesicular bodies. Retrograde transport requires the function of Pincher and Rac and the resulting endosomes avoid degradation to mediate signaling events leading to gene induction. Despite its ability to also retrogradely transport, EGFR cannot mediate the same signaling events as Trk. It follows that retrograde signaling depends upon the mechanism of retrograde transport and on the nature and processing of retrogradely transported endosomes. When the lifetime of p75NTR-bound BDNF was examined, it was found that after 5hrs of internalization the vast majority of BDNF was degraded (Hibbert et al., 2006). In our case we see EGF-Qdot in non-lysosomal MVBs even after 12 hrs of ligand treatment (unpublished observation). It will be interesting to determine whether this difference is due to the distinct pathways mediating endocytosis of p75NTR and Trk.
Figure 21. E/Trk is retrogradely transported in sympathetic neurons.
Sympathetic neurons grown in compartmentalized cultures were infected with an adenovirus containing the E/Trk chimera for 48hrs. Cells were then starved, treated with EGF-Qdot605 for 2 hrs at the side compartment (paradigm shown in A), fixed and stained with an antibody against the extracellular domain of EGFR. E/Trk (anti-EGFR, green, B and C) and EGF-Qdot605 (Qdot605, red, B and C) were visualized.
Figure 22. E/Trk is retrogradely transported in multivesicular bodies (MVBs).
SCGs grown in compartmentalized cultures were infected with an adenovirus containing the E/Trk chimera for 48hrs. Cells were then starved, treated with EGF-Qdot605 for 2 hrs at the side compartment, fixed and processed for EM (materials and methods). Quantum Dot was silver enhanced, also as described in materials and methods. EGF-Qdot accumulated in partially filled MVBs in the cell body (A) and in the axons (B). Scale bar=2um in A, 500nm in B.
Figure 23. E/Trk retrograde transport requires Pincher and Rac.
SCGs grown in compartmentalized cultures were infected with an adenovirus containing the E/Trk chimera for 48hrs. They were then treated with EGF-Qdot525 for 6hrs at the side compartment and then infected with an adenovirus expressing dominant-negative Pincher (PincherG68E-HA) (B-middle panel) or dominant negative Rac (RacN17-T7) (B-lower panel) or not infected (B-upper panel). Three days later the side compartments were treated with EGF-Qdot525 for 2hrs, fixed and stained. The paradigm is depicted in (A). EGF-Qdot525 (Qdot525, green, B), EGF-Qdot605 (Qdot605, cyan, B), PincherG68E (anti-HA, red, B-middle panel) and RacN17 (anti-T7, red, B-lower panel) were visualized.
**A**

Day 1: E/Trk virus

Day 3: EGF-Qdot525

Day 3-6hrs later: Wash

Day 6: EGF-Qdot605

*PincherG68E or RacN17 virus*

**B**

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Figure 24. E/Trk colocalizes with Pincher and RacV12.
SCGs grown in compartmentalized cultures were infected with an adenovirus containing the E/Trk chimera and an adenovirus containing either RacV12-GFP (A) or Pincher-HA (B). 48 hrs after infection cells were starved and treated the next day with EGF-Qdot605 at the distal axon compartment for 2hrs. E/Trk (anti-EGFR, red, A and B), EGF-Qdot605 (Qdot605, cyan, A and B), RacV12-GFP (GFP, green, A) and Pincher-HA (anti-Pincher, green, B) were visualized.
Figure 25. EGFR is retrogradely transported in MVBs in sympathetic neurons.

(A) SCGs grown in compartmentalized cultures were infected with an adenovirus containing EGFR for 48hrs. Cells were then starved, treated with EGF-Qdot605 at the distal axons for 30 min at 4°C, washed three times with DMEM and then moved to 37°C for 90 min. EGFR (anti-EGFR, green, A) and EGF-Qdot605 (Qdot605, red, A) were visualized.

(B, C) SCGs grown in compartmentalized cultures were infected with an adenovirus containing EGFR for 48hrs. Cells were then starved, treated with EGF-Qdot605 at the distal axons for 2 hrs, fixed and processed for EM (materials and methods). Quantum dot was silver enhanced, also as described in materials and methods. Individual examples of MVBs found along axons (C) and in cell bodies (B). Scale bar=500nm
Figure 26. EGFR retrograde transport is Pincher-independent.
SCGs grown in compartmentalized cultures were infected with an adenovirus containing PincherG68E-HA and an adenovirus containing either E/Trk (B-upper panel) or EGFR (B-lower panel) and treated with green fluorospheres at the distal axon compartment for 48hrs. Cells were then starved and the next day treated with EGF-Qdot605 at the distal axons for 2hrs. The paradigm is depicted in (A). E/Trk (anti-EGFR, red, B-upper panel), EGFR (anti-EGFR, red, B-lower panel), PincherG68E (anti-Pincher, cyan, B), fluorospheres (green, B) and EGF-Qdot605 (Qdot605, purple, B) were visualized by confocal microscopy.
Day 1
Viruses: E/Trk or EGFR + HA-PincherG68E

Day 3
Fluorospheres
EGF-Qdot605

B
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Figure 27. E/Trk preferentially associates with Rab5 while EGFR preferentially associates with Rab7.

SCGs grown in compartmentalized cultures were infected with either an adenovirus containing E/Trk (A-upper panel, B-upper panel) or EGFR (A-lower panel, B-lower panel) and an adenovirus containing either Rab5-GFP (A) or Rab7-GFP (B). 48hrs after infection cells were starved and the next day treated with EGF-Qdot605 for 2hrs at the distal axon compartment, fixed and stained. E/Trk (anti-EGFR, red, A and B upper panels), EGFR (anti-EGFR, red, A and B lower panels), EGF-Qdot605 (Qdot605, cyan, A and B), Rab5-GFP (GFP, green, A) and Rab7-GFP (GFP, green, B) were visualized by confocal microscopy.
Figure 28. Cathepsin associates preferentially with EGFR retrograde endosomes.

SCGs grown in compartmentalized cultures were infected with an adenovirus containing either E/Trk (upper panel) or EGFR (lower panel). 48hrs after infection, cells were starved and the next day the distal axon compartments were incubated with EGF-Qdot605 at 4°C for 30 min. The distal axons were then washed with DMEM three times and the cells were moved to 37°C for 4 hrs, fixed and stained. E/Trk (anti-EGFR, red, upper panel), EGFR (anti-EGFR, red, lower panel), EGF-Qdot605 (Qdot605, cyan) and cathepsin (anti-cathepsin, green) were visualized by confocal microscopy.
Figure 29. EGFR retrograde MVBs are processed faster than E/Trk MVBs.
SCGs grown in compartmentalized cultures were infected with an adenovirus containing either E/Trk (A and B, upper panels) or EGFR (A and B, lower panels). 48hrs after infection, cells were starved and the next day the distal axon compartments were incubated with EGF-Qdot605 for 2hrs, fixed and processed for EM as described in Materials and Methods. Ligand-labelled E/Trk MVBs (A-upper panel) do not appear as electron-dense as EGFR ones (B-lower panel). Proximal dendrites of EGFR (B-lower panel), but not E/Trk (B-upper panel), contain lysosomes. Scale bar=200nm in A, 2um in B.
Figure 30. Trk retrogradely transported MVBs retain Rab5.
SCGs grown in chamber cultures were co-infected with an adenovirus expressing E/Trk and one expressing Rab5-GFP. 48 hrs after infection the distal axons were treated with EGF conjugated to fluoronanogold (EGF-FNG) for 2hrs. Cell bodies and proximal axons were then fixed and processed for EM. Fluoronanogold was gold enhanced and the cells were post-embed, immuno-gold-labeled for GFP as described in materials and methods. Scale bar=50nm.
Figure 31. Activated ERK5 preferentially associates with Trk endosomes. SCGs grown in compartmentalized cultures were infected with an adenovirus containing either E/Trk (upper panel) or EGFR (lower panel). 48hrs after infection, cells were starved and the next day the distal axon compartments were treated with EGF-Qdot605 for 2hrs. Cells were then fixed and stained. E/Trk (anti-EGFR, green, upper panel), EGFR (anti-EGFR, green, lower panel), EGF-Qdot605 (Qdot605, cyan) and phospho-ERK5 (anti-phospho-ERK5, red) were visualized by confocal microscopy.
Figure 32. NGF, but not EGF, treatment at distal axons induces VGF expression in a Pincher-dependent manner.

(A) Uninfected (upper panel) or EGFR-GFP adenovirus infected (lower panel) SCGs grown in compartmentalized chamber cultures were starved in the presence of anti-NGF for 60 hrs. The distal axons were then treated with either fluorospheres and NGF (upper right panels) or EGF-Qdot605 (lower panel) for 10 hrs or left untreated (upper left panel). VGF (anti-VGF, red), fluorospheres (green-upper panel), EGFR-GFP (GFP, green, lower panel) and EGF-Qdot605 (Qdot605, cyan, lower panel) were visualized by confocal microscopy.

(B) SCGs grown in compartmentalized chamber cultures were starved for 60 hrs in the presence of anti-NGF and at the same time infected at distal axons with a herpes simplex virus containing PincherG68E-HA. Cells were then treated with fluorospheres and NGF for 10 hrs at the distal axon compartments. VGF (anti-VGF, red), PincherG68E (anti-HA, green) and fluorospheres (cyan) were visualized by confocal microscopy.
Chapter VI

Pincher-mediated macroendocytosis underlies generation of Trk signaling endosomes in hippocampal neurons

Introduction

According to the Neurotrophin (NT) hypothesis for synaptic plasticity, repetitive neuronal activity stimulates NT expression, secretion and activity to facilitate activity-dependent synaptic plasticity (Schinder and Poo, 2000). Of the neurotrophins, BDNF and its receptor TrkB have been extensively studied in the context of changes in synaptic strength, as exemplified by Long Term Potentiation (LTP), as well as in the context of hippocampal-dependent long-term memory. BDNF has proven to be extremely versatile and has been implicated both in the early, short lasting form of LTP (E-LTP) and the protein synthesis-dependent late phase LTP (L-LTP). It has also been implicated in memory acquisition, retention and retrieval (for review see Lu et al., 2008; Yamada and Nabeshima, 2003).

The evidence for the involvement of BDNF and TrkB in synaptic plasticity and memory is overwhelming. Both BDNF heterozygous knockout and TrkB knockout mice show impaired L-LTP (Korte et al., 1995; Minichiello et al., 1999), but the deficit in BDNF knockout mice can be reversed with application of exogenous BDNF (Korte et al., 1996). These animals also show impairments in hippocampal dependent memory tasks such as the water maze (Linnarsson et al., 1997). Deficits in L-LTP are also observed after treatment of hippocampal slices with antibodies against TrkB or BDNF or the application of the BDNF scavenger TrkB-Fc (Kang et al., 1997). The importance of BDNF in synaptic plasticity is underscored by the fact that application of exogenous BDNF is sufficient to induce L-LTP in the presence of protein synthesis inhibitors (Pang et al., 2004).

What are the downstream signaling mechanisms that contribute to BDNF actions in synaptic plasticity? Both presynaptic axonal terminals and postsynaptic dendrites
contain BDNF secretory vesicles (Fawcett et al., 1997) that upon high electrical
stimulation release BDNF into the synaptic cleft (Hartmann et al., 2001). Secreted BDNF
can then bind TrkB, both presynaptically and postsynaptically (Drake et al., 1999).
Tetanic stimulation also causes an increase in TrkB mRNA and protein levels in
dendrites (Tongiorgi et al., 1997), activity-dependent insertion of TrkB into the plasma
membrane (Du et al., 2000; Meyer-Franke et al., 1995), enhancement of BDNF-mediated
TrkB endocytosis (Du et al., 2003) and an increase in TrkB phosphorylation levels (Du et
al., 2003). Induction of L-LTP ultimately causes an activation of cAMP-dependent
protein kinase (PKA) and ERK kinases (Kandel, 2001) that phosphorylate transcription
factors resulting in the transcription of downstream genes, including BDNF. Activity-
induced signaling results in long term increase in synaptic transmission that can last for
days (Abraham and Williams, 2003).

The role of TrkB endocytosis in the regulation of synaptic plasticity is not clear.
Inhibition of TrkB internalization inhibits TrkB translocation to lipid rafts (Pereira and
Chao, 2007) which has been shown to be critical for the potentiating effects of BDNF on
synaptic responses to tetanus in hippocampal slices (Suzuki et al., 2004). Furthermore,
electrical activity enhances TrkB endocytosis (Du et al., 2003). Despite the vital role of
retrogradely transported Trk signaling endosomes in other systems, such as survival of
peripheral neurons (for review see Zweifel et al., 2005), their role in synaptic potentiation
has not been examined.

We have previously demonstrated that hippocampal neurons express the
membrane trafficking protein Pincher, that mediates Trk endocytosis in PC12 cells and
sympathetic neurons, and that ligand-bound Trk colocalizes with Pincher along
hippocampal processes (Valdez et al., 2005). Here we show that ligand-bound Trk is
trafficked along hippocampal dendrites in a Pincher- and Rac-dependent manner. We also
show that Trk endosomes are preferentially associated with Rab5 over Rab7 and are also
associated with a number of Pincher- and Rab5- binding partners. Finally we show that
Trk endosomes contain activated ERK5, making them a potential mode of transmitting
signals from dendritic terminals to the hippocampal cell body.
Results

**Trk endosomes are trafficked along hippocampal dendrites.**

Trk signaling endosomes move retrogradely along axons of peripheral neurons to reach the cell body and these endosomes are the primary Trk signaling platform in these neurons. Since Trk/BDNF signaling has been implicated in hippocampal functions such as L-LTP, we decided to investigate whether Trk signaling endosomes along hippocampal dendritic processes could possibly mediate Trk signaling. In order to examine if Trk endosomal structures can be localized at dendrites of hippocampal neurons we expressed the E/TrkB chimeric receptor in cultured hippocampal neurons and treated the cells with EGF-Qdot605 for 20 min to induce internalization of the receptor. Both receptor and ligand were found indiscriminately at all neuronal processes (Fig. 33). This demonstrates that Trk endosomes are not selectively localized at neuronal axons but can be found in dendrites as well.

In order to define the structures that contain Trk/ligand complexes along hippocampal dendrites we transfected cultured hippocampal neurons with the E/TrkB chimeric receptor, treated the cells with EGF-Qdot605 for 20 min and then processed the cells for electron microscopy. Quantum dots were silver-enhanced to increase their visibility. EGF-Qdot605 was found along the surface of hippocampal processes as well as in clear endosomes and multivesicular bodies (Fig. 34). EGF-Qdot605 was also found concentrated at membrane ruffles at the cell surface of these cells (Fig. 34A). Multiple receptor-ligand complexes appear to be internalized in the same endocytic structure, since multiple EGF-Qdot605 signals are found in all endosomes, as depicted in the multivesicular body in figure 34C. Both surface and internalized label was observed.

Since Trk/ligand complexes are found in endocytic structures along hippocampal neurites, we wanted to examine whether these structures are mobile and thus can transmit signals to the cell body. In order to do so, we infected hippocampal neurons with E/TrkB, incubated them with EGF-Qdot605 for 20 min and then performed live imaging in a temperature-controlled chamber kept at 37°C using a high-speed camera (see materials and methods). While the majority of EGF-Qdot605 foci appeared to be immobile, we found two populations of mobile endosomes. The first population was a fast moving
population, with an average speed of 8.2 mm/hr (marked by asterisk in fig. 35). The second population was slower, with an average speed of 3.4 mm/hr (marked by arrow in fig.35). Movements observed by individual endosomes were unidirectional, although both movements to and from the cell body were observed. Immobile endosomes showed negligible movement along both directions but did not show a net displacement over the course of our movies (total time=5 s). Our average speeds are consistent with the reported speeds of Trk signaling endosomes along the axons of peripheral neurons that vary between 3 mm/hr and 20mm/hr (Howe and Mobley, 2004), suggesting that perhaps the same dynein/microtubule-dependent mechanisms are responsible for the retrograde transport of these endosomes both along sensory/sympathetic axons and hippocampal dendrites.

**Trk endosomes are derived via Rac- and Pincher-dependent macroendocytosis.**

We have previously demonstrated that Trk signaling endosomes in PC12 cells and sympathetic neurons are generated via a macroendocytic mechanism that requires Rac and Pincher (Valdez et al., 2007). In order to determine whether the same molecular processes govern the formation of Trk endosomes in hippocampal processes, we investigated the association of these endosomes with molecular markers of the Rac/Pincher pathway. Pincher-mediated macroendocytosis shares common characteristics with fluid phase macropinocytosis. Fluorescently labeled dextran, a fluid phase marker, cointernalizes with Trk after NGF treatment in PC12 cells (Shao et al., 2002). To examine whether dextran also marks Trk endosomes along hippocampal processes we expressed E/TrkB and Rab5-GFP (to mark endosomes—see fig.37 and table 1) in hippocampal neurons and treated the cells with EGF-Alexa555 and dextran-Alexa647 for 15 min. EGF and dextran were extensively colocalized in Rab5-positive endocytic structures (Fig. 36A, Rab5-GFP not shown, but almost 90% of EGF-Alexa555 colocalized with Rab5 after 15min of treatment—see table 1). 62.6% of EGF containing endosomes also exhibited uptake of fluorescent dextran (n=332).

To examine the involvement of Pincher in the generation of Trk endosomes at hippocampal neurites we transfected hippocampal neurons with TrkA and Pincher constructs and stained the neurons for activated (phosphorylated) Trk and Pincher. After
20 min of NGF treatment phospho-TrkA and Pincher colocalized in endosomal structures along neurites (Fig. 36A). To determine whether these endosomes are derived via Rac-mediated macroendocytosis, as it has been demonstrated in PC12 cells (Valdez et al., 2007) we expressed E/TrkB and RacV12 in these neurons. Treatment with EGF-Qdot605 for 20 min resulted in the coaccumulation of receptor, ligand and RacV12 along hippocampal processes (Fig. 36C).

Trk endosomal complexes associate with Rab5 and Rab5-binding partners.

We have previously demonstrated (see chapters III and IV) that the Rab5 adaptor APPL links Trk to the Pincher endocytic machinery and that Rab5 and its binding partners Rabankyrin and Rabenosyn, which are also Pincher-binding partners, are all associated with Trk endosomes. Given that the presence of these early endosomal markers defines the nature of the Trk endosome we decided to investigate whether these proteins are also components of hippocampal Trk endosomes. The fact that APPL links Trk to Pincher makes it a vital component of the Trk/Pincher endocytic complex, so we first asked whether APPL is found in Trk hippocampal endosomes. We infected hippocampal neurons with E/TrkB and APPL-YFP and treated the cells with EGF-Qdot605 for 20 min. APPL was found to colocalize with both E/TrkB and EGF-Qdot605 along hippocampal neurites (Fig. 37A).

The presence of Rab5 on Trk endosomes, both in PC12 cells and sympathetic neurons, defines the ability of these endosomes to avoid lysosomal degradation (see previous chapters). Therefore we examined if Rab5 is present on Trk endosomes along hippocampal neurites. Hippocampal neurons were transfected with E/TrkB and Rab5-GFP and treated with EGF-Alexa555 for 15 min. We found that the overwhelming majority of ligand was localized in Rab5 positive endocytic structures (Fig. 37B, see table 1-15 min timepoint).

Rabenosyn and Rabankyrin are Rab5 effectors that are also Pincher-binding partners. This dual interaction could serve to form a stable complex between Trk, Pincher and Rab5. Since these two proteins could play an important role in Trk endosomal processing, and since they have been shown to associate with Trk endosomes in PC12 cells, we sought to determine whether they are also a component of the dendritic Trk
endosomal complexes. Hippocampal neurons were transfected with TrkA, Pincher and either Rabenosyn or Rabankyrin and treated with NGF for 20 min. Both Rabankyrin and Rabenosyn colocalized with Trk and Pincher after NGF treatment (Fig. 37C, D-84.7% of TrkA clusters were found colocalized with Rabankyrin (n=177), along 31 neurites with a total length of 1.1 mm and 75% of TrkA clusters were found colocalized with Rabenosyn (n=48), along 10 neurites with a total length of 312 µm).

In order to eliminate the possibility that Rabankyrin and Rabenosyn were recruited to Trk endosomes because of Pincher or their own overexpression, we decided to look at endogenous Rabankyrin and Rabenosyn distribution in the absence of Pincher overexpression. Hippocampal neurons were infected with E/TrkB virus, treated with EGF-Qdot605 for 20 min and then stained for endogenous Rabankyrin or Rabenosyn. Both endogenous Rabankyrin and Rabenosyn were found together with E/TrkB and EGF-Qdot605 (Fig. 38).

**Trk endosomes retain Rab5.**

Given the above data, Trk endosomes appear to be associated with Rab5, Pincher and their common effectors, at least at early stages of ligand treatment (15-20 min). A vital property of Trk endosomes in PC12 cells and sympathetic neurons is their ability to retain Rab5 even after longer ligand treatments. This property distinguishes them from EGFR endosomes that tend to acquire Rab7 early and be processed for degradation. We wanted to determine whether dendritic Trk endosomes also preferentially associate with Rab5 over Rab7 and whether this property will distinguish them from EGFR dendritic endosomes. To that end we expressed either E/TrkB or EGFR in hippocampal neurons along with either Rab5 or Rab7. After 48hrs of transfection we treated the cells with EGF for various times and then quantitated the association of each receptor with both Rab5 and Rab7 (Table 1). At 15 min the vast majority of ligand-bound E/TrkB (visualized by looking at EGF-Alexa555) is associated with Rab5 but not Rab7 (Fig. 39A). At early time points (15min) EGFR is also associated with Rab5 quite a lot (81.1% of receptor puncta, n=90, data not shown) but is also associated with Rab7 at high levels (63.2% of receptor puncta, n=245, data not shown). It is not clear why these percentages add up to more than a 100 for the 15 min time point (all other time points in table 1 add up close to
It is possible that at 15min the Rab5/Rab7 exchange is at its most dynamic stage and some endosomes contain both. That would also explain the rapid decrease of Rab5 positive EGFR endosomes from 15 to 30 min. At 45 min, both receptors show a decrease in Rab5 association and an increase in Rab7 association. However, Trk is still preferentially associated with Rab5 (59.6% of receptor puncta, n=119, fig. 39B-left panels) over Rab7 (40.5% of receptor puncta, n=333, fig. 39B-right panels) while EGFR is mostly associated with Rab7 (60.7% of receptor puncta, n=1252, fig. 39C-right panels) over Rab5 (37.4% of receptor puncta, n=99, fig. 39C-left panels). This indicates that, just as in the other systems examined, Trk endosomes along hippocampal dendrites are specialized to retain Rab5 and avoid lysosomal degradation.

**Trk endosomes contain activated signaling effectors.**

In order to establish that Trk trafficking along dendrites can mediate Trk signaling, we wanted to determine whether these endosomal complexes are associated with activated Trk signaling effectors. We have already observed that Trk receptors along dendrites are activated since they can be detected with a phospho-specific antibody (Fig. 36B and 37C). In order to identify the presence of other signaling effectors on these endosomes, we transfected hippocampal neurons with E/TrkB, treated them with EGF-Qdot605 for 15 min and stained them for phosphorylated ERK5. Phospho-ERK5 has been specifically implicated in endosomal signaling. We found that receptor/ligand complexes are in fact associated with activated ERK5 (Fig. 40), indicating that this dendritic endosomal population corresponds to signaling endosomes.

**Discussion**

Pincher-mediated Trk macroendocytosis is critical for Trk retrograde axonal signaling mediating neuronal survival in sympathetic neurons (Valdez et al., 2005). Here we show that Pincher-mediated macroendocytosis also results in the formation of signaling endosomes along hippocampal neurites. The role of Trk signaling endosomes in hippocampal neurons and synaptic potentiation has not been investigated. We have found
that the same molecular mechanisms that govern formation of axonal Trk endosomes (see Chapter V) also mediate the formation of dendritic Trk endosomes in the hippocampus.

By utilizing an EGFR/TrkB receptor (E/TrkB) we were able to demonstrate that ligand bound receptor is localized both along hippocampal axons and dendrites. Treatment of E/TrkB expressing neurons with EGF-Qdot605 resulted in endocytosis of the ligand along neurites and accumulation of multiple ligand/receptor complexes in endocytic structures such as multivesicular bodies. These structures move towards the cell body at velocities consistent with previous observations in peripheral neurons (Howe and Mobley, 2004). This may be a reflection of Trk receptors engaging the same microtubule/dynein transport system in both neuronal types. Given the fact that the juxtamembrane region of Trk receptors directly binds a dynein light chain isoform (Yano et al., 2001), they may be directed to a specified universal transport mechanism.

The uptake of fluorescently-tagged dextran as well as the presence of Pincher and RacV12 on Trk endosomes demonstrate that Trk endosomes along hippocampal dendrites are derived via Rac- and Pincher- dependent macroendocytosis. The adaptor protein APPL, which links Trk to Pincher (see Chapter IV), Rab5 and the Pincher/Rab5 effectors Rabankyrin and Rabenosyn are also present on these endosomes. This protein complex associates with endocytic Trk in sympathetic neurons and PC12 cells, suggesting that a very specific mechanism is in place for Trk endocytosis and transport, regardless of cell type. This protein network is nucleated around Pincher and Rab5, with multiple interactions assuring that both of these proteins remain associated with the Trk endosomes. APPL interacts with Trk at two distinct sites, which could reflect a mechanism for assuring Pincher recruitment to endocytic Trk. This redundancy could be an indication of the importance of Trk internalization through Pincher.

We have previously demonstrated that Pincher-mediated endocytosis results in long-lived degradation-resistant endosomes in sympathetic neurons (Chapter V). Since dendritic hippocampal endosomes are derived via the same molecular mechanisms they could also avoid lysosomal degradation by selective association with Rab5 over Rab7. Consistent with this, we found that Trk endosomes along hippocampal dendrites retain Rab5, unlike EGFR endosomes that rapidly associate with Rab7. Therefore, formation of these endosomes via Pincher-mediated macroendocytosis may serve to attenuate Trk
degradation and facilitate long term Trk signaling to the nucleus of these cells. A role of Trk endosomes in BDNF-induced signaling is evident by the association of these endosomes with phosphorylated ERK5.

L-LTP is associated with transcriptional responses to high frequency tetanic stimulation. In peripheral neurons, retrogradely transported Trk endosomes induce transcriptional changes by CREB phosphorylation (Riccio et al., 1997). Trk signaling endosomes may serve as signaling platforms that mediate sustained signaling in hippocampal neurons as well. We have identified a population of endosomes that are trafficked along hippocampal dendrites via a distinct mechanism and identified a number of endosomal proteins involved in this process. To what extent these endosomes contribute to BDNF-mediated changes in synaptic transmission remains to be determined. Characterization of these dendritically derived Trk endosomes provides a new set of tools by which to manipulate Trk endocytosis and trafficking in hippocampal neurons. It will be interesting to determine the effects of Pincher depletion or overexpression of dominant negative Pincher on L-LTP and hippocampal-mediated memory processing.
Figure 33. Trk/ligand endosomal complexes are found on all hippocampal processes. Hippocampal neurons were infected with an adenovirus expressing the E/TrkB chimeric receptor. 48 hrs after infection, cells were treated with EGF-Qdot605 for 20 min, fixed and stained. E/TrkB (anti-EGFR, green) and EGF-Qdot605 (Qdot605, red) were visualized by confocal microscopy. The entire neuron is depicted in (A) and different processes are shown separately in (B-E). Scale bar=5μm.
A

B

C

D

E
Figure 34. EGF-Qdot605 is localized at various endocytic structures along hippocampal neurites.

Hippocampal neurons were infected with an adenovirus expressing E/TrkB. 48hrs after infection cells were treated with EGF-Qdot605 for 20 min, fixed and processed for EM. EGF-Qdot605 was silver enhanced. EGF-Qdot was localized both on the surface and in endocytic structures along hippocampal neurites (A), on membrane ruffles (B) and in multivesicular bodies (C). Scale bar=500nm.
Figure 35. EGF-Qdot605 moves at multiple speeds along hippocampal neurites.
Hippocampal neurons were infected with an adenovirus containing E/TrkB. 48hrs after
infection cells were treated with EGF-Qdot605 for 20 min, washed and live imaged with
a high-speed camera (see materials and methods). EGF-Qdot was found to move at
different speeds along hippocampal dendrites. Scale bar=5um.
Figure 36. Trk neurite endosomes are derived from Pincher- and Rac- mediated macroendocytosis.

(A) Hippocampal neurons were cotransfected with constructs containing E/TrkB and rab5-GFP. 48hrs after transfection cells were treated with EGF-Alexa555 and dextran-Alexa647 for 15 min, fixed and stained. Dextran (Alexa647, green) and EGF (Alexa555, red) were visualized by confocal microscopy. Scale bar=5um.

(B) Hippocampal neurons were cotransfected with constructs containing TrkA and Pincher-HA. 48hrs after transfection cells were treated with NGF for 20 min, fixed and stained. Phospho-TrkA (anti-phospho-Trk, green) and Pincher (anti-HA, red) were visualized by confocal microscopy. Scale bar=5um.

(C) Hippocampal neurons were infected with adenoviruses containing E/TrkB and RacV12-GFP. 48hrs after infection cells were treated with EGF-Qdot605 for 20 min, fixed and stained. E/TrkB (anti-EGFR, red), EGF-Qdot605 (Qdot605, cyan) and RacV12-GFP (GFP, green) were visualized by confocal microscopy. Scale bar=5um.
Figure 37. Trk endosomes associate with Rab5 and Rab5-binding partners.
Hippocampal neurons were infected with adenoviruses expressing E/TrkB and APPL-YFP (A) or co-transfected with constructs containing E/TrkB and Rab5-GFP (B) or co-transfected with constructs containing TrkA, Pincher-HA and Rabankyrin-GFP (C) or co-transfected with constructs containing TrkA-YFP, Pincher-HA and Rabenosyn-myc (D). 48hrs after infection/transfection cells were treated with EGF-Qdot605 for 20 min (A) or EGF-Alexa555 for 15 min (B) or NGF for 20 min (C and D), fixed and stained. E/TrkB (anti-EGFR, red, A), EGF-Qdot605 (Qdot605, cyan, A), APPL (YFP, green, A), EGF-Alexa555 (Alexa555, red, B), Rab5 (GFP, green, B), phospho-TrkA (anti-phospho-Trk, red, C), Pincher (anti-HA in C, anti-Pincher in D, cyan, C and D), Rabankyrin (GFP, green, C), TrkA (YFP, red, D) and Rabenosyn (anti-myc, green, D) were visualized by confocal microscopy. Scale bar=5um.
Figure 38. Trk endosomes are associated with endogenous Rabankyrin and Rabenosyn.

Hippocampal neurons were infected with an adenovirus containing E/TrkB and 48hrs after infection treated with EGF-Qdot605 for 20 min. E/TrkB (anti-EGFR, red, A and B), EGF-Qdot605 (Qdot605, cyan, A and B), Rabankyrin (anti-Rabankyrin, green, A) and Rabenosyn (anti-Rabenosyn, green, B) were visualized by confocal microscopy. Scale bar=5um.
Figure 39. Trk preferentially associates with Rab5 over Rab7 along hippocampal neurites.

Hippocampal neurons were co-transfected with constructs containing E/TrkB and either Rab5-GFP (A and B-left panels) or Rab7-GFP (A and B-right panels) or co-transfected with constructs containing EGFR and either Rab5-GFP (C-left panels) or Rab7-GFP (C-right panels). 48 hrs after transfection cells were treated with EGF-Alexa555 for 15 min (A) or EGF for 45 min (B and C). EGF-Alexa 555 (Alexa555, red, A), Rab5 (GFP, green, A, B and C-left panels), Rab7 (GFP, green, A, B and C-right panels), E/TrkB (anti-EGFR, red, B) and EGFR (anti-EGFR, red, C) were visualized by confocal microscopy. Scale bar=5um.
Figure 40. Trk endosomes contain activated ERK5.
Hippocampal neurons were transfected with a construct containing E/TrkB and 48hrs after transfection treated with EGF-Qdot605 for 15 min. E/TrkB (anti-EGFR, red), EGF-Qdot605 (Qdot605, cyan), and phosphorylated ERK5 (anti-phospho-ERK5, green) were visualized by confocal microscopy. Scale bar=5um.
Table 1: Colocalization of E/TrkB and EGFR with Rab5 and Rab7 along hippocampal neurites at various time points after EGF treatment of hippocampal neurons.

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<th>Rab5 %</th>
<th>n</th>
<th>Rab7 %</th>
<th>n</th>
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<th>n</th>
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Hippocampal neurons were cotransfected with a construct expressing either E/TrkB or EGFR and a construct expressing either Rab5-GFP or Rab7-GFP. 48hrs after transfection, neurons were treated with EGF for various time points, fixed and stained with an antibody against the extracellular domain of EGFR. Receptor puncta along neurites (corresponding to endosomal structures) colocalizing with either Rab5-GFP or Rab7-GFP were counted and the above table was generated.

% = percentage of endosomal receptor structures associated with Rab5 or Rab7. 
n = total number of receptor endosomes.
Chapter VII

General Discussion

According to the widely accepted signaling endosome hypothesis, target-derived neurotrophins bind Trk receptors at nerve terminals and the receptor/ligand complex then undergoes endocytosis and is retrogradely transported in endosomes along microtubules to the cell body (Zweifel et al., 2005). As part of this thesis, I have developed an approach that allows for the direct visualization and selective manipulation of retrogradely transported Trk endosomes in cultured neurons. Using this approach, I was able to unravel the requirements for Trk retrograde transport and the nature of the retrogradely transported endosome (Chapter V). Using the PC12 cell line, I was also able to address questions regarding the formation, processing and signaling potential of Trk endosomes (Chapters III and IV). Finally, I identified a Trk endosomal population in hippocampal neurons that could have a potential role in signaling events relating to neurotrophin-induced synaptic plasticity (Chapter VI).

Retrograde signaling endosomes are multivesicular bodies.

By using an EGFR/Trk chimera I was able for the first time to selectively visualize a pure Trk endosomal population. Previous studies utilizing NGF as a ligand could not differentiate between Trk and p75NTR endosomes. Using this approach I identified multivesicular bodies (MVBs) as the retrograde signaling organelles for Trk. This finding is consistent with early in vivo and in vitro studies showing that the majority of axonally transported radiolabelled NGF was found in MVBs (Hendry et al., 1974). Using the E/TrkB chimera, I have demonstrated that ligand binding to p75NTR is not necessary for Trk retrograde transport and MVB targeting. Receptor progression into MVBs has been thought to be a means of attenuating signaling since the acidic pH of these structures favors ligand dissociation. While this might be the case for other receptors, NGF stays bound to Trk at relatively acidic pH (pH=6) (Zapf-Colby and Olefsky, 1998). We found that MVBs along axons and in cell bodies of sympathetic neurons are associated with phosphorylated ERK5, indicating that these MVBs are
signaling-capable. Strikingly, there is retention of Rab5 on these MVBs, indicating that they are a specialized population that is not yet targeted for degradation. Retention of Rab5 maybe mediated by the presence of Pincher on these MVBs (discussed below).

**Generation of retrogradely transported endosomes is Pincher- and Rac-dependent.**

Contrary to previous studies, we have found that generation of Trk signaling endosomes does not involve clathrin, but depends on Rac and Pincher. Overexpression of dominant-negative eps15 did not attenuate Trk endocytosis, but it did block clathrin-mediated EGFR endocytosis. While we cannot completely rule out a role for clathrin in Trk endocytosis, it is unlikely that clathrin-mediated endocytosis is the primary mechanism of Trk internalization. The use of dominant-negative dynamin as a specific inhibitor of clathrin-mediated endocytosis does no longer hold, as a number of clathrin-independent mechanisms have been shown to utilize dynamin for membrane scission (Sandvig et al., 2008). Consistent with this, we found that dynamin is required for Pincher-mediated Trk endocytosis as well. Recent findings have revealed that EHD proteins, including Pincher, share many similarities with the dynamin superfamily, including the ability for membrane scission (Daumke et al., 2007). Since both Pincher and dynamin are required for Trk endocytosis, either one of them could be responsible for the membrane scission event. In fact recent findings indicate that Pincher is sufficient to mediate such scission events since dynamin-independent, Pincher-dependent endocytic processes have been characterized, such as the endocytosis of Nogo (A. Hatkic, personal communication). One possibility is that dominant-negative dynamin blocks Trk endocytosis by interfering with Rac activity. We have shown that Rac-mediated actin remodeling is required for Pincher-mediated Trk endocytosis. Dynamin has been shown to modulate Rac localization and function, and overexpression of dominant negative dynamin-2 inhibits Rac-dependent macropinocytosis (Schlunck et al., 2004). Therefore the effects of dominant negative dynamin on Trk endocytosis may not be by directly inhibiting membrane scission but by indirectly interfering with Rac activity. Interestingly, a dominant negative form of dynamin-1 was much less effective at inhibiting cell spreading through Rac mislocalization, and a dynamin-2 mutant that also lacks the C-terminal PRD domain failed to do so. Both of these constructs were as effective as
dominant negative dynamin-2 in blocking clathrin-mediated endocytosis. In our experiments we used dominant negative dynamin-2, which blocks both clathrin-mediated endocytosis (by blocking membrane scission) and Rac-mediated cell spreading. One potential way to distinguish between a direct or an indirect (through Rac) role of dynamin is to use the dynamin-2 mutant lacking the C-terminal PRD domain instead, which blocks membrane scission without affecting Rac localization.

Additional evidence against clathrin involvement in Trk endocytosis comes from our EM studies. Extensive analysis of Trk-containing endosomal structures by electron microscopy (EM) never revealed clathrin-coated pits or vesicles (Chapter III). Because we need to overexpress Trk receptors to visualize them by EM, one might argue that this overexpression alters the endocytic/trafficking route of Trk. We think that this possibility is unlikely since overexpression of EGFR did not alter its trafficking to clathrin-coated pits and vesicles.

The comparison of Trk receptors to EGFR has been an invaluable tool during this thesis work. The EGFR endocytic pathway through clathrin-mediated endocytosis and the EGFR progression through different endosomal compartments are much better studied and understood than the corresponding processes for Trk. Furthermore, there is solid evidence for an involvement of clathrin in EGFR endocytosis; RNAi-mediated knockdown of clathrin or clathrin adaptors attenuate EGFR endocytosis, EGFR activation leads to clathrin phosphorylation and a number of adaptors have been shown to couple EGFR to the clathrin machinery (for review see Sorkin and Goh, 2009). This kind of concrete evidence does not exist for clathrin-mediated endocytosis of Trk. By using EGFR as a “standard”, I have been able to identify unique features of Trk endocytosis and processing, both in PC12 cells and sympathetic neurons.

**Trk vs EGFR endosomal processing**

Pincher-mediated macroendocytosis exhibits fundamental differences from clathrin-mediated endocytosis right at the start of each process. Distinct sets of proteins mediate generation of clathrin-coated pits/vesicles (such as clathrin, AP-2, eps15) and membrane ruffles/macroendosomes (Rac, Pincher), with dynamin being the only common feature between the two pathways. After internalization, the molecular
mechanisms that mediate subsequent processing appear to converge since Trk endosomes recruit Rab5, a marker of the early endosome and its binding partners Rabenosyn and Rabankyrin, all of which have been shown to exist on EGFR endosomes as well. Despite sharing these common components, Trk and EGFR endosomes are processed quite differently. We have shown that Trk has a much slower degradation rate and that Trk endosomal signaling through ERK 1/2 is sustained. Furthermore, Trk endosomes avoid lysosomal degradation and not associate with Rab7 as rapidly as EGFR. Therefore the two endosomal populations demonstrate differential processing despite using the same core Rab5/Rab7 endocytic pathways. The major difference between Trk and EGFR endosomes is the presence of Pincher on the Trk endosomal population. Pincher could slow the exchange of Rab7 for Rab5 by binding to Rab5 effectors. Pincher may serve to retain Rab5 on Trk MVBs by forming a stable complex with Rabenosyn and Rabankyrin. It has been proposed that Rab5 activity on endosomes increases until it reaches a peak and then Rab5 is displaced by Rab7 (Del Conte-Zerial et al., 2008). The temporal profile of this conversion can be shifted depending on the endocytosed cargo, indicating that this is a highly dynamic process and that there is crosstalk between the endocytic machinery and the signaling emanating from the internalized receptor. The fate of endocytosed receptors is also dictated by receptor modifications, with ubiquitination being the best example. It has been demonstrated for EGFR that alterations of its ubiquitination state by ubiquitinating (cbl) or deubiquitinating (UBPY/USP8) enzymes can dramatically shift its degradation rate. Therefore endosomal processing is defined both by the cargo and the endocytic machinery and can be quite fluid. Interestingly, it has been recently demonstrated that Rab7 activity is not required for multivesicular body biogenesis or cargo sequestration in intraluminal vesicles (Vanlandingham and Ceresa, 2009). This is consistent with our identification of Rab5 positive MVBs. The same study found that Rab7 recruitment to the MVB is critical for lysosomal fusion and RNAi-mediated knockdown of Rab7 results in cargo accumulation in MVBs. Increased Trk receptor lifetime could be achieved if Pincher serves to retard Rab7 acquisition at MVBs and therefore lysosomal fusion.

We have found that Trk multivesicular bodies retain their signaling potential as evidenced by the presence of activated ERK5 on these endosomes. In order for Trk
complexes to mediate signaling from MVBs they must remain in the outer limiting membrane and not be sequestered in MVB intraluminal membranes, as sequestration there has been shown to terminate receptor signaling. Pincher reduces the formation of intraluminal vesicles (Valdez et al., 2005) and could potentially act to localize Trk on the outer limiting membrane.

Ubiquitination is the major mechanism of receptor targeting to the intraluminal vesicles of multivesicular bodies. Ubiquitin moieties are recognized by components of the ESCRT complex, which orchestrate the events that sequester cargo for degradation in lysosomes (Williams and Urbe, 2007). The initial stages of EGFR sorting involve the ubiquitin-interacting protein Hrs, which also binds clathrin. Clathrin has been proposed to act as an organizer of the ESCRT pathway by restricting proteins of the complex into clathrin-enriched microdomains (Raiborg et al., 2002). By directing Trk away from clathrin-mediated endocytosis, Pincher may serve to retard ESCRT assembly on clathrin-devoid endosomes. Interestingly, different ubiquitin ligases are responsible for ubiquitination of EGFR, which is ubiquitinated by c-cbl, and Trk which has been proposed to be ubiquitinated by both TRAF6 and nedd4-2 (Arevalo et al., 2006b; Geetha et al., 2005; Waterman et al., 1999). C-cbl remains on EGFR endosomes and continuously ubiquitinates internalized EGFR receptors, directing them to lysosomes (Umebayashi et al., 2008). The Rab5 exchange factors GAPex 5 and RIN1 promote EGFR degradation by enhancing the interaction between EGFR and c-cbl or interacting with the ESCRT complex, respectively (Kong et al., 2007; Su et al., 2007), indicating there is direct coupling between ubiquitination mechanisms and endocytosis. The coordinated actions of endocytic proteins with the ubiquitin/sorting pathway must ultimately determine the rate of receptor degradation. Further studies are needed to elucidate the relationship of Pincher to the ubiquitin/ESCRT sorting pathway.

**APPL links Trk to the Pincher endocytic machinery.**

The adaptor protein APPL is the molecular link between Trk and Pincher (chapter IV). The existence of a molecular bridge between Trk and Pincher further illustrates that Pincher-mediated Trk endocytosis is a highly specific and regulated process. APPL binds Trk via two distinct mechanisms, perhaps indicating the importance of this interaction.
Disruption of the Trk/Pincher interaction by mutating the NPF motif of APPL results in increased lysosomal targeting of Trk and attenuation of endosomal signaling. This further supports the notion that the prolonged endosomal signaling and slow degradation rate of Trk are dictated by the presence of Pincher on Trk endosomes. Interestingly, APPL has not been found to interact with other EHD family members and it has been suggested, based on the residues surrounding its NPF motif, that it would exhibit weak or no binding to EHD family members (Grant and Caplan, 2008). We have shown, both by protein pulldown and coimmunoprecipitation assays, that APPL and Pincher interact, and that this interaction is specifically mediated by the EH domain of Pincher and the NPF motif of APPL. Preferential binding of APPL to Pincher, but not the other EHD family members, could in part explain the unique role of Pincher as compared to EHD1-3. Since APPL is a Rab5 binding partner as well it could also serve to stabilize Rab5 on multivesicular bodies, contributing to the increased lifetime of the endosomes. The effects of the APPL mutation on endosomal signaling and receptor lifetime of Trk suggest that the initial stages of Pincher recruitment and the formation of a complex between the receptor and Pincher at the plasma membrane is critical for mediating differential processing of these endosomes. Therefore, without APPL, Pincher cannot be effectively recruited to Trk endosomes at a later stage, despite the presence of Rab5, Rabenosyn and Rabankyrin. This could also explain why Pincher doesn’t get recruited to EGFR endosomes, even when they contain Pincher-binding partners, highlighting the importance of the receptor-Pincher connection.

**Pincher-mediated endocytosis in hippocampal neurons**

The identification of mobile Trk endosomes on hippocampal dendrites uncovers the possibility for a role of these endosomes in neurotrophin-induced synaptic plasticity, a fairly unexplored area. The molecular mechanisms that govern the formation and processing of Trk endosomes are conserved between peripheral and central neurons. This could indicate that Trk signaling and neurotrophin-induced phenotypes are critically dependent on the generation of long-lasting endosomes. Binding of NGF to the TrkA receptor causes receptor endocytosis with an observed half-life of 5 min (Zapf-Colby and Olefsky, 1998). Since neurotrophin treatment results in rapid internalization, signaling
endosomes may be the only signaling platforms in neurons. BDNF/TrkB local signaling at synaptic terminals may mediate some short-term effects on synaptic plasticity, but gene expression changes require a signal to reach the nucleus and signaling endosomes may be the only suitable mechanism for dendrite to soma signal transmission. Since we have already developed the tools for manipulating Pincher-mediated Trk endocytosis and trafficking in sympathetic neurons, the same tools can be used to decipher the role of these endosomes in hippocampal neuronal populations.

In this thesis, I have also established a role for Pincher-mediated retrograde transport in retrograde neurotrophin signaling and gene induction. This opens the door for exploring the role of this process in vivo and deciphering the effects of manipulating Pincher-mediated endocytosis on neurotrophin-induced phenotypes.
List of References


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