Extensive neuronal networks coordinate crucial processes within the human body. One of the key players in neuronal growth and differentiation, nerve growth factor (NGF), was discovered in the early 1950s [1]. Forty years later, an interaction between NGF and a tyrosine receptor kinase, TrkA, was identified [2-4]. The neurotrophic effects of NGF are initiated through its activation of TrkA [5]. NGF and TrkA are well-characterized oncogenes, whose expression is elevated in multiple cancer types [6-8]. NGF-TrkA processes are also disrupted in a number of neurodegenerative diseases and developmental disorders [9, 10].

In the developing neuron, TrkA activation by NGF at the distal axon results in a pro-survival signal that travels up to one meter along the axon to the neuronal cell body. The predominant model in the field is the signaling endosome hypothesis [11, 12]. In this model, NGF binding to TrkA induces receptor dimerization and clathrin-mediated endocytosis. An NGF- and TrkA-containing endosome activates downstream signaling pathways, while being transported to the cell body. Dynein binds TrkA in the early endosome, transporting TrkA and associated proteins to the cell body via microtubule-based transport [13]. However, axonal terminals contain a complex actin meshwork [14] and TrkA associates with the myosin VI-binding protein, GAIP-interacting protein (GIPC), at the uncoated vesicle (UCV) stage. We propose that an indirect interaction between TrkA and myosin VI is responsible for short-range transport of the endocytosed NGF-TrkA complex, prior to long-range microtubule-based transport. In our mechanism, we suggest another protein, APPL1, also mediates this interaction.

Direct binding of GIPC to TrkA [5], APPL1 [15], and myosin VI [16], as well as TrkA to APPL1 [17], is seen both in vivo and in vitro. Previous knockdown experiments of GIPC and APPL1, using siRNA, support a role in TrkA trafficking [15, 17]. Although APPL1 remains bound to TrkA at the early endosomal stage, GIPC dissociates [15]. We suggest that dynein and/or downstream effector binding to TrkA-APPL1 causes this dissociation. Previous expression and purification techniques for full-length, as well as domain constructs, provide us with important tools to understand this process.

A thorough understanding of this system has important human health implications. Disruption of TrkA trafficking in the tumor microenvironment could potentially inhibit tumor growth by suppressing NGF-induced survival. On the other hand, therapeutic targets for neurodegenerative diseases may involve strengthening NGF-TrkA signaling and trafficking processes.

Specific Aim 1: Biochemically assess binding mechanisms of TrkA trafficking components.

**Hypothesis:** Addition of myosin VI does not disrupt TrkA-APPL1-GIPC binding. However, dynein and/or signaling proteins compete with GIPC for TrkA binding.

Binding assays will be established using an in vitro system. Sequential binding experiments allow us to determine the relative binding affinities of TrkA, APPL1, and GIPC to each other. Addition of myosin VI, dynein, or Akt, a TrkA-APPL1 effector, show what effect this has on TrkA-APPL1-GIPC binding.

Specific Aim 2: Investigate a role for myosin VI in TrkA signaling in PC12 cells.

**Hypothesis:** Myosin VI associates with TrkA indirectly in UCV, and is required for transport.

Sequential immunoprecipitation (IP) experiments in fractionated cells probe players of TrkA trafficking. Knockdown of myosin VI using siRNA directly tests a role in TrkA signaling.

Specific Aim 3: Investigate transition of TrkA cytoskeleton transport in compartmented cultures.

**Hypothesis:** Microtubule- and actin-dependent transport is responsible for TrkA retrograde movement in sympathetic neurons.

Compartmented cell assays in the presence of microtubule depolymerizing or myosin ATPase inhibition drugs test if TrkA retrograde trafficking transitions from actin- to microtubule-dependent transport.
1. Background and Significance

1.1 TrkA and NGF play important roles in neuronal survival.

Everything from thought to memory to motor control depends on the actions of the human nervous system. This intricate array of neurons is constantly sending and receiving signals, both between other neurons and with surrounding tissues and organs. Development of this network is no less complicated [18]. Developing neurons are programmed to undergo apoptosis, unless stimulated to differentiate by a pro-survival signal [9]. This pro-survival signal is activated by small target-derived proteins called neurotrophins. Neurotrophins not only promote the survival of developing neurons, but regulate the resulting system throughout the life of the organism [11, 12].

Of these, nerve growth factor (NGF) was the first neurotrophin discovered and is crucial for survival of developing sympathetic and sensory neurons of the peripheral nervous system [2]. NGF binds the receptor tyrosine kinase, TrkA, at the axonal terminal, inducing dimerization and autophosphorylation at distinct residues of the receptor [19]. Subsequent phosphorylation, by the partner TrkA receptor, occurs at seven additional residues in the intracellular domain [10]. Once phosphorylated, these residues bind specific downstream effectors, initiating TrkA kinase activity and multiple signaling cascades [10]. Known pathways activated by TrkA activity include the phosphatidylinositol 3-kinase (PI3-K)-Akt, mitogen-activated protein kinase (MAPK)-ERK, and phospholipase-C-γ (PLC-γ) pathways [11, 12, 20]. In addition, the transcription factor cyclic AMP responsive-binding element (CREB) is activated upon NGF binding to TrkA [21].

In the presence of NGF-activated signaling, the neuron expresses survival genes and differentiates. Without it, the cell undergoes apoptosis. Neurons are not typical cells however. The NGF-TrkA interaction occurs at the distal axon, up to one meter away from the soma, or neuronal cell body. How does this retrograde survival signal transport to the soma? Aside from gaining understanding of developmental processes, answering this question has important implications to human life. Neurodegenerative diseases, such as Alzheimers or amyotrophic lateral sclerosis (ALS) [9], developmental disorders, such as Down Syndrome [10], and multiple cancers [6-8] can all contain imbalances in NGF-TrkA processes.

1.2 A NGF-TrkA signaling endosome undergoes retrograde transport to the neuronal cell body.

Although three similar models exist for retrograde NGF-TrkA signal transport, the signaling endosome hypothesis is the most broadly accepted in the field [11, 12, 22, 23]. The signaling endosome hypothesis suggests that NGF-TrkA is endocytosed following TrkA activation. This observation is supported by evidence showing quantum dot- or radioactively-labeled NGF bound to TrkA within the cell following activation [24-27]. Following clathrin-mediated endocytosis, TrkA associates with the cytoplasmic protein, APPL1, which binds the early endosomal marker, Rab5, as well as TrkA-APPL1 downstream effectors, such as Akt [28, 29]. At the UCV stage, TrkA and APPL1 also directly bind the PDZ protein GIPC. While APPL1 remains associated to TrkA at the early endosome stage and throughout retrograde transport, GIPC dissociates [15, 17]. NGF- and TrkA- signaling endosomes undergo microtubule- and dynein-dependent retrograde transport to the neuronal cell body [18]. The TrkA receptor directly binds a 14 kDa light chain of cytoplasmic dynein, also called Tctex-1 [29-32]. Inhibiting APPL1, GIPC, or dynein following TrkA internalization prevents the NGF-induced pro-
survival signal from reaching the cell body [15, 17, 32]. It remains unclear, however, at what point TrkA associates with dynein following endocytosis.

1.3 Myosin VI plays an important role in the trafficking of multiple membrane receptors.

We suggest that prior to microtubule-dependent transport, TrkA indirectly associates with myosin VI through GIPC, and undergoes actin-dependent transport. This interaction provides a mechanism by which NGF-TrkA signaling endosomes are transported away from a dense actin meshwork at the cell periphery. Actin filaments are known to localize to the tips of neuronal growth cones as bundles (see Figure 1 on previous page), with their plus-ends pointing toward the cell membrane [14, 33]. Myosin VI is an unconventional myosin that moves along actin in a minus-end directed manner [34-36], making it a strong candidate for early TrkA transport. Our hypothesis is supported by previous work showing a crucial role for myosin VI and dynein in the endocytosis and retrograde transport of another neurotrophin and its tyrosine receptor kinase [30, 37].

Brain-derived neurotrophic factor, BDNF, is a neurotrophin that contributes to synaptic strength and plasticity in the brain [37]. Its receptor, TrkB, is located at both pre- and post-synaptic sites [37]. Upon activation by BDNF, TrkB is internalized through clathrin-mediated endocytosis. Myosin VI binds the C-terminus of GIPC, which associates with the juxtamembrane region of both TrkA and TrkB through its PDZ domain [15, 37]. A TrkB-GIPC-myosin VI complex was identified in HEK 293 cells [37]. Additional experiments show that dynein is also important in TrkB transport following activation [30]. Mutations that alter dynein function disrupt BDNF-induced signaling [30]. In addition to TrkB, myosin VI also plays a role in the endocytic trafficking of glutamate receptors, GLUT1, a glucose transporter, also through an interaction with GIPC [16, 38, 39], and cystic fibrosis transmembrane conductance regulator (CFTR), through an interaction with the clathrin-coated vesicle (CCV)-associated PDZ protein disabled-2 (Dab2) [40]. While these previous TrkB experiments support our mechanism of myosin VI- and dynein-dependent transport following neurotrophin activation, they do not identify the endocytic compartment where this transition occurs.

Figure 2: Model of proposed mechanism. Following NGF activation, TrkA is rapidly internalized through clathrin-mediated endocytosis into clathrin coated pits (CCP). Subsequent movement to uncoated vesicles (UCV) induces actin-based transport through myosin VI. Upon dynein or TrkA-APPL1 substrate binding, microtubule-dependent retrograde transport traffics NGF-TrkA to the cell body. Figure adapted from Varsano T et al, 2006 and Zweifel LS et al, 2005.
1.4 A mechanism for retrograde transport of NGF-TrkA signaling endosomes at the UCV and early endosome stage following NGF activation.

Our proposed mechanism (see Figure 2 on previous page) is centered on two main components of NGF-TrkA signaling and retrograde transport: 1. Following activation and internalization, TrkA and its associated binding protein, APPL1, form a trafficking complex with myosin VI through the PDZ protein GIPC at the UCV stage and undergo actin-dependent transport, and 2. Dynein, TrkA-APPL1 substrates, and GIPC compete for TrkA binding at the early endosome stage, resulting in GIPC dissociation and the initiation of microtubule-based transport. We will perform a number of experiments to investigate our hypothesis. Initially, we will express and purify various constructs of these proteins to probe the biochemical interactions between the players through fluorescence resonance energy transfer (FRET) experiments. FRET will also allow us to probe for TrkA-binding competition by adding myosin VI, dynein or Akt to the TrkA-APPL1-GIPC complex to see if GIPC dissociates.

In our second specific aim, we will attempt to identify a TrkA-APPL1-GIPC-myosin VI complex within specific endocytic fractions of PC12 cells. While rat pheochromocytoma, PC12, cells are not neurons, they respond to NGF by inducing differentiation and growth arrest, mimicking NGF-activated signal transduction events in sympathetic neurons [41, 42]. Using sequential IP experiments, we will probe for the presence or absence of this specific trafficking complex, following activation by NGF and fractionation of the cells. Additionally, we will knockdown myosin VI in PC12 cells using siRNA to assess downstream NGF-induced signaling.

Our final specific aim uses the compartmented culture system to study a possible actin- to microtubule-based transition in NGF-TrkA transport from the axonal terminal. These experiments are important to probe filament-dependence within a sympathetic neuron system, which is more physiologically relevant. Combining all three approaches allows us to ask specific questions about direct and indirect protein-protein interactions, a potential role for myosin VI, and mechanisms of transport. Answering these questions could not only have important human health implications, but may provide insight into a widespread mechanism for myosin VI-dependent transport of receptors from the membrane following clathrin-mediated endocytosis.

2. Research Design and Methods

2.1 Specific Aim 1: Biochemically assess binding mechanisms of TrkA trafficking components.

**Rationale:** NGF-TrkA signaling and trafficking activity is dependent on a number of specific protein-protein interactions. At the UCV stage, TrkA binds APPL1 and GIPC [15, 17]. In the absence of these interactions, NGF-induced survival is disrupted [15, 17]. Although the binding domains are known, it is unclear what the relative affinities of these players are for each other, and what causes the dissociation of GIPC at the early endosome stage. Understanding these early events in NGF-TrkA trafficking is important to determine how the signaling endosome is transported from the axonal terminal, as well as to investigate when the TrkA-dynein interaction is established.

**Experimental Design:** Prior knowledge of the interaction domains allows us to express and purify relevant constructs for binding assays. The juxtamembrane domain of TrkA binds the phosphotyrosine binding (PTB) domain of APPL1 in a phosphorylation-independent manner [17], the PDZ domain of GIPC [5, 15, 17], and Tctex-1, a 14 kDa light chain of cytoplasmic dynein [32]. The C-terminus of APPL1 binds the PDZ domain of GIPC [17]. The C-terminus of myosin VI binds a domain on the C-terminus of GIPC [16, 34]. The N-terminus of GIPC is important for its dimerization [34].

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Using this information, we will express and purify specific constructs of these proteins (see Figure 3). A ΔN-term GIPC (GIPC$_{125-333}$) construct using rat cDNA will be cloned into the pGEX2 vector (GE Healthcare) [17]. The PTB domain and C-terminus of APPL1 (APPL1$_{472-709}$) protein will be cloned into the pGEX-4T-3 vector (GE Healthcare) [5, 17]. Additionally, APPL1$_{472-709}$ and GIPC$_{125-333}$ will be cloned into the pEYFP-N1 (enhanced yellow fluorescent protein) vector (BD Biosciences) [5], to express YFP-GIPC$_{125-333}$ and YFP-APPL1$_{472-709}$, respectively. The juxtamembrane region of rat TrkA (TrkA$_{448-552}$) will be subcloned into the pGEX-4T-1 vector  (Pharmacia) [5], as well as into the pECFP (enhanced cyan fluorescent protein) vector (Clontech), to express CFP-TrkA$_{448-552}$. The C-terminal tail domain of myosin VI (myo6$_{955-1254}$) will be subcloned into the pGEX vector [43]. Tctex-1 will be cloned into a pET3a vector (New England Biolabs) [44]. Full-length Akt will be cloned into a pGEX vector following previous protocols [45]. All vectors will be expressed in *Escherichia coli* and purified as previously described, including cleavage of the GST tag with the corresponding protease (e.g. Thrombin for pGEX-4T-1/3) where relevant [3, 16, 17, 32, 43, 44].

Following expression and purification of our protein constructs, we will establish a binding assay using FRET technology. In FRET systems, two proteins are tagged with a donor and acceptor chromophore. Cyan fluorescent protein (CFP), as donor, and yellow fluorescent protein (YFP), as acceptor, are the most commonly used [46]. In the absence of binding, CFP is excited by 442nm light, and emits 480nm light. When CFP and YFP come into close proximity, however, energy emitted by CFP excites YFP, resulting in a 530nm emission signal. This signal will be measured using a fluorescence spectrophotometer. After measuring the absorbance at 530nm of increasing protein concentrations, a binding curve can be fit to the data. This is used to compare relative affinities. By adding in myosin VI, dynein or Akt, we can assess the effect of additional components on binding affinities.

In the first experiment, 1:1 increasing concentrations of CFP-TrkA$_{448-552}$ and YFP-APPL1$_{472-709}$ will be measured. Proteins bind PDZ domains with an affinity in the micromolar (μM) range [47]. Since we hypothesize that TrkA and APPL1 bind each other with higher affinity than either binds GIPC, we will start our experiments using concentrations in the high nanomolar (nM), low μM range. Once the binding curve for TrkA$_{448-552}$-APPL1$_{472-709}$ is established, we will repeat the experiment using CFP-TrkA$_{448-552}$ and YFP-GIPC$_{125-333}$. Here, we expect to use μM concentrations of both components.

Following initial binding assays, we will assess the effect of adding multiple components. As a baseline, we will establish a binding curve for CFP-TrkA$_{448-552}$ and YFP-GIPC$_{125-333}$, in the presence of APPL1$_{472-709}$. Again, these concentrations should be in the μM range, although these components may bind each other with higher affinity than TrkA$_{448-552}$-GIPC$_{125-333}$ alone. Following establishment of a range of concentrations that give a reliable signal, we will add Myo6$_{955-1254}$, Tctex-1, or purified Akt to our *in vitro* system.
Expected Results: As explained in the experimental setup, we expect that TrkA448-552 will bind APPL1472-709 with a higher affinity (high nm, low μM range) than it binds GIPC125-333 (μM range). When APPL1472-709 is included, we expect the relative binding affinity of TrkA448-552 for GIPC125-333 to increase, suggesting formation of a TrkA-APPL1-GIPC complex is favorable. According to our hypothesis, myosin VI is also a part of this early trafficking complex. Adding Myo6955-1254 should either increase the binding affinity of TrkA448-552 for GIPC125-333 further, or have no effect. However, we expect that adding Tctex-1 and/or Akt will cause GIPC to dissociate from TrkA-APPL1. This would result in a loss of FRET signal, or greatly decreased relative binding affinity. Rab5 could be used as a positive control, since it binds APPL1 at the UCV stage, but does not affect TrkA-APPL1 binding [11].

Alternative approaches/Possible pitfalls: In these experiments, we are using purified components in an in vitro assay. Potentially, multimeric forms of these proteins (specifically, TrkA and GIPC) are required for binding. Furthermore, TrkA is a transmembrane receptor. While binding experiments of juxtamembrane constructs of TrkA to APPL1 and GIPC were performed previously [15, 17], we must keep in mind that physiologically, the membrane and/or phosphorylation state of TrkA may affect relative binding affinities of these components.

Binding affinity experiments contain a number of hurdles and pitfalls. FRET signals can be hard to detect and expression of target and fluorescent protein fusions can be difficult. If FRET proves to be a technically difficult approach, we could alternatively perform in vitro pull-down assays. Using a purified construct of the intracellular domain of TrkA, we could pull-down APPL1 and GIPC, as previously shown [15, 17]. Following initial experiments, we could add purified myosin VI, dynein, or Akt to our system and see if APPL1 and/or GIPC still bind TrkA. To probe this system, we would resolve the pull-down elution samples on SDS-PAGE gels, followed by Western blot, and quantification of the data.

If dynein or Akt are unable to dissociate the TrkA-APPL1-GIPC interaction, this does not show that downstream effectors do not disrupt this complex. It simply suggests that dynein and Akt are not the molecules that do. Furthermore, TrkA-APPL1 binding is phosphorylation-independent, but effector binding may not be. Another approach could be fractionating endosomal components following NGF activation (see Section 2.2.1) and performing IP experiments using the separate fractions. Mass spectrometry could identify potential proteins responsible for GIPC dissociation. This could be beneficial for two reasons: TrkA is phosphorylated following NGF activation in PC12 cells, and all downstream effectors are present in an in vivo system.

2.2 Specific Aim 2: Investigate a role for myosin VI in TrkA signaling in PC12 cells.

Rationale: We hypothesize that an indirect interaction between TrkA and myosin VI is present in uncoated vesicles. If this interaction exists, it suggests a mechanism of short-range transport by the NGF-TrkA signaling endosome that is actin-dependent. Considering the dense actin meshwork (see Figure 1) of axonal terminals, guided transport of NGF-TrkA from the cell surface may be crucial to deliver the pro-survival signal to the cell body quickly after activation.

In order to test our hypothesis, we will perform sequential IP experiments in PC12 cells to probe for the presence of a TrkA-APPL1-GIPC-myosin VI trafficking complex. We will also knockdown myosin VI using siRNA. Previous experiments using PC12 cells show TrkA binds APPL1 and GIPC at the UCV stage [15, 17], where GIPC also binds myosin VI [34]. Additionally, siRNA knockdown of APPL1 or GIPC disrupts TrkA signaling in these cells, as assessed by the phosphorylation state of downstream TrkA-APPL1 effectors [15]. However, the TrkA-APPL1-GIPC-myosin VI complex has not been identified nor a direct role for myosin VI probed in TrkA trafficking.
2.2.1. Probe for a TrkA-APPL1-GIPC-myosin VI trafficking complex using sequential IPs.

**Experimental Design:** There are two major considerations to these sequential IP experiments: 1. We will transfect a GIPC construct with a tandem affinity purification (TAP) tag into the cells prior to NGF activation, allowing us to cleave off the initial antibody after the first IP, and 2. We need to isolate the endocytic fractions in order to identify the compartment where this complex is forming.

Full-length GIPC will be cloned into a pcDNA3.1 vector with a C-terminal TAP tag, followed by transfection into PC12 cells as previously described [48]. To isolate different endosomal fractions, we will use the OptiPrep system (Sigma-Aldrich) [13, 49, 50]. Thirty minutes after 50 ng/mL NGF activation [51, 52], the cells will be treated and the resulting lysate poured over a 5-20% (in 5% steps) OptiPrep gradient [13]. After resolution of lysate fractions by centrifugation, the interphases will be separated. The UCV and early endosomes are expected to be in lower OptiPrep concentrations. Each fraction will be resolved by gel electrophoresis, followed by Western blot to probe for endosomal markers. Antibodies against clathrin heavy chain, Rab5, EEA1, Rab7, and Rab11 (antibodies from Abcam) will probe for CCV, UCV, early, late, and recycling endosomes, respectively.

IPs will be performed in two sequential steps. The TAP tag contains a tobacco etch virus (TEV) protease cleavage site, followed by Protein A, which strongly binds IgG. For initial IP experiments, the fractionated lysate will be incubated with beads coated with IgG (Invitrogen). IP wash steps and elution will be performed as described previously [5, 37]. Samples will be resolved by SDS-PAGE gel electrophoresis, followed by Western blot, where we will probe for APPL1 (Cell Signaling), TrkA GIPC, and myosin VI (Santa Cruz).

Following the first IP, TEV protease will be added to the immunoprecipitate. After cleavage, the resulting complex will be transferred to Protein A-Sepharose beads (Thermo Scientific) previously incubated with anti-TrkA or anti-myosin VI antibody. IP experiments will be repeated, following previous protocols [15, 43]. The immunoprecipitate will again be resolved by SDS-PAGE gel, followed by Western blot, to probe for TrkA, GIPC, myosin VI, and APPL1. In addition, non-sequential IPs of NGF-activated PC12 lysate will be performed in the presence or absence of GIPC to investigate if the TrkA, myosin VI interaction is indirect. GIPC will be knocked down using siRNA (see Section 2.2.2). In these experiments, an antibody to TrkA will be used to probe the lysate.

**Expected Outcomes:** We expect to identify a TrkA-APPL1-GIPC-myosin VI trafficking complex in the UCV fraction(s), following NGF activation. A positive result is indicated by the presence of all four proteins after each round of IP. Furthermore, we expect that this complex will not appear in fractions predominantly composed of CCV, early, late and/or recycling endosomes. Another important consideration involves additional proteins involved in TrkA trafficking. For example, Dab2 contributes to NGF-induced neurite outgrowth in PC12 cells [53]. Although *in vitro* experiments with the PTB domain of Dab2 failed to show significant binding to TrkA, it does bind myosin VI at the CCV stage [30, 54]. A negative result in our IP experiments does not conclusively show that TrkA and myosin VI are unable to indirectly associate, but that Dab2 or an additional protein may be an intermediate protein between the two.

These experiments also probe specifically for an indirect interaction of TrkA and myosin VI. We expect that immunoprecipitates of PC12 lysate, following NGF activation, will show TrkA, APPL1, GIPC, and myosin VI when an anti-TrkA antibody is used. However, when GIPC is knocked down with siRNA, we expect to see only APPL1 associated with TrkA in UCV fractions.

**Alternative Approaches/Possible Pitfalls:** A sequential IP protocol will allow us to detect our complex in a straight-forward, endocytic compartment-specific manner. However, an important aspect to consider is the on-off rate of these protein-protein interactions. The complex may not be stable enough to sustain
multiple IP washes and sequential experiments. Additionally, there will be at least low levels of contamination among the endocytic fractions, and the results may not be immediately clear. This could be a serious concern if the CCV and UCV fractions are indistinguishable, and a majority of TrkA is not binding GIPC, native or tagged, in our assay. Along these same lines, GIPC has multiple binding partners as well, and may be bound to other proteins.

An alternative approach is to systematically perform single IP experiments, following knockdown of specific components. Although this approach is similar to our sequential IPs, it does not contain the potential problems of probing with a non-endogenous tagged protein. To identify a TrkB-GIPC-myosin VI complex, coimmunoprecipitations (co-IPs) were performed in cultured hippocampal neurons to probe for a TrkB-GIPC interaction, followed by GIPC-myosin VI [37]. Following these experiments, co-IPs were repeated in the presence or absence of TrkB, GIPC, or myosin VI, to show that TrkB and GIPC could associate with one another in the absence of myosin VI, but the TrkB, myosin VI interaction was lost in the absence of GIPC [37].

### 2.2.2 Knockdown of myosin VI in PC12 cells using siRNA.

**Experimental Design:** Previous studies looked at APPL1 and GIPC knockdown in PC12 cells using siRNA [17]. After 48 hours of targeted siRNA treatment, GIPC and APPL1 levels were reduced 90% and 60%, respectively [17]. Upon NGF activation in the siRNA-treated cells, NGF-induced downstream signaling was assessed through Western blot. Phosphorylated Erk1/2 (pErk1/2), MEK (pMEK), and Akt (pAkt) levels were greatly reduced as compared to non- or control siRNA treated cells [17]. To support our hypothesis of a crucial role for myosin VI in TrkA trafficking, we will perform a similar experiment, using siRNA targeted to myosin VI (Sigma).

Following 48 hours of myosin VI siRNA treatment, NGF will be added to the PC12 cells. Thirty minutes after activation, the cells will be treated and lysates resolved by gel electrophoresis. These gels will be probed by Western blot for pErk1/2 (Promega), pMEK, and pAkt levels (Cell Signaling). As a negative control, the cells will be incubated with a scrambled siRNA. Additionally, siRNA- and scrambled siRNA-treated cells will be tested in the absence of NGF activation. As a positive control, siRNA treatment targeted at APPL1 and/or GIPC should confirm previous results.

**Expected Outcomes:** According to our hypothesis, the outcome of this experiment should mimic those of previous APPL1- and GIPC- siRNA treatment experiments. When myosin VI levels are reduced in the cell, TrkA should be unable to traffic from the UCV. This results in a reduction of signaling, seen by reduced levels of pErk1/2, pMEK, and pAkt in the cell. Probing the cell for total levels of Erk1/2, MEK, and Akt should show equivalent concentrations with or without siRNA treatment. If myosin VI knockdown does not have an effect on TrkA signaling, it could be that dynein transports the NGF-TrkA signaling endosome from the cell periphery or that diffusion is responsible for early endocytic transport.

**Alternative Approaches/Possible Pitfalls:** Ideally, knockdown of myosin VI would be performed in compartmented cultures (see Specific Aim 3). However, knockdown and overexpression techniques appear to be technically difficult in this system. Possible experiments to inhibit myosin VI or disrupt its expression in sympathetic neurons are explained in Specific Aim 3.

Expressing a dominant negative myosin VI mutant is an additional method to probe its possible role in TrkA trafficking. A dominant negative myosin VI mutant, with mutations in residues 1107-1109 (from RRL to AAA), is unable to bind GIPC [55]. Previous studies with this mutation were done in MDCK cells, whose protein expression can be induced by ZnCl2 [55]. An attempt to subclone this dominant negative myosin VI mutant into a vector with an inducible promoter is possible. This vector could be transfected into PC12 cells and expressed.
2.3 Specific Aim 3: Investigate transition of TrkA cytoskeleton transport in neurons.

**Rationale:** To test whether or not filament transport changes during TrkA trafficking, we will use the compartmented culture system [56]. Regardless of the outcome of Specific Aim 2, this is an important question to investigate because, 1. These experiments will directly address whether or not there is a role for myosin ATPase activity in sympathetic neurons, and 2. The initiation of microtubule-dependent NGF-TrkA transport will be probed. We hypothesize that initially actin-based transport guides the NGF-TrkA signaling endosome away from the axonal terminal, where TrkA encounters dynein and transitions to microtubule-dependent transport.

Addition of microtubule-depolymerizing and myosin-inhibiting agents to the compartmented cultures investigates this possible transition. This experiment also allows us to probe specifically whether or not myosin ATPase activity is required in TrkA trafficking. We will assess NGF-TrkA signaling and transport in two ways: first, by probing for activated TrkA effector molecules through Western blot, and second, by using a radioactively labeled NGF molecule to track movement through the sympathetic neuron.

**Experimental Design:** The compartmented culture system segregates distal axons (DAx) from cell bodies and proximal axons (CB/PAx) using Teflon dividers (see Figure 4). Rat sympathetic neurons from the cervical ganglia are plated in the central compartment of a compartmented culture dish. Neuronal elongation from these neurons in the CB/PAx compartment is guided by grooves along the collagen-coated culture dish surface [56]. Within 5-7 days, distal axons are apparent in the DAX compartment [56]. Since the fluid environments of the compartments are also separate, differential treatment of distal and proximal axons is possible.

Prior to 50 ng/mL NGF treatment [51, 52], 5 μM nocodazole or 40 mM 2,3-butanedione monoxime (BDM) [13, 57, 58] will be added to the DAx or CB/PAX compartments. These drugs cause microtubule depolymerization and inhibit myosin ATPase activity, respectively.

One hour after NGF treatment, both compartments will be probed for pTrkA (pY490) (Abcam), pErk1/2, and pAkt by SDS-PAGE gel and Western blot [25]. Total Erk1/2 and Akt (antibodies from Santa Cruz) levels should not change, and will be assessed in the CB/PAX compartment as a control. Untreated cultures will be probed as an additional control. We will also activate TrkA in the distal axons using iodinated NGF, ¹²⁵I-NGF, to track NGF-TrkA trafficking following BDM or nocodazole treatment. Iodination of NGF will follow previous protocols [59, 60]. Quantification of the radiolabeled NGF one hour after activation and treatment will be compared to levels following ¹²⁵I-NGF activation in the absence of BDM or nocodazole, in both compartments. The fluid environment of the CB/PAX compartment must also be tested, since NGF gets secreted from the cell body after trafficking [56].

Additional experiments involve adding the inhibitory agents after NGF treatment, at specific time points. These time points will range from 0-30 minutes. Previous studies have established that within 10 minutes of NGF activation, NGF and TrkA are internalized and present within endocytic organelles [17]. Therefore, intervals within a 30 minute timeframe should capture a possible actin-to-microtubule transport transition.
**Expected Outcomes:** We expect that applying BDM to the distal axons of compartmented cultures will disrupt TrkA trafficking at all time points following NGF activation. Also, the levels of $^{125}$I-NGF in the CB/PAx are expected to be dramatically decreased, as compared to non-BDM treated cultures. Western blots probed for phosphorylated effectors should also show a substantial decrease in CB/PAx signaling. Likewise, BDM treatment in the CB/PAx compartment should have no effect. In this experimental setup, we are not able to determine that the myosin ATPase activity required is specifically that of myosin VI. The outcome of Specific Aim 2 will aid in our analysis. If myosin VI is shown to affect TrkA trafficking through siRNA knockdown, and is present in a complex with TrkA, this suggests that BDM-treatment on TrkA trafficking is targeting myosin VI activity. Nocodazole treatment on the distal axons should inhibit TrkA trafficking to and signaling within the cell body, but not the distal axons. Treatment of nocodazole in the CB/PAx compartment should disrupt signaling and trafficking locally. We expect that the nocodazole treatment data will confirm results from previous studies [13].

If BDM has no effect on NGF-TrkA signaling and trafficking, and/or myosin VI does not play a role in TrkA trafficking, there are a number of possible explanations. It could be that myosin ATPase activity is important for TrkA trafficking, however not through a myosin VI-specific interaction. Dynein may bind TrkA shortly after endocytosis. Time course experiments with BDM treatment in the distal axons will investigate dynein-transport initiation. At some point within the 0-30 minute time range, BDM treatment in the distal axons should no longer have a dramatic effect in either compartment. Likewise, at the same time point nocodazole treatment in the distal axons should disrupt DAx signaling.

**Alternative approaches/Possible pitfalls:** Further experiments to probe this system could include specific myosin VI overexpression and/or knockdown in compartmented cultures, as suggested in Section 2.2.2. A possible idea to accomplish this knockdown is to add a myosin VI tail antibody [43] to the distal axons prior to NGF activation. There are a number of potential pitfalls to this approach however. The antibody may not disrupt myosin VI-GIPC binding, and/or may not have an effect on myosin VI activity in this system. Another possible approach is to transfect a dominant negative mutant into the system (see Section 2.2.2). Once subcloned, this vector could then be transfected into the cell body of compartmented cultures. Additional experiments would be required to verify that the dominant negative myosin VI is able to reach the distal axons after expression.

A risk of filament-depolymerizing experiments is that secondary effects can overshadow the experimental results. A myosin ATPase inhibitor is used, as opposed to an actin-depolymerizing agent to avoid this situation. Actin is involved in endocytosis and axonal growth [33]. Actin depolymerization might inhibit NGF-TrkA endocytosis, as opposed to UCV movement. Previous microtubule depolymerization experiments in compartmented cultures, using nocodazole, showed disrupted TrkA trafficking, without gross secondary effects [13]. Another possible pitfall is the BDM drug itself. There is controversy about the effect of BDM on non-muscle myosins. While BDM inhibits partially purified Drosophila myosin VI [57], it can also negatively affect non-myosin proteins [58]. Finally, if $^{125}$I-NGF gives an unreliable or weak signal, additional tools to assess TrkA trafficking include NGF bound to quantum dots [25] or a TrkA-GFP fusion protein [51].

3. **Timeline**

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<th>Experiment</th>
<th>Year 1 (Month 1-6)</th>
<th>Year 1 (7-12)</th>
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4. Literature Cited