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THE ARF-GEF ARNO AND ARF6 REGULATE DENDRITIC AND AXONAL DEVELOPMENT IN CULTURED RAT HIPPOCAMPAL NEURONS

by

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of **Doctor of Philosophy**

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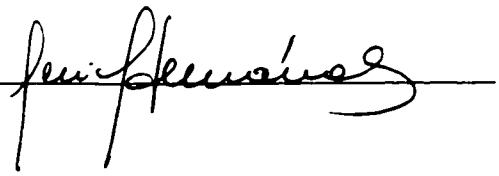
SIGNED: 

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Dedicated To:

Those, I love most

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ABSTRACT

The unique morphology of nerve cells is a key feature of the complex organization of the nervous system. Neurite extension and branching are dynamic processes that take place throughout the life of a neuron. The development of dendritic and axonal processes requires the coordination of membrane-cytoskeletal rearrangements to ensure the establishment of proper neural connections. These events are thought to be largely regulated by signaling pathways involving the Rho-family of small guanine triphosphatases (GTPases). Another family of small GTP-binding proteins, the ARF (ADP-ribosylation factor) family, has been implicated in the regulation of membrane transport and actin filament reorganization in non-neuronal cells. However, it is unknown what role ARF family members play in the modulation of these events during neurite extension and branching. Therefore, we have analyzed the role of the ARF-guanine exchange factor (GEF) ARNO (ARF-nucleotide binding site opener) in dendritic arbor development and axonal elongation and branching by overexpressing wild-type and mutant forms of ARNO and ARF6 in cultured rat hippocampal neurons. The over-expression of catalytically inactive ARNO and dominant-negative ARF6 result in increased dendritic branching as well as enhanced axonal extension and branching. Expression of inactive ARNO caused a redistribution of a subset of endosomes to the axonal plasma membrane and displacement of the profilin-binding protein Mena from the growth cone plasma membrane. These results suggest that ARNO through ARF6 negatively regulates dendritic branching and axonal elongation and branching during neural development.

CHAPTER ONE: INTRODUCTION

1.1 Development of the nervous system

The complex organization of the nervous system arises from the morphological complexity of neurons and the development of functional neuronal circuitry. In order to acquire its unique organization, the developing nervous system undergoes several morphological events, including neuronal migration, the extension of dendrites and axons to proper target regions and synapse formation with appropriate partners. Understanding how developing neurons elongate processes in order to acquire a particular shape that determines their function is still an open question in neurobiology. For my dissertation project I have explored the role of the ARF guanine exchange factor, ARNO, and its downstream effectors during dendrite and axonal development in cultured rat hippocampal neurons.

During vertebrate embryogenesis, the central nervous system (CNS) develops as a hollow cylinder from a flattened structure called the neural plate. The neural plate is a one-cell thick ectodermal layer on the dorsal surface of the embryo. The ridges of the neural plate, the neural folds, bend toward the midline, where they fuse giving rise to the neural tube. The initial formation of neural structures is called neurulation (Purves and Lichtman, 1985; Zigmond, 1999). During neurulation, three distinct swellings can be distinguished at the rostral end of the neural tube: the forebrain vesicle, which gives rise to the cerebral hemispheres, the midbrain vesicle, which gives rise to the adult midbrain, and the hindbrain vesicle, which gives rise to the lower brain stem (pons and medulla) and cerebellum (Cowan et al., 1984). These swellings are the result of rapid and

disproportionate cell proliferation along the neural tube. The inner surface of the neural tube, the neuroepithelium, is where most of the cell proliferation takes place (Rakic, 1974). This layer is called the ventricular zone and it will eventually become the epithelial lining of the ventricles of the brain. Once born, postmitotic neurons migrate from the ventricular germinal zone towards the cerebral surface as early as embryonic day 13 (E13) in the rat forebrain where they assemble in such a way that neurons that are born first are located in deeper layers and those that are born later are located in more superficial layers (called, "inside-out" sequence) (Angevine, 1965; Rakic and Sidman, 1970). As a result, four transient zones can be recognized from the ventricle outward, name, the ventricular, subventricular, intermediate, and marginal zone (Jacobson, 1991). The final location of nerve cells is critical to establish correct connections for proper nervous system function. At early stages of neurogenesis, young neurons migrate past those that originated earlier, guided towards the surface by moving along the surface of specialized supporting cells named radial glia (Jacobson, 1991). In some regions of the CNS, the neurons of the mantel layer become segregated into laminae that lie parallel to the surface of the CNS whereas in other regions neurons form uniform clusters that are structurally distinct called nuclei (Zigmond, 1999).

1.2 Neurite initiation

Once nerve cells migrate to their correct position they must elaborate dendrites and axons. The first step of neuronal morphogenesis is the initial budding of the neuritic processes from the cell body known as neurite initiation. The molecular mechanisms underlying neurite initiation likely involve an organized sequence of events which may

include the selection of a location at the plasma membrane that will serve as a nucleation center for the initial bud, the delivery of membrane components to this specific site, cortical actin reorganization and finally consolidation of the neuritic process by microtubule tracks. Little is known about the molecular mechanisms underlying neurite initiation (Luo, 2002). After neurite initiation, their polarized outgrowth will give rise to two morphologically and functionally distinct cytoplasmic processes: dendrites and axons. The use of dissociated neuronal cultures has provided some insight about how the polarized growth of neuronal processes is initiated. Neurons first send out several minor processes and soon one of these processes exhibits rapid outgrowth and becomes an axon (Dotti et al., 1988). Recent experiments have found that the growth cone (see below) of a neurite destined to become an axon exhibits significant increases in actin dynamics and instability. In fact, when cytochalasin was applied to one of the growth cones to destabilize the actin cytoskeleton it was found to be sufficient to convert this minor process into an axon-like process (Bradke and Dotti, 1999).

1.3 Growth cone structure

Soon after completing migration, postmitotic neurons are round with short cytoplasmic extensions. Changes in cell shape require the generation of plasma membrane protrusions, which are based on the coordinated reorganization of the actin and microtubule cytoskeleton as well as the polarized insertion of membrane components into the expanding end of the cell (Craig et al., 1995; Lockerbie et al., 1991; Pfenninger and Friedman, 1993). Receptors at the cell surface are activated by extracellular factors which trigger intracellular signaling pathways that initiate actin cytoskeleton changes.

These actin cytoskeleton rearrangements result in the sprouting and elongation of neuronal processes (da Silva and Dotti, 2002). These neuronal processes named dendrites and axons function to receive, process, and transmit information. Dendrites and axons grow towards their specific partners guided by a motile fanned-shaped plasma membrane extension named, "the growth cone" (Ramon y Cajal, 1892). This specialized structure at the tip of elongating neurites is formed by a core (or central) and peripheral domain. The core domain is filled of membrane organelles and growing microtubules that can extend into the actin filament (F-actin) rich regions. The peripheral domain is formed by veil-like structures named lamellipodia, which are composed of a meshwork of F-actin that is often located between filopodial extensions. Filopodial extensions are finger-like membrane protrusions containing bundles of F-actin (da Silva and Dotti, 2002; Luo et al., 1997). This actin-rich peripheral domain is highly motile and continuously extends and retracts lamellipodia and filopodia, changing the shape of the growth cone. The morphology of neuronal growth cones and the morphology of dendritic and axonal arbors depend on the organization and dynamics of actin filaments, microtubules, and membrane traffic. Cytoskeleton dynamics at the growth cone are characterized by actin polymerization which occurs at the membrane cortex, followed by a myosin driven retrograde movement of microfilaments away from the leading edge, whereas depolymerization occurs in the interface between the peripheral and the central domain. Actin polymerization is controlled by proteins (such as profilin) that regulate nucleation and addition of actin monomers (Korey and Van Vactor, 2000) whereas proteins such as cofilin (actin depolymerizing factor) mediate F-actin depolymerization

(Kuhn et al., 2000). The microtubule array appears to depend on the transport of microtubule polymers and assembly at the plus end of microtubules and both processes are necessary for elongation (Baas, 1997). Microtubule dynamic instability is important to ensure the delivery of organelles from the core region to the peripheral domain (Gallo, 1998) and the insertion of membrane into the growth cone plasma membrane (Zakharenko and Popov, 1998). The rearrangement of the actin and microtubule cytoskeleton may influence each other to allow the recruitment of vesicles for fusion to the targeted membrane necessary for neurite extension (Fukata et al., 2002).

1.4 Neurite pathfinding

The lamellipodial and filopodial protrusions sent out by the developing growth cone are highly dynamic structures that sample their surroundings. The guidance forces that the growth cone encounters in the environment consist of short-range (or local) and long range (or diffusible) cues, that are either attractive or repulsive (Goodman and Tessier-Lavigne, 1997). Extending neurites are guided at short-range by contact-mediated mechanisms that involve nondiffusible cell surface and extracellular matrix (ECM) molecules that provide a permissive substrate for growth or cause a repulsive effect which can result in neurite arrest, growth cone collapse and retraction. Examples of contact attraction molecules are cell adhesion molecules of the immunoglobulin and cadherin superfamilies, and ECM molecules such as laminins. Examples of contact repulsion cues are transmembrane molecules such as Eph ligands and semaphorins, and ECM molecules such as tenascin. Neurites elongating over long distances can also respond to long-range chemoattraction or chemorepulsion, a process by which target cells

secrete diffusible attractive (for example, netrins) or repellent (for example, secreted semaphorins and netrins) substances that have a permissive and attractive or an inhibitory and repulsive effect (Tessier-Lavigne and Goodman, 1996). It is important to note that some guidance molecules can be attractive or repulsive, and some families of guidance cues have both diffusible and nondiffusible members. Therefore, the coordinate actions of these molecules instruct the growth cone to advance, turn, or retract; and membrane receptors at the growth cone transduce these signals to the underlying cytoskeleton. Thus, for a neurite to grow there must be a balance between cytoskeleton dynamics and membrane trafficking events and these processes must be tightly regulated to allow correct navigation.

1.5 Neurite elongation

With time, the dendritic branches grow extensively and the axon elongates towards its targets and sends out collateral branches. The growth cone translates the extracellular signals in the environment into rounds of extension, retraction and turning to enable growth cones to find their way. These guidance signals involve both cell surface bound and diffusible cues, each of which can be either attractive or repellent (Song and Poo, 1999; Tessier-Lavigne and Goodman, 1996).

1.5.a Dendrite elongation

The extracellular signals that have been found to influence dendrite growth and branching during development are the neurotrophins, semaphorins, osteogenic proteins, slits and ephrins (McAllister, 2001; Scott and Luo, 2001). The neurotrophin BDNF (brain-derived morphogenetic factor), semaphorin 3A, osteogenic protein-1 (OP-1) and

slits have all been shown to stimulate dendritic growth (Polleux et al., 2000) and branching (Horch et al., 1999; Le Roux et al., 1999; Whitford et al., 2002) in cortical pyramidal neurons. In contrast, the expression of EphA receptor in cultured ferret visual slices suggests that Ephrins negatively regulate dendritic branching (Butler et al., 1999). In addition to growth-regulating molecules, the shape of the dendritic arbor can be regulated by synaptic activity (Cline, 2001; McAllister, 2000). In fact, spontaneous waves of action potential activity are present in the developing retina, hippocampus, cerebral cortex and spinal cord (Feller, 1999).

1.5.b Axonal elongation

Axons also rely on guidance molecules such as semaphorins, slits, netrins and ephrins during axonal navigation, elongation and branching (Brose and Tessier-Lavigne, 2000; Song and Poo, 1999; Yu and Bargmann, 2001). In contrast to their stimulatory effects on dendrite morphogenesis, semaphorin 3A and slit proteins have been found to repel cortical pyramidal axons (Polleux et al., 2000; Whitford et al., 2002). Molecules such as netrins have been shown to be bifunctional, inducing repellent or attractive responses in the neural midline depending on the type of receptors expressed (Brose and Tessier-Lavigne, 2000; Song and Poo, 1999). In fact, Netrin 1 has been associated with the establishment of the hippocampal commissural system as they attract commissural fibers to the contralateral hippocampus during development (Skutella and Nitsch, 2001). Slit proteins are best known for their chemorepellent effects since they repel both spinal cord motor axons and hippocampal axons from the dentate gyrus (Brose and Tessier-Lavigne, 2000; Nguyen Ba-Charvet et al., 1999). Recent work, however, has shown that

they can also be positive regulators as they promote axonal elongation and branching in dorsal root ganglia (Wang et al., 1999).

1.6 From surface receptors to the actin cytoskeleton

How are extracellular cues translated into cytoskeletal changes linked to dendritic and axonal development? Evidence is emerging that they influence dendritic and axonal growth by interacting with membrane receptors such as Plexin B, EphA, ephrins B, Notch and Robo (Patel and Van Vactor, 2002; Redmond and Ghosh, 2001) and subsequently regulate the activity of small GTPases (Luo, 2002).

1.6.a Small GTPases

During neuronal morphogenesis, extracellular cues trigger signalling pathways from membrane receptors to small GTPases that result in membrane traffic and actin cytoskeleton changes (Song and Poo, 1999). Like other small GTP binding proteins, Rho proteins act as molecular switches, transducing extracellular signals into rearrangements of the actin cytoskeleton (Hall, 1998). Their activity is facilitated by interacting proteins including guanine exchange factors (GEFs) and guanine activating proteins (GAPs) which in turn are regulated by upstream signals. GEFs stimulate the release of GDP and the binding of GTP, thereby switching the GTPase 'on'. By contrast, GAPs switch them 'off' by increasing the intrinsic GTPase activity. The Rho family of small GTPases (such as Rac1, RhoA and Cdc42) is known to play an important role in the regulation of actin cytoskeleton polymerization in non-neuronal cells (Hall, 1998). Most of what we know about Rho GTPases functions comes from microinjection experiments in nonneuronal cells where they have been found to exert changes in the actin cytoskeleton (Hall, 1998).

In fibroblasts, Rho has been found to regulate actin-stress fiber formation and focal adhesion. Rac1 has been found to regulate membrane ruffling and lamellipodia formation and Cdc42 to regulate filopodia formation (Hall, 1998). Recent studies have shown that they also play a critical role in different aspects of neuronal development including migration, dendritic and axonal growth, and axon guidance (Dickson, 2001; Luo, 2002; Redmond and Ghosh, 2001). Their role in migration has been shown through mutation of Cdk5 (cyclin-dependent protein kinase 5) and its p35 subunit which results in abnormal corticogenesis as it converts the "inside-out" migration pattern into an "inside-in" pattern (Chae et al., 1997; Ohshima et al., 1996). The p35 protein is an essential subunit of Cdk5, and recently it has been found to be a downstream effector for Rac (Nikolic et al., 1998). The effects of Rac1 on neurons have been contradictory, as increased or decreased Rac1 activity has been shown to cause effects on axonal growth (both initiation and elongation), guidance and branching but not on dendritic growth (Jin and Strittmatter, 1997; Luo, 2000; Luo et al., 1996a; Luo et al., 1994; Ng et al., 2002). However, other experiments have implicated Rac1 in the regulation of dendritic growth and complexity, spine morphogenesis and branch dynamics (Albertinazzi et al., 1998; Hernandez-Deviez et al., 2002; Nakayama et al., 2000; Tashiro et al., 2000; Threadgill et al., 1997). Guanine-nucleotide exchange factors (GEFs) for members of the Rho family have been also implicated in the regulation of neuronal morphogenesis including cell morphology, axonal outgrowth, collapse and guidance (Kunda et al., 2001; Leeuwen et al., 1997; Shamah et al., 2001). In summary, small GTPases have shown to be good

candidate molecules to translate the information from specific guidance signals into actin cytoskeleton changes in different aspects of neuronal morphogenesis.

1.6.b Signal transduction in dendrite development

Neurotrophic factors such as nerve growth factor (NGF) and neurotrophins exert their effects through the Trk family of tyrosine kinase receptors. Neurotrophins have been shown to increase the dendrite complexity of pyramidal neurons by increasing total dendrite length, the number of branch points, and/or the number of primary dendrites (Baker et al., 1998; McAllister et al., 1995; Niblock et al., 2000). Although little is known about the intracellular pathways mediating these effects, studies in neuronal cell lines indicate that NGF activates the small GTPase Rac1, and this activation is necessary for neurite elaboration (Yasui et al., 2001). In addition, Notch signaling has emerged as a key regulator of dendritic growth and branching (Whitford et al., 2002). Recent studies indicate that activation of Notch by its ligands (such as Delta) positively influences dendritic branching while decreasing dendritic length in cortical neurons. These effects are likely mediated by the activation of Rac1 and Cdc42 and RhoA GTPases, respectively (Redmond and Ghosh, 2001).

1.6.c Signal transduction in axonal development

The Eph receptors and their ligands, the ephrins, have been shown to be critical in modeling topographic maps (where the spatial arrangement of cells of origin is reflected in the spatial arrangement of their axon terminals) in the nervous system (Patel and Van Vactor, 2002). For example, Eph receptors have been implicated in the formation of the retinotopic map (Feldheim et al., 2000; Feldheim et al., 1998) by guiding retinal ganglion

cell axons to their proper target regions in the tectum. These effects are mediated mainly by their actions as repulsive factors. The most compelling evidence that an extracellular cue can directly modulate the activities of Rho GTPases comes from the work of Wahl (Wahl et al., 2000) and Shamah (Shamah et al., 2001), which link Eph receptors to the actin cytoskeleton. Ephexin, a novel Eph receptor interacting protein, is a guanine nucleotide exchange factor (GEF) for Rho GTPases (Shamah et al., 2001). The stimulation of EphA receptors by Ephrin-A1 has been found to inhibit ephexin activity toward Cdc42 and Rac1 and potentiate its activity toward RhoA resulting in growth cone collapse (Shamah et al., 2001). Another receptor that has been shown to regulate small GTPases is Robo. Robo transduces the signal for axon repulsion from its ligand Slit. In the context of neuronal migration, Robo has been found to directly interact with the Slit-Robo guanine activating protein, srGAP, therefore inhibiting Cdc42 activity (Wong et al., 2001). In addition, migrating neurons expressing dominant negative srGAP lose their ability to respond to the repulsive activity of Slit (Wong et al., 2001). These data suggest that axon repulsion also depends on the downregulation of Cdc42 activity via the Slit-Robo-srGAP pathway.

1.7 ARF proteins and neuritogenesis

Despite the increasing work in the field, little is known about the role of other small GTPase families during neuronal morphogenesis. In addition to the Rho-family of GTP-binding proteins, members of the ARF family of small GTPases play an essential role in membrane trafficking, organelle structure and cytoskeletal rearrangement. Like members of the Rho family, ARF proteins belong to the Ras superfamily of small GTP-

binding proteins. Mammalian ARFs are divided into three classes based on their sequence. The class I ARFs (ARF1-3) is the best understood and have been shown to be involved in trafficking in the endoplasmic reticulum-Golgi and endosomal systems. Little is known about the function of class II ARFs (ARF4 and 5). The class III ARF (ARF6) has been shown to function in the endosomal-plasma membrane system (Donaldson and Jackson, 2000). ARF6 has been shown to regulate membrane traffic between the plasma membrane and an early endosomal compartment (Radhakrishna and Donaldson, 1997), to alter cortical actin, and to influence the ability of Rac1 to form lamellipodia (Boshans et al., 2000; D'Souza-Schorey et al., 1997; Frank et al., 1998b; Radhakrishna et al., 1999; Radhakrishna et al., 1996). The morphological and behavioral similarities between a migrating fibroblast and the growth cone of advancing neurites suggested that these molecules participate in the regulation of neuronal morphogenesis. In fact, recent studies in developing neurons suggest that ARF proteins regulate axonal elongation since treatment with brefeldin A (BFA) selectively inhibited axonal growth in cultured hippocampal and dorsal root ganglia neurons (Hess et al., 1999; Jareb and Banker, 1997). However, the brefeldin A-sensitive GEF that is responsible for this effect has not yet been identified. Another class of ARF GEFs, ARNO is insensitive to brefeldin A, and has been shown to catalyze nucleotide exchange on ARF1 and ARF6 (Chardin et al., 1996; Frank et al., 1998a). We have shown that ARNO is present during early events in neuritogenesis (Hernandez-Deviez et al., 2002), and both morphological and biochemical studies have shown high levels as well as differential expression, of ARFs and their GEFs isoforms in the embryonic rat brain (Hernandez-Deviez et al.,

2002; Suzuki et al., 2001; Suzuki et al., 2002). The mRNAs encoding ARF-1, -4, -5, and -6 as well as the ARF-GEF msec7-1 were shown to be enriched in the ventricular germinal zone (Suzuki et al., 2001), a layer where cells are actively proliferating prior to migration in the developing brain. In addition, immunofluorescence analysis of the ARNO showed ARNO to be enriched in cells in both the ventricular and marginal zones of the hippocampus embryonic day 17, a time when neurons are actively dividing and migrating into superficial layers of this structure (Hernandez-Deviez et al., 2002). The presence of these proteins in the developing brain strongly implicates a role for these molecules in the regulation of many aspects of neuronal morphogenesis including neuronal migration, neurite elongation and branching. Recent work has shown a role for ARNO, ARF6, and Rac in modulating the actin cytoskeleton and membrane traffic during cell migration in wound healing (Santy and Casanova, 2001). However, little is known about the role for ARNO in the coordination of actin filament rearrangement and membrane dynamics during neuritogenesis. We have studied the role of ARNO and its downstream effectors during dendrite and axonal development in rat hippocampal neurons in culture. We found that in developing hippocampal neurons, ARNO, through downstream activation of ARF6 negatively regulates dendritic branching and axonal elongation and branching.

CHAPTER TWO: REGULATION OF DENDRITIC DEVELOPMENT BY THE ARF EXCHANGE FACTOR

Dendritogenesis is a dynamic process that continues during the development and life of a neuron; correct dendritic arbor development and stabilization is central to the ability of neurons to receive, integrate and process information (Scott and Luo, 2001). Members of the Rho-family of small GTPases act as molecular switches to control dendrite elongation, branching and spine morphology (Li et al., 2000; Luo, 2000; Nakayama et al., 2000; Threadgill et al., 1997). Here, we analyzed the role a member of the ADP-ribosylation (ARF) family of small GTPases in dendritic arbor development in rat hippocampal neurons in culture. We show that over-expression of the inactive form of the GTP-exchange factor (GEF) ARNO (ARF nucleotide binding site opener) as well as inactive ARF6 resulted in increased dendritic branching. Co-expression of either Rac1 or active ARF6 with inactive ARNO eliminated the increased branching. These results show that the ARF family of small GTPases provides an additional level of regulation of dendritic branching and that ARF6 activation turns on two independent pathways, one through Rac1 and the other through ARF6 effectors, both of which act to suppress dendritic branching *in vivo*.

During development neurons migrate into the hippocampus and actively extend processes to establish precise connections (Altman and Bayer, 1990). We found that ARNO is present during these early events in dendritic development. Immunolabeling showed that on day 17 there was strong ARNO labeling in the ventricular and marginal zones (Fig. 2.1a) and weaker labeling within the substance of the hippocampus. Both

MAP-positive and negative cells were labeled (not shown), consistent with ARNO's role as a ubiquitous ARF regulator. At day 18, concentrated labeling of cells at the periphery was absent, but more cells within the substance of the hippocampus were strongly labeled (Fig. 2.1 b), perhaps reflecting migration into this region. Labeling was found throughout the cell bodies and in processes (Fig. 2.1 c). Importantly, the subcellular distribution of ARNO positions it at sites of active process extension. In addition to its localization in the perinuclear region at all stages, ARNO was concentrated at lamellar extensions and ruffling areas at early stages (Fig. 2.1 d), and later ARNO was concentrated at the tips of processes (Fig. 2.1 e). Immunoblot analysis and recent results (Suzuki et al., 2002) show that ARNO is present in both embryonic and adult hippocampus (Fig. 2.1 f), and at day 18 partitions predominantly into the membrane fraction. Since ARNO functions at membrane surfaces, this partitioning into embryonic membranes indicates that ARNO is highly activated during early dendritogenesis. Further immunoblot analysis, showed that ARF1/3 and ARF6 were present in embryonic and adult hippocampus (Fig. 2.1 f), indicating a role for these molecules in developing and mature brain. These morphological and biochemical localizations are consistent with a direct role for ARNO and its ARF substrates in dendritic initiation and remodeling.

To directly test the role of ARNO in dendritogenesis, we over-expressed wild-type and inactive forms of ARNO (Frank et al., 1998b) in neurons in culture. ARNO is recruited to membrane phosphoinositides via its pleckstrin homology domain and, when over-expressed, the inactive form would be expected to displace endogenous ARNO, resulting in inhibition of nucleotide exchange on ARF molecules. Untransfected cells

contained a few thick dendrites (Fig. 2.2 a), and neurons over-expressing wild-type ARNO showed no quantifiable effects upon dendritic complexity (Fig. 2.2 b, h). However, over-expression of inactive ARNO (ARNO-E156K) resulted in a dramatic change in the dendritic arbor (Fig. 2.2 c, h). Cells expressing ARNO-E156K had many dendritic branches, and these branches formed a meshwork around the cell body (Fig. 2.2 c), causing a 4.5-fold increase in the number of dendritic tips (Fig. 2.2 h). These results indicate that ARNO could be a powerful regulator of dendritic development and branching.

ARNO has GEF activity on both ARF1 and ARF6 *in vitro* (Chardin et al., 1996; Frank et al., 1998a). We found that dominant negative ARF6 (ARF6-T27N) resulted in a morphology similar to that seen with inactive ARNO. Cells expressing ARF6-T27N contained many short dendrites with a complex dendritic arbor (Fig. 2.2 d, h). In contrast, over-expression of ARF6 caused no quantifiable effects upon complexity (Fig. 2.2 h). However, over-expression of the constitutively active form of ARF6 (ARF6-Q67L) resulted in a small decrease in the number of dendritic tips (Fig. 2.2 h).

If the effects of ARNO-E156K were being mediated through ARF6, we would expect that co-expression of active ARF6 and ARNO-E156K would reverse the enhanced dendritic complexity. In fact, co-expression of these molecules resulted in a complete loss of dendritic processes (Fig. 2.2 e, h). Although these results do not rule out other ARF6 GEFs, they are consistent with the hypothesis that the effects of ARNO were mediated through ARF6.

Even though ARF6-Q67L completely reversed the effect of ARNO-E156K upon dendritic branching, it is possible that ARF1 also has a role in these events. However, attempts to test this hypothesis by over-expression of wild-type and mutant forms of ARF1 resulted in cell death.

During cell migration, Rac1 is activated downstream of ARF6 (Santy and Casanova, 2001). To determine if Rac1 is a downstream effector of ARF6 during dendritogenesis, we first analyzed the effects of expression of Rac1 or dominant-negative Rac1 (Rac1-N17) on dendritic morphology. Interestingly, over-expression of Rac1-N17 resulted in a phenotype (Fig. 2.2 f) that was very similar to cells over-expressing ARNO-E156K, including a 3-fold increase in the number of dendritic tips (Fig. 2.2 h). In contrast, over-expression of wild-type Rac1 had no effect upon dendritic complexity (Fig. 2.2 h). Interestingly, in mature hippocampal neurons Rac1-N17 had no significant effect upon dendritic branching (Nakayama et al., 2000), suggesting that regulation of dendritogenesis in mature neurons differs from early stages of arbor development.

Our hypothesis predicts that dendritic branching is negatively regulated by ARNO and ARF6 signaling. ARF6 activation turns on two independent pathways, one through Rac1 and the other through phosphoinositide kinases (Honda et al., 1999), both of which contribute to suppress dendrite branching. Thus, if the effects of Rac-N17 expression upon dendrite complexity were mediated through the ARNO and ARF6 pathway, we would predict that co-expression of Rac1 with ARNO-E156K would suppress the increased dendritic complexity. In these experiments, neurons co-transfected with ARNO-E156K and Rac1 had reduced dendritic complexity compared to cells expressing

only ARNO-E156K (Fig. 2.2 g, h), suggesting that increased complexity was in part due to ARNO effects upon the Rac-dependent pathway. However, in a parallel suppression experiment, neurons co-expressing active ARF6 and Rac1-N17 resulted in loss of dendritic extensions (Fig. 2.2 h). These results suggest that signaling pathways that stimulate ARF6 can act through ARF6 effectors directly or through Rac1 to modulate dendritic dynamics.

The control of dendritic arborization has important implications for nervous system function (Cline, 2001; Scott and Luo, 2001). ARF6 and ARNO could provide precise developmental control of both initiation and branching early in dendritogenesis, as well as rapid changes induced by synaptic activity or neurotrophic factors. This regulation could influence the extent of synaptic inputs and information integration crucial for normal function of the nervous system. It is now critical to determine the factors that regulate ARNO activity in developing neurons.

2.1 Acknowledgements.

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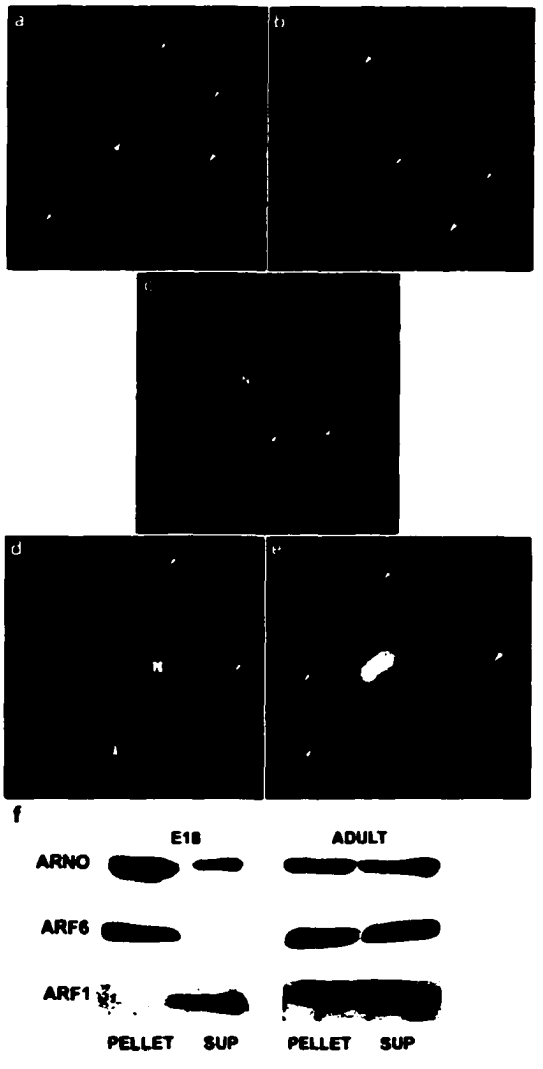

Figure 2.1. **ARNO is expressed in developing hippocampus.** Anti-ARNO antibodies (Frank et al., 1998a) (red), DAPI (blue). (**a**), embryonic day 17, cells in the ventricular and marginal zones with high ARNO expression (arrows). In the substance of the hippocampus, there was weaker labeling (arrowheads). (**b**), day 18, strongly labeled cells throughout hippocampus (arrows) and some with weaker labeling (arrowheads). (**c**), neuron *in situ*. ARNO was present in the cell body and an extension (arrows). (**d-e**), Neuronal cultures immunolabeled with anti-ARNO (green), propidium iodide (red). (**c**), Early, ARNO at lamellar extensions (arrows) and ruffles (arrowhead). (**d**), later, ARNO in the tips of processes (arrows). Arrowhead, axon; N, nucleus. Scale bars: (**a**, **b**) 50μm, (**c-e**) 5μm. (**f**), Supernatant and pellet from embryonic and adult hippocampi immunoblotted using anti-ARNO, ARF6 and ARF1/3 antibodies. All experiments were approved by the Institutional Animal Care and Use Committee.

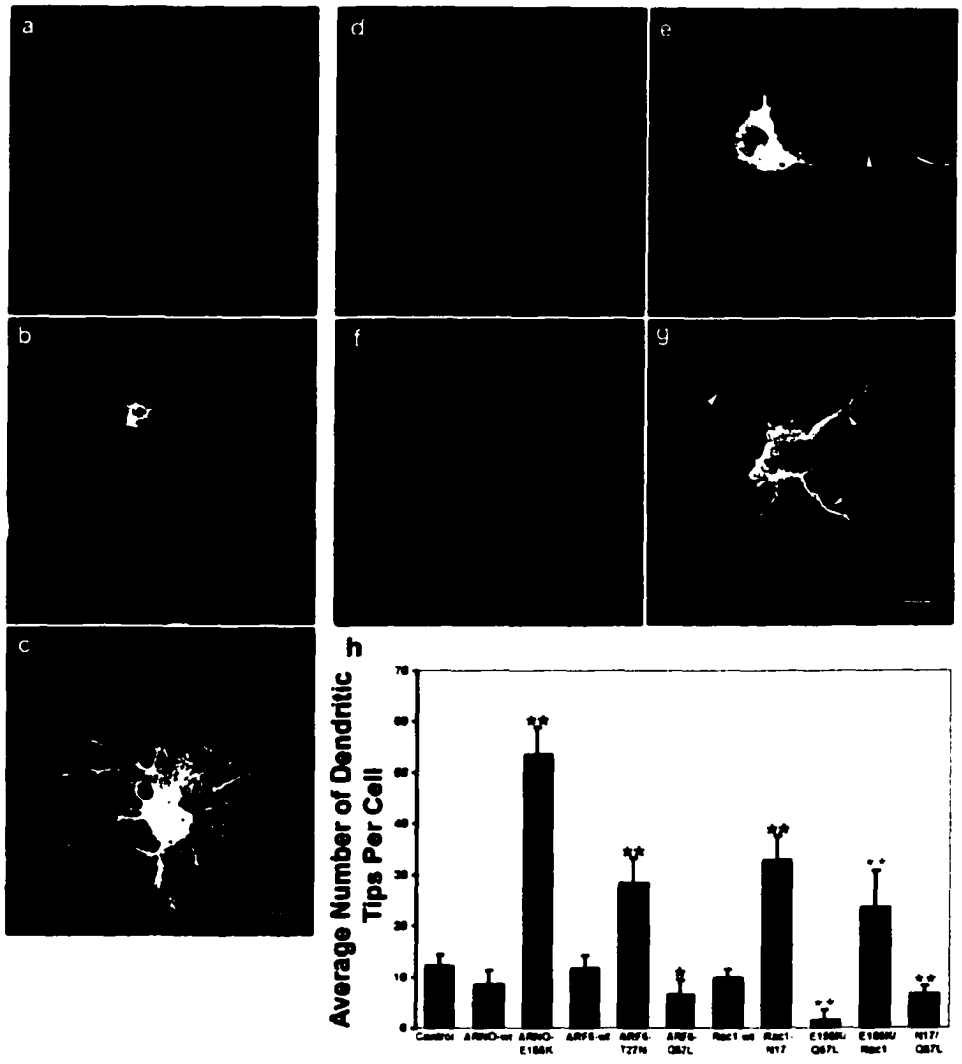

Figure 2.2. **ARNO mediates dendrite initiation and branching through ARF6 and Rac1. Left panel**, Neurons transfected after 1 day in vitro and immunolabeled after 9 days with anti-MAP2 (green) and anti-*myc* (ARNO), anti-HA (ARF6), or anti-FLAG (Rac1) (red). Mock transfectants (**a**) and cells expressing wild-type ARNO (**b**) or ARNO-E156K (**c**). **Right panel**, Cells expressing ARF6-T27N (**d**) or Rac1-N17 (**f**). **e**, Neurons expressing ARNO-E156K and ARF6-Q67L. Axon, (arrowhead). **g**, Neuron expressing ARNO-E156K and Rac1. Dendritic branches, (arrows); axon, (arrowhead). Scale bars, **a-g**: 10μm. **h**, Dendrite complexity was quantified by counting dendritic tips. Changes were compared using one-way analysis of variance (ANOVA) and expressed as the mean number of dendritic tips per cell. Data are means ± standard deviation (n = 10). $P < 0.05$ (*), and $P < 0.01$ compared to control (**), $P < 0.01$ compared to ARNO-E156K (**, red), $P < 0.01$ compared to Rac1-N17 (**, blue). Cells illustrated are near the mean. Cell survival was 65% (ARF6 plasmids) to 85% (other plasmids).

CHAPTER THREE: THE ARF EXCHANGE FACTOR ARNO AND ARF6 NEGATIVELY REGULATE AXONAL ELONGATION AND BRANCHING

3.1 Abstract.

In the developing nervous system, neurite extension and branching are critical to establish highly complex connections and ensure adequate function and these events require coordination of membrane-cytoskeleton interactions. Small GTPases of the Rho family have been shown to regulate different aspects of axonal development through their effects upon actin cytoskeleton dynamics. We have previously shown that the ARF-GEF ARNO is present in hippocampal neurons during early development and, through downstream activation of ARF6, negatively regulates dendritic branching. To investigate the role of ARNO and ARF6 on axonogenesis, we overexpressed wild-type and mutant forms of ARNO and ARF6 in cultured rat hippocampal neurons. Over-expression of catalytically inactive ARNO and dominant-negative ARF6 resulted in greatly enhanced axonal extension and branching and this effect was abrogated by co-expression of constitutively-active ARF6. These effects may be mediated by coordinated changes in membrane trafficking and the cytoskeleton, as expression of inactive ARNO caused a redistribution of a subset of endosomes to the axonal plasma membrane and displacement of the profilin-binding protein Mena from the growth cone plasma membrane. These results suggest that ARNO, through ARF6, is a negative regulator of axonal elongation and branching during neural development.

3.2 Introduction.

During the development of the nervous system, neurite outgrowth is necessary for the formation of the highly specific pattern of connections between nerve cells and the precise pattern of connections is vital for proper nervous system function. Developing neurites reach their proper targets in response to a complex set of environmental cues. During navigation, the growth cone translates these cues into cyclical extensions of filopodia and lamellipodia by changing the dynamics of the plasma membrane surface and the organization of the actin cytoskeleton (Craig et al., 1995; Gallo and Letourneau, 2000; Kamiguchi and Lemmon, 2000). These events consequently result in neurite outgrowth, retraction, or branching (Song and Poo, 1999; Tessier-Lavigne and Goodman, 1996). The small GTPases of the Rho subfamily are critical regulators of the actin cytoskeleton (for review see (Hall, 1998)) and have been implicated in the regulation of different aspects of neuronal development (for review see (Luo, 2000)). Increased or decreased Rac1 activity has been shown to result in effects on axon outgrowth (both initiation and elongation), guidance and branching (Luo et al., 1996b, Jin, 1997 #27; Luo et al., 1994; Ng et al., 2002). Guanine-nucleotide exchange factors (GEFs) for members of the Rho family have also been implicated in the regulation of neuronal morphogenesis including cell morphology, axonal outgrowth, collapse and guidance (Kunda et al., 2001; Leeuwen et al., 1997; Shamah et al., 2001).

In addition to the Rho-family of GTP-binding proteins, members of the ADP-ribosylation factor (ARF) family of small GTPases play an essential role in membrane trafficking and cytoskeletal rearrangement. ARF6 has been demonstrated to regulate

membrane traffic between the plasma membrane and an early endosomal compartment, to alter cortical actin, and to influence the ability of Rac1 to form lamellipodia (Boshans et al., 2000; D'Souza-Schorey et al., 1997; Frank et al., 1998b; Radhakrishna et al., 1999; Radhakrishna and Donaldson, 1997; Radhakrishna et al., 1996). Recent work has shown a role for ARNO, ARF6, and Rac in modulating the actin cytoskeleton and membrane traffic during cell migration in wound healing (Santy and Casanova, 2001). In neuronal development, ARF proteins have been suggested to regulate axonal elongation since treatment with brefeldin A (BFA) selectively inhibited axonal growth in cultured hippocampal and dorsal root ganglia neurons (Hess et al., 1999; Jareb and Banker, 1997). However, the brefeldin A-sensitive GEF that is responsible for this effect has not yet been identified. Another class of ARF GEFs is insensitive to brefeldin A, and ARNO (ARF nucleotide-binding site opener) is a member of this group (Chardin et al., 1996; Frank et al., 1998a). We have shown that ARNO is present during early events in neuritogenesis (Hernandez-Deviez et al., 2002), and both morphological and biochemical studies have shown high endogenous expression of ARFs and their GEFs in the developing hippocampus, strongly implicating a role for these molecules in the regulation of neurite elongation and branching (Hernandez-Deviez et al., 2002; Suzuki et al., 2001; Suzuki et al., 2002). However, the role for ARNO and ARF6 in the coordination of actin filament rearrangement and membrane dynamics during axonogenesis remains unclear. In this study, we have explored the role of ARNO and its downstream effectors during axonal development in rat hippocampal neurons in culture. We find that over-expression of catalytically inactive ARNO and dominant-negative ARF6 results in enhanced axonal

elongation and branching. These changes in axonal development are accompanied by changes in the distribution of endosomal membranes along the axon and by displacement of the profilin-binding protein, Mena, from the growth cone plasma membrane. These results suggest that during development ARNO and ARF6 serve as negative regulators of axonal extension and branching.

3.2 Results.

3.2.a Over-expression of catalytically inactive ARNO affects axonal length and complexity.

To test the role of ARF family members upon axonogenesis, we first over-expressed wild-type and catalytically inactive forms of the ARF-GEF ARNO in embryonic day 17 (E17) rat hippocampal neurons in culture. Since ARNO acts as a nucleotide exchange factor for ARF6, expression of wild type ARNO increases the amount of activated ARF6 (Santy and Casanova, 2001). In contrast, since ARNO is recruited to membrane phosphoinositides via its pleckstrin homology domain, expression of inactive ARNO may displace endogenous ARNO, consequently inhibiting nucleotide exchange on ARF6 and decreasing the amount of activated ARF6. Dendritic and axonal processes were distinguished using antibodies against the neuronal markers MAP2 (microtubule associate protein 2) or tau and transfected cells were identified using antibodies against the epitope tag. Untransfected cells exhibited a single axonal extension that emerged from the cell body and formed approximately 4-5 branches (Fig. 3.1 a, c, e). Neurons overexpressing ARNO wild-type had no quantifiable effects upon axonal length and complexity (Fig. 3.1 a, c, f), suggesting downstream effectors are limiting in the effect of ARNO upon axonogenesis. However, over-expression of catalytically inactive ARNO (ARNO-E156K) resulted in a dramatic effect on axonal elongation and branching (Fig. 3.1 a, c, g). Cells expressing ARNO-E156K had a long axon that emitted numerous collateral branches that were also long, resulting in a nearly

6-fold increase in total axonal length and a 4-fold increase in the average number of axonal branches.

3.3.b The effects of ARNO upon axonogenesis are mediated by downstream activation of ARF6.

ARNO has been shown to have GEF activity on both ARF1 and ARF6 *in vitro* (Chardin et al., 1996; Frank et al., 1998a; Monier et al., 1998), and both ARF proteins are present in developing hippocampus (Hernandez-Deviez et al., 2002; Suzuki et al., 2001; Suzuki et al., 2002). Therefore, we next examined the consequences of over-expression of wild-type and mutant forms of ARF6 upon axonogenesis. Cells expressing wild-type ARF6 or constitutively-active ARF6 (ARF6-Q67L) showed slightly shorter axons and no quantifiable effects upon axonal branching (Fig. 3.1 a, c). In contrast, cells expressing dominant-negative ARF6 (ARF6-T27N) showed an axonal phenotype similar to that seen with expression of inactive ARNO (Fig. 3.1 a, c), resulting in a 2-fold increase in axonal length (Fig. 3.1 a), and nearly 2-fold increase in axonal complexity (Fig. 3.1 c).

If expression of ARNO-E156K leads to reduced activation of ARF6, we would expect that co-expression of constitutively-active ARF6 would reverse the observed effects of ARNO-E156K by circumventing the need for an exchange factor. Under these conditions, co-expression of these molecules resulted in a dramatic reduction in the total length and in the average number of collateral branches of the axonal process (Fig. 3.1 b, d). These results are consistent with the hypothesis that the effects of ARNO upon axonal elongation and branching are mediated through ARF6.

We attempted to directly study the effects of ARF1 expression upon axonogenesis. Unfortunately, expression of either wild-type or mutant forms of ARF1 resulted in cell death, undoubtedly due to inhibition of the secretory pathway.

Rac1 has been shown to regulate axonal outgrowth (Albertinazzi et al., 1998; Jin and Strittmatter, 1997; Kaufmann et al., 1998; Kuhn et al., 1999; Luo et al., 1996b; Luo et al., 1994), and has also been shown to be activated downstream of ARF6 in MDCK cells during cell migration (Santy and Casanova, 2001). Recently, we showed that ARF6 can regulate dendritic growth via downstream activation of Rac1 (Hernandez-Deviez et al., 2002). To determine if Rac1 is a downstream effector of ARF6 during axonogenesis, we first investigated the effects of over-expression of wild-type or dominant-negative mutant Rac1 (Rac1-N17) on axonal length and branching. As shown by others (Albertinazzi et al., 1998; Jin and Strittmatter, 1997; Kaufmann et al., 1998; Kuhn et al., 1999; Luo et al., 1996b; Luo et al., 1994), over-expression of wild-type Rac1 resulted in a 3-fold increase in axonal length (Fig. 3.1 a), but no quantifiable effects upon axonal branching were detected (Fig. 3.1 c). Somewhat paradoxically over-expression of Rac1-N17 resulted in a 4.5-fold increase in axonal length and a 2-fold increase in axonal branching (Fig. 3.1a, c).

If the effects of ARNO upon axonal branching were mediated through activation of ARF6 and consequently Rac1, we would predict that co-expression of Rac1 with ARNO-E156K would reduce the enhanced axonal branching seen with ARNO-E156K and ARF6-T27N. To test this, hippocampal neurons were co-transfected with ARNO-E156K and Rac1 plasmids. Interestingly, co-expression did not cause an additive effect

upon axonal length and resulted in a small decrease in branching that was not statistically significant (Fig. 3.1 b, d), suggesting that ARNO effects upon axonal branching are not mediated by ultimate downstream activation of Rac1. However, Rac1 activation could require a GEF whose function is regulated by ARF6 (Santy and Casanova, 2001), which would not be activated under these conditions. To determine this, cells were co-transfected with ARNO-E156K and constitutively-active Rac1 (Rac1-V12) plasmids. Co-expression did not cause an additive effect upon axonal length instead resulted in a reduction in both axonal length and branching (data not shown), suggesting that the increased branching may be regulated through a Rac1 pathway. However, if only the Rac1 pathway is mediating these changes, co-expression of dominant-negative Rac1 with activated ARF6 would be expected to result in increased branching and length. In fact, in a parallel suppression experiment, co-expression of constitutively-active ARF6 with dominant-negative Rac1 resulted in reduction in both axonal length and branching (Fig. 3.1 b, d). These results suggest that regulation of axonal length and branching by ARF6 could be triggered through two independent pathways, utilizing both Rac1 and/or other ARF6 downstream effectors (see discussion) (Honda et al., 1999).

Because neurite outgrowth involves membrane-cytoskeleton interactions, the ARNO-E156K phenotype may be due to changes in actin polymerization, cytoskeletal proteins and/or membrane insertion. ARNO and ARF6 have been shown to modulate the reorganization of the cortical actin cytoskeleton in fibroblast cell lines (D'Souza-Schorey et al., 1997; Frank et al., 1998b; Radhakrishna et al., 1996) although the mechanism for this reorganization remains unclear. Recent studies have shown the importance of the

Ena/VASP family of profilin-binding proteins in controlling actin dynamics, growth cone motility, axonal guidance, and fibroblast motility (Bear et al., 2000; Bear et al., 2002; Lanier et al., 1999; Wills et al., 1999). This prompted us to examine the effects of ARF6 inhibition on the distribution of Mena in the growth cone. To test this we chose stage 2 or 3 neurons (Dotti et al., 1988) since they frequently have expanded, flattened growth cones at the tips of their extending processes. In untransfected cells, as well as cells over-expressing wild-type ARNO, Mena is localized at the tips of growth cones (Fig. 3.2 a, b; Table 3.1). In contrast, in neurons expressing inactive ARNO, Mena is displaced from its location at the growth cone leading edge (Fig. 3.2 c; Table 3.1). Quantification of Mena distribution showed that in untransfected cells, as well as cells expressing wild-type ARNO, Mena was always at the leading edge lamellae and filopodia. However, in cells expressing inactive ARNO Mena was localized either at the growth cone base or was completely depleted from the growth cone (Table 3.1). To test whether these effects were specific to Mena, we studied the distribution of the cytoskeletal proteins vinculin, ERM (ezrin, radixin, moesin), actin filaments, and microtubules in the growth cone. Vinculin is believed to mediate interactions between integrins and the actin cytoskeleton, and has been found to be concentrated at the growth cone (Renaudin et al., 1999; Varnum-Finney and Reichardt, 1994). PC12 cells deficient in vinculin have been shown to extend unstable lamellipodia and filopodia and have a slow rate of neurite outgrowth (Varnum-Finney and Reichardt, 1994). In untransfected cells, as well as cells over-expressing wild-type ARNO and inactive ARNO, vinculin is consistently localized at the distal lamellar region of the growth cones (Fig. 3.2 d-f). We next examined the actin

binding proteins ERM; ERM proteins localize to a variety of cortical structures where they have been proposed to act as plasma membrane-actin filament cross-linkers (Algrain et al., 1993; Tsukita et al., 1994). In addition, anti-ERM antibodies have been found to label the growth cones of neurons (Birgbauer et al., 1991; Goslin et al., 1989) and its proper localization depends upon intact microtubules (Goslin et al., 1989). In untransfected cells, as well as cells over-expressing wild-type ARNO and inactive ARNO, ERM proteins were found in a normal distribution in the growth cone filopodia (Fig. 3.2 g-i). We also found the distribution of filamentous actin and microtubules to be unaffected by ARNO-E156K expression. In untransfected cells as well as cells over-expressing wild-type ARNO and inactive ARNO, F-actin was seen in the distal filopodial and lamellipodial extensions of growth cones (Fig. 3.2 j-l) and microtubule bundles ran along the axonal shaft without penetrating the proximal region of the growth cone (Fig. 3.2 m-n). These results show that displacement of Mena by over-expression of ARNO-E156K was specific and are consistent with the hypothesis that ARNO regulates axonal extension by affecting events that maintain Mena associated with the growth cone plasma membrane.

In addition to effects on the cytoskeleton, ARF6 activation has been shown to modulate membrane traffic in the endocytic pathway (D'Souza-Schorey et al., 1998; Radhakrishna and Donaldson, 1997), and to control synaptic vesicle trafficking and calcium-regulated exocytosis in neuroendocrine cells (Powelka and Buckley, 2001; Vitale et al., 2002). To test the effect of ARNO-E156K upon endosomal traffic it was necessary to mark the endosomal compartment of neurons. Axonal endosomes do not

contain many of the classical endosomal markers such as transferrin receptor and EEA1 (Mundigl et al., 1993; Wilson et al., 2000). Therefore, we utilized a plasmid encoding endotubin-GFP. Endotubin is an early endosomal marker that targets to endosomes in polarized and non-polarized cells (Gokay and Wilson, 2000; Wilson and Colton, 1997). Upon expression in neurons, endotubin-GFP localized to vesicular and tubular structures in the cell body, dendrites and axon (Fig. 3.3 a). However, double-labeling studies with antibodies against the synaptic vesicle marker SV2 showed that endotubin-GFP was not targeted to SV2 containing vesicles (Fig. 3.3 b). To determine if the endotubin-labeled structures were endosomes, endosomes were labeled by incubation with the lectin wheat germ agglutinin (WGA) conjugated to rhodamine for 30 minutes at 37°C (Mundigl et al., 1993). Imaging showed that endotubin (Fig. 3.3 c) and the internalized WGA (Fig. 3.3 d) co-localized extensively, indicating that endotubin-GFP was targeted to endosomes. Higher resolution imaging of the axons of neurons expressing endotubin-GFP alone showed labeling concentrated in punctate structures along the axon (Fig. 3.4 a). Interestingly, expression of ARNO-E156K caused a redistribution of endotubin-containing endosomes to a peripheral location along the axon (Fig. 3.4 b), but did not affect the distribution of SV2 (Fig. 3.4 d). To determine if over-expression of ARNO-E156K increased the amount of endotubin present at the plasma membrane, immunolabeling of neurons co-expressing endotubin (not fused to GFP) and ARNO-E156K was carried out to visualize cell surface endotubin. In these experiments, immunolabeling for endotubin was performed without permeabilization. After incubation with secondary antibody, cells were re-fixed, permeabilized, and

immunolabeled for ARNO-E156K. Cells co-expressing inactive ARNO and endotubin showed an increase in plasma membrane labeling of endotubin (Fig. 3.5 b, d) throughout the axon (Fig. 3.5 b) and at the growth cone (Fig. 3.5 d) compared to cells expressing only endotubin (Fig. 3.5 a, c). To further confirm that endotubin-containing membranes were redistributed to the plasma membrane in ARNO-E156K expressing cells, the plasma membrane was labeled by incubating the cells in rhodamine-WGA for 30 minutes at 4°C, rinsed, fixed, and processed for immunofluorescence. In cells expressing only endotubin-GFP, endotubin-containing endosomes were seen as punctate structures along the axon (Fig. 3.5 e). In contrast, cells co-expressing inactive ARNO and endotubin showed an extensive colocalization of endotubin and WGA at the plasma membrane (Fig. 3.5 f). These results suggest that ARNO could control outgrowth through selective insertion and retrieval of specialized endosomal membrane during axonogenesis. It is of interest to note that the distribution of endotubin-containing endosomes in the somato-dendritic domain is not affected by the over-expression of ARNO-E156K, suggesting that the two populations of endosomes are differentially regulated.

3.4 Discussion.

Several lines of evidence support a role for the Rho-family of small GTPases and their accessory molecules (GAPs and GEFs) in the regulation of axonal elongation and branching (Luo, 2002). We have shown here that the ARF-GEF ARNO and ARF6 are also essential for controlled extension and branching of the axonal process. Cells expressing catalytically inactive ARNO and dominant-negative ARF6 show significant enhancement of axonal length and branching. These effects may be mediated by changes in both membrane trafficking and the cytoskeleton as these cells show a redistribution of endosomal membranes along the axon and a specific displacement of the profilin-binding protein Mena from the growth cone plasma membrane. These data suggest that during development, ARNO and ARF6 play an important role in the regulation of axonal elongation and sprouting.

During axonal development three separable events occur: elongation, pathfinding, and branching. Small GTPases of the Rho family have been implicated in the regulation of all of these aspects of axonal development (Luo, 2000) as progressive deletion of the three *Drosophila* Rac GTPases result in differential defects in axon outgrowth, guidance, and branching (Ng et al., 2002). We found that inactive ARNO and dominant-negative ARF6 caused both an increase in axonal length and in axonal collateral branches. These events were not affected by co-expression of inactive ARNO and wild-type Rac1 but co-expression with activated Rac1 reduced the branching phenotype. These results indicate that activation of Rac1 requires a GEF that is activated by ARF6. Alternatively, it may indicate that activated Rac1 alone is sufficient to suppress the branching phenotype.

In addition to effects on Rac1, ARF6 could modulate axonal branching through its effects on membrane structure. Membrane domains enriched in $PI(4,5)P_2$ have been suggested to establish sites for actin polymerization (Martin, 2001). $PI(4,5)P_2$ and activated Cdc42 have been shown to bind to WASP/N-WASP (Wiskott-Aldrich syndrome protein) therefore stimulating the activity of Arp2/3 (actin-related protein) complex for de novo actin polymerization (Higgs and Pollard, 2000; Rohatgi et al., 1999; Rozelle et al., 2000). Since ARF6 stimulates phosphatidylinositol 4-phosphate 5-kinase (Honda et al., 1999) and PIP_2 has been shown to be enriched at sites of ARF6 activation (Brown et al., 2001), it may be that ARNO/ARF6 controls sites of axon branching by establishing PIP_2-rich domains at the plasma membrane, therefore recruiting activated Cdc42, WASP/N-WASP and Arp2/3 to nucleate actin filaments (Korey and Van Vactor, 2000). Once the new actin filament is formed, cytoskeletal proteins such as profilin may be engaged to promote filopodial extension and the sprouting of a new branch (Korey and Van Vactor, 2000). It has recently been shown that dominant-negative Type Iα PIP 5-kinase promotes neurite elongation, suggesting that $PI(4,5)P_2$ negatively regulates neuritogenesis in neuroblastoma cells (van Horck et al., 2002; Yamazaki et al., 2002). These findings are also consistent with our finding that the inactive forms of ARNO and ARF6 increase axonal branching, perhaps by restricting $PI(4,5)P_2$ synthesis and promoting localized dynamic assembly/disassembly of actin filaments resulting in branch formation.

Ena/VASP proteins have been found to promote the actin based motility of the intracellular pathogen *Listeria* (Cameron et al., 2000). Somewhat paradoxically, they

have also been shown to negatively regulate growth cone motility, axonal guidance, and fibroblast motility (Lanier et al., 1999; Wills et al., 1999). Recently, it has been found that in the absence of Ena/VASP, lamellipodial processes protruded at slower rate but more persistently, contained shorter and highly branched actin filaments, and this was associated with increased cell motility (Bear et al., 2000; Bear et al., 2002). These findings suggest that Ena/VASP proteins regulate cell motility by controlling the actin filament length. We found that in neurons expressing catalytically inactive ARNO, Mena, but not other cytoskeletal binding proteins, was displaced from the growth cone plasma membrane. The mechanism of this displacement remains unclear. However since it has been shown that free barbed ends of actin filaments are required to target Ena/VASP proteins to the filopodial tips (Bear et al., 2002) it may be that ARNO/ARF6 effects upon cytoskeleton dynamics results in more barbed ends and subsequently Mena localization at the plasma membrane.

Axonal growth also requires insertion of plasma membrane components. We found a redistribution of a subset of endosomal membranes to the plasma membrane in cells expressing inactive ARNO. In HeLa cells, ARF6 has been found to cycle between the plasma membrane and an endosomal recycling compartment (Radhakrishna and Donaldson, 1997). Results obtained in fibroblasts showed that constitutively-active ARF6 blocks endocytosis whereas dominant-negative ARF6 inhibits recycling back to the plasma membrane (D'Souza-Schorey et al., 1995; Radhakrishna and Donaldson, 1997). In contrast, in epithelial cells, ARF6 has been shown to promote endocytosis at the apical, but not basolateral domain (Altschuler et al., 1999). In this case, both

constitutively-active and dominant-negative ARF6 promote apical endocytosis, suggesting that ARF6 needs to cycle to regulate endocytosis. Because, we have explored ARF6 effects upon membrane dynamics using ARNO, therefore allowing the normal GTPase cycle, our results may differ from those reported in the literature. Our finding that inactive ARNO causes redistribution of specialized endosomes to the axonal but not dendritic plasma membranes suggests that ARNO regulates a unique endosomal compartment in neurons. Under these conditions, ARF6 activation may control elongation either through regulation of cell surface receptor recycling or increased membrane availability for growth.

Axon regeneration after injury is blocked by various growth-inhibitory proteins and efforts have been made to develop strategies to promote axonal regrowth in injured adult neurons. Recently it has been found that injured axons regrow on inhibitory substrates when Rho GTPase is inactivated (Lehmann et al., 1999). Our study suggests that manipulation of the active state of ARNO is a potential target to promote axonal regeneration after injury.

3.5 Materials and Methods.

3.5.a Reagents, antibodies, and DNA constructs

Cell culture media were obtained from Gibco-BRL, Gaithersburg, MD. The following antibodies were used: mouse anti-myc 9E10, mouse anti-MAP2, mouse anti-FLAG M2, mouse anti-vinculin, mouse anti-β tubulin (Sigma-Aldrich, Inc., St. Louis, Missouri), mouse anti-tau (Zymed Laboratories, Inc., San Francisco, CA), mouse anti-HA (Roche Molecular Biochemicals, Indianapolis, IN), mouse anti-Mena (BD Biosciences, San Diego, CA), mouse anti-endotubin (5F11) (Wilson et al., 1987), rabbit anti-myc (Upstate Biotechnology, Lake Placid, NY), mouse anti-ERM 13H9 (gift from Dr. Frank Solomon). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, West Grove, PA. Rhodamine-conjugated phalloidin was from Molecular Probes, Eugene, OR. Rhodamine-conjugated wheat germ agglutinin (WGA) was obtained from Vector Laboratories, Burlingame, CA. C-myc-tagged ARNO and HA-tagged ARF6 constructs were generated as described previously (Altschuler et al., 1999; Frank et al., 1998b). FLAG-tagged Rac1 constructs were provided by Dr. J. Settleman (Harvard Medical School, Boston, MA).

3.5.b Cell culture and Transfection

Primary neuronal and glial cultures were prepared from day 17 or 18 fetal rats (Banker, 1991). Hippocampi were dissociated by treatment with trypsin (0.05% for 15 min at 37°C), followed by trituration. Dissociated cells were plated at low density

(350,000 cells/60mm dish) on poly-L-lysine treated coverslips in glia-conditioned Neurobasal medium in the presence of supplement B27 and kept at 37°C under 5% CO_2.

cDNAs were expressed in the hippocampal neurons by transient transfection using Effectene transfection reagent according to manufacturers directions (QIAGEN, Valencia, CA). Single transfection with cDNAs encoding ARNO, ARNO-E156K, Rac1 and Rac1-N17, and co-expression of ARNO-E156K and Rac1 were performed after 1 day in vitro. Transfection with ARF6, ARF6-T27N, ARF6-Q67L and co-expression of ARNO-E156K and ARF6-Q67L plasmids were performed after 5 days in vitro.

3.5.d Uptake assay

Neurons were transfected with cDNA encoding endotubin-GFP at 10 days in vitro and allowed to express for 2 days. Cells were rinsed with serum-free medium and WGA conjugated to rhodamine (5μg/ml) was added to the culture medium followed by 30 minutes incubation at 37°C under 5% CO_2 (Mundigl et al., 1993). After incubation, cells were briefly washed and then fixed.

3.5.e Immunofluorescent Microscopy

For quantification analysis, neurons were fixed after 9 days in vitro in 4% paraformaldehyde. After fixation, cells were permeabilized, and blocked with 10% goat serum, 0.05% saponin, and then incubated with specific antibodies followed by incubation with fluorophore-conjugated secondary antibodies. After washing, coverslips were mounted in Aqua Poly/mount (Polyscience, Inc., Warrington, PA). For endotubin

labeling at the membrane, immunolabeling was carried out in buffer lacking saponin. Fluorescent images of single optical sections were obtained using a Leica TCS 4D laser scanning confocal microscope (Arizona Research Laboratory, Division of Biotechnology, University of Arizona) using a 20X (NA 0.6), 40X (NA 1.0) and 100X (NA 1.4) objectives. Simultaneous two-channel recording was performed with pinhole sizes of 40-90 μm using excitation wavelengths of 488/588 nm, a 510/580 double diachronic mirror, and a 515-545 band-pass FITC filter together with 590 nm long-pass filter. Images were processed and merged using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

For cytoskeletal protein and endotubin studies, fluorescent images were acquired using the DeltaVision restoration microscopy system (Applied Precision, Inc, Issaquah WA). The z-stacks were software deconvolved on a Silicon Graphics Workstation (SGI, Mountain View, CA) using measured point-spread functions to create the final images. Images were processed and merged using Adobe Photoshop software (Adobe Systems, Mountain View, CA). To facilitate comparison, identical imaging and processing parameters were used for all confocal illustrations.

3.5.f Quantification of axonal length and branching

Morphometric analysis of axonal length and branching were performed by analyzing single optical sections of confocal images with a SimplePCI Image Analysis System (Compix Incorporated, Pittsburgh, PA). Confocal images were acquired with the 20X (NA 0.6) objective. The software was calibrated to the microscope magnification.

Axons were identified based on their characteristic morphology or as MAP2-negative processes. Axonal length was determined by tracing the entire length of the process and total length was calculated. Axonal branching was determined by counting the number of branch points along the axon. For each construct, 10 transfected cells were analyzed.

One-way analysis of variance (ANOVA) was used to compare mean changes in the relative length of axons. Results were expressed as the mean of axonal length in microns. Data are presented as means ± standard deviation (n = 10). The p value of < 0.0001 was considered significant.

3.5.g Quantification of Mena distribution in the axonal growth cone.

Hippocampal neurons were transfected with *myc*-tagged ARNO wild-type and ARNO-E156K labeled with anti-Mena antibody and visualized by deconvolution microscopy. Sixteen axonal growth cones were imaged per treatment and the distribution of Mena was scored as present at the leading lamellae and filopodia, at the growth cone based, or depleted from the growth cone. Identical imaging and processing parameters were used.

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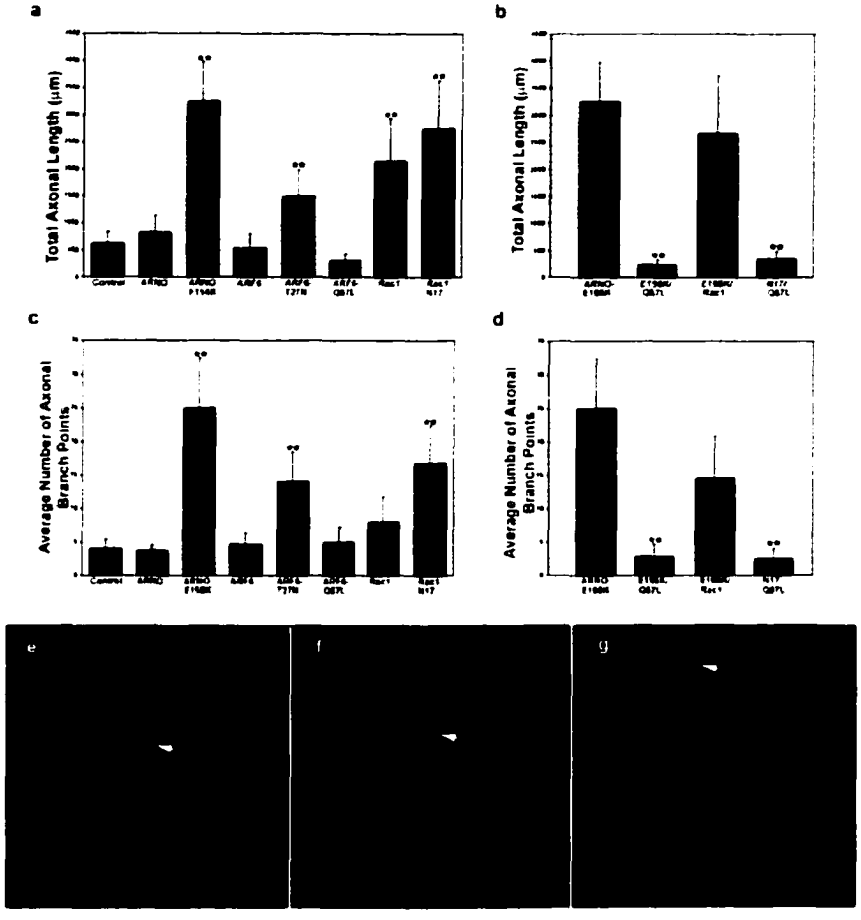

Figure 3.1. **ARNO mediates axonal length and branching through an ARF6-dependent pathway.** Morphometric analysis of the effects of over-expression of wild-type or inactive mutant forms of ARNO, ARF6, and Rac1 on axonal outgrowth and branching. (**a**) Effects on total axonal length. Expression of ARNO-E156K, ARF6-T27N, Rac1-N17, and Rac1 all resulted in increased axonal length. (**b**) Co-expression of ARF6-Q67L reversed the effect of ARNO-E156K and Rac1 upon axonal length. (**c**) Effects on the number of axonal branch points. Expression of ARNO-E156K, ARF6-T27N, and Rac1-N17 increased the number of branch points. (**d**) Co-expression of ARF6-Q67L reversed the effect of ARNO-E156K and Rac1 upon axonal branching. Data are presented as the means ± standard deviation (n = 10). $p < 0.0001$ (**). (**e-g**) Hippocampal neurons were transfected with GFP (**e**), GFP-ARNO wild-type (**f**) and GFP-ARNO-E156K (**g**), fixed after 6 days in culture and visualized by confocal microscopy. (**e**) Cell expressing GFP. The axon (arrow) extends some distance away from cell body emitting some collateral branches. (**f**) Cell expressing GFP-ARNO wild-type. The axon (arrow) is short with very few collateral branches. (**g**) Cell expressing GFP-ARNO-E156K. The axonal process (arrow) is very long with numerous collateral branches. Bars, **e-g**, 25μm.

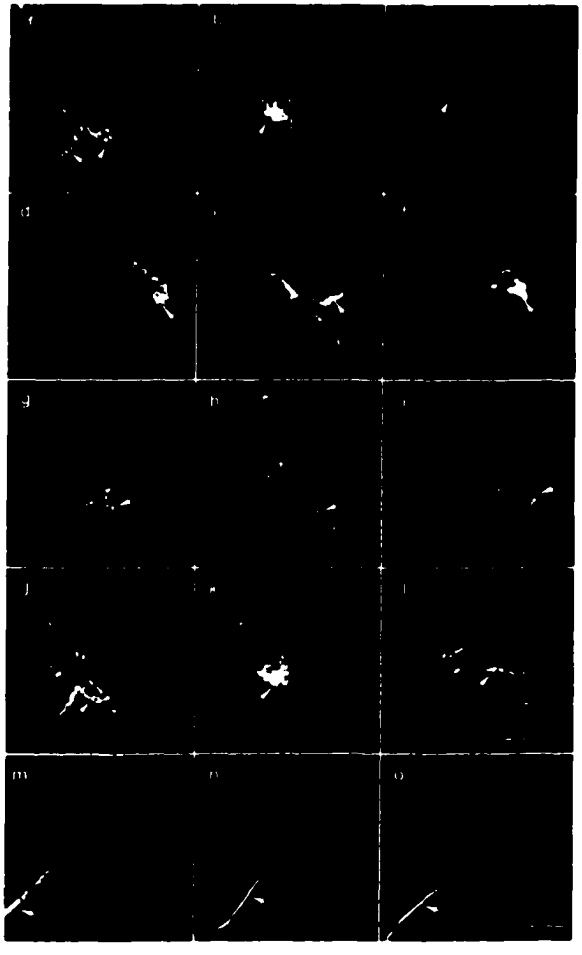

Figure 3.2. **Displacement of Mena from the growth cone plasma membrane in cells overexpressing inactive ARNO.** Hippocampal neurons were transfected with *myc*-tagged ARNO wild-type and ARNO-E156K labeled with anti-Mena antibody (**a-c**), anti-vinculin antibody (**d-f**), anti-ERM antibody (**g-i**), rhodamine-conjugated phalloidin (**j-l**), or anti-β-tubulin antibody (**m-o**), and visualized by deconvolution microscopy. The left column show growth cones of untransfected cells, the middle column show growth cones of cells overexpressing ARNO wild-type, and the right column show growth cones of cells overexpressing ARNO-E156K. All images were taken and processed using identical parameters. (**a,b**) In untransfected cells or cells overexpressing ARNO wild-type Mena is localized at the growth cone plasma membrane (arrows). (**c**) In the growth cone of a cell overexpressing ARNO-E156K, there is no concentration of Mena at the growth cone plasma membrane (arrow). (**d-f**) Vinculin is localized at the distal region of the growth cone in all cells (arrow). (**g-i**) ERM proteins are localized at the growth cone filopodia (arrow). (**j-l**) F-actin is localized at the filopodial/lamellipodial extensions of growth cones (arrow). (**m-o**) β-tubulin labeling is present in axonal shafts but not in the central domain of the growth cone. Bars, 5μm.

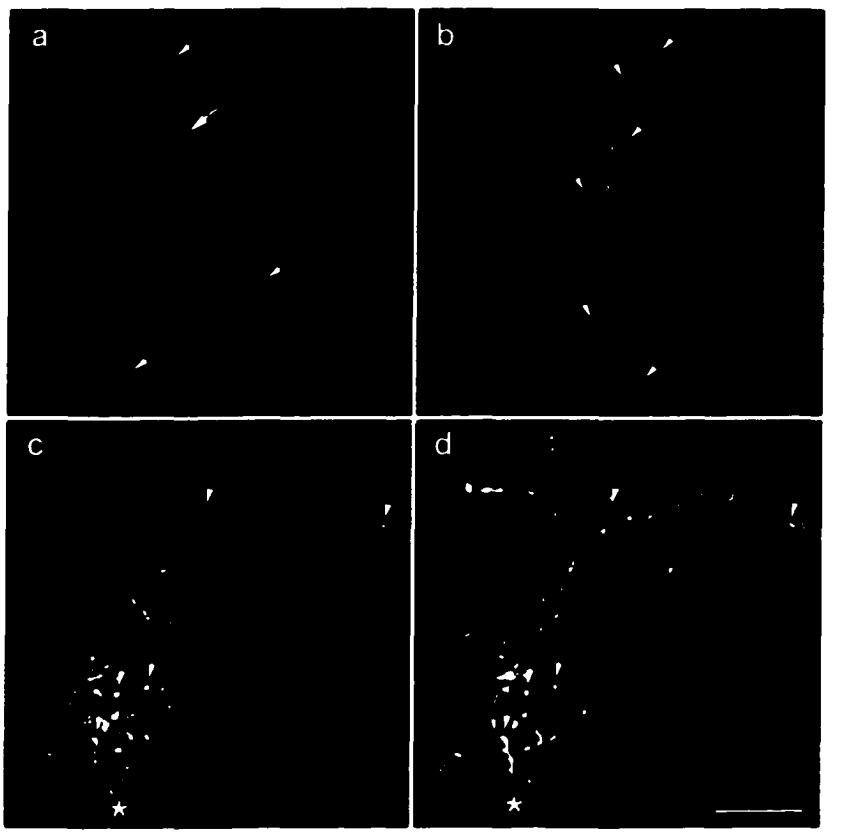

Figure 3.3. **Endotubin is targeted to an endosomal compartment.** Hippocampal neurons were transfected with endotubin-GFP (**a, b**, green) and were either double labeled with an antibody against the synaptic vesicle protein SV2 (**b**, red) or incubated with rhodamine-conjugated WGA (**d**). (**a**) Neuron at stage 3 of development (Dotti et al., 1988) expressing endotubin-GFP. Endotubin-GFP is localized to punctuate structures throughout cell body and dendrites and along the axon (arrows). (**b**) High resolution image of an axon of a neuron expressing endotubin-GFP and immunolabeled with anti-SV2. Endotubin-GFP (arrows) is localized to a compartment distinct from the SV2 (arrowheads). (**c, d**) Growth cone of a neuron expressing endotubin-GFP (**c**) that has been incubated with the endocytic marker WGA (**d**). Endotubin-GFP (**c**) shows abundant colocalization with internalized WGA (**d**) in the "palm" region (arrows) and filopodia (arrowheads), although some WGA endosomes do not contain endotubin. Bars, 25μm (**a**) and 5μm (**c-d**).

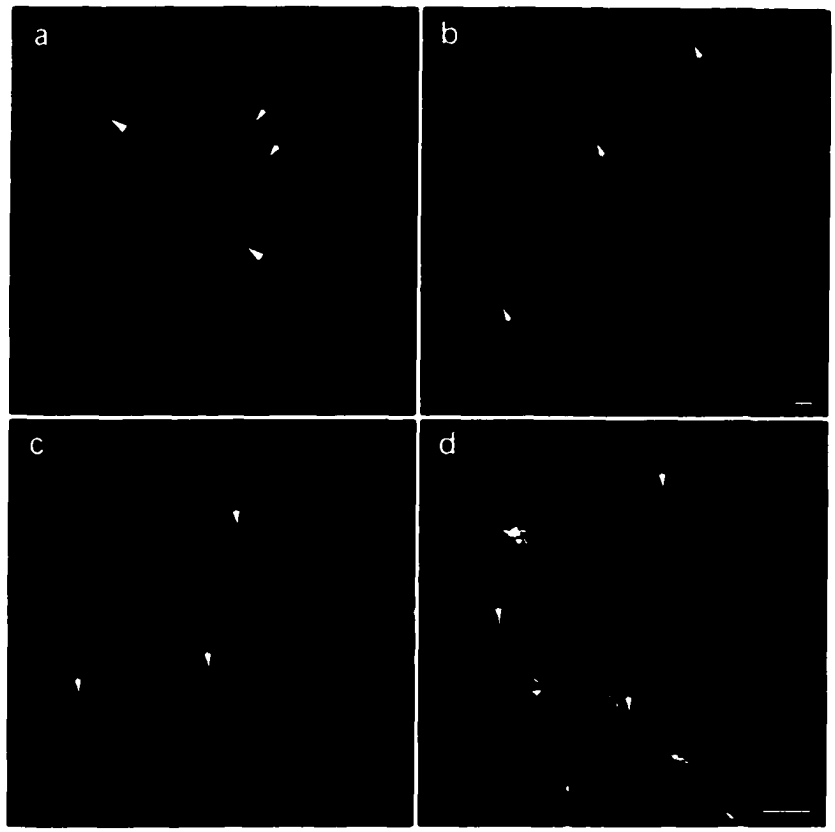

Figure 3.4. **ARNO-E156K causes redistribution of specialized endosomes along the axonal plasma membrane.** Hippocampal neurons were transfected with *myc*-tagged ARNO-E156K and/or endotubin-GFP, immunolabeled and visualized by confocal microscopy. (**a**) Axon of a neuron expressing endotubin-GFP. Endotubin-GFP is targeted to punctate structures along the axon (arrowheads) and at varicosities (arrows). (**b**) Axon of a neuron co-expressing ARNO-E156K (red) and endotubin-GFP (green). Endosomal structures are redistributed to the plasma membrane along the axonal length, and it appears to be more endotubin-GFP present on the surface (arrows). (**c, d**) Axon of an untransfected (**c**) and ARNO-E156K (**d**) expressing neuron labeled for SV2. SV2-positive endosomal structures are distributed throughout the length of the axon (arrows) an expression of ARNO-E156K did not change the distribution of SV2. Bars, 5μm.

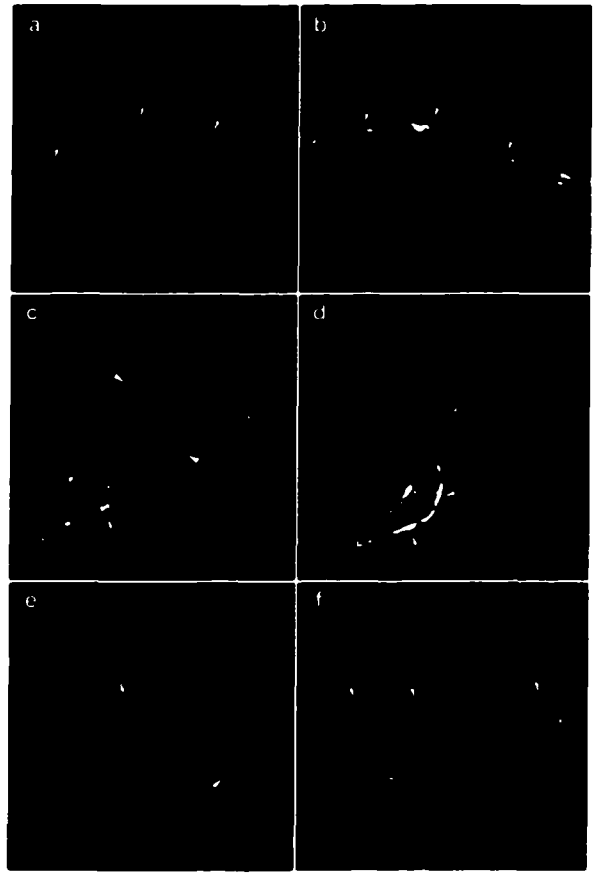

Figure 3.5. **ARNO-E156K causes an increase in the amount of endotubin labeling at the plasma membrane.** Hippocampal neurons were transfected with *myc*-tagged ARNO-E156K and/or endotubin and cell surface endotubin was visualized. All images were taken and processed using identical parameters. (**a**, c) Immunolabeling was performed without permeabilization. (**a**) Axon of a neuron expressing only endotubin. There is little plasma membrane labeling of endotubin (arrows). (**b**) Axon of a neuron co-expressing ARNO-E156K and endotubin. There is strong plasma membrane labeling of endotubin (arrows). (**c**) Growth cone of a neuron expressing endotubin. Some endotubin labeling is present at the proximal region of the growth cone (arrow) however, the distal lamellaepodial and filopodial region of the growth cone shows little endotubin labeling at the plasma membrane (arrowheads). (**d**) Growth cone of a neuron co-expressing ARNO-E156K and endotubin. There is strong plasma membrane labeling of endotubin on the growth cone (arrows). (**e-f**) Plasma membrane was labeled by incubating the cells with rhodamine-conjugated WGA (red) at 4°C for 30 minutes. (**e**) Axon of a neuron expressing endotubin-GFP (green). Endotubin-GFP is present in punctate structures along the axon and there is no co-localization with WGA (arrows). (**e**) Axon of a neuron co-expressing ARNO-E156K and endotubin-GFP (green). Endosomal structures are redistributed to the plasma membrane along the axon, there is extensive co-localization of endotubin and WGA at the plasma membrane (arrows). Bars, 5μm.

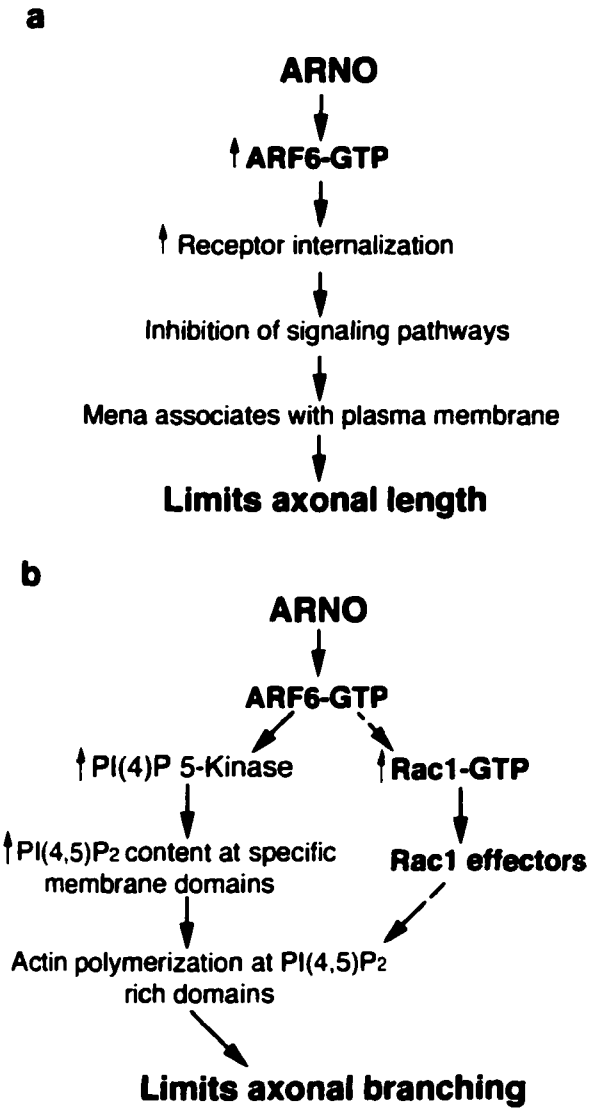

Figure 3.6. **ARNO and ARF6 negatively regulate axonal length and branching during development.** ARNO stimulates GTP exchange on ARF6, thereby increasing the amount of active ARF6 at specific plasma membrane domains. **(a)** Regulation of axonal growth. ARF6 increases cell surface receptor uptake. This increase in receptor internalization would inhibit signaling pathways that maintain Mena at the plasma membrane. Association of Mena with the plasma membrane affects cytoskeletal rearrangements thereby limiting axonal elongation (Bear et al., 2002). **(b)** Regulation of axonal branching. Activated ARF6 activates the lipid modifying enzyme phosphatidylinositol-4-phosphate 5-kinase (PI(4)P 5-kinase), leading to local increases in plasma membrane phophatidylinositol-4,5-biphosphate ($PI(4,5)P_2$). This local elevation of $PI(4,5)P_2$ determines site selection for axonal branches. Activation of Rac1 by ARF6-GTP results in stimulation of actin polymerization at these specific membrane domains.

Mena localization at the growth cone	Control	ARNO-wt	ARNO-E156K
At the leading edge lamellae and filopodia 	16	16	0
At the base of the growth cone 	0	0	5
Depleted from the growth cone 	0	0	11

Table 3.1. **Quantification of Mena distribution in the axonal growth cone.** Hippocampal neurons were transfected with *myc*-tagged ARNO wild-type and ARNO-E156K labeled with anti-Mena antibody and visualized by deconvolution microscopy. Mena was localized at the leading edge lamellae and filopodia in untransfected cells (16/16) as well as in cells over-expressing wild-type ARNO (16/16), at the base or proximal region of the growth cone in cells over-expressing ARNO-E156K (5/16), or depleted from the growth cone in cells over-expressing ARNO-E156K (11/16).

CHAPTER FOUR: FUTURE DIRECTIONS

We have shown that overexpression of catalytically inactive ARNO and dominant negative ARF6 resulted in increased dendritic branching and enhancement of axonal length and branching. These effects appear to be mediated by changes in both membrane trafficking and the cytoskeleton as these cells showed a redistribution of endosomal membranes along the axon and displacement of the profilin binding-protein Mena from the growth cone plasma membrane. These results demonstrate that the ARF family of small GTPases provides an additional level of regulation during neuritogenesis and that ARNO and ARF6 are essential for controlled extension and branching of dendritic and axonal processes. However, there are still many questions that remain unanswered.

Is PI(4)P 5-kinases α downstream of ARNO/ARF6 during dendritic and axonal development?

We have shown that ARNO and ARF6 negatively regulate neuritogenesis. Since ARNO activates ARF6, and ARF6 activates phospholipase D (PLD) and phosphatidyl-inositol 4-phosphate 5-kinase alpha (PI(4)P 5-kinase α) (Exton, 1997; Honda et al., 1999) it will be of interest to define further the signaling pathways downstream of ARF6 during dendritic and axonal development. Phosphatidylinositol-4,5-biphosphate ($PI(4,5)P_2$) is a signaling phospholipid that has been implicated in a variety of cellular events including vesicular traffic (Martin et al., 1997) and cytoskeletal remodeling (Yamamoto et al., 2001). $PI(4,5)P_2$ is synthesized through phosphorylation of phosphatidylinositol 4-phosphate by PI(4)P 5-kinase α at the D-5 position of the inositol ring (Honda et al.,

1999). ARF6 activates PLD, resulting in the production of phosphatidic acid (PA). This PA, with ARF6, activates PI(4)P 5-kinases α (Exton, 1997; Honda et al., 1999). In non-neuronal cells, ARF6 colocalizes at the plasma membrane with mouse PI(4)P 5-kinase α during membrane ruffle formation (Honda et al., 1999). In a neuronal cell line, overexpression of type I PI(4)P 5-kinase α caused an inhibition of neurite outgrowth, implicating PI(4)P 5-kinase in neurite remodeling (van Horck et al., 2002). To analyze the effects of PI(4)P 5-kinase α upon dendrite and axonal development, cultured rat hippocampal neurons would be transfected with plasmid encoding mouse PI(4)P 5-kinase α. If ARNO's effects upon dendritic and axonal development are mediated by the activation PI(4)P 5-kinase α, we would expect that neurons overexpressing PI(4)P 5-kinase α would exhibit a phenotype similar to that seen in cells overexpressing constitutively active ARF6. We would conclude from this experiment that activation of PI(4)P 5-kinase α affects dendrite and axonal development and its activation may be through ARNO and ARF6. To test whether this lipid modifying enzyme is downstream of ARNO/ARF6 in the regulation of dendritic and axonal development we could co-express PI(4)P 5-kinase α with catalytically inactive ARNO. If activation of PI(4)P 5-kinase α mediates ARNO's effects upon dendrite complexity and axonal branching and length we would predict that co-expression of PI(4)P 5-kinase α with inactive ARNO would reverse the dendritic and axonal phenotype seen in cells overexpressing inactive ARNO. These experiments would further dissect the signaling pathway downstream of ARNO and ARF6 in the regulation of dendritic arbor and axonal length and branching development.

Does adhesion to the substrate affect ARNO-mediated neurite branching?

The extracellular matrix comprises a complex environment that includes molecules such as laminin, tenascin, collagen, and fibronectin (Goodman and Tessier-Lavigne, 1997). These molecules have been found to affect neuronal adhesion, growth, and neurite morphology (Huang et al., 1995; Stahlhut et al., 1997). During development, most neurons establish multiple connections with target cells by sending out collateral branches. Neurite branching appears to depend on interactions with the extracellular substrate, as adhesive substrates (such as laminin with apolipoprotein E, and NCAM) increased neuritic branching of hippocampal neurons (Huang et al., 1995), and dendritic and axonal arborization of spinal motoneurons (Stahlhut et al., 1997). ARNO and ARF6 have been shown to promote migration in epithelial cells (Palacios et al., 2001; Santy and Casanova, 2001). Since ARF6 regulates membrane traffic (Altschuler et al., 1999), these findings suggest that ARF6 activation may influence adhesion to the substrate by affecting cell surface recycling of cell adhesion molecules (CAM). It would be of interest to investigate whether ARNO's effects upon neuritogenesis are due to increased adhesion to the substrate. This could be tested by overexpressing inactive ARNO in hippocampal neurons grown on different adhesive substrates. If the increased dendritic and axonal branching phenotypes seen in cells overexpressing inactive ARNO are due to an increase in adhesion we would predict that growth on a less adhesive substrate would result in a less complex dendritic tree and axons would be shorter with fewer collateral branches. It also would be of interest to determine whether ARNO's effects upon neuritogenesis are due to a correlative effect on the turnover of membrane receptors for

extracellular matrix molecules (Rhee et al., 2002). We could test this by examining the concentration of membrane receptors in cells overexpressing inactive ARNO. If the phenotype seen in cells overexpressing inactive ARNO is due to an upregulation of membrane receptors and consequent increase in adhesion to the substrate, we would expect to see an increase in the expression of these receptors at branching points. These results would suggest that ARNO modulates adhesion to the substrate and thus could influence dendritic and axonal branching during development.

What is the upstream signal of ARNO-mediated neurite branching?

Neurotrophic factors such as NT-3, neurotrophin, BDNF, and Slits have been found to stimulate neurite branching (Gallo and Letourneau, 1998; Horch and Katz, 2002; Schnell et al., 1994; Wang et al., 1999); for example, BDNF increases dendritic branching in the ferret visual cortex (Horch and Katz, 2002) and Slit2 induces axonal elongation and branching in rat dorsal root ganglion (Nguyen Ba-Charvet et al., 2001; Wang et al., 1999). Slits are secreted proteins that bind to Robo receptors, and play a role in axon guidance and branching (Brose and Tessier-Lavigne, 2000). In both invertebrates and vertebrates their repellent activity prevents axons from crossing the midline (Brose and Tessier-Lavigne, 2000); and recent studies in mammals showed that both full length and N-terminal fragment of Slit2 (Slit2-N) stimulate the formation of axonal collateral branches on sensory axons (Ozdinler and Erzurumlu, 2002; Wang et al., 1999). Extracellular agonists such as the epidermal growth factor (EGF) trigger nucleotide exchange and activate ARF6 in fibroblast cell lines (Boshans et al., 2000; Venkateswarlu and Cullen, 2000). We have shown that hippocampal neurons overexpressing inactive

ARNO have increased dendritic and axonal branching; the upstream signal that triggers these effects remains unknown. It would be of interest to determine whether BDNF and Slit2-N are modulating ARNO activity. To address this, hippocampal neurons overexpressing wild-type ARNO-GFP could be exposed to either BDNF- or Slit2-N-coated beads. If ARNO is downstream of BDNF or Slit2-N during dendritic and axonal branching, we would predict that exposure of cells overexpressing wild-type ARNO to either factor would result in no increase in axonal or dendritic branching. These experiments could provide some insights about the upstream agonist of the ARNO/ARF6 signaling pathway during dendritic and axonal branching.

Is ARNO a component of the *DAbl-Ena* signaling pathway during axon path-finding?

In addition to their role in branching, the slit-robo system plays a role in choice point navigation in *Drosophila* as they prevent axons from crossing the ventral midline (Brose and Tessier-Lavigne, 2000). Robo receptors associate with the non-receptor tyrosine kinase *Ableson* (*Abl*) and its substrate the profilin-binding protein *Ena*. In addition, loss of *Ena* function has been shown to partially disrupt Robo-mediated midline repulsive effect, suggesting that *Ena* is in part required for Robo's repulsive effect (Bashaw et al., 2000). *Abl* and *Ena* are also required in the intersegmental nerve b (ISNb) choice point navigation, as overexpression of *D-abl* or deletion of *Ena* result in ISNb 'bypass' phenotype (Lanier and Gertler, 2000; Wills et al., 1999). These experiments suggest that choice point pathfinding uses different ligand-receptor complexes, but shares similar downstream regulatory pathways. In mammals, Mena

(mammalian Ena) has been implicated in cell and growth cone motility, and commissure formation (Bear et al., 2000; Lanier et al., 1999). We have found that hippocampal neurons overexpressing inactive ARNO have long axons and Mena is displaced from the growth cone leading edge. In *Drosophila*, the ISNb motor axon has a stereotypical outgrowth pattern and has been widely used as a model system of axon guidance. It would be of interest to genetically analyze axonal outgrowth in the *Drosophila* embryo to determine whether ARNO is a component of the *DAbl-Ena* signaling pathway during pathfinding. We would predict that if ARNO is in this pathway, a *D-abl* loss-of-function background together with overexpression of inactive ARNO would result in a 'by-pass' phenotype. In addition, in an *Ena* gain-of-function background, overexpression of inactive ARNO would result in a 'stop-short' phenotype. These results would suggest that in *Drosophila*, ARNO is a component of the Abl/Ena signaling pathway during motor axon pathfinding.

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