

Roles of endosomal trafficking in neurite outgrowth and guidance

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Membrane trafficking and cargo delivery are essential for axonal and dendritic growth and guidance. Neurons have numerous diverse post-Golgi vesicles and recent advances have clarified their identity and regulation. Combinatorial approaches using *in vivo* imaging of 'intracellular cargo address labels' and functional perturbation have provided insight into these processes. In particular, the UNC-51 kinase regulates the trafficking of early endosomes and their axon guidance molecular cargos in several types of neurons in multiple organisms. Vesicular compartments bearing features of recycling endosomes, late endosomes or lysosomes also contribute to membrane addition and protein trafficking during neurite outgrowth and extension. New work shows that ubiquitylation of cargos and Rab effectors further specifies the trafficking routes of post-Golgi vesicles. These findings have begun to provide a more detailed view of the molecular mechanisms involved in neurite outgrowth and guidance. Additionally, high-resolution light microscopy imaging promises greater temporal and spatial understanding of vesicular exchange and maturation in neurons in the near future.

Introduction

Neurons are highly polarized cells that employ various mechanisms to initiate, maintain and regulate their biological function. Neurons have up to 10 000 times as much surface area as other cell types. Growth, remodeling and maintenance of the axonal and dendritic processes of neurons rely heavily on membrane trafficking. Delivery of membranes and receptors demands an extensive post-Golgi trafficking network (Box 1). Coordination must occur among processes of protein sorting, vesicle budding, transport along the cytoskeleton and vesicle fusion.

Because neuronal vesicular compartments possess a vast number of diverse cargos, a general strategy in recent studies has been to take advantage of known vesicular resident components and Rab small GTPases that are hypothesized to act as 'intracellular cargo address labels' [1,2]. By definition, 'cargos' usually refer to membrane vesicles that transport a set of molecules, commonly proteins or RNAs, between different locations within a cell. The identification of cargos depends on the reagents and methodology of visualization. For most cargos, it is difficult, and often impossible, to determine the precise molecular composition. Nonetheless, emerging consen-

suses are that vesicular cargos are compartmentalized in neuron- and neurite-specific manners and that cargos deliver guidance receptors and regulators in a temporally and spatially regulated fashion to meet the demands of neurite outgrowth, guidance and maintenance. Here, we focus on recent findings from multiple organisms, emphasizing endosomal trafficking in neurite outgrowth and guidance after the establishment of neuronal polarity and initiation of nerve processes. For recent progress on neurite initiation and polarity establishment, or remodeling and receptor turnover in mature dendrites, we refer the readers to other excellent reviews [3–7].

General rules of membrane trafficking

In non-neuronal cells, lipid addition to membranes occurs through both the Golgi secretory network and non-Golgi pathways [8,9], whereas proteins are trafficked to the membrane primarily through the post-Golgi secretory pathway. Proteins are sorted within the trans-Golgi network (TGN) to particular post-Golgi membrane compartments based on sorting motifs on their cytosolic tails [10]. These motifs recruit protein complexes that act as adaptors between cargo proteins and clathrin, which is required for vesicle budding. Commonly, before vesicle budding occurs, tubules derived from the TGN are elongated through the action of cargo-specific microtubule-based motor proteins. The tension generated by this elongation process promotes vesicle budding, facilitated by lipids that deform the membrane, such as diacylglycerol, and proteins that promote fission [11]. Once carrier vesicles have budded from the trans-Golgi tubules, they can be transported directly to the plasma membrane or enter the endosomal networks for further sorting [10] (Box 1, Figure 1).

In neurons, the endoplasmic reticulum (ER) extends into all dendrites, whereas Golgi bodies are outposted in a subset of dendrites [4]. Lipids and proteins from the dendritic ER might either be trafficked through the Golgi outposts in dendrites or be transported long-distance back to the peri-nuclear Golgi network before proceeding through the secretory pathway [12]. Trafficking through the Golgi is required for dendritic growth. However, disrupting Golgi trafficking with brefeldin A or inhibiting the function of protein kinase D (PKD) via overexpression of dominant-negative mutants does not affect axon outgrowth [13–15]. Therefore, although the role of Golgi secretion in dendrite formation is being elucidated, less is known about the membrane trafficking that underlies

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Box 1. The endosomal trafficking pathways

Vesicular compartments of the endosomal networks are classically defined by the amount of time needed for tracers such as internalized transferrin receptors (TfR) to reach them [101]. Such kinetic studies indicate that vesicles endocytosed from the plasma membrane are first transported to and fused with early endosomes. From there, proteins targeted for degradation move to late endosomes and lysosomes, whereas other proteins are transported to recycling endosomes for eventual reinsertion into the plasma membrane [102–104]. Although defined by their role in transport from the plasma membrane, the early endosomes, recycling endosomes and late

endosomes also receive vesicular traffic from the TGN and have been more recently defined by the presence of molecular markers, particularly Rab GTPases. In general, Rabs, when in a GTP-bound state, bind to specific membrane compartments through their C-terminal prenylation motifs. For example, Rab5 is mainly present in early endosomes, whereas Rab7 marks late endosomes. Rab11 and Rab8 are frequently associated with recycling endosomes. Rab GTPases regulate membrane trafficking by recruiting effector proteins that bind proteins responsible for budding and fusion (Figure 1).

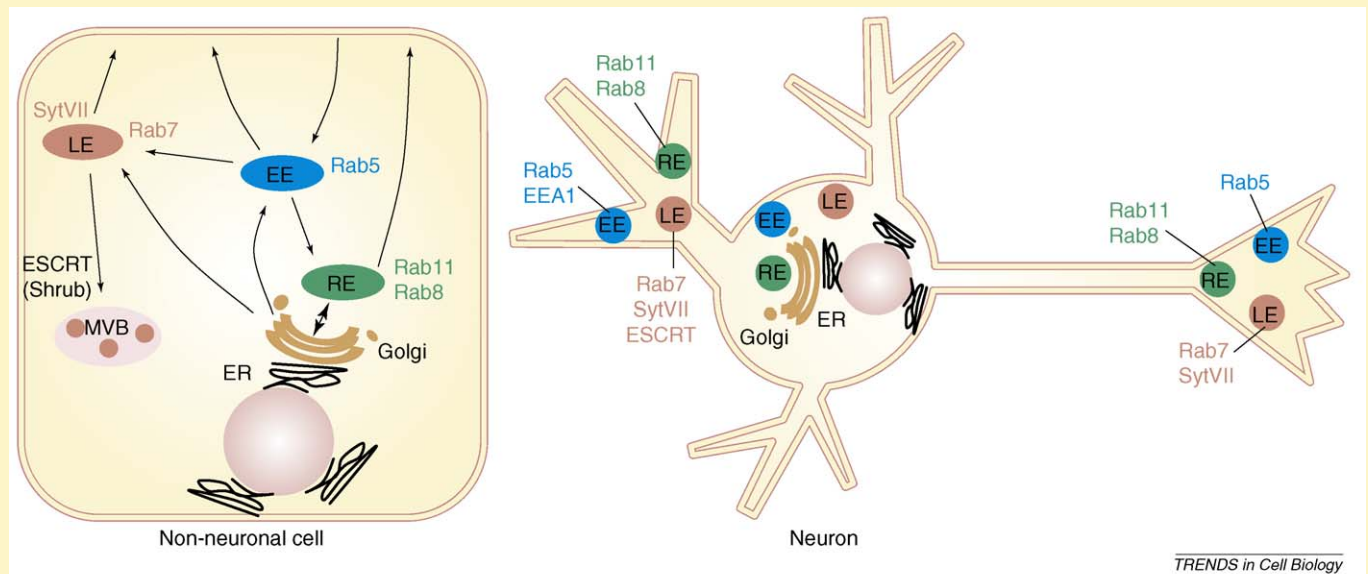


Figure 1. Vesicular trafficking in a non-polarized non-neuronal cell and in a polarized neuron. Compared with non-neuronal cells, Rab proteins and their associated vesicles in polarized neuronal cells have more compartmentalized functions. Rabs in different compartments can use different effectors. Neurons can have unique proteins and regulators not present in non-neuronal cells. Dendrites and axons can require different levels of activity in membrane trafficking.

axonal growth. However, the exocytic processes in both axons and dendrites probably share some similarities because disrupting the function of soluble NEM-sensitive-factor (NSF) attachment protein (SNAP) receptor (SNARE) complexes using the C terminus of tetanus neurotoxin insensitive vesicle-associated membrane protein (TI-VAMP or VAMP-7) prevents outgrowth of both axons and dendrites (see later) [16]. Additionally, an increasing number of studies have shown that neurons probably possess more diverse cargos and employ more complex regulatory mechanisms for vesicular regulation than non-neuronal cells. Here, we discuss general approaches and several consensus findings on the role of endosomal trafficking during neurite outgrowth and guidance.

Approaches for studying the post-Golgi trafficking and endosomal network in neurons

Understanding the vesicular contributions to neurite dynamics depends crucially on the use of reagents specifically labeling particular compartments and vesicular membranes. The advent of non-invasive fluorescent-protein-based reporters has greatly enhanced our knowledge in the past few years.

Numerous recent studies using reporters for Rab and other vesicular proteins demonstrate a neuron- and neurite-specific presence of vesicles with different affinities to Rab GTPases. For example, several Rabs are localized to

distinct membrane compartments in the soma of *Caenorhabditis elegans* gamma-amino-butyric-acid (GABA)ergic motor neurons and the transport of these membrane compartments to the synapse is differentially regulated [17]. Similarly, various Rabs and several organelle proteins exhibit distinct expression patterns in *Drosophila* photoreceptors, olfactory neurons and synapses of motor neurons [18–20]. In rat hippocampal neurons, at least some Rab-labeled compartments are differentially present in their dendrites and axons [21–23]. Moreover, labeling with Rab effectors reveals further restricted localization. For example, a known Rab5 effector, EEA1, is present on a select population of Rab5-positive endosomes in dendrites but is absent in axons [24].

Fluorescently labeled Rab markers combined with various Rab mutants enable functional monitoring and manipulation of endosomal dynamics in live cells and, thus, have greatly advanced our understanding of the endocytic and exocytic pathways. Rab GTPases cycle between an active GTP-bound, membrane-associated state and an inactive GDP-bound cytosolic state. Fluorescently labeled constitutively active or inactive Rab GTPases enable the study of their subcellular localization in relation to their cycling states [17,20,25]. Dominant-negative and loss-of-function Rab mutants enable further investigation of their roles in membrane trafficking [20,26–28]. Tracking fluorescence recovery after photobleaching (FRAP) reveals

the dynamic movement of various Rab compartments [17,21]. *In vitro* biochemical reconstitution of endosomes and lipid compartments make it possible to study how Rabs and other proteins mediate membrane formation and budding [29,30]. Fluorescence resonance energy transfer (FRET)-based *in vivo* imaging can further reveal the time and space of protein–protein interactions [31,32]. Recent advances in high-resolution microscopy, such as photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and stimulated emission depletion (STED), have begun to enable the observation of protein and organelle interactions at sub-100 nm level [33,34]. Several new studies using a combination of genetic and cellular strategies have investigated endosomal cargos in neurite outgrowth and guidance.

Neurite growth and guidance through early endosomal trafficking involving the UNC-51 kinase

Studies of genetic mutants in model organisms have uncovered crucial regulators of neuronal morphogenesis. The simple morphology of *C. elegans* neurons has a particular advantage in revealing precise defects in mutant animals at single neurite resolution. Insight into the mechanisms by which membranous vesicles contribute to axon outgrowth came from a set of mutants originally isolated based on movement defects. One such mutant is *unc-51* (*uncoordination*), which defines a family of conserved serine/threonine kinases that are related to yeast autophagy protein, Atg1 [35,36]. In loss-of-function mutants of *unc-51*, multiple types of neurons display axon defects, including premature termination, abnormal trajectories and extra axon branches [36]. Developing neurites also accumulate abnormal vesicles and cisternae [37]. UNC-51 binds UNC-14, a RUN domain protein (for 'RPIP8, UNC-14 and NESCA') [38]. Mutations in *unc-14* exhibit neurite defects similar to those of *unc-51*. RUN domain proteins can act as effectors of Rap and Rab GTPase members and influence membrane trafficking [39]. This interaction suggests possible involvement of vesicular components in the growth and guidance of neurites.

Several studies have shown that UNC-51 helps to deliver specific receptors for axon guidance cues in different types of neurons. In ventral guidance, *unc-14* and *unc-51* mutations mislocalize UNC-5 to the cell soma rather than axons, thus impairing the ability of UNC-5 to mediate repulsion of axons away from the netrin-rich ventral midline [40]. Guidance for anterior–posterior trajectories of mechanosensory neurons depends on both the netrin receptor UNC-40/DCC and the Slit receptor SAX-3/Robo and involves the interaction between UNC-51 and a novel protein VAB-8 [41–44]. The gene *vab-8* encodes two proteins with the long variant (VAB-8L) coding an N-terminal kinesin-like motor domain and a novel C terminus [43]. VAB-8L is required for posterior axon guidance of touch neurons and posterior migration of some cells. Interference of the *vab-8L* and *unc-51* interaction disrupts posterior axon projection [44]. Furthermore, overexpression of VAB-8 in touch neurons causes rerouting of a normally anteriorly projecting axon to the opposite direction. VAB-8 controls the cell-surface expression of SAX3/

Robo in touch neuron growth cones by working through the Rho and Rac guanine exchange factor (GEF) TRIO/UNC-73 [42]. Similar regulation might also occur to the netrin receptors UNC-40/DCC [41].

The role of UNC-51 in neurite outgrowth and guidance is conserved across species. There are two mammalian homologues, Unc51.1/ULK1 and Unc51.2/ULK2. In mouse, dominant-negative disruption of Unc51.1/ULK1 in cultured cerebellar granule cells inhibits neurite formation and extension [45]. Mammalian Unc51.1/ULK1 binds to the synaptic GTPase-activation protein (SynGAP) and Syntenin, an endocytic vesicular membrane protein with PDZ domains [46]. Syntenin serves as a scaffold protein to hold Unc51.1 and Rab5 to the vesicular membrane. SynGAP stimulates Rab5 GTPase activity *in vitro* and overexpression of SynGAP in cultured cerebellar granule neurons leads to truncated neurites and disorganized vesicular compartments. This complex of Unc51.1, SynGAP, Syntenin and Rab5 serves to regulate vesicular organization during neurite outgrowth, although the guidance receptors remain to be identified.

In mouse dorsal root ganglia (DRG), Unc51.1 and 2/ULK1 and 2 are also expressed in vesicle-like structures in axons and growth cones [47]. Knocking down Unc51.1 and 2/ULK1 and 2 leads to increased neurite branch formation and shortened axons in cultured DRG neurons. In this type of neuron, Unc51.1 acts by promoting endocytosis of the nerve growth factor (NGF)-bound TrkA receptor through a non-clathrin mediated pathway. NGF stimulates the polyubiquitylation of Unc51.1, which enables Unc51.1 to form a complex with TrkA, the NGF receptor, and p62, the atypical protein kinase C interacting protein required for TrkA internalization. This complex could then undergo non-clathrin-mediated endocytosis, presumably through the interaction of Unc51.1 with SynGAP and Rab5, and the internalization of the NGF–TrkA complex could maximize the elongation of DRG axons.

The vesicular localization of Unc-51 in developing neurites and mature axons implies an underlying role in transport regulation. A series of studies has begun to elucidate the interactions between Unc-51 with kinesin motors and their cargos. In *C. elegans*, UNC-14 binds UNC-16, which is a cargo adaptor for the kinesin motor proteins and a scaffold protein for the Jun N-terminal kinase (JNK)–MAP kinase pathway [48]. Loss-of-function mutations of *unc-14* or *unc-51*, like those of kinesin and *unc-16*, affect trafficking of synaptic vesicle precursors in motor and sensory neurons in *C. elegans* [48,49]. The fasciculation and elongation protein Zygyn/zeta (FEZ) family of proteins is identified through studies of the *C. elegans* gene *unc-76*, which was originally described for its roles in axon elongation [50]. UNC-76/FEZ proteins bind kinesins and contribute to the transport of synaptic vesicle precursors [51]. Similar observations have also been reported for the *Drosophila* homologs of UNC-76 and UNC-51. DUNC-76 interacts with kinesin-1 [52] and DUNC-51 binds to and phosphorylates DUNC-76 [53]. Loss-of-function mutations in *DUNC-51* lead to axon morphology defects, with vesicles accumulating along axons, reminiscent of those in *C. elegans* *unc-51* mutants. Phosphorylated DUNC-76 binds directly to the synaptic vesicle protein Synaptotagmin-1

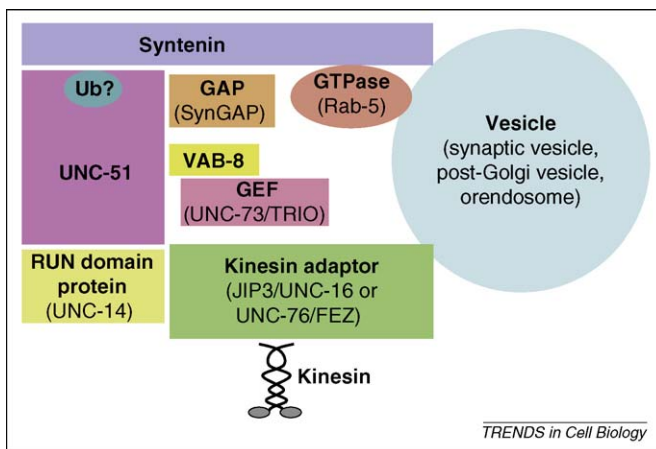


Figure 1. UNC-51 mediates vesicle formation and transport in neurite outgrowth. Shown is a generalized schemata of connections between Unc-51 and its binding partners. Unc-51 directly or indirectly interacts with RUN domain proteins and adaptor proteins; such interactions probably help to attach vesicles to kinesin motors. Scaffolding proteins such as Syntenin connect complexes of *unc-51* and GTPases such as Rab-5 to vesicles. Guanine exchange factors, such as UNC-73, and GAPs, such as SynGAP, regulate the cycling state of GTPases, controlling budding, binding and fusion of vesicles. Ubiquitylation of UNC-51 by vesicle cargo such as NGF and TRKA can regulate the complex. See details in the main text for the specific pairs of interactions in particular cell types.

[53], suggesting that the adaptor proteins achieve specificity by binding directly to cargos in a phosphorylation-dependent manner. Nonetheless, it is unlikely that the transport of synaptic vesicles directly contributes to neurite outgrowth because neurite outgrowth and extension occur normally in both *C. elegans unc-104* mutants and *Drosophila Imac* mutants in which synaptic vesicles fail to reach presynaptic terminals as the result of loss-of-function of the kinesin motor [54,55].

In summary, the studies on UNC-51 kinase illustrate how a conserved protein has multifaceted functions in different types of neurons and organisms. These findings, along with those demonstrating that impairing Unc-51 function disrupts the delivery of axon guidance receptors, indicate that the Unc-51 kinase probably regulates multiple types of post-Golgi vesicles (Figure 1). Such regulation could be important for pairing of UNC-51 associated cargos with motors at different stage of neurite outgrowth and guidance.

Regulation of extension and guidance by late endo-lysosomal cargos

Extension of neurites requires addition of new membrane [56]. However, the membrane sources that contribute to the growing axons have remained unidentified. Neurite outgrowth in cultured mammalian CNS neurons and in *C. elegans* does not require synaptobrevin, the vesicle-SNARE (v-SNARE) necessary for synaptic vesicle fusion, indicating that the vesicles exocytosed for neurite extension are separate from that for neurotransmitter release [57,58]. Instead of synaptobrevin (also known as VAMP2), TI-VAMP (also known as VAMP7) seems to serve as a v-SNARE for exocytosis during neurite extension [16,59]. Disrupting the function of TI-VAMP/VAMP7 by expressing a dominant-negative C-terminal peptide, which interferes its binding to the SNARE complex, prevents outgrowth of both axons and dendrites in mouse hippocampal neurons

[16]. TI-VAMP/VAMP7-positive vesicles are frequently labeled with the lysosomal glycoprotein Lamp-1 and can undergo Ca^{2+} -dependent exocytosis mediated by Synaptotagmin 7 (SyT VII) in primary sympathetic neurons [60,61]. These studies suggest that late endosomal and lysosomal compartments are crucial for neurite outgrowth.

Studies in *C. elegans* also indicate a role of late endosomes in neurite outgrowth. Mutations in the Rab guanine exchange factor *glo-4* or its target Rab38-like GTPase, *glo-1*, cause aberrant axon outgrowth in touch neurons [62]. *glo-4* mutants show mislocalized Rab7-marked late endosomal compartments, suggesting that the axon outgrowth defects might result from mis-trafficking of these vesicular compartments. GLO-4 binds to and acts downstream of the regulator of presynaptic morphology (RPM-1). The RPM-1 and GLO-4 pathway could serve to regulate the trafficking of the netrin receptor UNC-5 and the SLIT receptor SAX-3/Robo [63]. Disruption of this pathway leads to aberrant anterior-posterior and dorsal-ventral axon guidance.

In *Drosophila*, midline crossing of commissural axons depends on cell-surface expression of Robo receptors [64–66]. Robo cell-surface expression is downregulated when axons cross the midline and upregulated afterwards to prevent re-crossing. This reversible regulation is mediated by transient cell-autonomous expression of an endosomal protein Commisureless (Comm) that binds to Robo. When axons cross the midline, Comm is expressed and targets newly synthesized Robo to late endosomes to silence SLIT/Robo repellent signaling [65]. After axons have crossed the midline, Comm is extinguished, thus freeing Robo to be trafficked to the cell surface.

Collectively, these studies indicate that late endosomes can regulate neurite outgrowth and axon guidance by directing axon guidance receptors away from cell surface. Such mechanisms can exert a spatial control of guidance accuracy by coupling the endosomal trafficking during neurite elongation with local changes in guidance environment.

Contributions of recycling endosomes in neurite outgrowth

Recycling endosomes (REs) are multi-functional intracellular organelles mediating both canonical endocytic and non-canonical post-Golgi secretory trafficking in non-neuronal cells [67] (Box 1). RE-associated Rab GTPases Rab11 and Rab8 are present in a spatially regulated pattern in many types of invertebrate and vertebrate neurons [18,68,69]. The periaxonal subcellular localization of Rab11-marked REs differs from that of Rab5-marked REs, which largely colocalize with synaptic vesicles, consistent with the notion that these two endosomal compartments have differential functions in axons and presynaptic terminals.

The functions of REs in neuritogenesis have been revealed by studies using various strategies to interfere with RE trafficking. Segregated RE vesicles labeled by syntaxin 13, an endosomal SNARE mediating RE fusion, travel into both dendrites and axons of developing and mature neurons [70]. Overexpressing syntaxin 13 enhances neurite outgrowth in NGF-stimulated PC12 cells [71]. Inhibiting Rab8 function in embryonic hippocampal

neurons completely blocks neurite outgrowth, suggesting that secretory trafficking from REs is crucial for neurite formation [72]. In chicken embryonic retinal neurons, membrane materials supporting neurite outgrowth seem to originate from Rab11-positive REs [73]. In the *Drosophila* embryo, disrupting Rab11 function leads to disorganization, misrouting and collapse of developing axons in the ventral nerve cord [74]. The adult fly eye of Rab11 homozygous mutants also displays retinal abnormalities, missing photoreceptors and associated bristles [75]. In mammals, Protrudin, a Rab11-GDP-specific binding protein, regulates axonal outgrowth [76]. Overexpression of Protrudin promotes axon extension in cultured hippocampal neurons. By contrast, knocking down Protrudin inhibits NGF-induced neurite formation. Taken together, these studies indicate that REs and RE-associated regulators have important roles in neurite outgrowth.

Several studies have also revealed coordinated activity among endosomes, cytoskeletons and the exocyst complex in membrane addition and neurite dynamics. In the developing *Drosophila* neuromuscular junction (NMJ), synaptic growth requires Rab11-dependent endosomal trafficking and actin polymerization controlled by a small Rho GTPase Cdc42 and Nervous Wreck (Nwk), a conserved neuronal GTPase-activating protein (GAP) with an F-BAR domain. These proteins colocalize at periaxial zones of the *Drosophila* NMJ [18]. *Rab11*, *nwk* and *cdc42* loss-of-function mutants exhibit axon overgrowth with excess branches, increased bouton number and abnormal bouton pattern [18,77]. The exocyst complex, a macromolecule that targets secretory vesicles to specific sites on the plasma membrane for exocytosis, might bridge Rab-mediated vesicle interactions with Rho-targeted exocytosis [78,79]. For example, studies in *Drosophila* show mutations in specific components of the exocyst complex cause defects in neurite outgrowth and neuromuscular junction formation [80], loss of synaptic specificity and inappropriate synaptic connections [81]. Several components of the mammalian exocyst are present at sites of neurite outgrowth in NGF-treated PC12 cells and nascent synapses in cultured embryonic hippocampal neurons [82,83]. NGF induces the interaction between Exo70, an exocyst component, and TC10, a Rho-like GTPase. This interaction specifically antagonizes Cdc42-dependent neuronal Wiskott-Aldrich syndrome protein (N-WASP) activation and filopodia formation [84,85] and favors TC10-dependent membrane addition [86], providing a mechanism for switching between morphologically distinct forms of membrane addition and neurite outgrowth.

In addition to membrane addition during neuritogenesis, REs are crucial for polarized protein sorting. In developing *Drosophila* photoreceptors, newly synthesized rhodopsin enters Rab11-positive vesicles at the base of the rhabdomere after release from the ER, followed by exocytic delivery to the surface of the rhabdomere [87]. Disrupting the function of Rab11 and its interacting partners dRip1 and myosin V causes the failure of rhodopsin delivery to the surface of developing eyes [88]. In addition, several exocyst components are implicated as Rab11 effectors and facilitate secretory trafficking to the rhabdomere [78,79,89]. A similar role of REs has also been found in

vertebrate retina [73,90,91]. In mammalian neurons, axonal accumulation of the cell adhesion molecule L1/NgCAM enables the elongation of axons of embryonic neurons and regeneration of adult neurons [92]. Recent work shows that L1/NgCAM is trafficked to the cell surface of dendrites through the trans-Golgi network and then endocytosed into REs in the dendrites and trafficked to the axon in a process referred to as 'transcytosis' [93]. Proper sorting of NgCAM is dependent on the endosomal regulator NEEF21. Interfering with RE trafficking causes mis-sorting of NgCAM to the somatodendritic membrane [76,93].

Thus, these studies have given us a glimpse of multifaceted functions of recycling endosomes in neuritogenesis and targeted delivery of surface proteins. The trafficking of NgCAM also exemplifies the complex route of neuronal proteins and sets a model for understanding regulation of the exocytic and endocytic pathways needed for axon outgrowth. They imply that neuronal recycling endosomes probably have much more complex subtypes depending on protein and, possibly, lipid compositions.

Ubiquitin and endosomal regulation through the endosomal sorting complex required for transport

A universal protein modification often associated with membrane dynamics is ubiquitylation [94]. Binding of proteins to ubiquitin triggers various steps in endocytic or degradation pathways. Ubiquitylation occurs through the association of a target protein with the series of ubiquitylating enzymes categorized as E1, E2 and E3. The manner in which a substrate is ubiquitylated defines its functional outcome, with monoubiquitylation usually targeting plasma membrane proteins for endocytosis and polyubiquitylation serving as a recognition signal for proteasomal degradation.

Many known effectors of Rab proteins contain ubiquitin-binding domains, implying roles of ubiquitylation in vesicular regulation. For example, Rabring7, a Rab7 target protein has E3 ubiquitin ligase activity and can ubiquitylate itself and the epidermal growth factor receptor, a regulator of neuronal morphogenesis [95]. Rabex-5, a guanine exchange factor for Rab-5, possesses both a monoubiquitylation site and a motif that can interact with ubiquitin (designated as MIU) [96]. When Rabex-5 is monoubiquitylated, it binds to its own MIU, rendering the Rabex-5 cytosolic. When Rabex-5 is deubiquitylated, the MIU is free to bind monoubiquitylated cargo of endosomes and associate with the vesicular membrane. There it can activate Rab-5. Although these studies were conducted in HeLa and HEK293 cells, it is an intriguing possibility that similar mechanisms of regulation might exist in neurons, particularly because many neuronal cargos are ubiquitylated.

For example, neural cell adhesion molecule (NCAM) signaling is likely to be regulated by monoubiquitylation because increased rates of ubiquitylation lead to higher rates of NCAM endocytosis [97]. Polyubiquitylation of TrkA directed by the E3 ligase TRAF6 has been shown to direct TrkA signaling, internalization and NGF-dependent neurite outgrowth [98]. As discussed earlier, polyubiquitylation of Unc-51 kinase is also involved in TrkA signaling [47]. These studies highlight the importance of

regulatory roles of ubiquitylation in tuning signaling outputs of receptors.

Among the recent studies, of particular interest is the endosomal sorting complex required for transport (ESCRT), which functions in chaperoning ubiquitylated cargos through late-endosomal, lysosomal and multivesicular body (MVB) compartments. ESCRT has long been implicated in diseases associated with pathological neurons [99] but, more recently, it has been implicated in neurite outgrowth in the developing nervous system. The *Drosophila* coiled-coil protein Shrub is homologous to yeast Snf7, a component of the ESCRT-III complex involved in the formation of endosomal multivesicular bodies [100]. The embryonic neurons of *Shrub* mutants showed severe morphological abnormalities, such as ectopic appearance of axonal branches. Furthermore, in *Shrub* mutant neurons or in cells expressing *Shrub* RNAi, various endosomal markers were mislocalized, including spinster-RFP (a late endosomal and lysosomal marker), Rab7-GFP and Rab5-GFP, whereas recycling endosomal markers were unaltered. These results support a role for the ESCRT complex in neuronal morphogenesis via trafficking regulation through the endosomal network. Importantly, these studies have brought an organelle that is traditionally perceived as a 'degradative or scavenging station' into the forefront of normal neuronal development.

Conclusion and perspectives

The complexity of neurite morphogenesis and guidance remains one of the curiosities in neuronal development. Membrane addition and proper delivery of signaling molecules are integral functions of intracellular vesicular trafficking. Cataloging of intracellular compartments is greatly aided by the knowledge of cargo-specific molecules. A combinatorial functional perturbation begins to tease apart the steps involved in neuritogenesis and guidance. The example of the UNC-51 kinase illustrates how a conserved protein functions in a neuron-type specific manner by interacting with different effectors. However, we do not know where and when the kinase activity of UNC-51 comes into play in the vesicular movement in neurites. The implication of diverse endosomal compartments in neurite extension and guidance is intriguing. However, we have little knowledge of the specific effectors for distinct pools of vesicular cargos. Understanding modification of vesicular dynamics by ubiquitylation also requires the identification of the protein targets. A deeper understanding of effectors and modification of these proteins is of particular interest to elucidate how a general pathway could transport different cargos with temporal and spatial specificity. Moreover, addressing these questions requires technical advances in detecting cargo movement and membrane exchange with high resolution. For example, FRET analysis between effectors and cargos will reveal spatial activities crucial for guidance decisions during neurite extension. Increased efforts on developing better labeling probes and methodologies with greater sensitivity are making headway towards *in vivo* dissection of vesicular dynamics. Combining the use of chemically synthesized or genetically encoded sensors with sub-100 nm resolution of light microscopy imaging will yield unprecedented observations.

Finally, understanding how external stimuli and signals direct membrane addition required for appropriate neurite guidance and synaptic connections is essential for an integrated view of neuronal development.

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