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Roles of membrane trafficking in nerve repair and regeneration

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Successful axonal repair following injury is critical for nerve regeneration and functional recovery. Nerve repair relies on three functionally distinct events involving membrane trafficking. First, axonally transported vesicles accumulate, while others are generated at the cut end to restore a selective barrier to the severed axon. Then, retrograde transport of vesicles along microtubules informs the cell body that damage has occurred in the distal axon. Finally, membrane addition to a newly formed growth cone, or to the axonal membrane is required to promote axonal re-growth and elongation. Yet, how these membrane trafficking events are regulated and what are the identities of the molecules and organelles involved remains largely unknown. Several potential factors have been recently identified. Members of the SNARE machinery appear to regulate fusion of vesicles in a calcium-dependent manner to promote axolemmal resealing. Retrograde transport of endosomes powered by the dynein-dynactin molecular motor complex represents a potential injury-signaling platform. Several classes of secretory and endocytic vesicles may coordinate axonal membrane extension and re-growth. Here we discuss recent advances in understanding the mechanisms of the membrane trafficking involved in nerve repair.

Introduction

The extreme polarized morphology of neurons poses a challenging problem for intracellular membrane trafficking. The transport of numerous distinct classes of organelles and vesicles is required to establish and maintain the polarized morphology of neurons. This transport system also controls survival and stress signaling between the cell body and the distantly located dendritic and synaptic terminals. Similarly to non-polarized cells, membrane trafficking in neurons can be subdivided in two main categories, the biosynthetic and the endocytic pathway. In the biosynthetic pathway, proteins and lipids are synthesized in the endoplasmic reticulum and then trafficked through the Golgi apparatus where a series of post-translational modifications occur. Vesicles exiting the Golgi are targeted to the plasma membrane or join elements of the endocytic pathway. Endocytosis is the process

by which cell surface molecules, membrane proteins, receptors and extracellular solutes are sequestered away from the plasma membrane and delivered to early endosomes. Then, some membrane proteins and receptors are recycled back to the cell surface through the recycling endosomes, while others are transported to late endosomes and lysosomes for degradation, or join the biosynthetic pathway.¹ Given their morphological complexity, neurons have mastered the art of membrane specialization.² Neurons appear to possess a more complex population of endosomal carriers than non-neuronal cells^{3,4} and unlike non-polarized cells, trafficking through the endoplasmic reticulum and the Golgi complex is not restricted to the cell body but also occurs in axons⁵ and dendrites.⁶

Recent studies have begun to reveal the identity of organelles and membrane compartments that contribute to neurite extension and axon guidance during development (reviewed in refs. 7 and 8). Early endosomes play a role in axon guidance, while recycling and late endosomes were shown to contribute to membrane addition during neurite outgrowth and extension.⁸ The mechanisms regulating neurite outgrowth during development may be recapitulated during nerve regeneration. However, given the significantly greater distances that axons typically need to regrow in adult animals, the mechanisms may differ. In addition to growth per se, membrane trafficking events are required for membrane resealing and intracellular signaling. A three-step process requiring distinct membrane trafficking events can be proposed to drive nerve regeneration (Fig. 1). First, following nerve transection, the axonal plasma membrane, also known as axolemma, must be locally repaired. Second, retrograde injury signals traveling from the injury site back to the cell body increase the intrinsic growth capacity of injured neurons to promote successful regeneration. Third, membrane trafficking and cargo delivery contribute to membrane expansion during axonal re-growth and elongation. Here we discuss recent findings on the distinct roles of membrane trafficking in nerve repair. Based on the current knowledge of membrane trafficking during neuronal development, we also discuss the mechanisms that might be involved in axonal extension during regeneration.

Axolemmal Repair

One of the earliest responses of neurons to axotomy is the resealing of cut axons. Failure to reseal or a delay in resealing may be detrimental to a neuron's ability to survive axotomy or to regenerate a

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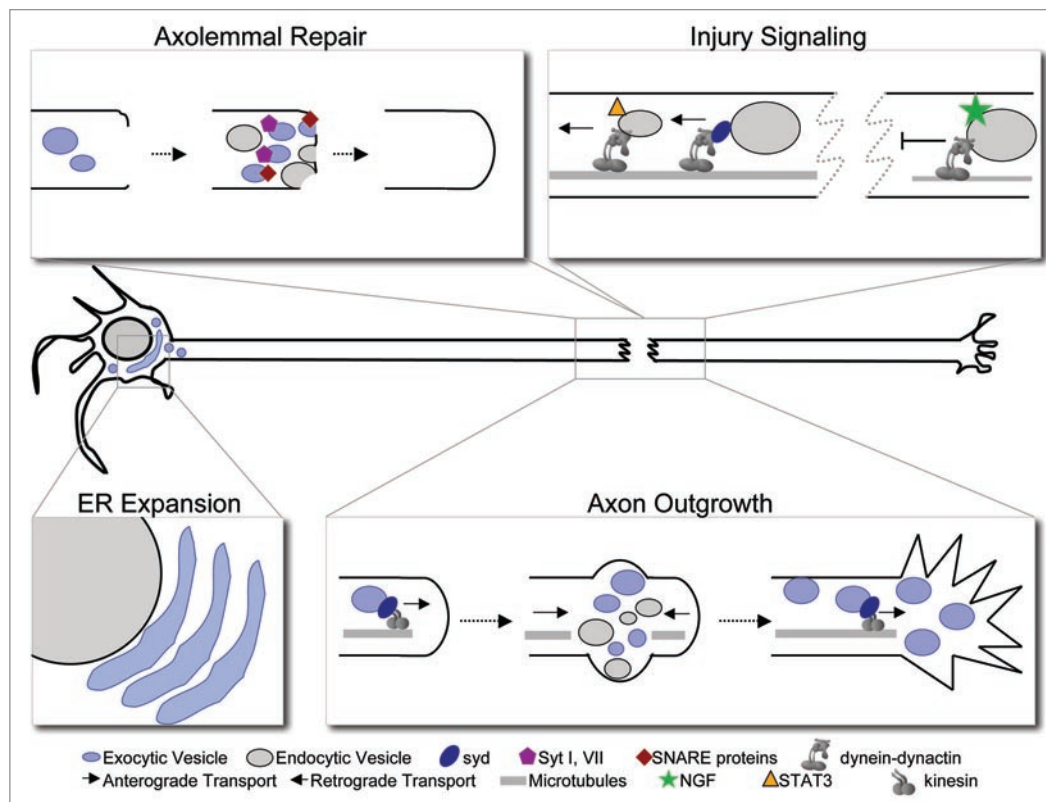


Figure 1. Model of membrane trafficking events during axon repair and regeneration. Repair of the axolemma is mediated by the accumulation and fusion of exocytic vesicles derived from the Golgi complex or lysosomal/secretory organelles. Calcium-dependent fusion mechanisms involving synaptotagmin I and VII, SNARE proteins TI-VAMP/VAMP-7 and syntaxins 1 and 4 are important for successful membrane resealing. Similarly, calcium-dependent endocytic mechanisms also play a role in membrane repair. Retrograde injury signaling informs the cell body that damage has occurred in the distal axon. Retrograde transport of Sunday Driver (syd)-associated endocytic vesicles powered by the dynein-dynactin molecular motor complex represents a potential injury-signaling platform. Lack of NGF delivery to the cell body represents other mechanisms to signal injury. Expansion of the endoplasmic reticulum allows for increased synthesis of proteins and lipids necessary for repairing the lesion and extending the axon over a large distance to successfully reconnect with its target. Establishment of a new growth cone is mediated by the accumulation and fusion of Golgi-derived and endocytic vesicles just proximal to the cut end. Continuous supply of anterogradely transported vesicles then contribute to the addition of membrane at the growth cone and potentially along the length of the axon.

new axon. Although restoring a selective barrier to severed axons is a prerequisite for restoring function, it has received relatively little attention during a century of research on axon regrowth after injury. In the early 1990s, confocal and electron microscope studies demonstrated the accumulation of membranous, injury-induced vesicles that appeared to occlude the open, cut ends of isolated squid giant axons.^{9,10} Further studies in the pseudo-myelinated axons of the earthworm, *Lumbricus terrestris*, revealed a role for calcium in vesicle fusion with each other or with the plasma membrane for resealing.¹¹ Membrane resealing in lesioned mammalian axons was shown to take up to several hours, and depend on axon diameter and on calcium in the extracellular environment.¹² The level of intra-axonal calcium is also important for membrane resealing. In *Aplysia* neurons, calcium influx after axotomy occurs by direct entrance through the disrupted plasma membrane at the lesion site and also through voltage-gated calcium channels.^{13,14} An increase in intra-axonal calcium induced by injury was also observed in rat sciatic nerve.¹⁵ This rapid shift in calcium concentrations induces at least two major events that are necessary for plasmalemmal repair: proteolysis and membrane

fusion/fission events. Calcium-dependent activation of proteases such as calpain is a necessary event for membrane resealing in rat septal neurons¹⁶ and in myelinated dorsal root axons.¹² Cleavage of submembrane spectrin skeleton by calpain is required for the formation of a competent growth cone after axotomy.^{17,18} This protease activity presumably releases membrane tension allowing for more efficient vesicle fusion with the plasma membrane.¹⁷

Since the observation 20 years ago that vesicles accumulate at the axon cut end^{9,10} (reviewed in ref. 19), progress has been made towards defining the nature of these vesicles and the molecules mediating their trafficking. It appears that both exo- and endocytosis are triggered upon elevated intra-axonal calcium to mediate membrane resealing. Calcium-dependent exocytosis is known to mediate membrane resealing in non-neuronal cell types like fibroblasts,²⁰ and also in the squid and crayfish giant axons.²¹ Recently, Norma Andrews group has identified the lysosomes as exocytotic vesicles involved in membrane resealing and synaptotagmin VII, a member of the synaptotagmin family of Ca^{2+} -binding proteins, as a regulator of this process.²² The machinery regulating vesicle fusion has

been extensively studied in the case of synaptic vesicle exocytosis. It involves an interaction between synaptotagmin and the SNARE machinery, which includes vSNAREs on the vesicle and the t-SNAREs such as syntaxin on the target membrane. Synaptotagmin VII-dependent lysosomal fusion in fibroblasts requires the v-SNARE TI-VAMP/VAMP7 and the t-SNARE syntaxin4.²³ Whether synaptotagmin VII is also involved in membrane resealing in mammalian neurons remains to be determined. However, the recent finding that in peripheral nerves, synaptotagmin VII is anterogradely transported in vesicles bearing the molecular motor binding protein Sunday Driver (syd), also known as JIP3 or JSAP1, suggests a mechanism for targeted delivery of synaptotagmin VII to distal nerve sites.²⁴ Synaptotagmin VII thus emerges as a strong candidate regulating membrane repair in vertebrate axons. This does not exclude the possibility that other organelles and molecules participate in resealing. Synaptotagmin1 and syntaxin1, which are known to regulate fusion of synaptic vesicles for neurotransmitter release, are also needed for membrane repair in the squid and crayfish giant axons.²¹ In addition to exocytosis, calcium-dependent endocytosis mediates plasma membrane repair in the squid giant axon.^{19,25} Endocytic vesicles bearing synaptotagmin on their surfaces are formed at the lesion site and promote membrane resealing.²⁵ Calcium-dependent endocytosis is also necessary for wound removal induced by the pore-producing bacterial toxin streptolysinO in NRK cells or in mechanically wounded cells.²⁶ Future work is needed to understand whether the organelles involved in membrane resealing are specialized for this task or whether several types of molecules and organelles present at the injury site randomly facilitate fusions events essential for the repair of damaged axolemmal membranes.

Transformation of a transected axonal tip into a growth cone is a critical step in the cascade leading to neuronal regeneration. Some of the vesicles that accumulate at axonal cut ends arise from continuous axonal transport from the cell body. In addition to assisting with the necessary immediate repair, the accumulation of vesicles at the cut end contributes to growth cone formation. Spira and co-workers have shown that microtubules form a trap in which anterogradely and retrogradely transported vesicles accumulate just proximal to the cut end and assist with growth cone formation after axotomy.²⁷ Cleavage of the submembrane spectrin skeleton by calcium-activated proteases is crucial to allow membrane expansion and growth cone formation.^{17,18,28} Failure to remove the spectrin barrier results in end-bulb formation, a classical structure observed in central nervous system (CNS) neurons, which generally fail to regenerate.¹⁷ Interestingly, axonal resealing is slower in central than peripheral axons, which may contribute to their different ability to regenerate.²⁹ In addition to intrinsic differences, chronic, non-disruptive axonal injury that often occurs in the central nervous system may not elicit the necessary changes, such as increased calcium and proteolytic activity required for repair and may instead lead to endbulb formation or axonal degeneration.³⁰ A detailed analysis of the molecular processes that enable vesicles to restore a plasma membrane seal in central and peripheral neurons could help understand the poor regenerative capacity of CNS neurons.

Injury Signaling

Injury to peripheral nerves provokes a series of morphologic and biochemical changes in neuronal cell bodies that were initially described more than 40 years ago. The most prominent change was described as chromatolysis and refers to the disruption and dispersion of Nissl Bodies due to disintegration of stacked rough endoplasmic reticulum.³¹ In addition to chromatolysis, rearrangement of the cytoskeleton and increased protein synthesis was also reported.^{31,32} In 1970, Cragg proposed several injury signaling mechanisms that could account for initiating the cell body reaction.³² Later studies in *Aplysia*³³ and in mice³⁴⁻³⁶ led to a model in which three types of injury signals functioning in a temporal sequence assist with nerve regeneration (reviewed in ref. 37): injury-induced discharge of axonal potentials, interruption of the normal supply of retrogradely transported target-derived factors, and retrograde injury signals traveling from the injury site back to the cell body, also called positive injury signals. While some components of the positive retrograde injury signals, including the importin complex, directly associate with the retrograde motor machinery (reviewed in ref. 38), others hitchhike on axonal vesicles. For a more complete discussion of the current knowledge of injury signals, see ref. 38-40.

Endosomes might represent the carrier of choice to convey information about distant event in the axon back to the cell body. Distinct endosomal populations exist in neurons.⁴¹ Each specialized endosome may facilitate the formation of signaling platforms by clustering a selected pool of signaling components, therefore providing a precise temporal and spatial regulation of signal transduction.⁴ Early studies on intra-axonal organelle transport have revealed an anterograde to retrograde conversion after injury,⁴² which could in part depend upon local proteolysis at the site of injury.⁴³ Since these early studies on axonal transport, much research has been devoted to understanding how these organelles move by characterizing the molecular motors they are associated with, dynein-dynactin and kinesin. However, we still know little about how motors discriminate among their many potential cargoes and how transport directionality is achieved. The motor-binding protein syd represents a potential motor adaptor on axonal endosomes²⁴ to convey information about axonal injury back to the cell body. In peripheral axons, the stress-activated protein kinase JNK is present on syd-associated vesicles and is transported in both anterograde and retrograde directions.⁴⁴ Nerve injury induces a transport switch, such that syd and JNK preferentially move retrogradely, most likely due to a preferred interaction between syd and the retrograde motor complex dynein-dynactin.⁴⁴ JNK signaling has been implicated in promoting nerve regeneration, as sciatic nerve transection induces a rapid and prolonged increase in activated JNK in the cell body, which returns to basal levels once regeneration is completed.⁴⁵ These studies suggest that syd mediates the axonal transport of endosomes carrying JNK on their surface, and that the directional switch induced by axon injury provides a mechanism for propagation of retrograde injury signals back to the cell body. Whether syd-endosomes are derived from the endocytic events at the nerve terminal or emerge at the site of injury from the local endocytic events²⁵ remains to be determined.

In addition to activation of mitogen-activated protein kinases such as JNK⁴⁴ and Erk⁴⁶ at the site of injury, axonal damage activates transcription factors, such as STAT3, which is important for regeneration. Indeed, STAT3 activation through the Jak2 signaling pathway occurs in DRG neurons cell body after peripheral, but not central lesion,^{47,48} strongly supporting a role for STAT3 in neuronal regeneration. The retrograde transport of locally activated STAT3 has been suggested^{49,50} and STAT3 associates with early endosomes in Hep3B liver cells.⁵¹ However, whether STAT3 itself is associated with vesicular structures in axons remains unknown. Nevertheless, STAT3 signaling following injury is likely to depend in part on vesicular transport since in vitro studies using compartmentalized cultures have suggested a signaling endosome model in which the gp130/JAK complex is endocytosed and then retrogradely transported to activate STAT3 in the cell body.⁵² Whether the gp130/JAK complex travels on the classical signaling endosome or another specialized organelle originating at the site of injury will require further studies.

Another type of endosome that plays an indirect role in injury signaling is the so called “signaling endosome”. Signaling endosomes contain neurotrophic factors secreted by the target tissue and are transported retrogradely back to the cell body. Loss of such signal has the potential to initiate a regenerative response and was first postulated by Cragg to explain chromatolysis.³² Later experiments have indeed shown that loss of trophic factors might act as an injury signal. Sciatic nerve axotomy leads to decreased levels of retrogradely transported nerve growth factor (NGF)⁵³ and artificial interruption of the NGF supply by injections of anti-serum to NGF mimics the changes in gene expression induced by axotomy.⁵⁴ The signaling endosome hypothesis has been put forward to explain retrograde neurotrophin signaling, amongst a number of other possible models.⁵⁵ The precise identity of such signaling endosome remains controversial and includes both early and late endosomes, as well as lysosome and multivesicular bodies (reviewed in ref. 55). Yet the role of multivesicular bodies in neurotrophic signaling has been recently challenged⁵⁶ and they may instead represent a population of organelles that arise upon injury in axons.⁵⁷ This is an interesting concept since storage of signaling molecules within intraluminal vesicles of multivesicular bodies may prevent their deactivation during the long journey from the axon back to the cell body.⁵⁸ As intraluminal vesicles are not always destined for lysosomal degradation, but can fuse back with the limiting membrane of late endosomes,⁵⁹ multivesicular bodies may offer a protected environment during transport and release their cargo at destination in the cell body. The identity of the various endocytic vesicular carriers, and the regulatory mechanisms that determine their transport direction remain unclear. Future proteomic studies, such as those carried out for synd-endosome²⁴ or dynein associated axonal vesicle⁶⁰ are needed to shed light on the precise nature of organelles transported along axons.

Axon Outgrowth and Elongation

The balance between constitutive plasma membrane retrieval and insertion that determines the shape and dimension of a neuron

has to be modified to allow a neuron to regrow its axon after injury.⁶¹ The importance of membrane trafficking, especially endocytosis, for neurite outgrowth and guidance during development is being unraveled (reviewed in ref. 62). Sadly, virtually nothing is known about how membrane trafficking assists with regeneration in vivo. Regenerative axonal outgrowth may recapitulate a developmental program.⁶³ However, given the significantly greater distances that axon need to cover to reconnect with their target in an adult animal, it is conceivable that axonal regeneration depends on somehow distinct mechanisms. What are the mechanisms regulating membrane insertion, and how is the vast amount of membrane material required for axonal extension following injury supplied to the growing axon? The classical paradigm implicates that proteins and lipids are synthesized in the cell body through the endoplasmic reticulum and the Golgi complex and are then co-transported along microtubules in the form of vesicles to reach the membrane insertion site. This paradigm largely rests on the long-standing assumption that only the cell body contains the necessary biosynthetic activity to synthesize protein and lipids. During the last 10 years, it became clear that axons possess components of endoplasmic reticulum and Golgi, and are capable of local protein⁵ and lipid synthesis.⁶⁴ Merianda et al. report that inhibition of Golgi function in isolated adult DRG axons attenuates translation-dependent axonal growth responses.⁵ Furthermore, they show that the capacity for secreting locally synthesized proteins in axons appears to be increased by injury. This is in contrast with earlier studies showing that protein synthesis in distal axons is not required for axon growth in the embryonic spinal cord,⁶⁵ but may emphasize a difference in outgrowth mechanisms in peripheral versus central neurons.

How and where locally synthesized membrane proteins are inserted into the plasma membrane remains largely unknown. In cultured developing neurons, the site of membrane insertion for axonal outgrowth appears to be primarily at the growth cone.⁶⁶ This is not the case for mature neurons, in which, at least in dendrites, membrane appears to be added to multiple sites along the dendritic surface.⁶⁶ Dendritic Golgi outposts distributed along dendrites may play a role in membrane insertion given their requirement for dendritic growth.⁶ It is conceivable that equivalent Golgi outposts along axon may play a similar role in membrane addition during regenerative axonal growth. Indeed, in growing *Xenopus* neurites, membrane is inserted at the cell body and all along the neurite, creating an anterograde bulk membrane flow that correlates with neurite elongation.⁶⁷

The massive demand for membrane biogenesis in regenerating neurons must be different to that of neurons not actively growing. The expansion of the endoplasmic reticulum in the cell body that occurs after injury reflects the increase in membrane synthesis. The mechanisms regulating this increase in membrane biogenesis are not completely understood. Recently, it has been proposed that transcription factors may play new, non-genomic roles in regulating regeneration. The transcription factor *c-fos* has, in addition to its transcription factor activity, the capacity to activate the biosynthesis of phospholipids and glycolipids necessary for membrane biogenesis.^{68,69} This activity is required for neurite elongation, at least in PC12 cells.⁶⁸ Whether this

non-transcriptional c-fos activity is required to stimulate membrane biogenesis in vivo has to be further examined, given the observation that c-fos expression is not induced in DRG neurons following nerve injury.⁷⁰

Axolemmal expansion requires fusion of vesicles with the plasma membrane. Similarly to its role in new membrane addition for extension of developing neurites,⁷¹ synaptotagmin VII may function in regenerative outgrowth. A population of axonally transported vesicles carrying syd and synaptotagmin VII on their surface²⁴ may mediate the delivery of synaptotagmin VII to distal axons. This would agree with the observed axonal growth defects observed in syd knockout animals.^{72,73} Another recently identified molecular pathway that may control membrane extension is the IGF1-exocyst pathway. In cultured hippocampal neurons, membrane expansion at the axonal growth cone is regulated by IGF-1 via a signaling cascade involving TC10 and the exocyst complex.⁷⁴ Interestingly, IGF-1 is required for peripheral nerve regeneration,⁷⁵ indicating that IGF-1 may regulate a signaling cascade for membrane insertion.

In addition to proteins, axon elongation requires insertion of lipids to the growing axons. Axons do not need to obtain all of their lipids from the cell body since they have the capacity to synthesize most phospholipids in the axon. Importantly, axonal synthesis of phospholipids is required for axon elongation.⁶⁴ In

contrast, cholesterol is not made in axons, but exclusively in the cell body. While endogenous supply of cholesterol may be sufficient for developmental growth, it may not be the case for regenerative growth. Several studies (reviewed in ref. 64) have suggested that lipoproteins such as apoE may function in lipid recycling following injury to repack and reuse lipids to promote membrane assembly and regeneration. The transfer of lipids from axon to Schwann cells⁷⁶ together with the transfer of ribosomes from Schwann cells to axons⁷⁷ reflect the intimate relationship between the Schwann cell and its ensheathed axon. In case of very long axons in which the cell body may not be able to provide sufficient material to support regeneration at the axon tip, the possibility that the distal axon can rely on non-neuronal sources to support its autonomy from the cell body and regenerate effectively is intriguing.

The field of axonal repair and extension remains filled with open questions. Future work will need to define the mechanisms regulating membrane trafficking in axonal repair in peripheral nerves and their implications to the lack of regenerative capacity of CNS neurons.

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