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Title

Regulation of plasma membrane expansion during axon formation

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ABSTRACT

Here will review current evidence regarding the signaling pathways and mechanisms underlying membrane addition at sites of active growth during axon formation.

This Review is dedicated to Karl H. Pfenninger who pioneered work on growth cone membrane addition and passed away on February 9, 2015.

Neurons represent an extreme example of polarized cells. This polarization arises because neurons generate cytoplasmic extensions, designated as axons and dendrites, which acquire highly elaborated shapes and differ in cytoskeletal organization, organelle type and plasma membrane composition. Axons and dendrites are crucial for nervous system development and maturation, as the establishment of precise synaptic connections (e.g. brain wiring), and hence the appropriate functioning of brain circuits is totally dependent on their elaboration and maintenance (Cáceres et al., 2012; Funahashi et al., 2014; Takano et al., 2015; Rolls & Tegla, 2015).

A large series of studies carried out over the past decade have begun to shed light on the mechanisms underlying neuronal polarization. Major components of the cellular machinery and signaling pathways involved in axon/dendrite formation have been identified (Arimura & Kaibuchi, 2007; Schelski & Bradke, 2017). These studies have established that cytoskeletal assembly (Conde & Cáceres, 2009; Kapitein & Hoogenraad, 2015; van Beuningen & Hoogenraad, 2016) and membrane protein trafficking (Horton & Ehlers, 2003; Bentley & Banker, 2016; Wojnacki & Galli, 2016) are two key events underlying the generation of axons and dendrites. Microtubule (MT)-microfilament (MF) organization, dynamics and its regulation by extrinsic and intrinsic factors, both in developing and mature neurons, have been the subject of several excellent reviews and won´t be considered here (Arimura & Kaibuchi, 2007; Conde & Cáceres, 2009; Van Beunigen & Hoogenraad, 2016; Jones & Svitkina; Schelski & Bradke, 2017). This review will focus, on neuronal membrane trafficking and particularly on our current understanding of the mechanisms underlying membrane addition/expansion (Pfenninger, 2009) in growing axons.

Neuronal membrane addition in growing axons

The trafficking of membrane components is an essential step for axon growth and differentiation. It involves organelles of the exocytic pathway, such as the rough endoplasmic reticulum (RER) and the Golgi apparatus (Bentley & Banker, 2016; Wojnacki & Galli, 2016), as well as endosomes (Villaroel-Campos et al., 2016; Wojnacki & Galli, 2016). At the *trans Golgi* network (TGN), or eventually at recycling endosomes (RE), axonal membrane proteins are sorted and packed into appropriate carriers. After budding and exiting from the TGN, tubulo-vesicular carriers are shipped away towards the plasma membrane by MT-based transport (Bentley & Banker, 2016; Wojnacki & Galli, 2016). The final step of this journey is the delivery of the transported cargoes into the plasma membrane by exocytosis, and event referred as membrane addition and that entails membrane expansion (Pfenninger, 2009). Current evidence suggests that in neurons membrane addition/expansion preferentially occurs at the axonal tip (e.g. the growth cone).

The first line of evidence suggesting that the growth cone is the site of preferential membrane addition comes from work pioneered by Karl Pfenninger and collaborators. Electron microscopy of isolated growth cones obtained from embryonic brain (Pfenninger & Friedman, 1993) led to the discovery of collections of large (150 nm) pleiomorphic vesicles, located close to the plasma membrane that were designated as plasmalemmal precursor vesicles or PPVs. Membrane expansion assays performed using isolated growth cones (e.g. growth cone particles or GCPs) revealed that membrane addition and plasmalemmal expansion were associated with a decrease in PPVs (Lockerbie et al., 1991).

Later on, it was shown that axonal growth cones of cultured hippocampal neurons contain PPVs (Deitch & Banker, 1993). Then, a seminal study by Banker and collaborators (Craig et al., 1995) demonstrated that in these neurons newly synthesized membrane proteins first appear at the axonal growth cone surface; it was also observed in young dendrites, but not in mature ones. Subsequent work provided further support to this idea (Vogt et al., 1996; Futerman & Banker, 1996; Zakharenko & Popov, 1998) raising the possibility that the increase in growth cone size that parallels the transformation of a minor neurite into an axon (Bradke & Dotti, 1997) is the consequence of membrane expansion due to preferential membrane addition at this location. Not surprisingly, MT assembly and dynamics,

required for axon growth also occur at the growth cone (Conde & Cáceres, 2009) and regulate membrane addition (Zakharenko & Popov, 1998); these authors demonstrated that inhibition of MT dynamics at the growth cone, halted the insertion of vesicular carriers into the growth cone plasma membrane. They also suggested that both events are tightly coordinated and that common signaling pathways might regulate them (see below).

The PI3K pathway and the regulation of growth cone membrane addition.

The localized growth cone activation of phosphatidyl-inositol 3´ kinase (PI3K) has emerged as a critical factor for driving axon specification and axon growth (Shi et al., 2003; Wiggin et al., 2005; Cáceres et al., 2012). Activation of PI3K by external stimuli (e.g. IGF1, Sosa et al., 2006) generates PI (3,4,5) P3, leading to recruitment and activation of atypical protein kinase C (aPKC)-PAR6-PAR3, the so-called PAR polarity complex, at the tip of the growing axon (Shi et al., 2003). Accumulation of PAR6-PAR3 at the axonal growth cone results in Cdc42-induced Rac activation, an event that involves binding of the Rac guanosine nucleotide exchange factor (GEF) STEF/Tiam1 to PAR3 (Nishimura et al., 2005); it has been proposed, yet not demonstrated in developing neurons, that Rac-GTP could activate PI3K, thus generating a positive feed-back loop that maintains high PI3K activity during axon specification (Arimura & Kaibuchi, 2007). Inhibition of PI3K activity (Shi et al., 2003) or silencing of PAR3-PAR6 expression (Shi et al., 2003; Chen et al., 2013), or Tiam1 or Rac (Kunda et al., 2001; Ng et al., et al 2002; Nishimura et al., 2005; Chuang et al., 2005) activities prevent axon formation in cultured hippocampal neurons. Conversely, ectopic expression of a fast cycling mutant of Cdc42 (Sosa et al., 2006) or STEF/Tiam1 (Kunda et al., 2001; Nishimura et al., 2005) promotes axon formation, including the extension of supernumerary ones. Besides, a growing body of evidence suggests that activation of the PI3K-polarity complex-Cdc42-Rac signaling pathways regulate effector proteins (e.g. GSK3β, CRMP2, Stathmin, APC, CLASP2, LIMK1, cofilin, etc.) that control key aspects of cytoskeletal organization and dynamics at the growth cone, such as MT assembly, protrusion, capture, stabilization, and transport, as well as the generation of a loose actin network (increased actin dynamics), MT-MF interactions and feed-back loops,

which drive axon formation (Arimura & Kaibuchi, 2007; Conde & Cáceres, 2009; Schelski & Bradke, 2017).

Studies also indicate that PI3K is a master regulator of growth cone membrane addition during axon growth (Figure 1). This evidence stems from work aimed to understand the mechanisms underlying the involvementof IGF1 and a variant of the IGF1 receptor (IGF1R) designated as βgc, enriched in axonal growth cones (Quiroga et al., 1995; Mascotti et al., 1997) and required for axon growth, both in cultured hippocampal neurons (Sosa et al., 2006) and *in situ* (Nieto-Guil et al., 2017). IGF-1R is expressed in most CNS areas and developmentally regulated reaching its highest levels at late embryonic and early postnatal stages (Werner et al., 1991; Garofalo & Rosen, 1989). Transgenic mice deficient in IGF1 or IGF1R exhibit a general growth deficiency including severe defects in central nervous system development (Liu et al., 1993).

Initial observations aimed to analyze the dynamics of PPVs visualized by labeling with fluorescent ceramide in cultured neurons treated with IGF1 revealed that PPV clusters cleared from growth cones 2 times faster in IGF1-treated samples than in equivalent ones that received BDNF or were non-treated at all (Pfenninger et al., 2003); it was also shown that IGF1 stimulated in a dose-dependent manner plasmalemmal expansion in GCPs. Intriguingly, and despite the presence of activatable TrkB in GCPs, BDNF was not able to promote membrane addition; the significance of this difference is not known and has remained largely unexplored.

A variety of cell systems and model proteins have been used to demonstrate the requirement of PI3K for exocytosis in non-neuronal cells and/or in response to trophic factors (Bhattacharya et al., 2016). In accordance with this it was found that βgc, the insulin receptor substrate prevalent in growth cones, IRS2, the p80 regulatory subunit of PI3K, and the kinase AKT, a direct effector of PIP_3, became phosphorylated and activated upon addition of IGF1 to isolated growth ones (Laurino et al., 2005). More importantly, IGF1-induced growth cone plasmalemmal expansion was inhibited by the PI3K inhibitors, Wortmamin and LY294002 (Laurino et al., 2005), with the later also inhibiting axon specification and growth (Shi et al., 2003). Together, this information suggests that the IGF1-activated IRS/PI3K/AKT (Figure 1) pathway plays a major role in axon growth by regulating

membrane addition and most likely cytoskeletal dynamics (Oksdath et al., 2016; see also Schelski & Bradke, 2017).

As discussed above, axolemmal expansion occurs by exocytosis of PPVs primarily at the growth cone (Pfenninger and Maylié-Pfenninger, 1981; Pfenninger and Friedman, 1993, Futerman and Banker, 1996). Exocytosis requires two different processes: vesicle docking (Verhage M & Sørensen, 2008) or attachment and vesicle fusion (Lang & Jahn, 2008). The underlying mechanisms are mediated by different families of proteins. Docking and targeting are often regulated by the *exocyst* complex involved in neurite outgrowth and synaptogenesis in developing neurons, but not in pre-synaptic vesicle fusion (Murthy et al., 2003). Vesicle fusion, in turn, is primarily a function of the SNARE protein family (Lang & Jahn, 2008; Wickner & Sheckman, 2008). In the next sections will review current evidence about the nature of proteins involved in these two steps, the underlying mechanisms, signaling pathways and relevance for axon formation.

Mechanisms of PPV docking and tethering

In both yeast and mammalian cells the exocys*t*, a complex of eight polypeptides, is involved in docking and tethering exocytic vesicles at sites of fusion (Lipschutz & Mostov, 2002; EauClaire & Guo, 2003; Martin-Urdiroz et al., 2016). The exocyst complex has been implicated in a wide variety of cell functions, including morphogenesis, migration and tumor invasion; its subunits are direct targets of several small GTPases, such as Rabs, Cdc42, TC10 (a Cdc42 homolog), and RalGTPases (Wu & Guo, 2015; Zhu et al., 2017) that result in exocyst activation. The exocyst participates in polarized exocytosis in budding yeast (He et al., 2009 and references therein) and is required for epithelial cell polarity by mediating trafficking of cargoes destined to the basolateral membrane (Andersen and Yeaman, 2010; Wu & Guo 2015).

In Drosophila motor neurons mutation of Sec5, a component of the exocyst inhibits neurite growth by selectively impairing docking and delivery to the cell surface of membrane proteins required for cell growth (Murthy et al., 2003). While these neurons failed to elaborate neurites and show abnormal development of the neuromuscular junction, the remaining (fewer) synaptic buttons exhibit robust

synaptic transmission suggesting that different docking mechanisms mediate membrane addition and exocytosis of synaptic vesicles (Murthy et al., 2003).

Other studies also support the involvement of the exocyst complex in neurite/axon formation in developing mammalian neurons. For example, in PC12 cells NGF-induced neurite growth is paralleled by redistribution of Sec10, another component of the exocyst, towards the growth cone in association with MT (Vega & Tsu, 2001). Besides, inhibition of the Nerve Growth Factor (NGF)-MAP kinase pathway prevents exocyst redistribution and neurite growth; accordingly, expression of a Sec10 mutant halts neurite extension (Vega and Tsu, 2001). In cultured hippocampal pyramidal neurons, subunits of the exocyst complex, such as Sec6/8 (Hazuka et al., 1997) or Exo70 (Dupraz et al., 2009) or sec10 (Vega & Hsu, 2001) or TC10 (Dupraz et al., 2009) are also enriched in growth cones. The presence of these proteins at axonal growth cones offered an excellent opportunity to test if the IGF1-PI3K signaling pathway involved in delivery of PPVs to the plasma membrane requires a functional exocyst.

-*The IGF1-TC10-exocyst pathway*

In both GCPs and cultured hippocampal neurons IGF1 induces activation of TC10, and translocation of Exo70 (and presumably other exocyst components) to the plasma membrane (Dupraz et al., 2009). Besides, reducing the expression/activity of either Exo70 or TC10 halt growth cone membrane addition and inhibits axon growth (Dupraz et al., 2009). Even more important, both proteins are required for the membrane insertion of the IGF1R, which is required for axon specification (Sosa et al., 2006; Dupraz et al., 2009). These observations suggest that, in addition to self-reinforcing loops controlling cytoskeletal assembly/dynamics (Schelsi & Bradke, 2017), at least one positive feedback loop involving the IGF1R exists for the regulation of membrane addition required for axon specification and subsequent growth (Figure 1).

-*The Rap-Ral1 exocyst pathway*

It is likely that the PI3K-TC10-Exo70 pathway is not the only way to activate exocyst components to regulate membrane addition during neuronal polarization. It has been shown that the sequential activation of the small GTPase Rap1B and Cdc42 determines axon specification (Schwamborn & Püschel, 2004). Interestingly, a recent study has shown that Rap1B activates Ral1 (Nakamura et al., 2013), a

GTPase known for its ability to promote exocyst-mediated membrane addition in developing neurons (Lalli & Hall, 2005; Lalli, 2009). Of great interest is the demonstration of a Ral1-mediated association between exocyst components and aPKC-PAR3 (Lalli, 2009) or PAR6 (Das et al., 2014) required for polarization in both cultured neurons and during polarized migration of neural progenitors. Thus, the polarity complex may not only be important for instructing axon specification (Shi et al., 2003), but also for coordinating cytoskeletal dynamics (Cheng et al., 2013) with membrane addition (Lalli, 2009).

Conflictive results exist regarding the relationship (e.g. upstream vs. downstream) between Rap1B and PI3K. Since both proteins are required for axon specification, a network model might account for their mutual and coordinated regulation (Choi et al., 2013). Another possibility might be the activation of Rap1B independent of PI3K, by means of the IGF1R and its target, the Crk adaptor protein (Nakamura et al., 2013). Future studies should distinguish between this and related possibilities.

The fusion of PPVs at the axonal growth cone: identifying the SNARES

The role and identity of the SNARE proteins involved in synaptic vesicle fusion have been extensively studied (Sudhof and Rizo, 2011; Jahn and Fasshauer, 2012) By contrast, little was known until recently about the SNAREs involved in the regulation of PPV fusion with the neuronal plasmalemma, despite that some of them, such as SNAP25, Syntaxin1 and VAMP7 were implicated in axonal and/or dendritic growth in PC12 cells or primary neurons (Osen-Sand et al., 1993 and 1996; Martinez-Arca et al., 2001). Recently, it was shown that silencing of a different set of SNARES including VAMP4, Syntaxin6 and SNAP23 repressed axonal outgrowth and the establishment of neuronal polarity, by inhibiting IGF1R exocytotic polarized insertion (Grassi et al., 2015). Moreover, IGF1 stimulation triggered the association of VAMP4, Syntaxin6 and SNAP23 to vesicular structures carrying the IGF1R and ectopic expression of a negative dominant form of Syntaxin6 significantly inhibited exocytosis of IGF1R-containing vesicles at the neuronal growth cone (Grassi et al., 2015; Figure 1). It has been shown that VAMP4 also localizes to enlargeosomes, cytoplasmic vesicles/organelles capable of undergoing rapid exocytosis in response to μM rises in cytosolic Ca++ (Borgonovo et al., 2002). In two neuronal cells lines (PC12 cells and SH-SY5Y), microinjection

of VAMP4 antibodies or downregulation of VAMP4 expression considerably inhibit enlargeosome exocytosis.

A variety of results exist about two other SNARE proteins: VAMP2 and VAMP7. Silencing experiments using a shRNA targeted to VAMP2 or treatment of hippocampal pyramidal neurons with tetanus neurotoxin did not preclude neuron polarization (Grassi et al., 2015). These results are in line with a previous report suggesting that VAMP2 is required for axon guidance but dispensable for axon growth (Zylbersztejn et al., 2012); another study showed that VAMP2 participates in neurite initiation in cortical neurons growing on poly-D-lysine (Gupton & Gertler, 2010). Regarding VAMP7, suppression of this SNARE does not significantly altered neuronal polarization in hippocampal neurons growing on polylysin but inhibited axonal outgrowth in cells growing on laminin (Grassi et al, 2015; Gupton & Gertler, 2010); these results closely resemble those found in cultured hippocampal neurons from VAMP7 knockout mice (Zylbersztejn et al., 2012; Danglot et al., 2012). The possibility that different set of proteins participate in axon growth depending on the substrate where neurons attach and grow is possible. For example, axon growth rate is significantly higher in neurons growing on laminin than in polylysine with different complement of microtubule-associated proteins (MAPs) regulating axon extension (DiTella et al., 1996; Dawson et al., 2001). Regardless, it is now evident that axon specification and growth requires specific SNARE-mediated PPV fusion. Plasmalemmal expansion at growth cones is also required for axon guidance and evidence exists linking guidance receptors with SNARES. For example, the Netrin receptor deleted in colorectal cancer (DCC) forms a protein complex with Syntaxin1 that associates with TI-VAMP (Cotrufo et al., 2011). In accordance with this blockade or suppression of Syntaxin1 or TI-VAMP disrupts Netrin-mediated chemoatraction and/or results in abnormal axon guidance patterns (Cotrufo et al., 2011, 2012; Ros et al., 2015; Barrecheguren et al., 2017).

Microtubules and the machinery involved in membrane addition

The regulation of MT dynamics is key for neuronal polarization (Conde & Cáceres, 2009). During development, the prospective axon is distinguished from the remaining neurites for its higher content of long-lived (stable) polymer; more importantly, MT stabilization is sufficient to induce axon formation (Witte et al.,

2008). Several recent reviews have addressed in detail the significance and importance of MT stability for neuronal polarization (Neukirchen & Bradke 2011; Sakakibara et al., 2013; Liu & Dwyer, 2014; Penazzi et al., 2016) and therefore will only refer here to the possible link between stable MT and membrane addition.

The relationship between stable MT and fusion of PPVs, including membrane insertion of the IGF1R, might involve the microtubule-based motor KIF5C (Oksdath et al., 2016). It has been shown that the preferential translocation and accumulation of KIF5C in a selected neurite marks axon specification (Jacobson et al., 2006). In addition, KIF5C exhibits a preference to interact with axonal over dendritic microtubules (Nakata and Hirokawa, 2003; see also Nakata et al., 2011) and preferentially drives Syntaxin6 and VAMP4 to the axon, "walking" on stable MT (see Nakata and Hirokawa, 2003). It has been proposed that once Syntaxin6 and VAMP4 accumulate at the neurite enriched in stable MT, it is able to trigger the insertion of PPVs (including those containing IGF1R) by exocytosis in the growth cone plasmalemma (Oksdath et al., 2016). Upon insertion in the membrane, IGF1R can be activated by IGF-1 and induce membrane insertion of more IGF1R (Dupraz et al., 2009). All together, these molecular events generate a self-reinforcing mechanism deemed necessary for initial axonal outgrowth and the establishment of neuronal polarity. Aside from this, other possibilities exist. For example, the discovery that the N-terminal domain of PAR3 binds and stabilizes MT and that disruption of this interaction stops axon specification, place the polarity complex directly linking PI3K signaling with MT stability. The Ral1-mediated association among exocyst components and aPKC-PAR3 (Lalli, 2009) or PAR6 (Das et al., 2014) could represent another important link between stable MT and the machinery involved in membrane addition.

Membrane addition by the recycling endosome in growing axons

A number of distinct types of endosomal compartments have been described (Wandinger-Ness and Zerial, 2014). Endocytic vesicles rapidly targeted to a distinct membrane-bound endocytic organelle referred as the early endosomes (EE). From this location cargos can either traffic to late endosomes (LE) and lysosomes (Lys) for degradation or recycle directly to the plasma membrane; they may also shuttle to RE for subsequent delivery to the plasma membrane. In

neurons, endosomal pathways are involved in many important physiological processes such as receptor recycling, intracellular signalling (Yap and Winckler, 2012) and membrane turnover (Hausott and Klimaschewski, 2016) and therefore play a decisive role in axonal growth and differentiation (Tojima and Kamiguchi, 2015, Villarroel-Campos et al., 2016; Wojnacki and Galli, 2016).

Among the main regulators of endocytic pathways are the large family of RabGTPases, which localize on the surface of various intracellular compartments. By regulating its GTPase activity, Rabs temporally interacts with multiple effectors and control membrane budding, vesicle formation and movement along cytoskeleton, as well as membrane fusion at the target compartment (Li and Marlin, 2015). The most prominent RE marker to date is the small GTPase Rab11, which has a crucial role in axonal growth. Optogenetic experiments aimed to control Rab11 localization within axonal growth cones revealed that axon growth and growth cone dynamics directly depend on Rab11 functioning near the axonal tip (Van Bergeijk et al. 2015). Rab11 controls axonal growth and neurite formation in PC12 cells, adult DRG neurons, and cortical neurons. Expression of Rab11 dominant negative or its suppression inhibits axon outgrowth, whereas Rab11 constitutively active enhances axon elongation (Shirane & Nakayama 2006, Eva et al., 2010, Takano et al. 2012). Rab11 have been implicated in the recycling and surface localization of the axonal cell adhesion molecule L1/NgCAM and β1-integrin (Shirane & Nakayama 2006, Eva et al., 2010). Recent studies have begun to shed light onto the possible mechanisms underlying RE-mediated membrane addition. In this regard Rab11-L1-containing endosomes colocalize with TC10 in growth cones and neurite shafts and undergo exocytosis via an Exo70-containing exocyst complex to promote neurite outgrowth (Fujita et al., 2013). GRAB, a GEF for Rab8, which regulates trafficking of secretary vesicles from the Golgi apparatus, can bind active Rab11 and activates Rab8 to recruits it to Rab11-endosomes. This occurs in growth cones, allowing vesicles driven by Rab11 to be passed off to Rab8 for fusion with plasma membranes (Furusawa et al., 2017). Together, these results suggest a common mechanism involving the exocyst for the insertion of PPVs and RE-derived vesicles. Rab35, another resident recycling endosomes GTPase has been proposed to promote neurite outgrowth in PC12 cells and axon elongation in cultured hippocampal neurons by regulating Cdc42 activity (Chevallier et al. 2009,

Kobayashi and Fukuda, 2012, Villarroel-Campos et al, 2016b). By contrast, the RE resident GTPase, Arf6 negatively regulates axon outgrowth and trafficking of β1-integrin-containing RE (Hernandez-Deviez et al. 2004; Eva et al. 2012). During neurite outgrowth Rab35 recruits its effector, the GTPase-activating proteins (GAPs) centaurin-β2 that inhibits Arf6 activity (Kobayashi & Fukuda 2012) and MICAL-L1 which functions as a scaffold protein for Rab8, Rab13, and Rab36, resulting in multiple Rab signalling at the same time (Kobayashi et al., 2014a, Kobayashi et al., 2014b). Taken together, multiple RabGTPases and Arf6 activities cooperatively regulate trafficking of RE to control neuronal differentiation and axonal growth axon development.

Non-conventional secretory pathways in developing neurons

Unconventional membrane exocytosis has also emerged as a potential mechanism for driving neuronal membrane expansion. For example, a recent study showed that several transmembrane proteins, including neurotransmitter receptors, guidance and adhesion proteins among others, have immature glycosylation profiles at the cell surface of hippocampal neurons (Hanus et al., 2016). Besides, it was shown that core-glycosylated proteins access the cell-surface in a Golgi-independent manner. Together, these data suggest that immaturely glycosylated proteins might travel to the plasma membrane using a non-conventional secretory pathway that bypass the Golgi apparatus (Hanus et al., 2016). Newly synthesized proteins in the soma can also enter neurites through ER membranes, by lateral diffusion (Cui-Wang et al., 2012) or by active dynein mediated transport (Valenzuela et al., 2014), since the ER network is contiguous throughout axons and dendrites. Using an ER aggregation-inducible release system to synchronize exit of plasma membrane proteins through the secretory pathway, Bowen and colleagues showed, that at least the AMPA-type glutamate receptor GluA1 and the postsynaptic cell adhesion molecule neuroligin 1 accumulate in RE located in dendrites and dendritic spines following ER exit. These results suggest that RE mediates another specialized Golgi-independent exocytic trafficking pathway in neurons (Bowen et al., 2017). Another proteins may use mixed mechanisms. For example, the rectifier voltage-gated potassium channel Kv2.1, which localizes to cell surface clusters at the somatodendritic compartment and at the axonal initial

segment (AIS) uses two different trafficking pathways to reach its final localization (Jensen et al. 2017). While Kv2.1 channels destined to the soma and dendritic membrane traffic through and exit from the Golgi apparatus, those targeted to the AIS use a non-conventional Golgi independent trafficking pathway (Jensen et al. 2017).

Another recently proposed unconventional secretory mechanism involved in neurite outgrowth is a nonvesicular lipid transfer by ER-plasma membrane (PM) contact sites at growth cones (Petkovic et al., 2014, Gallo et al., 2015). The ER-resident SNAREs Sec22b and Syntaxin1, at the PM, generate a non-fusogenic bridge where lipids might flow between these compartments. Interestingly, dominant negative and knockdown of Sec22 inhibit axonal and dendritic growth suggesting the idea that nonvesicular lipid transport has a critical role in plasma membrane expansion and neuritic growth (Petkovic et al. 2014). Moreover, insertion of polyproline linkers between the SNARE and transmembrane domain of Sec22 that increases the distance between the PM and ER surface also impairs neurite growth, without affecting VSV-G secretion. Thus, non-vesicular lipid transfer mediated by non-fusogenic SNARES is another mechanism contributing to membrane expansion in .growing neurites.

Concluding remarks

There is solid published information indicating that cytoskeletal assembly, including MT and MF organization, dynamics, and its regulation by extrinsic and intrinsic factors, as well as membrane protein trafficking are essential for axon specification and the establishment of neuronal polarity. In contrast, questions about the involvement of plasmalemmal expansion in axon specification and initial elongation necessary for the establishment of neuronal polarity remain open. A good part of the published information reviewed here strongly suggest that membrane expansion at the axonal growth cone is not just a read-out of polarity but an essential step in its estabishment. This adds a new and exciting dimension to the study of membrane biogenesis and trafficking in developing neurons.

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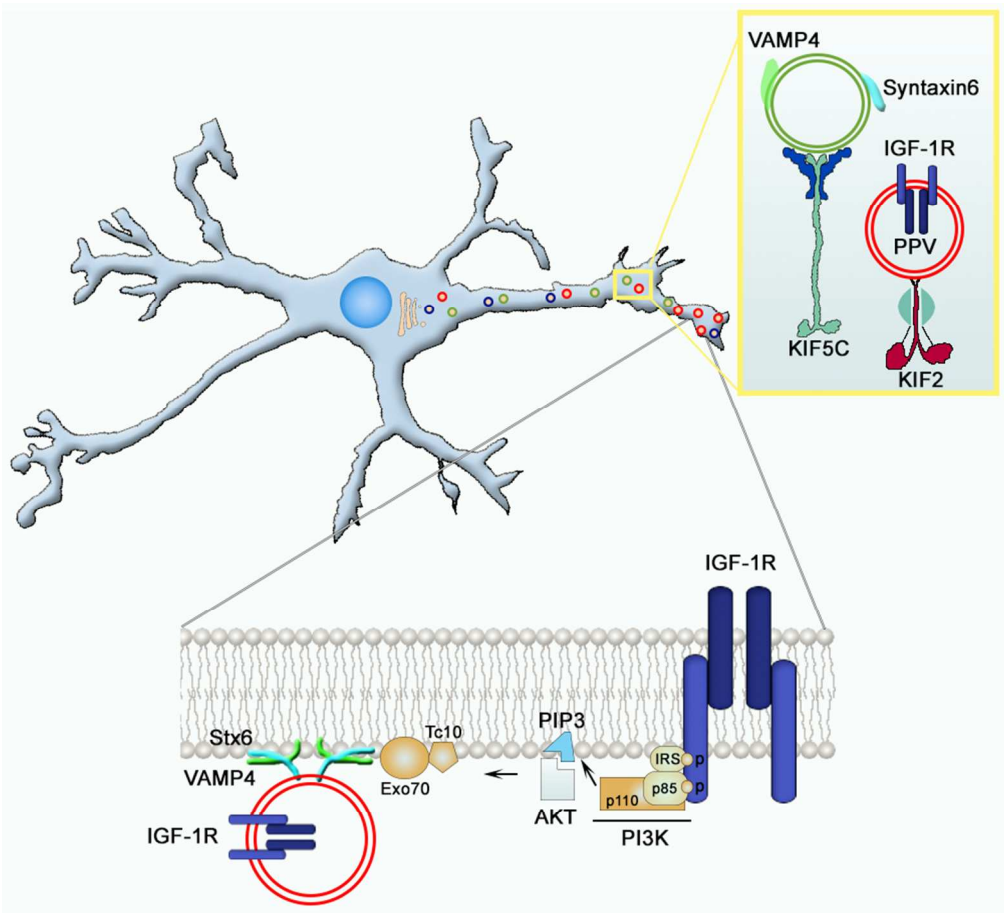
FIGURE

Figure 1: *A model for the regulation of plasmalemmal expansion in the growing axon***.** The growing neuron (top) synthesizes vesicles that contain insulin-like growth factor 1 (IGF1) receptor (IGF1R) and are transported to the axonal growth cone by a KIF2-dependent mechanism (red vesicles-Morfini et al. 1997). Moving on acetylated (stable) microtubules (Nakata and Hirokawa, 2003), the microtubular motor KIF5C transports the SNARE proteins Stx6 and VAMP4 (Oksdath et al., 2016) preferentially to the neurite where stable microtubules are enriched (green vesicles-see Jacobson et al., 2006). Other plasmalemmal precursor vesicles (PPVs) provide the bulk of new membrane (blue vesicles). Stimulation of IGF1R activates phosphoinositide 3-kinase (PI3K, comprised of a p110 catalytic and a p85 regulatory subunit) and the synthesis of phosphatidylinositol-3, 4, 5-trisphosphate (PtdIns(3,4,5)P3), which in turn increases AKT activity. This stimulates the small GTPase TC10 which can recruit the exocyst complex protein Exo70 (and most probably other proteins of the exocyst complex) to the plasma membrane and initiate vesicle (some of them containing the IGF-1R) docking (Dupraz et al., 2009). Activation of the fusion machinery (VAMP4 and syntaxin6; see Oksdath et al., 2016) subsequently triggers exocytosis. IRS, insulin receptor substrate.



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84x76mm (300 x 300 DPI)