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Neuronal polarization: From spatiotemporal signaling to cytoskeletal dynamics

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ABSTRACT

Neuronal polarization establishes distinct molecular structures to generate a single axon and multiple dendrites. Studies over the past years indicate that this efficient separation is brought about by a network of feedback loops. Axonal growth seems to play a major role in fueling those feedback loops and thereby stabilizing neuronal polarity. Indeed, various effectors involved in feedback loops are pivotal for axonal growth by ultimately acting on the actin and microtubule cytoskeleton. These effectors have key roles in interconnecting actin and microtubule dynamics – a mechanism crucial to commanding the growth of axons. We propose a model connecting signaling with cytoskeletal dynamics and neurite growth to better describe the underlying processes involved in neuronal polarization. We will discuss the current views on feedback loops and highlight the current limits of our understanding.

Contents

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1. Introduction

Neuronal cells function as complex computational units with specific input and output sites. The input is gathered by dendrites, processed, and the resulting output is relayed through the axon via action potentials to specific targets. This difference between dendrites and the axon is crucial to the function of neurons. It is a result of differences between dendrites and axons on many levels, down to the ultrastructure and molecular composition. The underlying separation of proteins is precise, with several proteins being exclusively present in one specific compartment. How can neurons establish a robustly polarized structure?

The process of neuronal polarization has been studied for decades - with dissociated hippocampal neurons being the most commonly used experimental system (Bradke and Dotti, 2000a; Arimura and Kaibuchi, 2007). The establishment of polarity in dissociated hippocampal neurons can be divided into five stages. Soon after plating, neurons develop a lamellipodium (stage 1), from which several small neurites emerge within a few hours (stage 2). Alternating neurites extend in brief growth phases while keeping similar lengths, until one of them breaks the symmetry, usually within 24 h after plating. This neurite grows for an extended period of time and will become the axon, while the other neurites pause (stage 3). Within one week, the remaining neurites branch and later develop into dendrites (stage 4). Eventually, the axon matures further and may form axonal branches, while synapses form (stage 5) (Dotti et al., 1988). Neuronal polarity in dissociated neurons is established without directional external cues - in a stochastic manner. This indicates the cell-inherent capability of these cells to polarize and the convenience of hippocampal neurons to study these mechanisms.

What drives cell-autonomous neuronal polarization? This review covers the connection between signaling pathways and cytoskeletal dynamics during neuronal polarization. For this purpose, we will first integrate feedback loops and axonal growth in one model of neuronal polarization. In turn, we will go into the details of the proposed model. We will highlight feedback loops as a concept responsible for robust induction of neuronal polarity. This will be followed by a discussion of cytoskeletal mechanisms during axonal growth. Lastly, we connect feedback-loop effectors to cytoskeletal dynamics and review actin waves as a mechanism for the induction of neuronal polarity.

2. Our concept of polarization

Axonal specification of a given neurite can be characterized by three sequential processes: initial molecular polarization, axonal growth, and complete molecular polarization. In stage 2, one or two neurites show an initial molecular polarization, which may or may not fluctuate between different neurites. This initial molecular polarization can probably be stabilized by neurite growth. Indeed, neurite growth is necessary for stabilization of KIF5C accumulation and in turn for complete molecular polarization (Yamamoto et al., 2012). This molecular polarization at first is not restricted to axon-specific factors but rather seems to be a vectorial flow (Bradke and Dotti, 1999). If this model should be true, it should not only explain physiological axon specification but also the development of multiple axons, induced by different means.

2.1. Induction of multiple axons by various manipulations

Different manipulations can induce multiple axons: (1) overexpression of polarity effectors, like Par3 (Schwamborn and Püschel, 2004), (2) overexpression of constitutively active or hyperactive polarity effectors, like Rap1 and Cdc42 respectively (Schwamborn and Püschel, 2004), and (3) bath application of drugs that stabilize microtubules or destabilize the actin cytoskeleton, like Taxol (Witte et al., 2008) and CytoD, respectively (Bradke and Dotti, 1999). Destabilizing actin

(Bradke and Dotti, 1999) or stabilizing microtubules (Witte et al., 2008) in a single neurite, or stretching a neurite with mechanical force (Lamoureux et al., 2002), induces axonal specification of this neurite. Of note, even in stage 3 neurons, destabilization of the actin cytoskeleton (Bradke and Dotti, 2000b), stabilization of microtubules (Gomis-Rüth et al., 2008) or stretching force (Lamoureux et al., 2002) can induce multiple axons or a second axon, respectively. Microtubule stabilization may induce axonal specification and multiple axons by increasing anterograde transport. Actin destabilization and mechanical force, on the other hand, probably do not have a direct impact on anterograde transport.

The fact that both, mechanical stretching and actin destabilization can induce axon specification in stage 2 and stage 3 neurons indicates a common effect triggered by both. One common effect is increased neurite length. This would support the idea that neurite growth is the crucial step for axon specification. But how can multiple axons and the induction of a second axon, after axon specification already took place, be explained?

2.2. Neurite growth as the putative requirement for multiple axons

Axon specification can be viewed as a process that requires a certain stable concentration of polarity effectors in a neurite. Usually, one neurite alone claims most of the polarity effectors and becomes the axon. Polarity effectors probably only accumulate in one neurite due to the efficiency of the feedback-loop network. This network keeps one neurite in a growth promoting state, while the others are maintained in a growth-inhibitory state (Fig. 3). In the following paragraphs we will explain how this model can explain the induction of multiple axons under different conditions.

2.2.1. Mechanical force and destabilization of the actin cytoskeleton

Applying mechanical force or destabilizing the actin cytoskeleton converts all neurites from a growth-inhibitory state into a growth-promoting state. During this process, polarity effectors accumulate similarly in all neurites. If the concentration of polarity effectors, necessary for axon specification, is only a fraction of the total amount within the cell, all neurites could surpass this concentration and thereby become an axon. This could explain the induction of multiple axons in the framework of one model.

2.2.2. Overexpression of polarity effectors

Overexpression of polarity effectors increases their availability and thereby prevents negative feedback on minor neurites. In turn, these overexpressed effectors might accumulate faster in more neurites, increase their growth and ultimately induce multiple axons. However, an alternative explanation for multiple axons in overexpression systems can be an adaptive increase in expression of many different factors by the cell. The concentration of a variety of polarity effectors would no longer be limited. Consequently, this would prevent the negative feedback on minor neurites.

2.2.3. Multiple axons after axon specification

Induction of multiple axons after axon specification could be caused by a dynamic regulation of the transport of polarity effectors. In turn, the transport may not be fixed to the axon. Changing the length of neurites could thus change transport. Therefore, even after one axon is specified, a length increase in another neurite may serve as a sufficient drive to redistribute polarity effectors. While neurite growth usually is inhibited in minor neurites after axon specification, this inhibition can be relieved by for example actin destabilization. This would drive the redistribution of polarity effectors and in turn the induction of a second axon or multiple axons.

3. Feedback loops enable robust neuronal polarization

During neuronal polarization, a spherical cell develops into a highly-compartmentalized cell with clearly defined borders between dendrites and the axon. The underlying signaling pathways generate a high morphological and molecular contrast between dendrites and the axon. High-contrast is crucial for a variety of biological phenomena on very different levels, including vision and cell differentiation. One effective way to accomplish high contrast of two processes is by generating a bi-directional negative feedback between both processes. In such a system, small, stochastic differences between the responses of both processes are amplified into an all-or-nothing response. For neuronal polarity, this means to break the symmetry at the transition to stage 3 by amplifying the difference of spatially confined signals within one neurite, the future axon, versus the other neurites, the future dendrites.

3.1. Local activation-global inhibition: the neurite length-dependent feedback

The first feedback loop to be proposed was solely based on neurite length. Goslin and Banker cut the longest neurite shortly after it outgrew the others and monitored which of the other neurites would become the longest and in turn the axon (Goslin and Banker, 1989). They found that whenever one neurite exceeded all other neurites by 10 μm just after cutting, it would almost always (96% of cases) become the new axon. However, if this criterion was not met, it was not possible to predict which neurite would become the axon. This indicates that neurite length acts as self-promoting feedback to enable axon generation. The authors mention one possible explanation: a growth-promoting protein that is available in limited amounts. This protein should be transported actively to the growth cone, while diffusing back to the soma. Since the rate of retrograde diffusion would be lower in longer neurites, the concentration of the protein would be higher, the longer the neurite. However, since the protein would only be available in limited amounts, an increased concentration in one neurite would lead to a reduced amount in other neurites. Thus, the growth would be increased in one neurite (local activation) but reduced in all other neurites (global inhibition). In fact, the increase of growth speed of one neurite during polarization results in a reduction of growth speed of the other neurites, supporting this model. Similar loops appear to act not only in neurons that undergo initial neuronal polarization but also in neurons that formed functional synapses and are well integrated in synaptic circuits, as these neurons transform a dendrite to an axon when cut close to the cell body (Gomis-Rüth et al., 2008).

To participate in these feedback loops, a certain factor (1) should be transported anterogradely to accumulate in the axonal growth cone, where it (2) should promote neurite growth. Several factors that fulfill these criteria have been identified:

3.1.1. Shootin1

Shootin1 fuels axonal growth by a neurite-length dependent increase of anterograde transport that further increases neurite-growth by force generation in the growth cone (Toriyama et al., 2006). While Shootin1 accumulation fluctuates between different neurites during stage 2, it stably accumulates in the future axon during stage 3. Knock down of Shootin1 inhibits polarization and localization of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on axonal tips (Toriyama et al., 2006). Shootin1 is transported by actin waves to growth cones (Winans et al., 2016; Toriyama et al., 2006), which causes the fluctuations in its localization. Actin waves are periodically occurring waves moving along the neurite shaft that are associated with protein transport and increased neurite outgrowth (Winans et al., 2016; Flynn et al., 2009; Toriyama et al., 2006). Shootin1 is regulated by p21-mediated kinase (PAK1)-mediated phosphorylation (Toriyama et al., 2013), which is a downstream target of Ras-Related C3 Botulinum Toxin Substrate 1 (Rac1) (Brown et al., 1996) and implicated in neuronal polarity

(Jacobs et al., 2007). However, the physiological role of Shootin1 during neuronal polarization is still unclear.

3.1.2. HRas

HRas is implicated in the promotion of neurite growth through a positive feedback loop involving PI3K. Knockdown of HRas inhibits axon specification (Fivaz et al., 2008). HRas induces axon specification by activating phosphoinositide 3-kinase (PI3K) (Yoshimura et al., 2006). In polarized neurons, HRas activity is increased in the tip of the axon, while inhibiting its downstream target PI3K abolishes this activity (Fivaz et al., 2008). This indicates the presence of a positive feedback loop between HRas and PI3K (Fig. 1, I). Moreover, HRas is recruited to the tip of the longest neurite by vesicular transport, in turn reducing the HRas concentration in minor neurites (Fivaz et al., 2008). The C-terminal palmitoylation motif in HRas is responsible for its axonal localization. This motif recruits proteins to the axon, hinting at a general sorting mechanism. (El-Husseini et al., 2001; Chai et al., 2013). However, neither the mechanism of axonal recruitment nor of HRas activation by PI3K is known. It is possible that HRas is recruited by PI3K through retrograde signaling of tropomyosin receptor kinase B (TrkB), which is involved in the brain derived neurotrophic factor (BDNF)-cyclic adenosine monophosphate (cAMP) - protein kinase A (PKA) loop described below (Cheng et al., 2011a). To date, the physiological role of the HRas - PI3K feedback loop is unclear.

3.1.3. BDNF-cAMP-PKA loop

BDNF activates TrkB and thereby adenylyl cyclase. This leads to increased production of cAMP, which activates PKA (Cheng et al., 2011a) and probably exchange protein directly activated by cAMP (Epac) (Muñoz-Llancao et al., 2015). This BDNF-induced signaling in turn increases the secretion of BDNF and TrkB insertion in the cell membrane. The positive feedback loop involving BDNF is depicted in Fig. 1, II. Importantly, TrkB signals to PI3K, which increases anterograde transport of TrkB from the soma. This indicates a mechanism by which a limiting pool of TrkB within the cell would be concentrated in the future axon. Indeed, while all neurites are able to secret BDNF when stimulated with BDNF in early stages, only the future axon is able to do so in stage 3 (Cheng et al., 2011a). Moreover, PKA (Shelly et al., 2010) and EPAC (Muñoz-Llancao et al., 2015) are important regulators of neuronal polarity via several signaling pathways. Besides TrkB receptors, the IGF-1 receptor was proposed to be involved in a positive feedback loop involving PI3K and regulating IGF-1 receptor insertion (Dupraz et al., 2009).

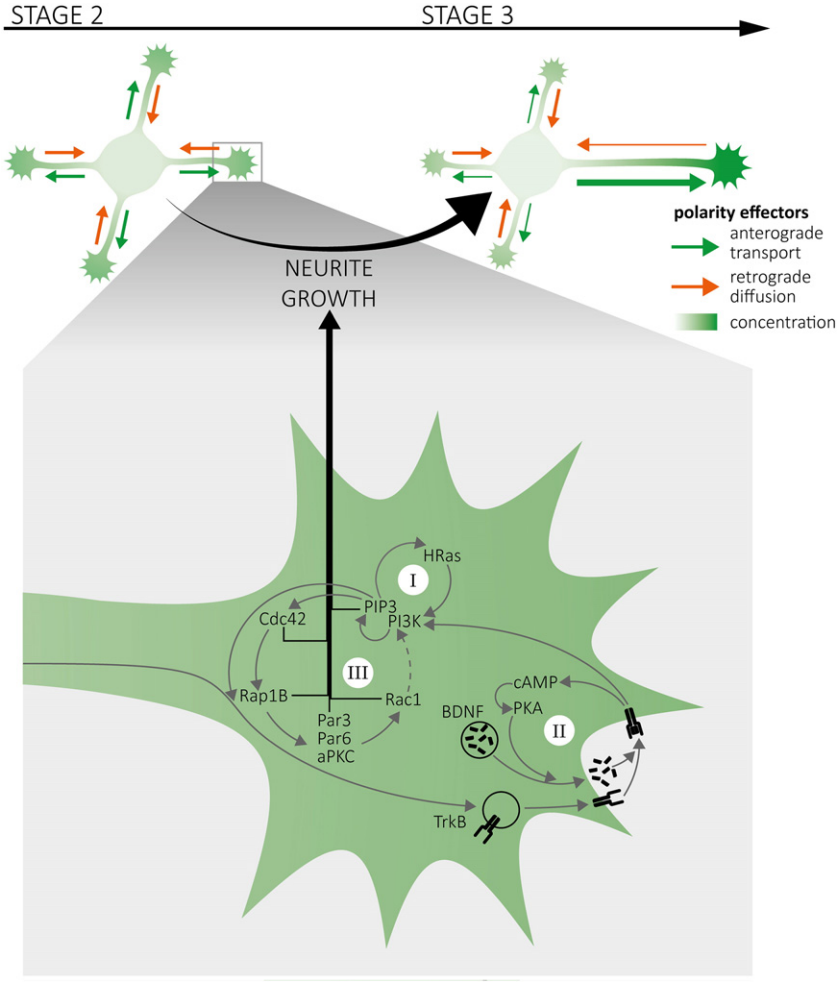

Fig. 1. Feedback loops during neuronal polarization. In stage 2, all neurites are approximately the same length. At one point, one neurite extends more than the others, marking the entry to stage 3. This is thought to be accomplished by a neurite-length dependent redistribution of polarity effectors that are available at limited quantity. Polarity effectors are transported anterogradely into the growth cones and diffuse back retrogradely. Retrograde diffusion is lower for longer neurites, thus increased growth leads to increased accumulation. Moreover, anterograde transport is increased upon axonal growth. Polarity effectors themselves increase growth and thereby fuel the positive feedback loop. Since polarity effectors are limited in quantity, accumulation in the growth cone of the future axon leads to a lower concentration in minor neurites. In turn, minor neurites grow less. To initiate the initial neurite growth, necessary to increase the accumulation of polarity effectors, small signaling fluctuations are thought to be increased by signaling feedback loops (I–III), depicted in the lower panel. The product of PI3K activity (PIP3), Rap1B, Cdc42, Par3 and Rac1 increase axonal growth by mechanisms illustrated in Fig. 2. Abbreviations: ras-related C3 botulinum toxin substrate 1 (Rac1), Cell division control protein 42 homolog (Cdc42), Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), Ras-related protein 1B (Rap1B), phosphoinositide 3-kinase (PI3K), tropomyosin receptor kinase B (TrkB), brain derived neurotrophic factor (BDNF), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA).

This indicates that an increase in receptor activation by a positive feedback loop might be a general mechanism during neuronal polarization. Nevertheless, knockout models of TrkB appear not to have an effect in neuronal polarization (Klein et al., 1993). The physiological role of the IGF-1 receptor in neuronal polarization remains to be shown in knockout models.

3.1.4. Amplification of local activation by signaling feedback loops

The neurite-length dependent feedback loop relies on the neurite-length dependent accumulation of growth-promoting factors. If these factors are implicated in growth-promoting signaling feedback-loops, this could further amplify the local increase in growth. As described above, HRas and BDNF indeed are part of positive feedback loops (Fivaz et al., 2008, Cheng et al., 2011a). Interestingly, both feedback loops activate PI3K, suggesting a synergistic interplay of both pathways and thus increased activation of PI3K. Importantly, downstream targets of PI3K play an important role in increasing neurite growth (Section 4). In summary, accumulation of growth-promoting factors might increase neurite growth supra-linearly by promoting local signaling feedback loops.

3.1.5. Global inhibition: antagonistic role of cAMP and cGMP in distant neurites

It was proposed that long-range growth-inhibition of other neurites is caused by an inhibitory factor (Shelly et al., 2010). However, experimental results that suggested this, can be explained without the presence of an inhibitory factor. In detail, it was found that stimulation of one neurite with the adenylyl cyclase activator forskolin, to locally induce cAMP production, leads to a reduction in cGMP in this neurite. Unstimulated, distant neurites on the other hand show the opposite effect. Most often, the forskolin-stimulated neurite becomes the axon. Instead of an inhibitory factor, neurite-length dependent feedback mechanisms could mediate these effects. An increase in cAMP levels would lead to BDNF release, activating TrkB, and in turn leading to PI3K activation and TrkB accumulation (Section 3.1.3). Since the HRas feedback loop also signals via PI3K, this would reduce the concentration of both targets in the other neurites, thereby reducing the baseline activity of the feedback loop. In turn, cAMP levels and PKA activity would be decreased. Experimental results could support this hypothesis: the onset of the reduction of cAMP in the other neurites is after 9 min, steadily falling until 18 min (Shelly et al., 2010). The transport of TrkB could be estimated by looking at the transport of HRas, as this is also specifically recruited in the axon (Fivaz et al., 2008). The increase in HRas concentration reaches 60% after 8 min, while the maximum is reached after over 15 min (Fivaz et al., 2008). A similar reduction of PKA activity in other neurites after 10 min was also observed when locally applying BDNF beads to one neurite, but to a lower extent (Cheng et al., 2011a). While this model is very appealing, the different time constants remain to be experimentally measured. Subsequently, the process should be modeled to show its accuracy. Moreover, its physiological relevance during neuronal polarization needs to be tested.

3.1.6. Influence of adhesion-induced signaling

Adhesion molecules, like laminin, can activate signaling pathways that speed up axon specification (Lein et al., 1992). Interestingly, the effect of laminin on neurite growth is a good indication of the neurite-length dependent feedback loop. Addition of laminin first increases growth of all neurites (Lochter and Schachner, 1993). Once one neurite outgrew the other neurites, growth of minor neurites stalls, while the major neurite continues to grow faster (Lochter and Schachner, 1993). Stalling of the growth of minor neurites seems to occur later without laminin addition. How is the preferred increase of growth in the major neurite by laminin accomplished? Laminin increases neurite growth through integrin-beta 1 (Itgb1) (Lei et al., 2012). Itgb1 signaling is increased in the nascent axon, probably due to Itgb1-accumulation in the axon (Easley et al., 2006). The resulting increase in axon growth probably accelerates accumulation of polarity effectors in the axon, and consequently depletion of polarity effectors in minor neurites. This could be the reason for the stalled growth of minor neurites, and would support the proposed neurite-length dependent mechanism of global inhibition. Importantly, also other adhesion molecules, like tenascin and fibronectin, influence neurite growth similarly to laminin (Lochter and Schachner, 1993, Lochter et al., 1995).

In summary, adhesion-induced acceleration of axon specification exemplifies the effect of evenly distributed extracellular cues on axon specification. This indicates that the multitude of extracellular signals in vivo, even if not spatially restricted, can speed up axon specification. The effects of laminin addition on cultured, undifferentiated neurons is a good experimental indication of the neurite-length dependent feedback.

3.1.7. Modelling the loop

Until now, we focused on the role of growth-promoting factors in feedback loops, which rely on anterograde transport and retrograde diffusion. However, growth-inhibiting factors could also play a role in establishing neuronal polarity via feedback loops. Indeed, overexpression studies suggest that Ras homolog gene family, member A (RhoA) is a negative regulator of neuronal polarity and is degraded in the axonal growth cone during neuronal polarization (Cheng et al., 2011b). Despite much work on RhoA and its role during neuronal polarization, its physiological role is still unclear. The effectors that trigger degradation might be available to neurites in limited amounts. Accumulation in the axonal growth cone would thus lead to a reduction in minor neurites. In turn, inhibiting factors would accumulate in minor neurites due to reduced degradation. Another, more direct mechanism has been proposed for a length-dependent negative feedback in the shorter neurites. For this, the inhibiting factor would anterogradely diffuse to the neurite tip from which it would be actively transported retrogradely back to the soma. However, to date no growth-inhibiting factor that is transported retrogradely has been found to drive neuronal polarity.

Several mathematical models showed that a positive regulator is sufficient to develop precisely one axon (Samuels et al., 1996, Fivaz et al., 2008, Toriyama et al., 2010, Naoki et al., 2011). For a detailed review of the different models see Inagaki et al., 2011. In a more recent model, anterograde transport, retrograde diffusion and degradation of effectors were included to model neuronal polarization (Naoki et al., 2011). This model can explain numerous experimental findings – like the loss of polarity upon knockdown of the kinesin KIF5C (Nariko et al., 2005), or upon inhibition of the proteasome system (Yan et al., 2006). Comparison of two earlier models (Samuels et al., 1996, Fivaz et al., 2008) using experimental data, indicated that the data did not fit well to any of the models (Wissner-Gross, 2012). However, these models do not include back diffusion or active transport of the polarity effector. Incorporating more recent models that include those mechanisms (Toriyama et al., 2010, Naoki et al., 2011) could have led to a better fit. In summary, the neurite-length dependent feedback probably involves various mechanisms – including transport, diffusion and degradation. Mathematical models help to explain whether the interplay between those mechanisms is sufficient to cause neuronal polarization. The robustness of the multitude of mathematical models exemplifies the robustness of neuronal polarization. However, experiments need to test whether the neurite-length dependent feedback is necessary for neuronal polarization.

3.2. Fueling neurite-length dependent feedback by trafficking

The neurite-length dependent feedback loop relies on the anterograde transport of effectors. An increase in anterograde transport in longer neurites would speed up the enrichment of effectors in the future axon and depletion of effectors in the minor processes. Importantly, several polarity effectors are transported anterogradely to the growth cone

by kinesins: Par3 and PIP3 by KIF3 (Nishimura et al., 2004, Horiguchi et al., 2006), Adenomatous polyposis coli (APC), Kinesin Associated Protein 3 (KAP3) and Collapsin response mediator protein 2 (CRMP2) by KIF5 (Kimura et al., 2005, Ruane et al., 2016). Membrane proteins, like the receptors IGF-1 receptor or TrkB, are transported in plasmalemmal precursor vesicles (PPVs) to neurites (Morfini et al., 1997, Arimura et al., 2009, Huang et al., 2011). Interestingly, membrane transport and transmembrane receptors like TrkB are polarized to the neurite that later develops into the axon (Gärtner et al., 2014). Indeed, anterograde transport of soluble proteins and vesicles to the nascent axon is increased (Bradke and Dotti, 1997). In specified axons, this transport is cargo-selective due to several filters and sorting mechanisms (Galiano et al., 2012, Maniar et al., 2011, Yau et al., 2014, Van Beuningen et al., 2015, Del Castillo et al., 2015). However, in unspecified neurites, anterograde transport seems to be an unspecific vectorial flow (Bradke and Dotti, 1997).

What mechanisms could drive the increase of the vectorial flow to the nascent axon? Different mechanisms seem to increase anterograde transport in the nascent axon: (1) increased number of microtubules, (2) unified directionality of microtubules and (3) post translational modification of microtubules.

3.2.1. Unified directionality and increased number of microtubules

Increased number and enriched plus-end-out orientation of microtubules in the nascent axon could play an important role in increasing anterograde transport. The number of microtubules is higher in the shaft of the nascent axon compared to minor neurites (Yu and Baas, 1994, Seetapun and Odde, 2010). Moreover, microtubules within neurites at stage 2 are bidirectionally oriented (Yau et al., 2016). Upon neuronal polarization, the amount of plus-end-out oriented microtubules gradually increases, until they are the sole species of microtubules in the axon (Yau et al., 2016, Kapitein and Hoogenraad, 2010). How is the directionality of microtubules regulated? An active mechanism based on TRIM46 starts to stabilize unidirectionality of microtubules only after an axon is already specified (Van Beuningen and Hoogenraad, 2016). A mathematical model, developed by Seetapun and Odde (2010), proposes a passive mechanism for the enrichment of the number of microtubules and microtubules with plus-end-out directionality. This model only relies on increased neurite length, it does not involve any other molecular mechanisms. However, the model does not provide a compelling explanation for the presence of entirely unidirectional plus-end-out microtubules in the axon. However, predictions of the model for the neurite-length dependent accumulation of microtubules are consistent with current data.

3.2.2. Post-translational modifications of microtubules

Post-translational modifications of tubulin could contribute to the increased anterograde trafficking in the nascent axon by influencing binding of kinesins to microtubules. Indeed, kinesin binding during axon specification is differentially regulated in the nascent axon and minor neurites, as indicated by kinesin overexpression studies. While KIF5C (kinesin 1) accumulates in the tip of the nascent axon, KIF1A is distributed to all neurite tips similarly (Jacobson et al., 2006). Accumulation of kinesin 1 can be redirected from the axon to all neurites by taxol treatment (Hammond et al., 2010). As taxol influences posttranslational modifications of microtubules, these modifications may be causal for axon-specific transport. In the nascent axon, post-translational modifications are increased compared to minor neurites (Witte et al., 2008, Hammond et al., 2010). Specifically, microtubules in the nascent axon show increased acetylation (Witte et al., 2008, Hammond et al., 2010) and de-tyrosination (Hammond et al., 2010). Tyrosinated tubulin inhibits and acetylated tubulin promotes kinesin 1 binding (Konishi and Setou, 2009, Hammond et al., 2010). Thus, both modifications lead to increased kinesin binding in the nascent axon. Apart from post-translational modifications, kinesin 1 binds stronger to GTP-tubulin and is increased in the nascent axon (Nakata et al., 2011). Importantly, increasing solely tubulin acetylation, by histone deacetylase (HDAC) inhibitors, is not sufficient to redirect kinesin 1 accumulation to all neurites. Interestingly, the ability to redirect kinesin 1 to all neurites correlates with the induction of multiple axons, as taxol induces multiple axons and HDAC inhibitors do not (Witte et al., 2008). Since taxol treatment redirects kinesin 1 to all neurites, several posttranslational modifications might be necessary to effectively regulate kinesin-binding in neurons.

3.2.3. Enhancing the effect of increased transport by local translation

All mathematical models of neuronal polarity assume a constant expression rate of polarity effectors and anterograde trafficking as the sole mean of protein accumulation at neurite tips. However, mRNA can also be transported anterogradely to the neurite tip, to be in turn translated there (Preitner et al., 2014, Perry et al., 2016). This would result in an additional trafficking-dependent accumulation of proteins that increase neurite growth. One of the targets that transports mRNA to the growth cone is APC (Preitner et al., 2014). APC is not only transported to the growth cone by kinesins (see above), but is also involved in neuronal polarization and axon growth (Zhou et al., 2004, Shi et al., 2004) (see Sections 4.7.4, 4.7.5). Among many other mRNAs, APC transports the mRNA for beta2B-tubulin, which itself is important for axon growth (Preitner et al., 2014). In addition to beta2B-tubulin, the polarity effector Par3 is locally translated in the growth cone (Hengst et al., 2009). Local translation of Par3 is activated by the growth factors Netrin-1 and NGF (Hengst et al., 2009). In summary, local translation of neurite-growth promoting factors might amplify the effect of increased axon-directed transport on axon specification.

3.3. Executing the signals: the PI3K signaling cascade

Several central regulators of neuronal polarity are part of the same signaling cascade (PI3K signaling cascade) that executes signals of the mentioned feedback loops (Fig. 1, III). The PI3K signaling cascade comprises PI3K, Ras-related protein 1B (Rap1B), the Par complex and the RhoGTPases Cell division control protein 42 homolog (Cdc42) and Rac1. These master regulators are connected to different networks of downstream effector molecules of neuronal polarity (reviewed in Namba et al., 2015). In addition, different feedback loops act via PI3K and thus activate the PI3K signaling cascade (see Sections 3.1.1, 3.1.3 and 3.1.4). Thus, the PI3K signaling cascade could act as an executer of different feedback loops.

The signaling cascade from PI3K via Cdc42 and the Par complex to Rac1 has been investigated in different studies (Bokoch et al., 1996, Schwamborn and Püschel, 2004). To date, one study showed that expression of constitutively active Rap1B rescues defects in polarity caused by PI3K inhibition (Schwamborn and Püschel, 2004). This could suggest that Rap1B is a downstream target of PI3K and thus part of the described signaling cascade. Alternatively, Rap1B may not be a downstream target of PI3K but may activate the PI3K downstream effector Cdc42 (Namba et al., 2015).

Interestingly, PI3K downstream targets may also activate PI3K, opening the possibility of a positive feedback loop. This is supported by the increased activity of PI3K in primary neurons upon expression of constitutively active variants of its downstream targets Rap1B and Cdc42 (Schwamborn and Püschel, 2004). Moreover, in growth cones of PC12 cells, a feedback loop between PIP3 and the RhoGTPases Cdc42 and Rac1 is reported (Aoki et al., 2005, Aoki et al., 2007). In non-neuronal cells, PI3K is directly activated by its downstream targets Cdc42 and Rac1 (Zheng et al., 1994, Bokoch et al., 1996, Yang et al., 2012). During axon specification, accumulation of PIP3 in the growth cone of the nascent axon may further support the presence of the PI3K feedback loop to fuel this accumulation (Shi et al., 2003, Arimura et al., 2004, Ménager et al., 2004). However, PIP3 accumulation was not proven to depend on a feedback loop involving the Par complex and RhoGTPases. Alternatively, the accumulation of PIP3 could be caused

by HRas (Fivaz et al., 2008) (Section 3.1.2) or by a neurite-length dependent increase of vesicular transport (Section 3.2) of PIP3 (Horiguchi et al., 2006).

In summary, the importance of a positive feedback loop between PI3K, the Par complex and RhoGTPases for neuronal polarity has yet to be proven in primary neurons. It is possible that a negative feedback, e.g. from GTPase-activating proteins (GAPs) to Cdc42 and Rac1, prevents signal amplification of the PI3K feedback loop (Arimura and Kaibuchi, 2007). Further experiments will be necessary to directly address the role of this feedback loop during neuronal polarization. Independent of its role as a feedback loop, the PI3K signaling cascade could connect the main signaling hubs of neuronal polarity, and thus might trigger their coordinated activation. It should be noted that some parts of the PI3K signaling cascade rely on overexpression studies. In fact, Cdc42 KO neurons do not show a change in Rac1 activation (Garvalov et al., 2007). Moreover, Par3 and Par6 do not regulate neuronal polarity in flies (Rolls and Doe, 2004). Further studies using knockout models will help to reveal the physiological relevance of this signaling cascade and its potential role as a feedback loop.

3.4. Neurite-length dependent global inhibition

Until now, we focused on neurite-length dependent local accumulation and global depletion of polarity effectors to increase neurite length. However, neurite growth is also restricted by a neurite-length dependent signal in the soma (Rishal et al., 2012, Albus et al., 2013). This signal restricts the maximum neurite length through kinesin and dynein (Rishal et al., 2012). Interestingly, knockdown of kinesin and dynein both result in an increased maximum neurite length (Rishal et al., 2012). This suggests that kinesin and dynein are both promoting the signal in the soma. Based on this data, it is possible that an effector is transported anterogradely to the neurite tip, where the effector is changed, to be retrogradely transported to the soma. A mathematical model confirmed the feasibility of this mechanism and predicted the inhibition of the anterograde transport by the retrogradely transported changed effector (Rishal et al., 2012). From start of the anterograde transport to finishing of the retrograde transport, there is a time delay. Due to this delay, the model showed that the inhibition of the anterograde transport varies periodically and leads to a sinusoidal variation of the signal at the soma (Rishal et al., 2012). The time delay depends on the length of the transport way, which is determined by the neurite length. Thus, the frequency of the signal at the soma decreases when the neurite elongates.

The main effector for the signal in the soma is importinBeta mRNA which is translated at the neurite tip (Perry et al., 2016). ImportinBeta is bound by nucleolin, which permits the association and the transport to the neurite tip by kinesins. In the soma, importinBeta may regulate neurite outgrowth by regulating gene expression. Blocking the association of nucleolin with KIF1A increases total outgrowth of all neurites already after 2 h of neurite growth in DRG neurons (Perry et al., 2016). This indicates that the mechanism does not only restrict the maximum neurite length but also early neurite growth. The influence of this mechanism on neuronal polarization has not been investigated. However, due to the reduction of axonal growth, it is possible that this mechanism slows down the specification of an axon. Further experiments and careful modelling will be necessary to unravel the role of the neurite-length dependent global-inhibition in axon specification.

3.5. Is the neurite-length dependent feedback loop necessary for neuronal polarization?

The described mathematical models of neuronal polarization rely on the contribution of neurite length as the driving force for an asymmetric accumulation of factors. However, before a single neurite starts growing more than the others (stage 2), several polarity effectors already accumulate in this neurite – for example activated IGF-1 receptor (Dupraz et al., 2009), activated cofilin (Garvalov et al., 2007), Rap1 (Schwamborn and Püschel, 2004), plasma membrane ganglioside sialidase (PMGS) (Silva et al., 2005), Liver Kinase B1 (LKB1) and Ste20 Related Adaptor (STRAD) (Shelly et al., 2007). We will refer to the accumulation of such effectors as initial molecular polarization. Morphologically polarized neurons (stage 3) in turn show an accumulation of several additional effectors in the nascent axon, for example dephospho-Tau (Mandell and Banker, 1996), CRMP2 (Inagaki et al., 2000), phosphorylated (inactivated) Glycogen synthase kinase 3 beta (GSK3beta) (Jiang et al., 2005), ILK (Guo et al., 2007), Par3 (Shi et al., 2003) and HRas (Fivaz et al., 2008). Initial molecular polarization, without concomitant axon growth, might be triggered by signaling feedback loops. These feedback loops could restrict the molecular changes to one neurite – possibly by a length-independent increase in transport. Indeed, in a third of stage 2 neurons, microtubule stabilization is increased in a single neuron (Witte et al., 2008), which could serve as a molecular basis for increased transport. However, initial molecular polarization without axonal growth is probably not sufficient to complete molecular polarization, like dephospho-Tau accumulation. When neurons were physically prevented from growing neurites longer than 20 μm, not a single neuron developed a dephospho-Tau positive neurite (Yamamoto et al., 2012). This suggests that there is an initial molecular polarization in one neurite that is predictive for axon development, but only when one neurite outgrows the other neurites, is molecular polarization complete. Thus, axonal growth seems to be imperative for complete molecular polarization. Disturbing axonal growth by the knockout of factors implicated in axonal growth noticeably reduces the number of neurons polarizing (Garvalov et al., 2007, Tahirovic et al., 2010). This exemplifies that factors that play a crucial role in axonal growth might have the inherent ability to contribute to neuronal polarity. This raises the important questions how axons grow and how this growth is regulated.

4. Axonal growth

4.1. Cytoskeleton in the growth cone

Axons grow at their tip, the growth cone. This is a highly dynamic structure able to sense and integrate a variety of signals to direct the axon to its target. Signals controlling axonal growth eventually converge on the microtubule and actin cytoskeleton (Lowery and Vactor, 2009). For an in-depth explanation of cytoskeletal dynamics see other reviews on actin (Pollard, 2016) and microtubules (Kapitein and Hoogenraad, 2015, Coles and Bradke, 2015). Structurally, the growth cone is highly compartmentalized, consisting of a central domain (C-domain), a peripheral domain (P-domain) and an intermediary transition zone (T-zone) (Schaefer et al., 2002, Dent and Gertler, 2003). The C-domain mainly comprises microtubule bundles entering the growth cone from the axon shaft (stable microtubules) (Dent and Gertler, 2003). The P-domain is dominated by dynamic actin structures: lamellipodia and filopodia, and a few microtubules (Dent and Gertler, 2003). The microtubules in the P-domain are sensitive to microtubule depolymerizing drugs, suggesting that they are highly dynamic (Bamburg et al., 1986). In the T-zone, myosin II-induced contraction of F-actin generates condensed actomyosin structures – called actin arcs (Dent and Gertler, 2003). Myosin II activity in actomyosin structures induces a retrograde-directed pulling force against the actin cytoskeleton. In addition, peripheral actin polymerization leads to a leading edge-directed force. Due to high membrane tension (Craig et al., 2012), however, this leading edge-directed force does not deform the membrane but instead exerts to a retrograde–directed force. Both, actomyosin-induced pulling force and peripheral actin polymerization lead to a retrograde-directed force that pulls actin into the T-zone (retrograde flow) (Dent and Gertler, 2003, Medeiros et al., 2006). There, actin filaments are severed into smaller pieces to be again available as G-actin for polymerization at the tip of the filament (Dent and Gertler, 2003). How are the actin

and microtubule cytoskeleton within the growth cone restructured to move the growth cone forward?

4.2. Generation of force during axonal growth

Growth cone advance happens in three phases: protrusion, engorgement and consolidation (Lowery and Vactor, 2009). It is still unclear which mechanisms drive force generation to enable axon growth. There are two main hypothesized mechanisms for force generation during neurite growth. The clutch hypothesis relies on generation of traction forces between the growth cone and the extracellular-matrix cell-adhesion molecules. Actin filaments anchor at adhesion sites in the P-domain which uncouples the actin filaments from retrograde flow (Mitchison and Kirschner, 1988). In turn, peripheral polymerization of actin filaments induces protrusions, while myosin II pulls the T-zone closer to the adhesion site, thereby generating traction forces (protrusion phase) (Mitchison and Kirschner, 1988). During this process, actin arcs reorient in the direction of growth (Schaefer et al., 2008). Stable microtubules can then grow up to the new adhesion site (engorgement phase) and the axon shaft is elongated by bundling of microtubules in the former C-domain (consolidation phase) (Lowery and Vactor, 2009). Several studies show evidence for the clutch hypothesis and thus traction forces in Aplysia bag cell neurons (Suter et al., 1998, Schaefer et al., 2008, Hyland et al., 2014) and in mammalian hippocampal neurons (Bard et al., 2008, Toriyama et al., 2013, Garcia et al., 2015).

However, experimental evidence supports the notion of additional mechanisms of force generation. In hippocampal neurons, growth spurts do not correlate with traction (Koch et al., 2012). Moreover, established neurites can still grow without filopodia and lamellipodia (Marsh and Letourneau, 1984, Bentley and Toroian-Raymond, 1986, Bradke and Dotti, 1999), structures necessary for actin-mediated force generation. Normally, microtubules are intimately connected to actin (Sections 4.7.7, 4.9.2, 4.9.3). For example, actin can inhibit microtubule growth by coupling to retrograde flow (Lin and Forscher, 1995, Schaefer et al., 2002, Burnette et al., 2007). Thus, the possibility of microtubules to generate force in the presence of only residual actin does not necessarily mean that microtubules generate force during physiological neurite growth. Still, studies independent of actin depolymerization indicate that microtubule-mediated force generation could noticeably contribute to neurite growth. These studies propose that force generation is either due to polymerization in the P-domain (Rauch et al., 2013), or due to pushing forces originating in the axon (Roossien et al., 2013, Lu et al., 2013). Further studies will be necessary to elucidate the contribution of microtubules and actin to force generation during neurite growth. In summary, it is still unclear how the force is generated to drive axon growth. However, we already know more about mechanisms that trigger axonal growth.

4.3. Mechanisms that trigger axonal growth

Mechanisms that increase neurite growth in the axon are reflected in its cytoskeletal architecture. The actin and microtubule cytoskeleton differ in the growth cone of the axon compared to the growth cones of minor neurites. The axonal growth cone shows a more dynamic actin cytoskeleton (Bradke and Dotti, 1999) and more microtubule polymerization in the P-domain (Witte et al., 2008). Moreover, more microtubules are present in the axon shaft (Seetapun and Odde, 2010) and these microtubules are more stable (Witte et al., 2008). Destabilizing the actin cytoskeleton within a single neurite, or alternatively stabilizing the microtubule cytoskeleton, causes that specific neurite to differentiate into the axon in most (70–80%) cultured neurons as demonstrated with cytoD (Bradke and Dotti, 1999) and taxol (Witte et al., 2008) respectively. This shows that differences in cytoskeletal dynamics have a functional impact on neurite growth.

What are the physiological mechanisms that induce these changes in the cytoskeleton of the nascent axon and how does this increase neurite-growth? During axonal specification, small stochastical changes in cell signaling are thought to be amplified by feedback loops (Section 3). These feedback loops increase neurite growth and maintain the growth cone of the nascent axon in a growth-promoting state, while keeping the other neurites in a growth-inhibiting state.

In the following sections, we will illustrate which cytoskeletal changes are induced by signaling pathways downstream of the feedback loops to increase neurite growth in the axon. We will only focus on downstream targets of effectors that have been shown to influence neuronal polarity. These downstream targets harness several mechanisms to increase growth cone advance: (1) increased translation of myosin II mediated force into growth cone advance, (2) increased actin-mediated protrusions, (3) increased microtubule mediated force, and (4) increased membrane insertion.

4.4. Increased translation of myosin II forces into growth cone advance

Myosin II exerts a retrograde force on actin. This force can be translated into growth cone advance by coupling F-actin to the extracellular matrix (clutch hypothesis). One means to couple F-actin stronger to the extracellular matrix is by promoting the coupling of adhesion sites with actin through Shootin1 (Toriyama et al., 2013). Rac1 as well as Cdc42 can increase this coupling (Toriyama et al., 2013). Both activate PAK1 and thereby mediate phosphorylation and activation of Shootin1 (Toriyama et al., 2013, Kubo et al., 2015). Phosphorylation of Shootin1 is necessary for its binding to L1 Cell Adhesion Molecule (L1-CAM) (Toriyama et al., 2013) and coupling to F-actin through cortactin (Kubo et al., 2015). The L1-shootin1-cortactin complex acts as a molecular clutch that confers force generation by myosin II (Toriyama et al., 2013, Kubo et al., 2015) (Fig. 2, III). Increased coupling of actin to the extracellular matrix also reduces retrograde actin flow, and thereby increases the outwards directed force exerted by actin polymerization. How does actin polymerization contribute to neurite growth and are there additional means of regulating it?

4.5. Increased actin-mediated protrusion by Rac1

Actin-mediated protrusions are the main means of pushing the cell leading edge forward and are therefore pivotal to axonal growth. Protrusions are influenced by two main factors: membrane tension and the force that pushes against the membrane. Decreasing the membrane tension, by membrane insertion, or increasing the pushing force leads to bigger protrusions (Craig et al., 2012). Pushing forces against the membrane can be strengthened by increasing the number of actin filaments pushing against the membrane. Rac1 is a physiological regulator of actin polymerization during neuronal polarization (Tahirovic et al., 2010). Expression of Rac1 or its guanine nucleotide exchange factor (GEF) T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1) promote neurite growth and induce the formation of multiple axons (Kunda et al., 2001, Nishimura et al., 2005). Suppression of Tiam1 inhibits increased actin dynamics, a hallmark of axonal growth cones (Bradke and Dotti, 1999, Kunda et al., 2001). Rac1-regulated actin dynamics are necessary for neuronal polarization and seem to be mainly transmitted via Wiskott–Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE) activation (Tahirovic et al., 2010). WAVE regulates the actin cytoskeleton via actin related protein 2/3 (Arp2/3) mediated branching of actin filaments to promote lamellipodia generation (Bailly et al., 1999, Prass et al., 2006, Pollard, 2007, Takenawa and Suetsugu, 2007, Urban et al., 2010). WAVE and Arp2/3 are recruited to the leading edge to promote growth cone protrusion (Miguel-Ruiz and Letourneau, 2014). Inhibition of Arp2/3 decreases the number of free barbed ends and the NGF-induced F-actin increase (Miguel-Ruiz and Letourneau, 2014). While NGF-induced protrusions still form during Arp2/3 inhibition, they are instable and quickly collapse (Miguel-

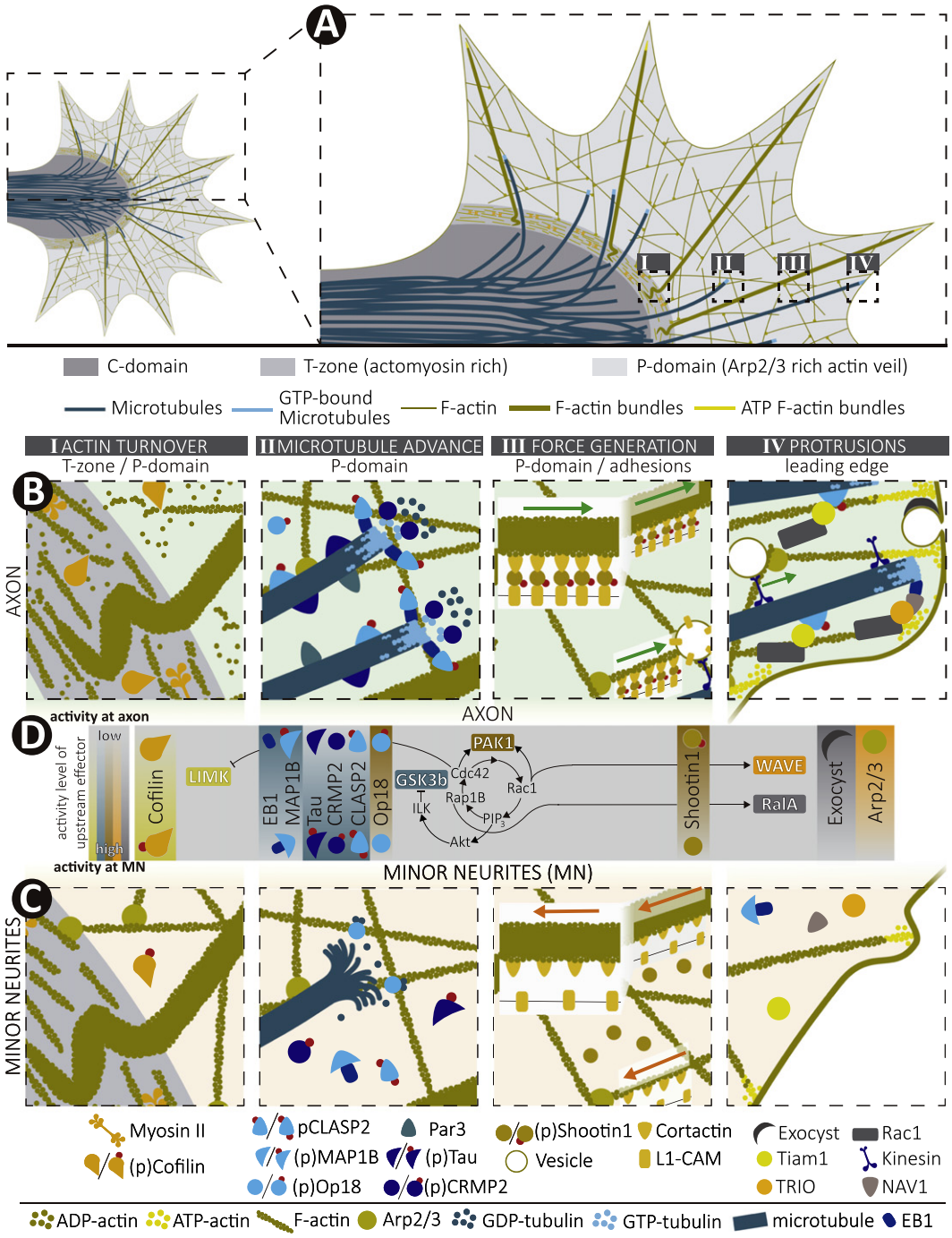

Fig. 2. How signaling drives changes in cytoskeletal dynamics in the axonal growth cone. Neurite growth is increased in the nascent axon and reduced in minor neurites. (D) Pathways from the feedback loops in Fig. 1 to cytoskeletal effectors are shown. Color gradients indicate activity of downstream factors (LIMK, GSK3b, PAK1, WAVE, RalA), directly regulating cytoskeletal effectors (grey = lower activity, color = higher activity). The direction of the gradient indicates whether activity is higher in minor neurites or the axon. (A) Overview of a growth cone including 4 areas that are depicted in detail in B and C. (B,C) (I) Cofilin promotes actin turnover by severing F-actin into smaller filaments and might compete with myosin II for actin binding. (II) P-domain microtubule advance is increased by stabilization via microtubule associated proteins (Tau, pMAP1B), Par3 and increased polymerization by +TIPs (EB1-pCLASP2), tubulin-dimer stabilizing CRMP2 and inhibition of microtubule destabilizing factor stathmin/Op18. (III) Force generation is increased by pShootin1-mediated coupling of F-actin-cortactin to L1-CAM and microtubule mediated transport and thus increase of concentration of L1-CAM. (IV) Leading edge protrusions are increased by actin polymerization and membrane insertion coordinated by P-domain microtubules. P-domain microtubules coordinate Arp2/3 activation via the microtubule associated protein MAP1B-Tiam1 and the EB1-NAV1-Trio complex. Membrane insertion is increased by vesicle-transport and activation of the exocyst complex. Abbreviations: phospho Cytoplasmic Linker Associated Protein 2 (pCLASP2), phospho Collapsin response mediator protein 2 (pCRMP2), phospho oncoprotein 18/stathmin (pOp18), phospho (p) MAP1B, L1-cell adhesion molecule (L1-CAM), microtubule associated protein 1B (MAP1B), T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1), triple functional domain (Trio), navigator 1 (NAV1), ras-related C3 botulinum toxin substrate 1 (Rac1), Adenosin diphosphate (ADP), adenosin triphosphate (ATP), filamentous actin (F-actin), actin related protein 2/3 (Arp2/3), guanosine diphosphate (GDP), guanosine triphosphate (GTP), end binding protein 1 (EB1), Cell division control protein 42 homolog (Cdc42), Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), protein kinase B (Akt), interleukin-like kinase (ILK), Glycogen synthase kinase 3 beta (GSK3b), LIM kinase (LIMK), Wiskott–Aldrich syndrome protein(WASP)-family verprolin-homologous protein (WAVE), Ras-related proteinA (RalA), Ras-related protein 1B (Rap1B), central domain (C-domain), transition zone (T-zone), peripheral domain (P-domain), p21-activated kinase (PAK1), minor neurites (MN).

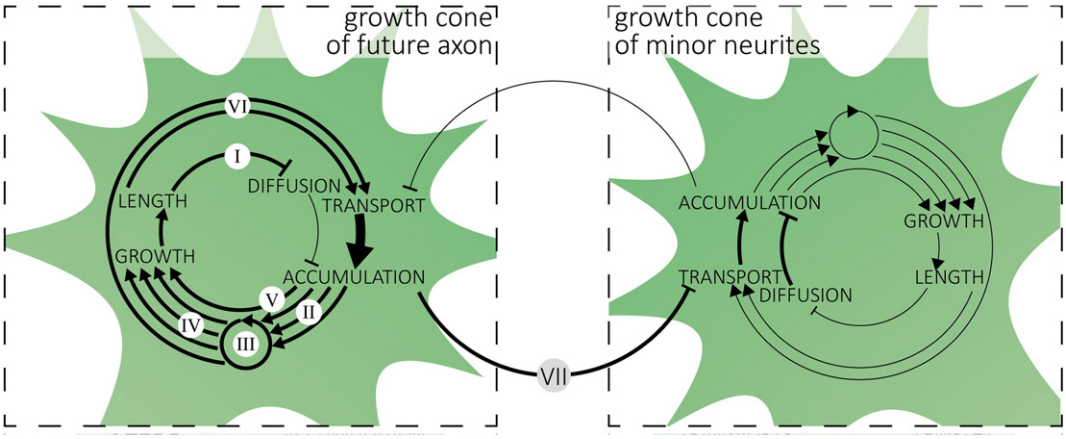

Fig. 3. Model for stabilization of neuronal polarization by feedback loops fueled by axonal growth. If one neurite grows extensively more than the other neurites, the increase in length will (I) lead to reduced retrograde diffusion and in turn increased accumulation of polarity effectors. These polarity effectors (III) activate feedback loops (some of those detailed in Fig. 1) which (IV) stimulate neurite growth via different downstream signaling cascades. (V) Accumulating polarity effectors also directly increase neurite growth. (VI) Feedback loops actively increase transport to the growth cone while neurite length passively increases transport. Increase in length and transport again increase the accumulation of polarity effectors. (VII) This accumulation decreases the available amount of polarity effectors for the other neurites, thereby reducing transport of polarity effectors to other neurites. Thicker and thinner lines of connecting arrows indicate a higher and lower activity, respectively.

Ruiz and Letourneau, 2014). This can be explained by considering that the membrane is constantly under tension and considerable force must be exerted to deform it during protrusion (Craig et al., 2012). Thus, it is conceivable that a reduced number of barbed ends pushing against the membrane destabilizes the protrusion. In summary, Rac1-mediated increase of the number of actin filaments pushing against the membrane promotes membrane protrusion. The physiological role of Arp2/3 during neuronal polarization has remained unclear.

4.6. Regulation of actin turnover by Cdc42

In addition to an increased number of actin filaments, actin polymerization to F-actin can increase the force contribution of each single actin filament to further enhance membrane protrusion. To form F-actin, a sufficient concentration of free G-actin needs to be available. During growth cone steering, the G-actin concentration increases on the protruding site of the growth cone (Lee et al., 2013). This indicates that growth cone protrusions can be increased by increasing G-actin concentration. One way to increase G-actin concentration is by increased actin turnover via cofilin. Interestingly, cofilin is important for neuronal polarization and is regulated by Cdc42 during this process (Garvalov et al., 2007). However, the mechanism by which Cdc42 regulates cofilin is not yet clear. On the one hand, Cdc42 knockout neurons show reduced polarization and decreased cofilin activity (Garvalov et al., 2007). On the other hand, increased Cdc42 activity in dendrites, caused by knockout of a Cdc42 GAP, also caused decreased cofilin activity (Rosário et al., 2012). How is it possible that loss of Cdc42 and increased Cdc42 activity both lead to decreased cofilin activity? In Cdc42 knockout neurons, no filopodia are present while peripheral microtubules are looping (Garvalov et al., 2007). It is possible that changes in the cytoskeleton cause the decrease in cofilin activity rather than direct signaling by Cdc42 (Garvalov et al., 2007). Nonetheless, cofilin activity itself is growth-promoting (Endo et al., 2003, Marsick et al., 2010, Zhang et al., 2012) and important for neuronal polarity (Garvalov et al., 2007). Ablation of the two members of the Actin depolymerizing factor (ADF)/cofilin, prevent neurite formation both in vivo and in cell culture (Flynn et al., 2012). During axonal growth, cofilin might be the main factor promoting actin turnover by actin disassembly. In fact, cofilin activity is specifically increased in the axonal growth cone (Garvalov et al., 2007). Actin disassembly takes place in most parts of the P-domain (Goor et al., 2012) but probably is also relevant in the T-zone (Medeiros et al., 2006). In migrating cells, cofilin is thought to compete with Arp2/3 for binding sites in lamellipodia (Chan et al., 2009), while Arp2/3 is thought to recruit cofilin (Koestler et al., 2013). This supports a possible role of cofilin in the lamellipodia-rich P-domain (Fig. 2, I.). Interestingly, in migrating cells, cofilin also competes with myosin II for F-actin binding (Wiggan et al., 2012, Kanellos et al., 2015), supporting a possible role in the T-zone of growth cones (Fig. 2, I.). Of note, cofilin is active throughout the axonal growth cone (Garvalov et al., 2007), while only acting on ADP-bound actin.

In summary, Cdc42-induced cofilin activation promotes actin turnover in the axonal growth cone, possibly to increase G-actin concentration and to thereby increase cell protrusions. While this increases the leading edge-directed force, mediated by actin, microtubules might also exert a leading edge-directed force.

4.7. Regulation of microtubule mediated force

The number of P-domain microtubules is increased at the site of growth cone steering and during axonal growth (Grabham et al., 2007, Lee and Suter, 2008). One mechanism by which microtubules facilitate increased growth might be the generation of force for growth cone advance. Less is known about the regulation of microtubule-mediated force generation, compared with actin-mediated force generation. Microtubule-mediated force generation could be influenced by the number, polymerization and stabilization of P-domain microtubules (Rauch et al., 2013). Thus, stabilization of P-domain microtubules by Taxol (Witte et al., 2008, Buck and Zheng, 2002) and the various physiological effectors that act on P-domain microtubules could affect force generation. Of note, influencing P-domain microtubules could also influence actin organization (Section 4.9) and transport of proteins and membrane (Section 4.10).

4.7.1. Regulation of the protrusion of microtubules into the P-domain

The number of P-domain microtubules is thought to be restricted by hindrance of microtubules from entering the P-domain by actin arcs in the T-zone (Lowery and Vactor, 2009, Tanaka et al., 1995, Schaefer et al., 2002, Medeiros et al., 2006). Consequently, disruption of actin arcs by myosin II inhibition (Turney et al., 2016) or reduction of retrograde flow by coupling of actin with adhesion sites (Grabham et al., 2007) can increase the number of microtubules protruding into the P-domain. Apart from these mechanisms, the protrusion of microtubules through the T-zone might be increased by pushing forces from dynein (Ahmad et al., 2000, Myers et al., 2006). After microtubules entered the P-domain, they are regulated by stabilization, destabilization, polymerization and depolymerization (Fig. 2, II.).

4.7.2. Rac1 mediated stabilization by inhibition of stathmin

Besides the role of Rac1 in actin dynamics, Rac1-mediated regulation of microtubules is also relevant to neuronal polarization (Watabe-Uchida et al., 2006). Dedicator of cytokinesis 7 (DOCK7)-induced Rac1 activation increases microtubule stabilization via oncoprotein 18/stathmin (Op18) inactivation (Watabe-Uchida et al., 2006) (Fig. 2, II.). Op18 acts on microtubules by sequestering tubulin dimers and promoting catastrophes (Belmont and Mitchison, 1996, Jourdain et al., 1997, Howell et al., 1999).

4.7.3. PI3K mediated stabilization of P-domain microtubules by MAPs and +TIPs

During neuronal polarization, PI3K is primarily responsible for regulating microtubule dynamics. PI3K induces phosphorylation and thereby inactivation of GSK3beta via Interleukin-like kinase (ILK) and protein kinase B (Akt) (Jiang et al., 2005, Yoshimura et al., 2006, Guo et al., 2007). Reduced GSK3Beta activity in turn leads to reduced phosphorylation and thereby to activation of CRMP2 (Yoshimura et al., 2005, Yoshimura et al., 2006), APC (Zumbrunn et al., 2001, Shi et al., 2004), Tau (Hong and Lee, 1997, Lesort and Johnson, 2000) and Cytoplasmic Linker Associated Protein 2 (CLASP2) (Hur et al., 2011). Moreover, GSK3beta phosphorylates MAP1B to activate, rather than to deactivate it (Río et al., 2004, Trivedi et al., 2005) (Fig. 2, II.).

4.7.4. Regulation of P-domain microtubules through MAPs by GSK3beta

P-domain microtubules are stabilized by the action of various MAPs which are regulated by GSK3Beta. In contrast to all other mentioned downstream targets, GSK3Beta activity towards MAP1B is preserved in the axonal growth cone (Hur and Zhou, 2010). This is likely due the influence of substrate priming events on GSK3eta activity. Substrates are referred to as 'primed' if they require phosphorylation by other kinases prior to GSK3Beta phosphorylation. 'Unprimed' substrates do not require previous phosphorylation (Hur and Zhou, 2010). MAP1B is an unprimed substrate of GSK3beta, in contrast to the other mentioned targets. MAP1b increases tubulin polymerization (Takemura et al., 1992, Pedrotti and Islam, 1995, Tymanskyj et al., 2011) but does not suppress dynamic instability (Vandecandelaere et al., 1996). Dephosphorylated (inactive) MAP1B sequesters EB1 and EB3 in the cytosol (Tortosa et al., 2013). Phosphorylation and activation of MAP1B probably increases tubulin polymerization by sequestering less EB1 and EB3, both of which promote microtubule growth (Tortosa et al., 2013) (Fig. 2, II.). MAP1B associates preferably to dynamic (tyrosinated) microtubules (Tymanskyj et al., 2011). Therefore, MAP1B is thought to be important for the maintenance of dynamic microtubules in the P-domain (Utreras et al., 2007, Tymanskyj et al., 2011, Tortosa et al., 2013). Moreover, MAP1B is importat for microtubule bundling in neuroblastoma cells (Feltrin et al., 2012). CRMP2 stabilizes tubulin heterodimers and promotes microtubule assembly (Fukata et al., 2002) (Fig. 2, II.). Interestingly, inhibiting CRMP2 activity on one side of the growth cone induces turning to the other side (Higurashi et al., 2012). Tau increases microtubule rigidity (Felgner et al., 1997) and induces microtubule bundles (Lewis et al., 1989). Tau binds longitudinally to microtubules (Al-Bassam et al., 2002) and is present on dynamic microtubules in the P-domain of the growth cone (Black et al., 1996) (Fig. 2, II.). There, Tau is required for Wnt5a mediated growth cone turning (Li et al., 2014). As explained below (Section 4.7.6), the physiological role of GSK3beta regulation of MAPs remains unclear.

4.7.5. Regulation of P-domain microtubules through + TIPs by GSK3beta

Microtubule plus-end binding proteins can accumulate in the axonal growth cone (Neukirchen and Bradke, 2011), where they stabilize P-domain microtubules. APC influences neuronal polarization by promoting the transport of Par3 to the growth cone (Shi et al., 2004). Moreover, APC promotes stabilization of microtubules by binding to their plus ends (Zhou et al., 2004). However, this mechanism has not been shown to be important for neuronal polarity. CLASP2 is a +TIP which is important for microtubule advance in the P-domain (Marx et al., 2013). While EB1 was not shown to influence axonal polarity, it does act as a scaffold for APC and CLASP2 for microtubule plus tip binding (Akhmanova and Steinmetz, 2008, Honnappa et al., 2009) (Fig. 2, II.).

4.7.6. Role of direct P-domain microtubule stabilization during neuronal polarization

The GSK3beta-CRMP2 pathway influences neuronal polarity (Yoshimura et al., 2005, Yoshimura et al., 2006). While the role of Tau (González-Billault et al., 2002), CLASP2 (Beffert et al., 2012) and MAP1B (Gonzalez-Billault et al., 2001, González-Billault et al., 2002) in neuronal polarity has been elucidated, the importance of their inactivation or activation by GSK3beta for neuronal polarization is not clear. Moreover, MARK2 and LKB1-SAD are important for neuronal polarity and thought to act on MAPs (Chen et al., 2006, Kishi et al., 2005, Shelly et al., 2007, Barnes et al., 2007). However, the importance of their regulation of MAPs remains to be convincingly demonstrated during neuronal polarization.

In summary, P-domain microtubules in the axonal growth cone are stabilized by Op18 inhibition, mediated by Rac1, and activation of MAPs and +TIPs, mediated by PI3K (Fig. 2, II). However, apart from this direct regulation of microtubule stability, microtubules are also affected by actin dynamics.

4.7.7. Regulation of P-domain microtubules by actin

Studies indicate that the actin cytoskeleton can influence P-domain microtubules. Depletion of actin bundles by the myosin light chain kinase (MLCK) inhibitor ML7 also leads to a loss of dynamic microtubules in the P-domain (Zhou and Cohan, 2001, Zhou et al., 2002). This indicates a connection of actin with microtubules. This connection might be important for neuronal polarity, as the microtubule cytoskeleton is changed upon knockout of classical actin-regulating polarity effectors. As such, double knockout of Rac1 and Rac3 destabilizes microtubules in neurons (Tivodar et al., 2015). Moreover, Cdc42 knockout neurons present many looped microtubules in the growth cone P-domain (Garvalov et al., 2007). Indeed, actin bundles seem to guide microtubules but also limit their advance by retrograde flow (Lin and Forscher, 1995, Schaefer et al., 2002, Burnette et al., 2007). Recent studies support the inhibitory role of the actin-network on dynamic microtubules, and suggest that actin bundles are not necessary for microtubules to extend into the P-domain (Burnette et al., 2007). Instead, uncoupling of microtubules from actin disrupts the influence of retrograde flow on microtubules (Lee and Suter, 2008), which enables microtubules to advance further (Burnette et al., 2007, Grabham et al., 2007). During neurite growth, microtubules are uncoupled from actin (Lee and Suter, 2008) while retrograde flow itself is reduced by coupling of actin to adhesion sites (Lee and Suter, 2008).

Coupling of microtubules to actin is mainly conveyed by +TIPs, like CLASP2. CLASP2 undergoes retrograde flow, indicating the coupling of microtubules to actin (Tsvetkov et al., 2007). How is CLASP2-mediated coupling regulated? CLASP2 actin-binding and microtubule-plus-end-binding activities are negatively regulated by separate phosphorylation sites (Hur et al., 2011). To promote axonal growth, CLASP2 actin-binding is inhibited by phosphorylation, while microtubule-binding is not inhibited (Hur et al., 2011). Moderate GSK3 activity leads to partially phosphorylated and growth-promoting CLASP2 (Hur et al., 2011). For a detailed review of actin-microtubule interactions in neurons see Coles and Bradke (2015).

In summary, one major mechanism to regulate P-domain microtubules is by their coupling to actin. While actin can guide microtubules to a certain extent, it mostly acts inhibitory on the advance of microtubules. One regulator of neuronal polarity, CLASP2, is involved in regulating microtubule-actin coupling. This indicates that uncoupling of microtubules from actin can be an important mechanism to increase axonal growth during neuronal polarization.

4.7.8. Microtubule dynamics during polarization

Most factors mentioned in the last sections accumulate in growth cones and particularly in the growth cone of the nascent axon. How does this impact microtubule dynamics? Velocity of EB1 comets in the growth cone is lower compared to the neurite shaft (Seetapun and Odde, 2010). However, velocity and lifetime of EB1 comets in growth cones of undifferentiated neurites and the axon are similar (Seetapun and Odde, 2010). This suggests that the factors mentioned before do not change microtubule polymerization considerably. An alternative explanation to the absence of differences in microtubule polymerization could be that only a small subset of microtubules shows different dynamics. This could be due to temporally and spatially restricted changes in microtubules dynamics. Large changes in dynamics for only a few microtubules would be harder to detect. Of note, earlier measurements of EB3 comet velocity and lifetime in growth cones of the nascent axon were two fold higher and did not differ in the shaft compared to the growth cone (Stepanova et al., 2003). Apart from this, it is conceivable that it is not microtubule polymerization, but rather depolymerization or mechanisms restricted to the leading edge of the growth cone that determine the impact on axonal growth. Indeed, reaching the leading edge might be an important factor for the growth-promoting effect of P-domain microtubules (Section 4.9.1). This mechanism seems to drive membrane insertion – another important determinant of neurite growth.

4.8. Regulation of membrane insertion

Membrane insertion is crucial to neurite growth since it reduces membrane tension (Craig et al., 2012). During neurite growth, membrane tension would quickly increase due to the increasing surface area of the cell. As explained in Section 4.5, increased membrane tension inhibits membrane protrusion (Craig et al., 2012). But membrane protrusion is necessary to move the growth cone forward. Thus, membrane insertion is necessary to overcome the growth-induced increase in membrane tension. In the axonal growth cone, increased membrane insertion leads to an excess of membrane and thus to reduced membrane tension (Dal and Sheetz, 1995, Craig et al., 1995). Increased membrane insertion is necessary for neuronal polarization (Dupraz et al., 2009). In addition, concentration of membrane vesicles is higher in the neurite developing into the axon than in the other neurite (Gärtner et al. 2014). How is membrane insertion increased during axon specification?

4.8.1. Membrane insertion during axonal growth

Membrane is inserted in the growth cone plasmamembrane by exocytosis of PPVs. These PPVs partly originate from the trans-golgi network (TGN) and are anterogradely transported to the growth cone (Section 3.2) (Wojnacki and Galli, 2016). In addition, PPVs form by endocytosis in the growth cone and are subjected to cycles of exocytosis and endocytosis. For this purpose, endocytosed vesicles may either be directly exocytosed as early endosomes or may first mature to recycling endosomes (Wojnacki and Galli, 2016). In Xenopus retinal ganglion cells (RGCs), most membrane recycling happens from early endosomes (Falk et al., 2014).

4.8.2. Membrane insertion mediated by Rap1B activation

Exocytosis of vesicles is stimulated by IGF-1 mediated activation of PI3K (Laurino et al. 2015) and possibly Cdc42 (Alberts et al., 2006). For exocytosis, vesicles are docked and targeted before membrane fusion is mediated by soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE). Targeting and docking of membrane vesicles in the growth cone are accomplished mainly by the exocyst complex (Murthy et al., 2003). During axonal specification, the exocyst complex could be activated by Rap1B. Rap1B activates Ras-related protein A (RalA) (Nakamura et al., 2013) which in turn activates the exocyst complex and thereby exocytosis (Fig. 2, IV.) via recruiting Par3 and Par6 (Lalli, 2009, Das et al., 2013). The resulting membrane insertion is crucial to neuronal polarization, due to the concomitant insertion of signaling molecules, like IGF-1 receptor (Dupraz et al., 2009) or PIP3 (Horiguchi et al., 2006). These signaling molecules not only promote further membrane insertion (Dupraz et al., 2009), but also activate several other signaling pathways, some of which increase force generation. This suggests that the interconnection of membrane insertion and force generation could play an important role in axonal growth. In the following sections, we will highlight why the connection of different pathways is important for axonal growth, and the mechanisms that might facilitate it.

4.9. Coordination of actin dynamics and membrane insertion by P-domain microtubules

Axonal growth requires the coupled-activities of force generation and membrane insertion. The necessity of this is obvious during growth cone steering, for which both mechanisms operate in the same spatially confined area. Microtubule stabilization on one site of the growth cone is sufficient to induce growth cone steering in that direction (Buck and Zheng, 2002). Indeed, P-domain microtubules might not only generate force in the growth cone but can also coordinate actin dynamics and membrane insertion. This indicates that stabilization of microtubules is sufficient to induce all mechanisms that are necessary to steer the growth cone: membrane insertion, force generation and actin-mediated protrusions. The tight interaction of membrane insertion and actin polymerization is exemplified by defective transport of VAMP7-positive vesicles upon Arp2/3 inhibition (Gupton and Gertler, 2009). Importantly, the connection of membrane insertion, microtubule stability and transport is important for neuronal polarization (Oksdath et al., 2016). This indicates that the coordination of those mechanisms is not only important for growth cone steering but also for neuronal polarization.

4.9.1. Membrane insertion mediated by P-domain microtubules

Direct support for the role of P-domain microtubules for membrane insertion comes from a recent study looking at growth cone steering (Akiyama et al., 2016). The authors showed that protrusions in the direction of stimulation are caused by the transport of vesicles on P-domain microtubules to the leading edge of the growth cone and subsequent exocytosis (Akiyama et al., 2016). This might be a general mechanism for membrane insertion during axonal growth. As explained in Section 4.8, membrane insertion reduces membrane tension to promote leading edge protrusion. In addition, membrane insertion, mediated by P-domain microtubules, might facilitate the localized insertion of different effectors in the plasma membrane. Insertion of effectors could lead to an increased generation of force.

4.9.2. Increase of actin-mediated force generation through P-domain microtubules

P-domain microtubules might regulate actin-mediated force generation by transporting two different kinds of effectors and mediating their insertion. Actin-mediated force generation can be promoted by increasing the number of adhesion receptors, by insertion of L1CAM (Shimada et al., 2008) or N-cadherin (Bard et al., 2008) in the plasma membrane (Fig. 2, III.). An influence of microtubule-mediated transport on adhesions is supported by a reduced stability of adhesion complexes upon depletion of P-domain microtubules (Suter et al., 2004). Indeed, P-domain microtubules transport VAMP7-positive vesicles, which can contain cell adhesion molecules like L1CAM (Alberts et al., 2003), to the leading edge (Akiyama et al., 2016). Moreover, Actin-mediated force generation could be increased by transporting factors that promote signaling cascades involved in Shootin1 activation (Section 4.4), like PIP3.

In Summary, P-domain microtubules might locally increase actin mediated force generation by guiding the insertion of adhesion complexes and signaling molecules into the plasma membrane.

4.9.3. Coordination of actin polymerization by P-domain microtubules

The influence of microtubules on the actin cytoskeleton is directly mediated by microtubule binding proteins. For example, depletion of the +TIP CLASP2 induced weakening lamellipodial structures (Marx et al., 2013). Moreover, P-domain microtubules could regulate actin polymerization by activating Rac1 at the leading edge. Rac1 activation, and subsequent actin polymerization, is coupled to microtubules by two separate mechanisms. First, the Rac1 GEF triple functional domain (Trio) is recruited to +TIPs via EB1 and navigator 1 (NAV1) (Van Haren et al., 2014) and in turn can promote Rac1 activation. Second, MAP1B recruits Tiam1 for Rac1 activation and increases Cdc42 activity by an unknown mechanism (Montenegro-Venegas et al., 2010, Chen et al., 2013). MAP1B knockout neurons display reduced axonal length that can be completely rescued by expressing constitutively active Rac1 or Cdc42 (Montenegro-Venegas et al., 2010, Henriquez et al., 2012). This indicates that the interaction of MAP1B with actin-regulating proteins might be the main mechanism by which MAP1B regulates axonal length.

Functionally, the interaction of the Rac1 GEFs Tiam1 and Trio with microtubules could provide a mechanism to increase cell-edge protrusions (Henríquez et al. 2012, Van Haren et al., 2014). Both interactions promote axonal growth (Montenegro-Venegas et al., 2010, Van Haren et al., 2014). The Trio-NAV1 complex increases Trio-mediated Rac1 activation (Van Haren et al., 2014). Rac1 in turn can increase the number of barbed ends produced by Arp2/3 and thereby increase the pushing force on the membrane. Interestingly, Trio-NAV1 interaction is increased upon microtubule stabilization (Van Haren et al., 2014). Thus, the Trio-NAV1 complex couples increased microtubule stability to increase actin polymerization (Fig. 2, IV.).

In summary, the role of P-domain microtubules goes beyond potential force generation during axonal growth. P-domain microtubules also constitute the main structure that spatially and temporally coordinates membrane insertion and actin dynamics. These mechanisms are important to propel the growth of the axon. Apart from this coordinating role, P-domain microtubules might also locally amplify signaling pathways by transporting signaling molecules.

4.10. Local signal amplification

Axonal transport leads to an accumulation of signaling molecules in the growth cones. This increases the concentration and thus signaling-pathway activity in this compartment. However, many signaling molecules propel axonal growth by acting in a sub-compartment of the growth cone, like the leading edge. Concentrating effectors in such a smaller sub-compartment would lead to an additional increase in effector concentration and thereby increased activity. An effective mechanism for local accumulation could be based on a spatially restricted feedback loop.

4.10.1. P-domain microtubule mediated transport

P-domain microtubules might increase their own stability by transporting signaling effectors in a positive feedback loop. Transported signaling effectors could trigger the activation of signaling cascades which increase the stability of microtubules, and thereby increase transport (Section 4.7). The resulting feedback loop between microtubules and signaling feedback loops could trigger a local increase of growth-promoting signaling.

One requirement is that membrane receptors and soluble proteins that can trigger such signaling cascades are transported on microtubules. Vesicles containing IGF-1 receptor (Grassi et al., 2015) and PIP3 (Horiguchi et al., 2006) are transported to the growth cone by microtubules, although their transport by P-domain microtubules has not been shown. During growth cone turning, PI3K activation is necessary for site-directed advance of microtubules (Akiyama and Kamiguchi, 2010). This supports the idea that signaling is involved in spatially restricted microtubule stabilization. Interestingly, PI3K is a common downstream target of membrane receptors like IGF-1 receptor and acts via the production of PIP3, both transported by vesicles.

Instead of vesicles, proteins could also be transported by motor proteins on P-domain microtubules. Several proteins involved in the same signaling cascades as PIP3 are transported anterogradely to the growth cone by kinesins: DOCK7 (Watabe-Uchida et al., 2006), Par3 (Nishimura et al., 2004), APC (Ruane et al., 2016) and CRMP2 (Kimura et al., 2005). These proteins could be transported into the P-domain to promote the stabilization and thereby the number of P-domain microtubules. Indeed, the localization of APC changes upon induction of growth cone-steering, from the central domain to the site of protrusion in the P-domain (Koester et al., 2007). This indicates that an induced transport of signaling factors to the P-domain might be important for neurite growth.

One study modeled the Rac1-Op18 pathway for microtubule stabilization and transport of cytosolic along microtubules (Xu and Bressloff, 2015). The model did not include the transport of Rac1 by microtubules and thus no feedback loop. However, the model predicted an increased microtubule density and increased concentration of cytosolic effectors depending on a preset distribution of Rac1. Another study developed a mathematical model for the redistribution of the gamma-aminobutyric acid (GABA) receptor depending on its interaction with microtubules (Bouzigues et al., 2010). The redistribution was modeled based on reduced diffusion during interaction of the GABA receptor with microtubules and increased microtubule stability due to GABA signaling. When applying a GABA gradient, these mechanisms were sufficient to redistribute the GABA receptor to the higher GABA concentration. This exemplifies that feedback loops could be sufficient to spatially increase the number of microtubule and in concert concentration of signaling effectors. It is conceivable that microtubule-mediated transport of effectors can actually be more efficient for effector-redistribution than the reduction of effector-diffusion in the second model.

In summary, P-domain microtubules might not only coordinate cytoskeletal dynamics and membrane insertion but also locally amplify growth-promoting signaling. This could localize the activity of different parts of signaling feedback loops efficiently to their place of action, for example the leading-edge.

4.10.2. Spatially targeted endocytosis and exocytosis

Redistributing membrane-receptors by endocytosis and subsequent targeted exocytosis may increase the efficiency of redistribution. Spatially directed exocytosis concentrates L1 to the growth cone periphery (Kamiguchi and Lemmon, 2000). For this purpose, membrane is endocytosed in the central domain of the growth cone. In turn, resulting vesicles move tangentially along microtubules to the growth cone periphery for exocytosis (Kamiguchi and Lemmon, 2000; Dequidt et al., 2007; Akiyama and Kamiguchi, 2010). This mechanism is important for axonal growth as the rate of L1 endocytosis correlates with the axon growth rate (Kamiguchi and Yoshihara, 2001). Once receptors are at their place, lateral diffusion is inhibited by binding to ankyrin (Gil et al., 2003). While these mechanisms have only been characterized for L1, they could also apply to other receptors like TrkB and the IGF-1 receptor. Cycles of Endocytosis and Exocytosis can decrease the concentration of these receptors in the C-domain of the growth cone while increasing their concentration in the peripheral growth cone. This mechanism could feed into the microtubule-dependent feedback loop for locally concentrating signaling effectors.

4.11. Conclusion

Altogether, feedback loops influence cytoskeletal dynamics by three main output nodes, the RhoGTPases Rac1 and Cdc42, and PI3K/PIP3. While the PI3K-GSK3beta pathway has mainly been involved in microtubule dynamics, RhoGTPases are the main regulators of the actin cytoskeleton. However, several connections from actin-regulating to microtubule-regulating proteins indicate that actin and microtubules

are tightly coupled in growth cones. During neuronal polarization, these pathways set the cytoskeleton within the axonal growth cone to a growth-promoting state by increasing the dynamics of actin filaments through Arp2/3 and cofilin, promote actin-mediated force generation by Shootin1 and increase exocytosis by the exocyst complex. Moreover, neuronal polarization is promoted by increased microtubule advance in the axonal growth cone, through MAP1B, Tau1, CRMP2 and CLASP2. Microtubule stabilization might spatially couple actin polymerization and membrane insertion and might enhance growth-promoting signaling in the P-domain via a transport-dependent positive feedback.

5. Actin waves

In a mathematical model of neuronal polarization, pulsed transport of shootin1 was found to be crucial for the spontaneous development of precisely one axon (Toriyama et al., 2010). Indeed, neurite growth in hippocampal neurons is often pulsed and occurs at only one to two neurites at a time. Interestingly, pulsed neurite growth coincides with the arrival of actin protrusions, so called actin waves, at the growth cone (Ruthel and Banker, 1999).

5.1. Regulation of actin waves

Actin waves develop at the soma and move along the neurite to the growth cone (Ruthel and Banker, 1999). During the initial growth phases of neurites, almost all growth events correlate with the arrival of actin waves, while this correlation decreases in later stages (Winans et al., 2016). Actin waves coincide with the fluctuating translocation of the Kinesin 1 motor domain (Winans et al., 2016). These Kinesin 1 motor domain fluctuations occur in different neuronal cell types and stabilize upon axon development (Jacobson et al., 2006, Randlett et al., 2010, Twelvetrees et al., 2016). Importantly, the frequency of actin waves is higher in the future axon than in other neurites (Flynn et al., 2009). How is the preferential occurrence of actin waves in the future axon accomplished? Waves are regulated by microtubule based transport (Winans et al., 2016) and wave frequency is reduced upon kinesin 12 knockdown (Liu et al., 2010). Kinesin-mediated transport can be regulated by microtubule stability (Hammond et al., 2010). Therefore, microtubule stability could ultimately regulate actin wave frequency. This would link actin wave frequency to neuronal polarization, since microtubule stability is increased in the nascent axon (Witte et al., 2008). In fact, microtubule-based transport directs accumulation of several polarity effectors to the axonal growth cone during neuronal polarization (Section 3.2). Actin waves move along the neurite by shootin1 - L1CAM - actin mediated force generation (Winans et al., 2016). Within actin waves, cofilin (Flynn et al., 2009) as well as Rac1 and Cdc42 activity (Winans et al., 2016) are increased. Using photoactivatable Rac1, actin waves can be spatiotemporally induced (Winans et al., 2016). However, the physiological mechanism of induction is still elusive. The increase in actin waves frequency in the future axon could indicate a possible role in neuronal polarization. Can the effects of actin waves on neurites thus account for changes observed in the future axon?

5.2. Effects of actin waves

It is proposed that actin waves act as a means for increased microtubule polymerization and kinesin mediated transport to the growth cone (Winans et al., 2016). In agreement with this, Shootin1 is transported to the growth cone in actin waves (Toriyama et al., 2006). Upon arrival of actin waves, the growth cone enlarges and becomes more dynamic (Ruthel and Banker, 1999, Flynn et al., 2009). The widening of the neurite shaft is likely extended on to the growth cone upon wave arrival due to the transported membrane. It is conceivable that microtubule polymerization also propagates until the C-zone and in turn might increase the number of peripheral microtubules. Moreover, the transport of different factors, like active Cdc42, Rac1 (Winans et al., 2016) and cofilin (Flynn et al., 2009), might directly influence actin dynamics to increase axonal growth. The mechanism of growth promotion is still elusive. Future studies need to address the specific changes in cytoskeletal dynamics within the growth cone upon actin wave arrival. The increase in transport, microtubule polymerization and neurite growth, induced by actin waves, point to an important role of actin waves in promoting neuronal polarization.

5.3. Actin waves in neuronal polarization

The role of actin waves during neuronal polarization is currently under debate. Katsuno et al. (2015) reported that a reduction of actin wave frequency to 20% by removing an adhesive stripe below the axon of stage 3 neurons led to an inhibition of further axonal growth. However, it was not clearly demonstrated that the removal of the adhesive stripe did only specifically inhibit actin waves, and did not affect additional events. It is conceivable that microtubule-based transport in general was disturbed. Moreover, these experiments do not address the importance of actin waves during the transition from stage 2 to stage 3. Importantly, 20% neurons do not show any actin waves but they still polarize and grow an axon (Flynn et al., 2009). Consequently, the importance of actin waves is still debated. However, it is possible that actin waves are a manifestation of the alternating growth typical observed in stage 2 neurons. This manifestation might not be fully penetrant, leaving a certain number of cells without actin waves. Still, further experiments that specifically inhibit actin waves in a neurite shaft, like photo-inhibition of Rac1 (Wu et al., 2009), in stage 2 neurons are necessary to probe the importance of actin waves for neuronal polarization.

6. Conclusion

Neuronal polarization is a robust all-or-nothing restructuring of neurites. Underlying mechanisms tightly control actin- and microtubule dynamics, membrane insertion and protein organization. Feedback loops seem to provide the driving force to polarize signaling and thereby restructuring. The basis for the underlying local-activation global-inhibition loop is neurite growth. Feedback loops eventually influence the cytoskeleton, especially in the growth cone, to drive neurite growth. Importantly, cytoskeletal effectors are connected in one signaling cascade, while the cytoskeleton itself provides a scaffold to coordinate different pathways to propel axonal growth. However, to date no study has directly examined the interconnection of the cytoskeleton and feedback loops thoroughly. Moreover, the physiological role of many of the proposed molecular feedback participants is still unclear. It will be the goal of future studies to reveal their precise role using knockout models. Furthermore, future studies should focus on the systems biology of neuronal polarization instead of only single effectors. These two approaches should shed light on how the connection between microtubule and actin regulating pathways, and the feedback of cytoskeletal dynamics on signaling pathways influence neuronal polarization. In vivo, the interconnection of different pathways might enable the integration of a multitude of cues while producing the same result, a polarized neuron. Understanding how feedback loops and cytoskeletal dynamics propel axon growth in young neurons could allow us to reactivate axon-growth in damaged, adult neurons. As it was shown for microtubule-stabilization after spinal cord injury (Hellal et al., 2011, Ruschel et al., 2015), this approach provides a promising therapeutic avenue to induce axon regeneration in disease.

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