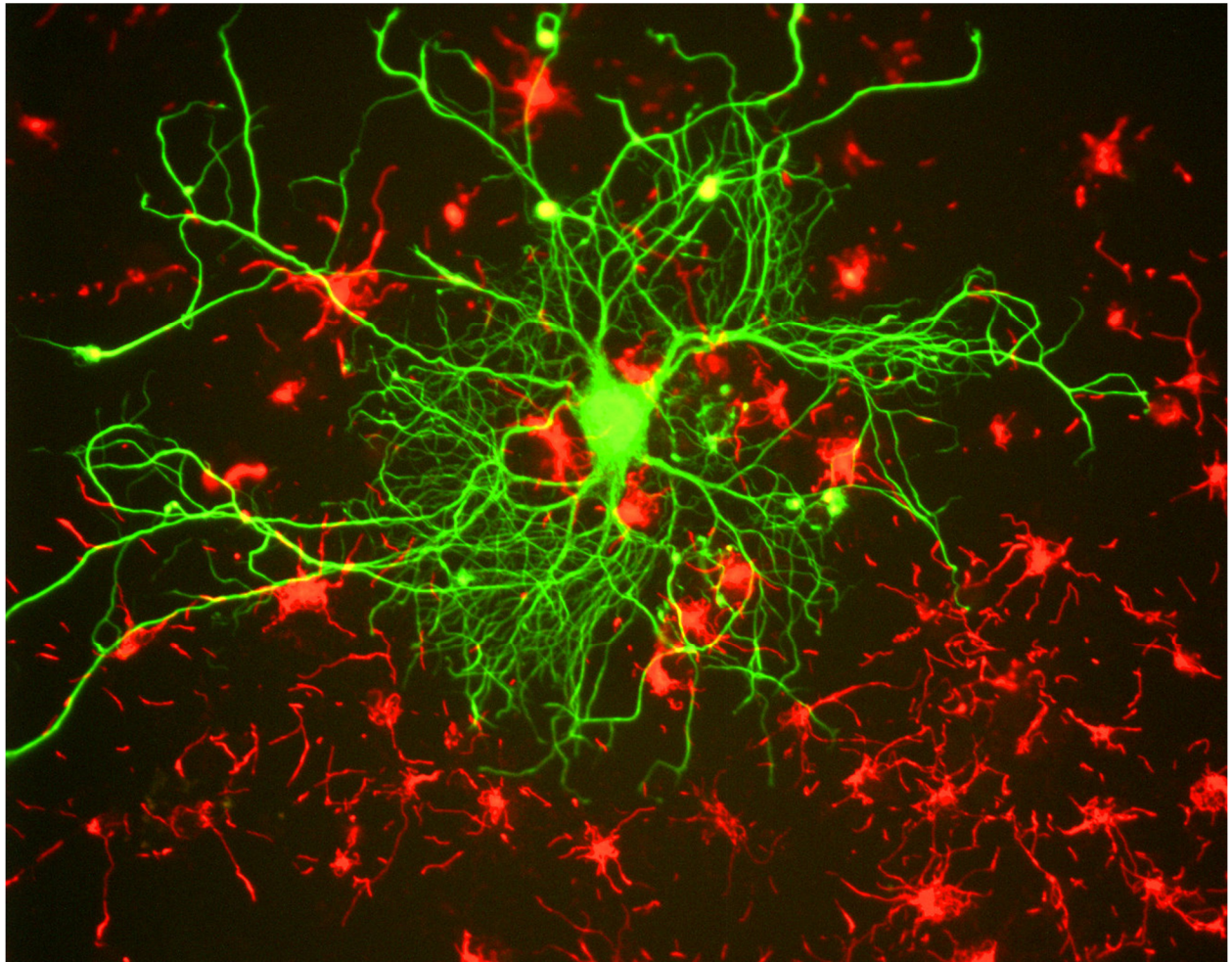Master thesis

# Intrinsic neuronal polarity: early events in symmetry breakage and neuronal polarization

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# Intrinsic neuronal polarity: early events in symmetry breakage and neuronal polarization

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# Content:

# Introduction

Cell polarity lies at the center of a wide variety of biological processes including cell migration, epithelial morphogenesis and chemotaxis. Among the most prominent polarized cells are neurons, cells compartmentalized in two functionally and morphologically distinct domains: axons and dendrites. Neurons typically consist of one axon and multiple dendrites, in which the dendrites receive neuronal signals which generate an axon potential at the level of the soma and propagates along the axon to release neurotransmitters at the axon terminal. In mammals, once cortical and hippocampal neurons have undergone differentiation, they start migrating to their final destination during which the morphological formation of axons and dendrites occurs. Though this is often described as the first break of symmetry[1-3], it is preceded by the formation of multiple morphologically equivalent neurites[4]. Neurons typically develop from the ventricular and sub-ventricular zone in the brain, where neuronal stem cells reside and give rise to neuronal precursor cells[5]. Immediately after the last mitosis, neuronal precursor cells generate their first neurites, followed by the migration to their final destination in the brain and the further development of neurites and eventually axons and dendrites[6].

Studies based on the use of cultured embryonic hippocampal neurons in the 1980s defined the morphological steps of polarization[4]. After plating, hippocampal neurons extend motile lamellipodia and filopodia around the cell body, which is defined as the 1st stage of polarization. During the 2nd stage, clusters of lamellipodia form small cylindrical processes, the 'minor' neurites, which are highly dynamic with periods of extension and retraction. One of the neurites will eventually initiate a sustained growth to form the neurons axon, known as stage 3. During the 4th stage, the remaining neurites will develop into dendrites. In stage 5, synaptic specializations and contacts are established. The establishment of polarization is often seen as the transition between stage 2 and 3, where one neurite becomes the axon. Until recently, it was thought that neurons in stage 2 could be seen as non-polarized cells with a random distribution of morphologically indistinguishable neurites along the cell body[1-3]. However, new evidence shows that the first neurite sprout is most likely to become the axon, which implies that this event defines the first break of symmetry[7].

All neurons undergo the above stages of polarization and are coordinated by the interaction between extracellular signals, intracellular signals and signaling pathways, which in turn influence the distribution of cellular compartments and determinants and the morphological state of the neuron. Evidence is accumulating that the cytoskeleton plays an important role in neuronal polarization[3,8,9], since the cytoskeleton can determine cell polarity not only by forming the structural scaffold for the cell shape, but also by organizing the polarized cell interior and intracellular traffic[3,8,9]. Recent advances have been made to unravel the specific mechanisms by which the cytoskeleton directs the shape of neurons. However, the extent of our knowledge on the complex actin and microtubule behavior on neuritogenesis and which molecular mechanisms and polarity regulators are involved is far from complete.

In this thesis, current knowledge about the formation of the first neurites and their involvement in establishing the polarity of mature neurons will be summarized. In the first chapter, the generation – and the location – of the first neurites will be discussed, with a focus mainly on the initial, instructive polarizing signals. The second chapter will comprise the latest findings and theories on how the cytoskeleton directs neuronal polarity, with an emphasis on the formation of neurites and the elongation of these processes. Finally, the stage 2-3 transition or the formation of the axon will be discussed, as well as the maintenance of the axon.

# 1 Initial regulators of neurite sprouting and location

The transition from neurite to axon has previously been described as a stochastic mechanism, where fluctuating distributions of intrinsic determinants stipulate which neurite becomes the axon[10]. However, recent findings suggest that the first formed neurite is most likely to develop into the axon[7], implying symmetry breakage is defined by the formation of the first neurite, or possibly even earlier on. It has also been shown recently that the second neurite forms opposite of the first[11]. The formation of the first two neurites on specific locations on the neuronal cell body has two important consequences for neuronal development: 1) it defines the axonal and dendritic domains, and 2) the initial polarity axis determines the axis of migration[11]. The first true polarizing signal in neurons therefore does not occur during the establishment of axons and dendrites, but directly after mitosis and the sprouting of the first neurites. Though axon/dendrite polarization is widely studied[1-3], little is known about these early events in neuronal polarity.

As in all cells, the morphological changes of neurons are brought about by the cytoskeleton. This chapter however will not go into detail on the cytoskeletal changes that result in morphological changes, but will instead focus on its upstream regulators. The events preceding neurite formation are most likely orchestrating the outgrowth of these very processes. Several proteins have been shown to be associated with microtubules (MAPs) or to bind actin (ABPs)[3]. These proteins are in turn regulated by a large variety of upstream regulators, such as Ras and Rho GTPases, Par polarity proteins, PI3K, GSK3 and many others[8]. An overview of different molecules known to be involved in neuronal polarity is summarized in Figure 1. The complex network of regulatory, signaling and effector proteins will not be described in detail here, but rather functions as an illustration of the complexity of the system. This first chapter comprises the earliest events in neuritogenesis, in which the aim is to unravel the first intrinsic polarizing signal that results in neurite formation and instruction of neurite outgrowth location. Furthermore, the observation that the location where the second neurite sprouts is opposite the first, will also be addressed.
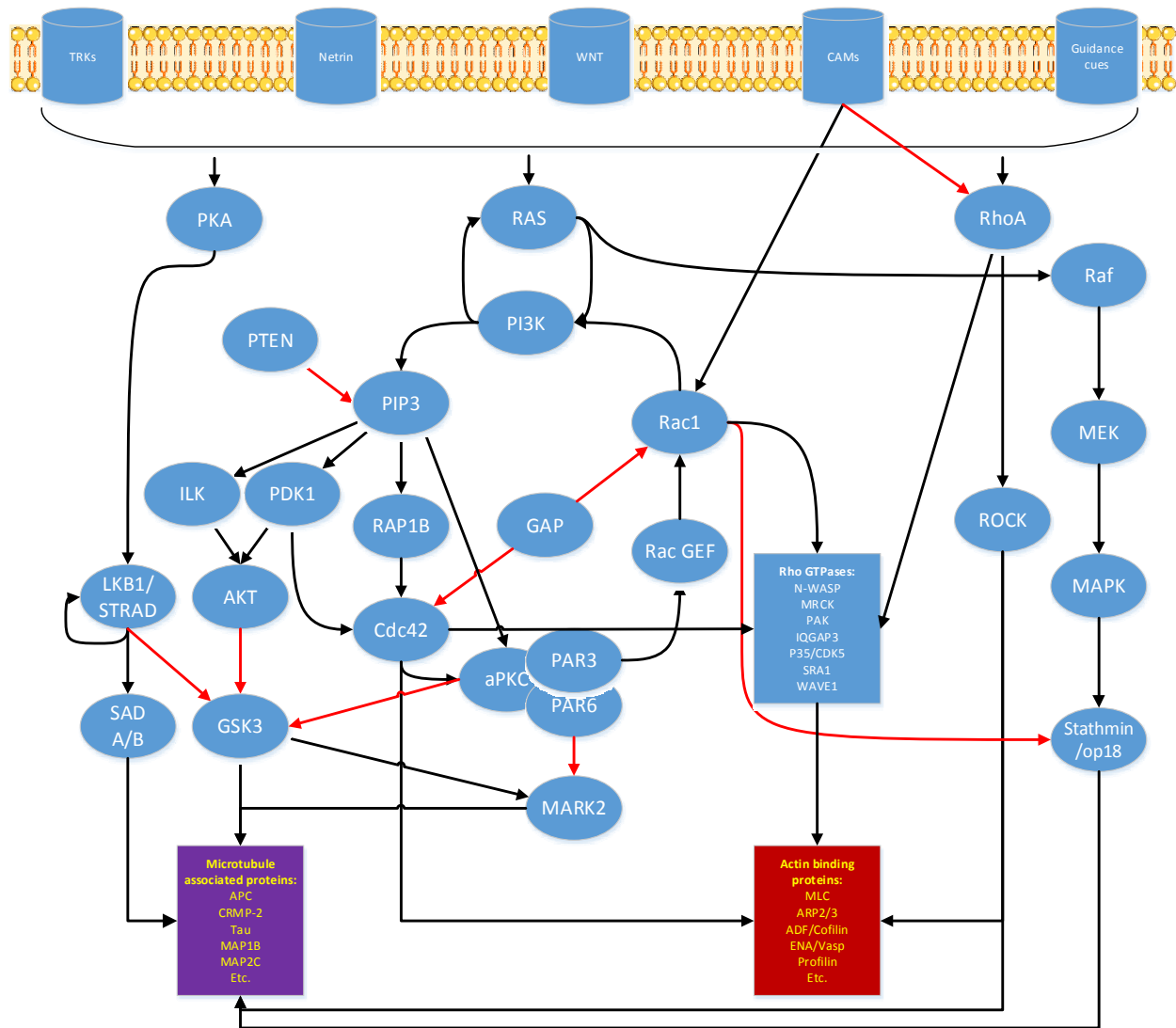
**Figure 1. Schematic representation of signaling pathways involved in neuronal development and neuritogenesis.** Different signaling pathways are involved in neuritogenesis and bring about cytoskeletal changes by influencing microtubule associated proteins (MAPs, purple box) or actin binding proteins (ABPs, red box). Cell surface receptors relay signals to intracellular signaling molecules, such as PKA, RAS and RhoA. Ras activates phosphatidylinositol 3-kinase (PI3K), which in turn produces phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 binds and activates cell division cycle 42 (Cdc42), as well as integrin-linked kinase (ILK) and phosphoinositide-dependent kinase 1 (PDK1). The latter two inactivate glycogen synthase kinase 3 (GSK3) via Akt. The former activates the PAR complex PAR3-PAR6-aPKC (atypical Protein Kinase C), in which PAR3 can activate Rac1 via Rac Guanine exchange factors (GEFs) and aPKC can inhibit GSK3 and Microtubule-affinity regulating kinase 2 (MARK2). Rac1 activates PI3K, and thereby forms a positive feedback loop. Cdc42 and Rac1 can activate different Rho GTPases, which in turn interact with ABPs. Ras can also activate mitogen activated protein kinase (MAPK) via Raf and mitogen activated protein kinase kinase (MEK). Rac1 inhibits and MAPK activate Stathmin/op18, a protein which destabilizes microtubules. Protein kinase A (PKA) is a protein activated by membrane receptors, and in turn activates liver kinase 1B (LKB1)/STRAD. This complex (in)activates MAPs via synapses of amphids defective (SAD) kinase A and B. LKB1/STRAD inhibits GSK3, where GSK3 inhibits different MAPs. Surface receptor signaling via RhoA activates RhoA activated kinase (ROCK), which in turn directs cytoplasmic changes via MAPs and ABPs. TRKs, tyrosine receptor kinases; CAMs, cell adhesion molecules; PTEN, phosphatase and tensin homolog; N-WASP, neural Whiskott-Aldrich syndrome protein; MRCK, myotonic systrophy kinase-related Cdc42-binding kinase; PAK, p21-activated kinase; IQGAP3, IQ motif containing GTPase activating protein 3; CDK5, p35-cyclin-dependent kinase; SRA1, specifically Rac1-associated protein 1; WAVE1, WASP-family verprolin homologous protein 1. MAPs and ABPs are mentioned in Figure 3 and 4 resp.

*1.1 Initial neuronal polarization is not determined by cytosolic asymmetry*

Neuronal polarization can be guided by either extrinsic or intrinsic cues. Early findings have shown that isolated hippocampal neurons *in vitro* can establish a normal axon/dendrite polarity in the absence of external cues[4]. These results suggest a cell-autonomous mechanism of neuronal polarization, determined by an asymmetrical distribution of cellular components. Several studies have shown that in hippocampal neurons, the centrioles, Golgi-apparatus and late/recycling endosomes cluster together at one pole of the cell[7,12,13]. It is hypothesized that the centrosome localization is a requirement for the initial neurite[12] and axon formation[13]. However, some recent studies, in which centrosomal functioning has been compromised, refute this concept. For example, fruit fly mutants lacking centrioles develop a rather normal nervous system[14], and *in vitro* laser ablation of the centrosome in hippocampal cells does not interfere with axon extension[15].

Furthermore, although the observation of these organelles clustering at the site where the first neurite/axon sprouts is correct, it has falsely been suggested that this cytosolic clustering precedes neurite formation[12]. Recent time-lapse studies with a higher temporal resolution allowed for a finer analysis of the organelle dynamics in newly formed neurons[16,17]. These studies reveal that the translocation of the Golgi apparatus and the centrioles is followed by first neurite formation. Consistently, the position of the centrosome is controlled by regulators of neuronal polarity including PI3K and Cdc42[10]. This implies that neuronal polarization is not determined by the asymmetric distribution of cytosolic cues.

*1.2 Cadherins determine first neurite position extrinsically*

Neuronal polarization *in vivo* occurs in a tissue environment where neurons are exposed to surrounding cell surfaces, secreted signals and the extracellular matrix. The influence of extracellular polarizing cues in axon and dendrite formation is well studied[10,18,19]. Multiple extracellular signals and their surface receptors have been identified to be involved in axogenesis (Figure 1). However, little is known about the function of these cues in mitigating early neuronal polarization and neurite formation. Many extracellular matrix and cell adhesion molecules, such as laminin and neuronal cadherin (N-cadherin) have also been implied in neurite outgrowth[20]. A recent study on the outgrowth of neurites investigated the role of extrinsic N-cadherin cues. N-cadherin is a cell-cell adhesion protein expressed in the brain and known to be involved in neuronal growth and migration[19]. This study showed that extrinsic N-cadherin cues are able to determine the site of first neurite outgrowth *in vitro* in mice hippocampal neurons[16]. Furthermore, this study revealed that the centrosome and Golgi moved to the pole induced by N-cadherin only after the neurite started to grow[16]. A similar order of events was seen in sensory neurons in the fruit fly, where localization of the centrosome was preceded by a (E-) cadherin landmark[17]. These results suggest that the generation of the first neurite is determined by extrinsic cues, namely the extracellular distribution of cadherins.

*1.3 Cadherins determine first neurite position intrinsically*
Cadherins are known to act both as a ligand (extracellular) and a receptor (membrane-bound). Extracellular cadherins seem to be triggering a directed neurite outgrowth and thereby function as an early hallmark for neuronal polarity. However, *in vitro* experimentation has also revealed the capability of neurons to polarize normally in the absence of extracellular cues. One possible explanation for this is that extrinsic cadherins have an instructive role rather than triggering neuronal polarity[21]. This hypothesis is in agreement with findings that *in vitro* N-cadherin expression on the membrane of hippocampal neurons is already polarized before neurite formation[16]. *In vivo,* this polarization has also been observed in sensory neurons in *Drosophila*, where DE-cadherin clusters at one pole of the neuron and precedes first neurite outgrowth[17]. These results suggest that intrinsic cadherins, on the membrane of the neuron itself, cluster together to determine the location of neurite formation. In the absence of asymmetrical external cadherin cues to determine polarity, intrinsic fluctuations of cadherin determine the position of the first neurite[11].

*1.4 Cytokinesis remnants define first neuronal asymmetry*
Recent evidence on the generation of the first neurite, and hence the location of the axonal domain of the neuron, suggests an early polarizing signal by membrane-bound cadherin[16,17]. Time lapse results of DE-cadherin clustering in *Drosophila* has shown that the clustering of cadherin occurs at approximately 2.8' after the last mitosis, which implies other upstream regulators might be involved in cadherin localization[17]. Besides its function in early neuronal polarity, DE-cadherin is known for being involved in epithelial polarity. In epithelial cells, DE-cadherin is recruited by Par-3 (Bazooka in *Drosophila*), and functions as an upstream regulator of cadherin[8]. Pollarolo *et al.* recently showed that cadherin and Bazooka clustering coincide, rather than precede each other[17]. In epithelial cells and neuroblasts, Bazooka localization is mediated by direct binding to phosphatidylinositol-4,5-biphosphate (PIP2), a phospholipid component of cell membranes[22]. PIP2 localization in newly formed sensory neurons occurs at the same place and prior to Bazooka localization[17]. Interestingly, the place of PIP2 expression on the cell membrane coincides with the place of mitotic cleavage of the cell[17]. This finding suggests that asymmetric cues for the formation of the first neurite are inherited from the mitotic cleavage and locate at the mitotic furrow. To investigate this further, Rho1 (RhoA in mammals), a GTPase known to be present at the mitotic furrow and to induce an increase in PIP2[23], was followed in hippocampal neurons directly after mitosis. Localization of another protein, AuroraA, also known to be located at the mitotic furrow and to induce neuritogenesis[24,25], has also been studied. Immediately after mitosis in *Drosophila* sensory neurons, Rho1 and AuroraA are localized at the mitotic furrow, where the first sprout appears[17]. Altogether, this recent study on initial neurite formation suggests an important role for cytokinesis remnants on neuronal polarization, in which Rho1 recruits PIP2, PIP2 recruits Par-3, and Par-3 recruits cadherins. However, direct evidence is still missing and future studies will help to elucidate the exact function of these and putative other furrow components in symmetry breakage.

*1.5 Localization of the second neurite is opposite of the first*
The formation of the first neurite has been shown to localize at the site of cleavage, where the second neurite forms opposite to the first[7]. Furthermore, *in vitro* analysis of hippocampal neurons shows that in 71% of the neurons the axon grows from the first neurite and in 23% from the second neurite[7]. These *in vitro* results suggest an intrinsic mechanism by which neuronal polarity is defined at the bipolar stage. It must be noted however, that all neurites on a multipolar body are capable of becoming the axon, yet with a homogenous environment, there is a preference for the first and second formed neurite.

The site from which the first neurite emerges is defined by the localized accumulation or activation of specific molecules that can induce neurite outgrowth. Cytokinesis remnants from the past division, such as RhoA and AuroraA and subsequent N-cadherin clustering, might be involved in the induction of polarized growth. However, it is not clear whether the formation of the second neurite also requires 'external' signals or is the consequence of a passive mechanism. A recent study used a mathematical model to test the possibility that the formation of the second neurite is determined just by the presence of the first neurite[11]. The model assumes that an autocatalytic mechanism which is local and self-reinforcing is brought about by variations in protein concentration on the membrane. Although it is irrelevant for the model which protein this is, N-cadherin might be involved. The model assumes that when the first neurite is sprouting, cell membrane and membrane proteins diffuse in such a way, that indeed the pole opposite to the first will accumulate the membrane protein that can reinforce neurite outgrowth[11] (eg. N-cadherin). To further support the outcome of this mathematical model, the authors also provide biological data that show that their data is in agreement with the molecular organization and distribution in cells.

*1.6 The formation of multiple neurites*
Stage 2 neurons typically form multiple neurites distributed uniformly over the cell body. Until recently, it was thought that the first polarization of the neuron was determined by a stochastic mechanism where one of the neurites would become the axon by chance[1,2]. The association of the first neurite with becoming the axon has only recently come to light. Till date, no studies have been performed investigating the order and localization of the other neurites on the cell body. Stage 2 neurons develop a multitude of dynamic neurites around the cell body, which all extend and retract. The extension of neurites is driven by four main steps: 1) an increase in the local concentration and activation of signaling molecules; 2) an increase in the amount of plasma membrane; 3) an increase in the dynamics of actin filaments; and 4) the enhancement of microtubule formation[10]. Subsequently, retraction of neurites is achieved by the reverse processes. These processes of extension and retraction continue until a cue leads to the activation of a continuous self-activation system or a positive feedback loop, by which a single neurite will elongate to become the axon. The exact cues and mechanisms of these positive and negative feedback loops leading to extension or retraction respectively, will be discussed further on.

# 2 Effectors of the cytoskeleton regulate neurite outgrowth

The sprouting of neurites is led by extracellular and intracellular signaling molecules, or polarity regulators. These polarity regulators and their downstream signaling pathways in turn lead to a change in cytoskeleton dynamics via effectors of the cytoskeleton: microtubule associated proteins (MAPs) and actin binding proteins (ABPs). The cytoskeleton has three important functions: it provides a structural scaffold for the cell shape, it organizes/polarizes the cell interior and is responsible for intracellular transport[3,9]. During neuritogenesis, when neurons transform from spheres to cells with neurites, the cytoskeleton plays an important role in engineering this morphological changes. This chapter will discuss the cytoskeletal mechanisms at work during neuritogenesis and will elaborate on the best studied MAPs and ABPs involved.

*2.1 Cytoskeletal organization in neurites*
The cytoskeletal organization of neurites comprises bundled microtubules over the length of the neurite and an actin-rich tip, called the growth cone[26]. This overall composition holds true for both dendritic and axonal precursors[9]. Actin filaments, enriched at the distal end of the neurites, regulate the directed growth and the shape of the growth cone, whereas microtubules give structure to the neurite shafts[9]. The growth cone is dominated by filopodia containing bundled actin filaments and lamellipodia containing an unbundled actin network[9]. Most actin filaments in the growth cone have their barbed ends facing the distal membrane, where polymerization can occur by the incorporation of G-actin to the filaments[27]. At the other end of the actin filaments we find the pointed ends, where depolymerization occurs. In the neurite shaft have their dynamic plus ends, where they polarize best, directed distally[28]. These microtubules are bundled and all polymerize in the same direction, towards the growth cone, where the microtubules interact with the pointed ends of the actin filaments[29]. Interestingly, the dendritic precursors have microtubules with their plus end directed both distally and proximally[30].

Although the overall composition in future dendrites and future axons seems alike, there are certain differences. Directly before an axonal precursor starts to grow, the growth cone increases in size and the actin filament network becomes more dynamic and less dense[20]. Microtubule stabilizing and actin destabilizing proteins are expressed in a larger quantity in axonal growth cones, and vice versa for dendritic growth cones (Fig. 2)[9]. Furthermore, the dendritic growth cones have actin filaments both radially oriented as well as perpendicular to the microtubule filaments[31]. These perpendicular 'actin arcs' consist of a dense actin meshwork and hinder microtubule filaments to protrude the growth cone (Fig. 2). An increase of the motor protein myosin II in the dendritic growth cone is thought to result in contraction and condensing of the actin network[32].

These and other recent observations have shed more light on the dynamic interactions between microtubules and actin in the growth cone and the mechanisms behind axon formation and elongation.
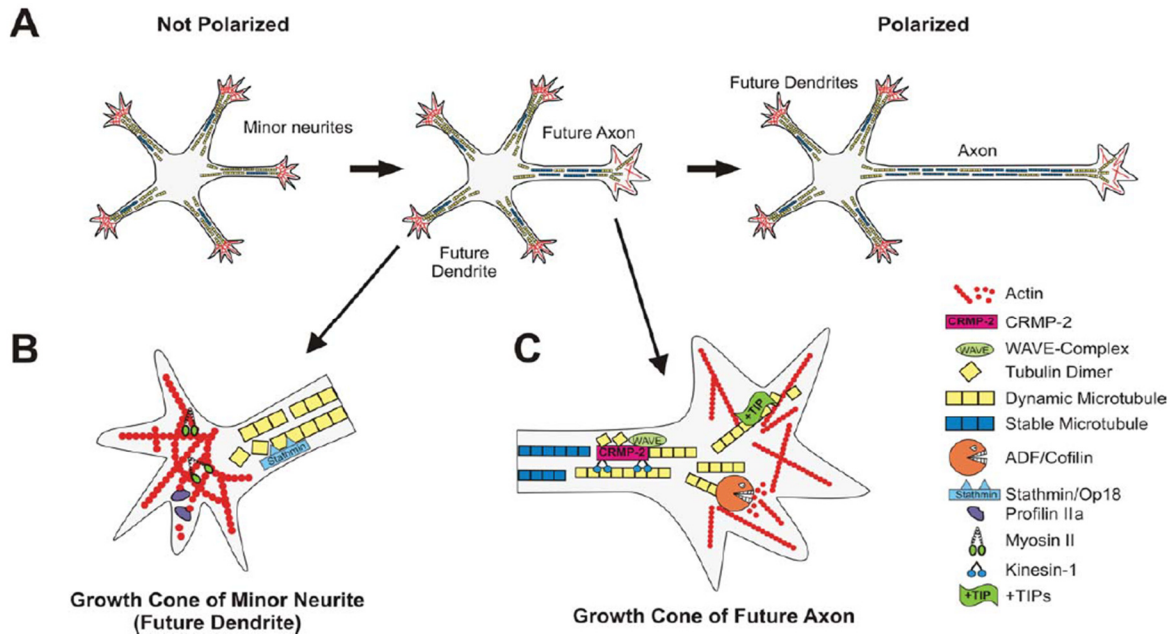
**Figure 2. Cytoskeletal organization in neurites.** A) During neuritogenesis, one of the neurites starts to elongate rapidly to become the axon. The axonal growth cone enlarges, cytoskeletal elements become more dynamic and the microtubules in the shaft become stabilized. B) The dendritic growth cone shows little cytoskeleton dynamics, caused by an increase in microtubule destabilizing Stathmin/Op18 and actin stabilizing Profilin IIa. The actin skeleton in the growth cone consists of actin filaments directed distally and perpendicular actin arcs, which prevent microtubule protrusion. C) The axonal growth cone shows a highly dynamic cytoskeleton. Distally directed actin filaments are less dense and are destabilized by ADF/cofilin. Microtubules are more stable due to proteins such as CRMP-2, the WAVE-complex and +TIPs and protrude the actin meshwork.

## 2.2 Cytoskeleton dynamics and neurite growth

After a multipolar body with multiple morphologically equal neurites has formed, one neurite will rapidly elongate to become the axon, whilst the other neurites remain short. As mentioned before, the actin filaments in the growth cone of the future axon are less dense and more dynamic compared to those in future dendrites. Microtubules however, are more stable in these axonal precursors, and interestingly it has been shown that either actin depolymerization[24] or microtubule stabilization[33] are sufficient to induce axon formation. Currently, it is believed that actin instability in the growth cone may cause reduced obstruction of microtubule protrusion and consequent neurite outgrowth[3]. Analogous to destabilizing actin in the growth cone, stabilizing microtubules also results in neurite outgrowth. These microtubules are able to overcome the actin barrier more readily[9]. Concordant with this theory, a large variety of MAPs and ABPs have been shown to have an effect on either neurite sprouting or elongation (Figure 3+4).

### 2.2.1 Microtubule associated proteins

Microtubule associated proteins have been implicated with neuritogenesis by stabilizing microtubule filaments to enhance growth (Figure 3). Some of these MAPs and their mode of action will be discussed here in short. For instance, MAP2 and tau are structural MAPs which function in the stabilization of microtubules in dendrites or axons, respectively[34]. Down-regulation of MAP2 inhibits neurite formation[35], whereas down-regulation of tau

inhibits axon formation[36]. The adenomatous polyposis coli protein (APC) interacts with and stabilizes microtubule plus ends[37], and knockdown of APC impairs axon growth[38]. Another MAP, collapse response mediater protein-2 (CRMP-2) binds to free tubulin subunits and promotes microtubule assembly[39]. CRMP-2 overexpression increases the formation of axons, while down-regulation impairs axon formation[40]. One last example of MAPs involved in neuronal polarization is stathmin/onco-protein 18(op18). Stathmin/op18 actively depolymerizes microtubule plus ends, and indirect downregulation of stathmin/op18 impairs axon formation, whereas overexpression induces multiple axons[41]. Taken together, microtubule stabilization by different MAPs enhances neurite and axon growth. However, as with actin regulators, still relatively little is known about the mechanisms of action and all the effectors involved.
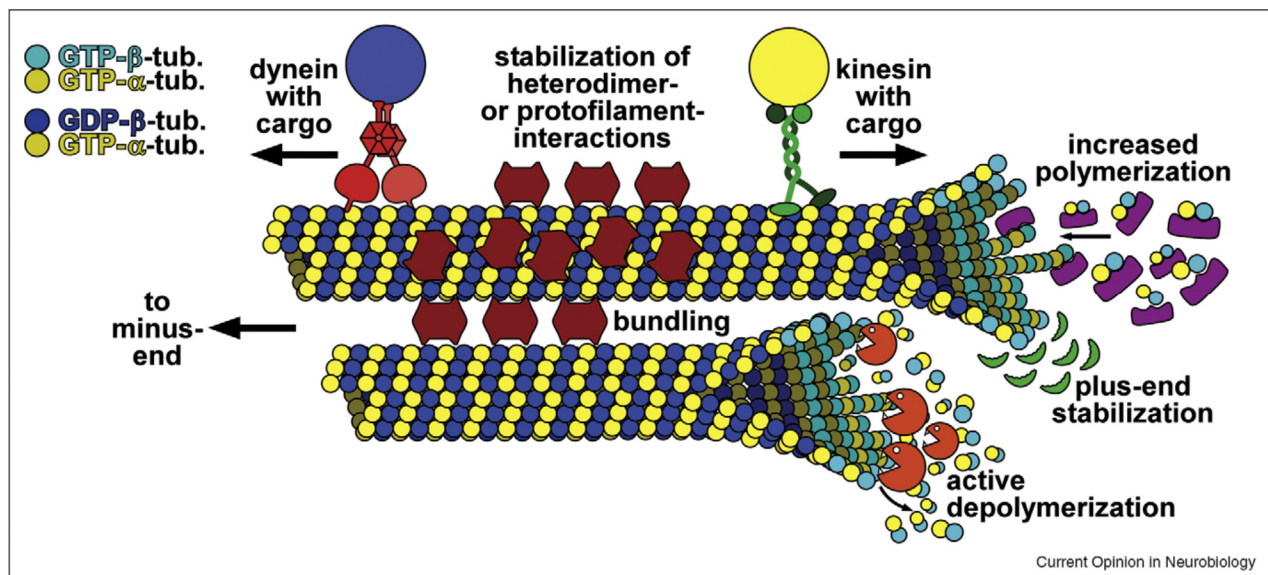


**Figure 3. Microtubule associated proteins.** Microtubule filaments consist of α/β-tubulin heterodimers. α-tubulin is always GTP-bound, while β-tubulin hydrolyzes GTP after incorporation into a filament. Different proteins control microtubule assembly and disassembly. Microtubule polymerization is controlled by proteins that bind α/β-tubulin heterodimers (such as CRMP-2 or MAP1B) or by plus-end binding proteins (such as APC, EB1, EB3). Stabilization of heterodimers within a filament is facilitated by Tau in axons and MAP2 in dendrites. Other proteins link or bundle neighboring filaments (such as doublecortin or MAP2c resp.). Depolymerization of microtubule filaments can occur direct or indirect via stathmin/op18, a protein that make α/β-tubulin heterodimers non-polymerizable.

### 2.2.2 Actin binding proteins

Different actin binding proteins have been associated with neuritogenesis (Figure 2). For instance, the family of actin-depolymerizing factor (ADF)/Cofilin (AC) are proteins enriched at the pointed end of actin filaments in the growth cone, where they depolymerize and severe actin[27]. For cofilin, it has been shown that overexpression promotes axon growth, whereas depletion of the protein impairs axon formation[42]. Recently, AC has been implicated in neurite formation (see 2.3). Profilin, a protein that promotes actin polymerization by accelerating ADP/ATP exchange and sequestering of G-actin monomers, stabilizes the actin cytoskeleton and negatively influences neurite outgrowth[43]. Similarly, the actin filament anticapping proteins, enabled/vasodilator-stimulated phosphoprotein

(Ena/VASP) increase filament elongation, and mouse neurons lacking these proteins do not form neurites[44]. Furthermore, the the actin related proteins 2 and 3 (Arp2/3) complex enhances actin density by branch nucleation, and scavenging the complex from its place of action enhances neurite outgrowth[45]. However, recently it has been shown that pharmacological inhibition of Arp2/3 impaired axon formation in granule neurons[46], which suggests that controlled actin polymerization is as important as the quantity of polymerization. Although few studies have been done on actin regulatory proteins, and few ABPs have been identified, the above findings suggest that increased actin dynamics and stability have a positive effect on neuritogenesis and axogenesis.
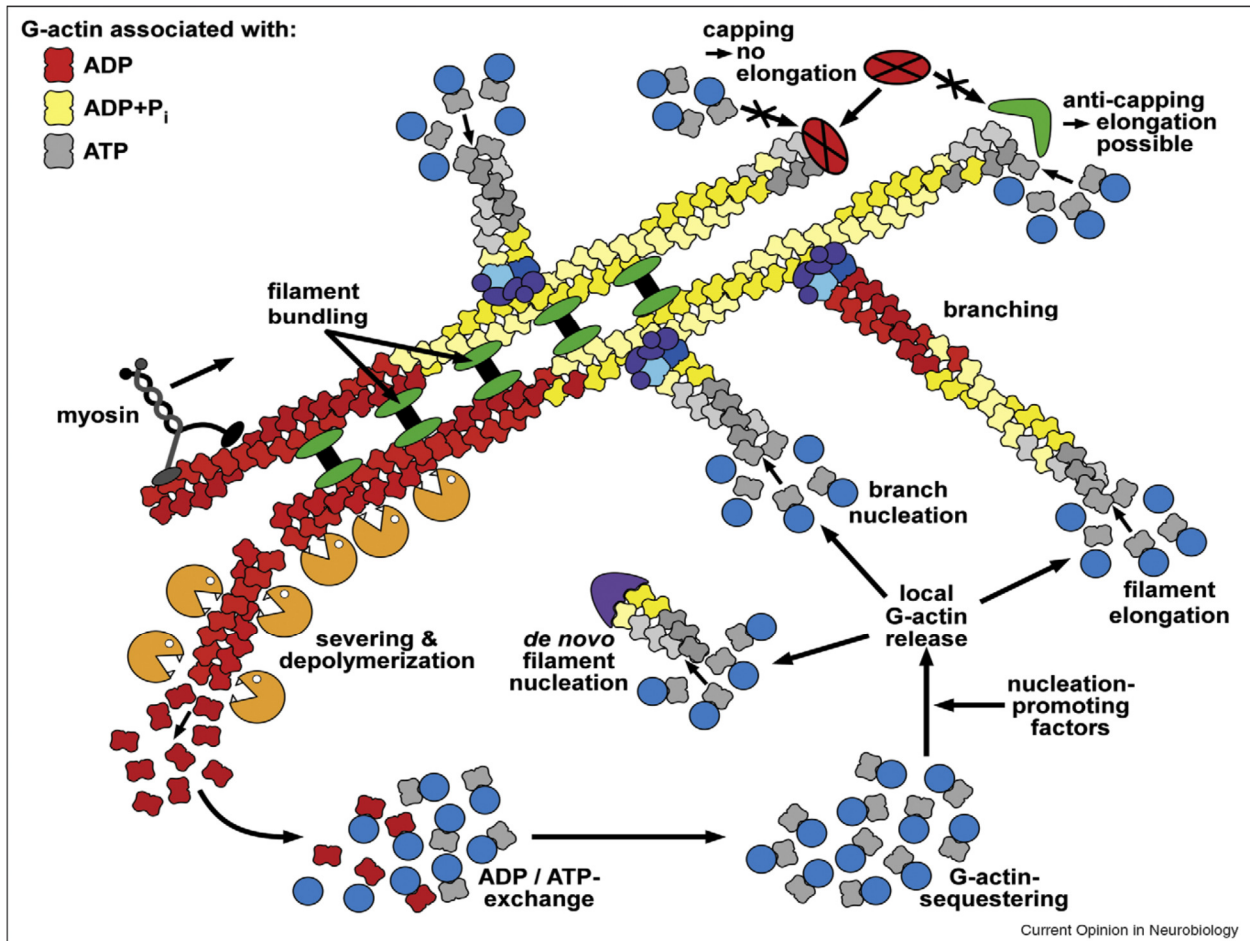


**Figure 4. Actin binding proteins.** Filamentous actin (F-actin) elongates by polymerizing ATP-bound globular actin (G-actin). Older F-actin dissociates inorganic phosphate groups. Actin dynamics are controlled by a large variety of processes and proteins. Filament elongation is a passive process and occurs with the energy within G-actin. Filaments can branch (initiated by the Arp2/3 complex) and bundle by fascin and Ena/VASP. Anti-capping proteins (such as Ena/VASP) at the barbed ends promote elongation by preventing the binding of capping proteins (such as neuromodulin and capZ), which in turn prevent filament elongation. The ADF/cofilin family of proteins severes and depolymerizes F-actin, which increases actin dynamics. G-actin units undergo ADP/ATP exchange, sequestering and local release, mediated by profilin or thymosin β4. Actin-based motor proteins (myosin) mediate transport and actin contractility.

## 2.3 Cytoskeleton dynamics and neurite formation

Directly after mitosis *in vivo* or plating *in vitro,* neurons are spherical cells and this symmetry is broken by the formation of neurites. The upstream regulators of neuronal polarity as depicted in Figure 1 interact with both MAPs and ABPs to alter microtubule and actin dynamics. The intrinsic polarity regulators are most likely inherited from the last mitotic division, where N-cadherin might be a key regulator[21]. The process of neurite and axon elongation is most likely the consequence of a decreased actin density at the growth cone and an increased microtubule stability[47]. Although several factors that regulate actin dynamics have been proposed to facilitate neuritogenesis, none of these ABPs have been shown to drive the intrinsic process of neurite formation. A recent study suggests that the family of actin regulatory proteins ADF/Cofilin (AC) alone is required and sufficient to induce neuritogenesis[48]. Both *in vivo* and *in vitro*, knockout of the AC proteins impairs neurite formation and disturbs actin organization. Actin filaments in neurons lacking AC do not point their barbed ends distally and are unable to bundle. Furthermore, it is shown that actin retrograde flow, a process where actin polymerization pushes the actin filaments and the adjacent membrane backwards, and actin turnover are decreased dramatically[48]. Thus, the ADF/Cofilin family of proteins is able to induce neurite sprouting in two ways. First, by their severing and depolymerizing activity at proximal end of the actin meshwork, they render the actin cytoskeleton permissive for microtubules to protrude. Second, the AC proteins regulate actin orientation due to their preference for older actin filaments. By preferentially severing and depolymerizing the older actin filaments, away from the leading edge, an organized actin network is perceived[48]. This is the first study to reveal an intrinsic determinant for neuritogenesis that directly interacts with the cytoskeleton.



**Figure 5. Model for neurite sprouting and elongation mediated by ADF/Cofilin.** In wild-type neurons (left) ADF/Cofilin (yellow) binds to older F-actin (dark-red) and subsequently severs and depolymerizes actin filaments away from the leading edge. Due to this preference for older F-actin, the actin filaments organize in a radial fashion. Furthermore, both the degradation and the orientation of F-actin allow microtubules to protrude the actin rich growth cone. In AC knock-out neurons F-actin severing and polymerization is impaired, which decreases actin turnover and retrograde flow, disorganizes F-actin and increases F-actin density. Subsequently, microtubules cannot protrude the dense and disorganized actin network, or microtubule elongation is improperly directed.

# 3 Neurite to axon transition: stabilization and maintenance

Directly after mitosis, spherical neurons put different mechanisms into action to extent neurites. A large variety of membrane and cytosolic molecules are involved in signaling pathways that – via microtubule associated proteins and actin binding proteins - lead to the alteration of the cytoskeleton. In turn, these cytoskeletal changes lead to the sprouting of multiple neurites, of which the first neurite is most likely to become the axon. Although molecules required for axon specification are present during early neuritogenesis, the axonal precursor has to commit to its identity as an axon.

Interestingly, cutting the original axon of young neurons close to the cell body can change a future dendrite to a new axon[49-51]. Besides this plasticity in early neuronal development, it has been shown that axotomy in functionally polarized neurons can turn a dendrite in an axon as well[52]. This neuronal plasticity shows that all neurites have to potential to become an axon, yet only the axonal precursor becomes stabilized and commits to the axonal fate. However, this dendrite-axon conversion only occurs when the axon is cut at a length of approximately 35μm or closer to the cell body[52]. If the cut is made more distally, the axon remains its identity and regrows. Interestingly, it has been shown that axotomized hippocampal neurons regrow their axon at the neurite opposite of the cut axon, again suggesting an intrinsic bipolarity axis[7].

These findings suggest a high plasticity in all neurites – and even mature axons and dendrites. After the initial establishment of neuronal polarity, how does the minor process that is selected to become the axon sustains growth and commits to its fate? Which mechanisms are involved in the stabilization and consolidation of the axonal precursor? The third chapter will elaborate on this question and will review the current knowledge on the maintenance of neuronal polarity, with an emphasis on the fate of the axon.

*3.1 Spatio-temporal expression of polarizing signals*
The early instructions for a minor process to develop into either an axon or a dendrite are discussed in Chapter 1 and 2. Initial polarizing signals determine the fate of a minor process to become the axon, albeit a reversible and plastic process. The differences in the final architecture between axons and dendrites arise because of their unique molecular and supramolecular organization. Aforementioned, future dendrites and future axons contain different molecules in the growth cone and shaft to drive cytoskeletal and morphological changes (Fig. 2). A wide variety of cell-surface receptors, cytosolic molecules and cytoskeletal elements show spatio-temporal expression patterns specific to either the dendritic or axonal fate[53]. One text-book example of this is the expression of two major neuronal MAPS, Tau and MAP2, which are generally thought to be polarized to the axons and dendrites, respectively, in mature neurons[54]. Two mechanisms of cellular distribution are involved in the polarization of the axon: intracellular transport and local feedback loops.

*3.2 Motor proteins and intracellular trafficking*

When one neurite of a multipolar neuron begins to grow to become the axon, the cytoskeletal foundation is laid for the mature neuron. Although all neurites have microtubules bundled in the shaft with their plus ends distally, the dendrites – and not the axon – will establish microtubules directed distally and proximally in roughly equal proportions[30]. Besides their functions in maintaining cell structure, neurite sprouting and growth, microtubules also play an important role in cellular transport. Kinesins and dyneins are microtubule motors that are mostly plus-end and minus-end directed, respectively (Fig. 3)[55]. Since axons have all their microtubules plus-end distally, unidirectional transport takes place, where the axon functions as a sink. The transport of molecules mediated by plus-end motors can therefore accumulate in the axonal growth cone, while depleting this compound in the soma and dendrites. Furthermore, some kinesins have a higher binding affinity for stable, acetylated microtubules, which are found in higher amounts in the future axon, thereby also directing trafficking[33].

The best studied motor proteins in neurogenesis are the kinesin superfamily of proteins (KIFs), which move to the plus end of microtubules, i.e. the distal axon. Kinesin-1 (comprising of two kinesin heavy chains, KIF5, and two kinesin light chains, KLC) is a motor that preferentially binds to acetylated microtubules[56] and several cargo proteins have been reported to be transported by it[10]. Interestingly, KIF5 localizes to the future axon even before this neurite begins to grow[57]. Consistent with the localization and binding preference of kinesin-1, it binds and transports several key molecules (directly or indirectly) involved in axon formation to the axon tip where they accumulate (Fig. 6)[10]. Kinesin-2 (comprising of KIF3 and KAP3) also accumulates in the axon and mitigates the transport of the PAR3-PAR6-aPKC complex by binding of PAR3 to KAP3[58], whilst guanylate kinase-associated kinesin (GAKIN) is reported to transport PIP3[59]. Besides transport of molecular complexes and enriched Golgi-derived vesicles (Fig. 6), KIFs are also involved in the transportation of mitochondria, peroxisomes and ribosomes into the future axon[60]. These and numerous other studies[61] show that intracellular trafficking has important roles in the establishment and maintenance of neuronal polarity by recruiting key regulators into one neurite and facilitate axogenesis.

*3.3 Feedback loops*

The intracellular distribution of polarizing signals is driven by both active transport via the microtubule skeleton and feedback mechanisms. It has been shown that either a brief actin depolymerization[24] or microtubule stabilization[33] are sufficient to bias the fate of a neurite to become the axon, which implies a self-reinforcing process at work during axon formation. Several positive and negative feedback loops have been identified to be involved in the generation of the axon and its subsequent stabilization and consolidation. Positive feedback loops, or amplification mechanisms, increase the production and/or localization of certain molecules, whilst negative feedback loops consist of protein degradation.
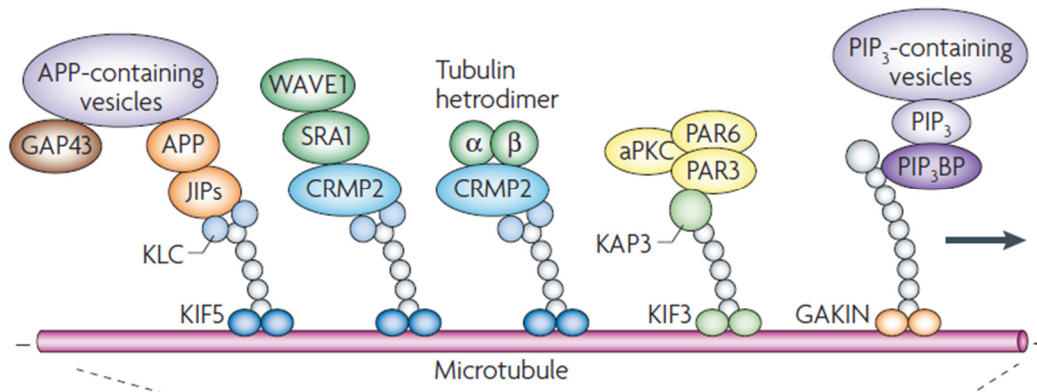
**Figure 6. Transport of key regulators in axogenesis by kinesins.** Kinesin-1 consists of two kinesin family of proteins 5 (KIF5) and two kinesin light chains (KLC). Cargo can bind either directly to the KLCs (JNK-interacting proteins (JIPs) and collapsing response mediator protein 2 (CRMP2)) or indirectly as a molecule or in a vesicle (amyloid precursor protein (APP), growth-associated protein 43 (GAP43), specifically Rac1 associated protein 1 (SRA1), WASP family verprolin homologous protein 1 (WAVE1) and tubulin dimers). Kinesin-2 consists of KIF3 and kinesin-superfamily associated protein 3 (KAP3) transports the PAR polarity complex by direct binding to PAR3. Guanylate kinase-associated kinesin (GAKIN) transports PtdIns(3,4,5)$P_3$ (PIP3) by binding it indirecty via PIP3 binding protein (PIP3BP). All three kinesins move along microtubules in the plus-end direction and accumulate in the axon.

One such reinforcing mechanism is the autocrine neurotrophin loop. Neurotrophins (NTs) are molecules that are secreted by the neuron itself and bind to Trk receptors (Fig. 1) to induce axon differentiation and growth[62]. Surface-bound NTs trigger both Trk transport and Trk insertion in the membrane, which enhances local Trk expression[63]. Furthermore, binding of NTs to Trks causes an elevated cAMP/PKA expression, which in turn increases the secretion of NTs[18]. Due to this feedback, a membrane patch enriched in Trks can originate.

Another putative mechanism of positive reinforcement at the level of the neuronal membrane is the above-mentioned accumulation of cadherins (Chapter 1). Fluctuating cadherin levels on the membrane cause the local elevation of this very cadherin expression[11]. p120-catenin (p120) is a protein that binds the cytosolic part of cadherin and functions in the stabilization of cadherin and downstream signaling pathways[64]. Knockout of p120 *in vitro* resulted in the rapid degradation of N-cadherin complexes[64]. These findings suggest that that binding of p120 is required for the maintenance of membranal cadherin expression. However, direct evidence of p120 mediated cadherin amplification is still missing.

Intracellular, PI3K-mediated production of PIP3 is implicated in the formation of the axon, and PIP3 is found to localize at the future axon during stage 2[8]. PIP3 expression activates the Rho GTPases Rap1B, Cdc42 and – via the PAR3-PAR6-aPKC complex – Rac1. Rac1 in turn can activate PI3K, thereby forming a positive feedback loop (Fig. 1)[18]. The proteins involved in this loop relay numerous signals downstream to the cytoskeleton.

Also, as seen in Figure 1, PKA can activate LKB1 by phosphorylating it, which is required for its downstream signaling. This complex is self-reinforcing in multiple ways. First, the LKB1/STRAD complex promotes LKB1 stability, thereby preventing its degradation[62].

Second, the complex promotes LKB1 phosphorylation by PKA, thereby activating LKB1[62]. Third, LKB1 recruits and binds STRAD to the scaffolding MO25, whilst this STRAD-MO25 complex promotes LKB1/STRAD formation. LKB1/STRAD then again promotes LKB1 stability and phosphorylation[62].

Besides feedback loops at the level of receptors and signaling molecules, positive reinforcement occurs at the cytoskeletal level as well. As previously mentioned, both the stabilization of microtubules and the destabilization of actin can induce neuritogenesis and neurite/axonal growth. In migrating fibroblasts, it has been shown that enhancing microtubule growth results in increased actin dynamics[65]. The growth of microtubules is hypothesized to activate Rac1, which in turn increases actin dynamics at the leading edge[65]. Subsequently, this increased actin dynamics readily enables microtubules to protrude further into the growth cone. Also, the increase in stable microtubules attracts motor proteins that transport growth-enhancing cargo, such as WAVE and CRMP-2 into the future axon[1]. Due to the polarity orientation of the microtubules, preferred transport of these cargoes into the future axon prevents the other neurites to grow[60].

Yet another mechanism to obtain localized expression of polarity regulators is by selective protein degradation. The inhibition of protein degradation with use of a proteosome inhibitor in neurons results in the formation of multiple axons[66]. AKT, a protein kinase that inhibits GSK3b and thereby promotes axon growth, has been shown to undergo selective degradation in future dendrites and not in the future axon[66]. This selective degradation is mediated by the classical ubiquitin-proteosome system (UPS), which tags proteins for degradation. A similar mechanism ensures that the Rho GTPase Rap1B (figure 1) is degraded in future dendrites[67]. The selective degradation of LIM kinase (LIMK) is also mediated by the UPS, albeit in the axon, since low LIMK levels are required for axon initiation[68]. Although the UPS is a conserved mechanism which has been studied extensively, its importance in the establishment of neuronal polarity and the factors involved remain to be unraveled. Further research has to be performed to identify all targeted proteins and the upstream regulatory signals involved during neurite and axon formation.

Taken together, feedback loops play an important role in the spatial localization of polarizing signals. A variety of mechanisms result in the local amplification or degradation of signaling molecules and the subsequent actin and microtubule dynamics. However, to which extent signaling loops are present during the development of neurites and the consolidation of the axon requires additional research. A complete understanding of all molecules involved remains to be elucidated.

*3.4 The axon initial segment and axon polarization*
Mature neurons comprise of two functionally distinct domains: the axonal and the somatodendritic domains. These domains are connected by a single structure locates at the proximal axon: the axon initial segment (AIS). When discovered, the AIS was implicated as the site of action potential initiation by integrating somatodendritic synaptic events[69]. More recently, it has been shown that the AIS functions as a molecular barrier and is involved in

the establishment of the axon[70]. The former is mediated by a high density of voltage-gated sodium and potassium channels at the AIS[71]. The latter involves a variety cell adhesion molecules, extracellular matrix molecules and cytoskeletal scaffolds which act as a functional barrier for membrane and cytoplasmic proteins[71].

It appears the scaffolding protein AnkyrinG (AnkG) is a master organizer of AIS assembly, since silencing of this protein prevents the AIS to form[72,73]. AnkG binds and recruits βIV spectrin to the AIS, which in turn has a high binding affinity for actin[74]. This actin accumulation at the AIS provides both a cytoplasmic barrier and a membrane barrier by anchoring a high density of L1 family cell adhesion molecules[75].
The rigid actin meshwork functions as a mechanical barrier between somatodendritic and axonal compartments, hindering cytosolic proteins to cross the AIS. Partial depolymerization of actin, as well as the silencing of upstream AnkG results in the free diffusion of cytosolic proteins between the two domains[72,76]. The membrane barrier located at the AIS is established by a high density of membrane proteins that hinder diffusion and a rigid CAM barrier, held together by the actin meshwork[75]. Actin disruption shows an increase in the mobility of phospholipids in the AIS membrane and the free diffusion of CAMs, which are normally restricted to the AIS[75,77]. Again, the loss of either actin or AnkG results in diffusion of somatodendritic and axonal membrane proteins between domains[71].

The AIS is a structure with an important function in maintaining neuronal polarity. Upon dismantling the AIS *in vitro,* the axon acquires many molecular and structural features of dendrites[72]. *In vivo,* similar results were found in AnkG deficient Purkinje neurons[78]. However, the axon was still identifiable as a single process opposite the dendrite, implying that AnkG is nog required to break symmetry, only to maintain it. Perhaps not surprisingly then, AnkG first becomes detectible at the proximal axon at stage 3. How AnkG is localized to the AIS and what its upstream regulators are, is still largely unknown, and might be determined at an earlier stage of neurogenesis.

# 4 Discussie

Neurons are amongst the most polarized cells. The formation of a mature neuron with highly specialized axonal and dendritic compartments involves numerous cellular interactions. The asymmetrical presence of extracellular cues *in vivo* can influence neuronal polarization by directing axonal and dendritic outgrowth[10,18,19]. However, most isolated neurons *in vitro* are able to develop into their mature state without external cues, which implies that the complex process of polarization is ultimately intrinsically regulated[4]. In this thesis, the mechanisms involved in early neuronal development – from the sprouting of the first neurites to the consolidation of the axon – are discussed.

Directly after its last mitosis, the spherical neuronal precursor develops several morphologically indistinct neurites, of which the first developed is most likely to become the axon. This notion underlines the importance of the earliest events in neuritogenesis, since the polarity axis of a neuron is determined during, or before the sprouting of the neurite. Recent evidence suggests that the first intrinsic polarizing signals are determined by the asymmetrical distribution of cytokinesis remnants[17]. Interestingly, this link between cytokinesis remnants and polarization has recently been suggested in budding yeast as well[79]. Since both the process of cytokinesis and of sprout formation require membrane and cytoskeleton remodeling, the core machinery of the former might be reused for the latter. The localized expression of mitotic furrow components is thought to recruit molecules required for neurite sprouting (Fig. 1). An intrinsic local feedback mechanism of neuronal cadherin might play a key role in neurite sprouting, resulting in the localized accumulation of N-cad[11,16].  The sprouting of the second, and possibly all neurites, is most likely the consequence of passive diffusion of membrane proteins (possibly cadherin) that instruct neurite outgrowth[11]. Although recent evidence links cadherin to intrinsic neurite sprouting, different molecules might be involved in early neuritogenesis. Future research should aim to identify all mitotic furrow components involved, and determine their individual contributions in neuronal polarization. Furthermore, increasingly more studies are identifying signaling molecules that relay signals to the cytoskeleton[8,10]. However, our knowledge on the specific interactions and mechanisms they are involved in remains far from complete. Redundancy, the sheer extent of molecules and the network of interactions involved in neuronal polarization complicate the field or research.

Morphological changes in any cell are mediated by changes in the cytoskeleton. A wide variety of signaling molecules relay instructions to the cytoskeleton, via actin binding proteins or microtubule associated proteins. The upstream signals most likely originate from the unequal distribution of cytokinesis remnants. ABPs act on the peripheral actin filaments to destabilize the actin meshwork and allow microtubules to protrude[9]. The actin filaments are directed with their barbed ends distally, thereby generating an outwards force as a consequence of increased actin turnover and retrograde flow. This outwards force is further enhanced by MAPs that increase microtubule elongation in the same trajectory as the actin filaments, thereby enhancing neurite outgrowth. Neurite sprouting and elongation are regulated by largely the same mechanism. Different cytoskeleton effectors have been found to contribute to neuronal polarity. As with upstream signaling

molecules however, our understanding of their exact chemical interactions and specific roles is far from complete. In recent years, particular progress has been made in understanding cytoskeleton dynamics in neurito- and axogenesis. However, no definitive conclusions have been made whether microtubules or the actin cytoskeleton are the driving force for neuritogenesis. The exact interactions between microtubules and the actin cytoskeleton remain enigmatic and future research will provide us with a more complete understanding.

One promising finding is the role of the ADF/cofilin family of proteins in steering the cytoskeleton by increasing actin turnover and retrograde flow[48]. It is thought that ADF/cofilin proteins arrange actin bundles distally, allow microtubules to protrude the growth cone and provide the force for elongation. Studies on other ABPs, such as Ena/Vasp, have shown to compromise neurite outgrowth[44]. However, neuritogenesis can be restored upon plating these cells on laminin, which suggests that the intrinsic mechanism of neurite formation does not rely on Ena/Vasp[80]. The ADF/cofilin family of proteins seems to be required for intrinsic neurite outgrowth. Interestingly, a recent study has shown that neuronal cadherin activates ADF/cofilin via the Rho GTPase Pak1[81]. Since both cadherin and ADF/cofilin have been labeled as intrinsic determinants of neurite outgrowth, this pathway might be of significant importance. Future studies have to explore the option if this holds true. When so, experiments aimed at other – instructive rather than intrinsic - polarizing cues should take this into account.

The formation of the axon starts with the rapid elongation of a single neurite. The instructions for the axonal precursor to enhance its growth and to obtain the axonal cell fate are regulated by a variety of mechanisms. The spatio-temporal localization of molecular determinants and the cytoskeletal architecture in the future axon is tightly regulated. The localized expression of signaling molecules and downstream cytoskeleton effectors (MAPs and ABPs) in neuritogenesis is regulated by molecular feedback loops and intracellular trafficking. Feedback loops are present both at the level of upstream signaling molecules and of the cytoskeletal. Intracellular trafficking promotes polarization mainly due to the unidirectional microtubules in the axon and bidirectional microtubules in the dendrites. This hypothesis is supported by the fact that dendrites converted to axons change their microtubule polarity accordingly within 24h[51]. Unfortunately, the mechanisms responsible for the development of these varying microtubule orientations in neuronal processes remain unclear.

The axon initial segment starts to develop during axogenesis and functions as a mechanical barrier between the somatodendritic and axonal compartments. The mechanical barrier formed by the AIS, sorting diffusing molecules by size, is well studied[71]. It is very likely however, that the AIS also functions as a chemical barrier through specific molecular recognition of cytosolic components. Furthermore, the initial positive and negative intracellular signaling pathways required to obtain a polarized neuron increase the metabolic demand of the cell. Although these pathways and feedback loops might be sufficient to acquire a polarized neuron, the AIS can take over most of these energy-dependent signaling pathways. Since mature neurons can live for decades in humans, a functional AIS allows neurons to consummate energy for their mature purpose. The AIS

might switch off and replace most early polarizing signals. Axotomy in mature neurons closer than 35um to the cell body resulted in the transformation of a dendrite into an axon[52]. Although it is proposed that this transformation is caused by microtubule deformation and destabilization, this might also be the result of disruption of the AIS. This option needs to be further explored.

*4.1 Future directions*

The function of a neuron is to receive and transmit information in the brain via the dendrites and the axon. Most of our knowledge on the polarization of these processes and the molecules involved is based on *in vitro* data, primarily using rodent hippocampal neurons. Recent progress has been made in identifying the numerous molecular mechanisms regulating neuronal polarization. Furthermore, different extracellular cues have been identified *in vitro* to be involved in this process. The functioning of these molecules and their implications in polarization are however inadequately studied *in vivo.* The function of a few molecules has also been confirmed *in vivo*, such as PI3K and several guidance cues[8,82]. Appropriate knockout models of all polarity regulators are needed to fully evaluate their function. Furthermore, *in vivo* studies will shed light on the instructive roles of extracellular cues and cell-cell interactions. This also creates the opportunity to assess which signaling cascades and molecules are relevant in physiological conditions, and what their contribution is to polarization. Nonetheless, we have acquired substantial knowledge on the polarization program of neurons with the use of *in vitro* approaches, and they will remain a powerful tool.

Different experimental techniques can be used to study neuronal polarity. Some drawbacks of the current techniques are however apparent. One major challenge in the field is to assign specific functions to the involved proteins and molecular pathways[26]. Currently, most studies performed have used simple, often morphological read-outs (such as no axon formation or multiple axons). Methods are needed for the quantitative analysis of cytoskeleton dynamics and the upstream regulators. Furthermore, improvements in imaging techniques allow us to monitor polarizing neurons on a higher spatio-temporal resolution. The molecular distribution of polarity determinants is subject to a high spatial and temporal variety due to the dynamic nature of neuronal polarity. Real-time imaging techniques will help to elucidate the complex interplay between signaling molecules, cytoskeleton effectors, motor proteins and the cytoskeleton. Although knowledge on the aforementioned factors is increasing rapidly, integrating them remains problematic. Incorporating and understanding all processes involved will be the challenge of the years to come.

The ultimate challenge of research on neuronal development is to apply our knowledge to pathological conditions such as spinal cord injury. Although *in vitro* manipulations of neurons can recapitulate their polarization program, *in vivo* lesions in nervous tissue are often not capable of recovery. Our understanding of neuronal development might lead to the reprogramming of defective neurons to cure disease. In this respect, a recent study has shown that moderate microtubule stabilization in rodents after spinal cord injury increased axon regeneration and functional recovery[83].

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