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Review

Moving through the crowd. Where are we at understanding physiological axon growth?

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ABSTRACT

Axon growth enables the rapid wiring of the central nervous system. Understanding this process is a prerequisite to retriggering it under pathological conditions, such as a spinal cord injury, to elicit axon regeneration. The last decades saw progress in understanding the mechanisms underlying axon growth. Most of these studies employed cultured neurons grown on flat surfaces. Only recently studies on axon growth were performed in 3D. In these studies, physiological environments exposed more complex and dynamic aspects of axon development. Here, we describe current views on axon growth and highlight gaps in our knowledge. We discuss how axons interact with the extracellular matrix during development and the role of the growth cone and its cytoskeleton within. Finally, we propose that the time is ripe to study axon growth in a more physiological setting. This will help us uncover the physiologically relevant mechanisms underlying axon growth, and how they can be reactivated to induce axon regeneration.

1. Introduction

Neurons are the building blocks of our central nervous system (CNS), possessing unique structures to fulfil their roles. Neurons receive chemical inputs (neurotransmitters) that are transformed by membrane polarisation/depolarisation into electrical signals. Such electrical signals trigger the release of neurotransmitters into neighbouring neurons, propagating signals throughout the neural circuit. These signals are received by dendrites and are then relayed by axons [1]. Neurons possess a highly branched dendritic tree and a single long axon. During CNS development, axons navigate extremely complex environments to reach their final destination and establish synaptic connections. Once axons find their synaptic partners and establish neural circuits, neurons of the CNS shift from a dynamic phase to a transmitting phase, losing their ability to grow and regenerate [2,3]. The ability of axons to manoeuvre and reach their synaptic partners is governed by both intrinsic and extrinsic factors [4]. In this review, we describe events occurring during axon development, outlining key contributors to axon growth and describing their role in this process. To this end, we will describe how axon growth proceeds when neurons are cultured in two dimensions (2D). We will further describe the recent progress of how neurons grow in three dimensions (3D) [5]. Finally, we provide an outlook on how these novel developments are advancing the field. In particular, we discuss how new models (3D collagen gels and organotypic slice cultures) can be exploited to better visualise detailed events taking place during axon development in more physiological contexts.

2. Neuronal polarity

Neuronal polarity is a complex process where neurons generate a single axon and dendrites, a process occurring in key stages which are also conserved *in-vivo*. It involves cellular and molecular events that are highly sophisticated and remain to be fully understood. In this review, we mostly focus on hippocampal neurons and excitatory cortical neurons as commonly used models to study neuronal polarity *in-vitro* and *in-vivo* respectively.

2.1. Neuronal polarity in-vitro

Dissociated hippocampal neurons are one of the most popular models used to study neuronal polarisation *in-vitro* (Fig. 1A). Their development is stereotypic. Morphologically, hippocampal neurons initially appear rounded and symmetrical, with lamellipodia surrounding the periphery of the cell (stage 1). Minor processes then start to

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develop at stage 2, maintaining an overall symmetric array. This symmetry breaks at stage 3, when one neurite experiences a burst in growth rate, elongating faster than the other neurites to become the axon. Finally, the remaining short neurites develop into dendrites and become highly branched (stage 4), allowing the neuron to transition functionally into an information-processing unit (stage 5) [6]. Understanding such intricate processes allows for studying growth *versus* non-growth states side-by-side. This greatly helped bring regeneration research forward [7–9].

Notably, the mechanisms underlying neuronal polarization are still largely unclear. It appears that several signalling events feed into this process. For example, several feedback loops locally activated in minor neurites have been associated with its specification for axonal fate - during stage 2–3 transition – including Shootin1, HRas, brain-derived neurotrophic factor (BDNF)- cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) and adhesion molecule loops. Global inhibition of remaining neurites is equally important to maintain polarity. This process – however - is far less understood [1,10]. One main feature of future axons is the accumulation of key components of the growth machinery even before overt morphological changes. For instance, the concentration of cAMP in a single neurite (the future axon) leads to a reduction of cAMP in all other neurites (future dendrites), thus acting as a global inhibitor [11]. However, it is worth mentioning that there is an overall traffic in the future axon including membrane traffic [12]. Another example is the insulin-like growth factor-1 (IGF-1) receptor, which activates phosphatidylinositol-3 kinase (PI3K). During neuronal polarisation, IGF-1 - along with activated PI3K -accumulates in the future axon [13]. Despite this wealth of possible molecular components, it is truly surprising that we still understand very little about a basic question: How does each neurite "know" whether and how the other neurites are growing. This remarkable coordination within a developing neuron has remained an enigma.

The cytoskeleton network, consisting of actin and microtubules (MTs), is the biological scaffold where intracellular forces converge and are balanced. Therefore, the cytoskeleton plays a key role during neuronal polarisation. In the future axon, actin filaments are more dynamic and less stable [14]. In fact, the destabilisation of actin filaments is causal to axon formation. Actin depolymerisation using cytochalasin D or latrunculin B releases growth restraint from minor neurites, allowing them to grow as axons [14,15]. Conversely, stabilisation of actin filaments using jasplakinolide prevents axon formation [15]. MTs play an equally important role in neuronal polarisation. MTs stabilisation *via* low doses of taxol induces axon growth in previously undifferentiated neurites [16] and in dendrites [17]. This knowledge was fundamental in understanding how one component of the neuronal cytoskeleton behaves in reaction to changes applied to the other, specifically within relevant compartments such as axons and growth cones. In fact, the interaction of MTs and actin filaments in neurons has remained fragmentary [18]. The role of actin and microtubules in the growth cone will be discussed in Section *3*.

Moreover, multiple axons have been induced with the overexpression of polarity-related factors, such as Par3, cell division cycle 42 (Cdc42) and Ras-related protein 1B (Rap1B), all of them directly influencing the neuronal cytoskeleton [19]. The latter is known to localise to distal tips of future axons and actin downstream of Cdc42. Cdc42 is a member of the Ras homolog gene family guanosine triphosphatase (Rho GTPase) a subfamily from the Ras superfamily of small GTPases. Cdc42 is well-known for its role in modulating cytoskeletal changes during neuronal polarity [20]. In fact, Cdc42 is essential for triggering axon formation as Cdc42 KO neurons fail to generate an axon [21]. Similarly, the Rho-GTPase Rac1 is essential for axon formation albeit using a different signalling pathway than Cdc42 [20]. Rac1 triggers axon growth by dynamizing the actin cytoskeleton through the Wave complex whereas Cdc42 dynamizes the actin cytoskeleton through cofilin [20,

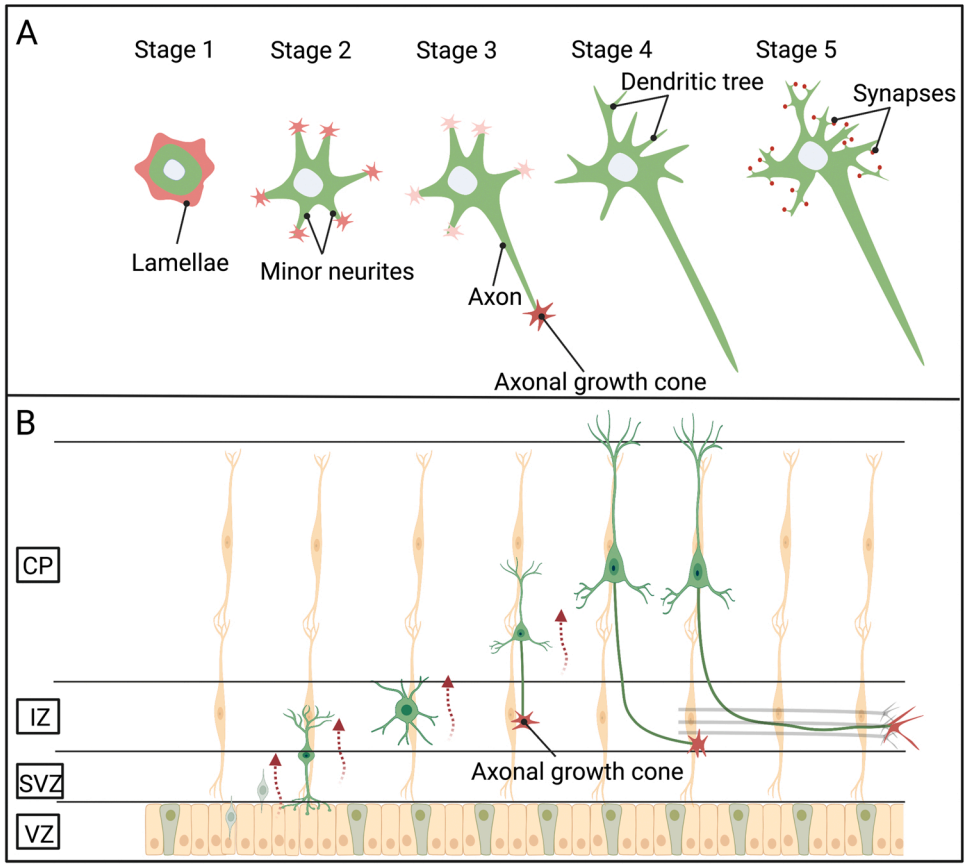

Fig. 1. Neuronal polarisation *in-vitro* and *in-vivo*. A. The different polarity stages of hippocampal neurons. Hippocampal neurons begin as a round cell surrounded by lamellipodia (stage 1). They then develop minor neurites (stage 2), one of which becomes the axon (stage 3). The remaining neurites polarise to become dendrites (stage 4). The neuron finally matures to become a compartmentalised cell (stage 5). B. Polarisation of excitatory cortical neurons. Neuronal progenitors proliferate in the ventricular zone (VZ) and migrate through the subventricular zone (SVZ) and intermediate zone (IZ) towards the cortical plate (CP) by climbing on radial glial cells (RGCs) (orange). During migration, neurons develop axons which extend into the IZ forming axon bundles (grey).

21]. Recently, the guanosine nucleotide-exchange factors (GEF), Tuba and the Rho GEF factor 7 (Arhgef7) were identified as activators of Cdc42 through Rab8 and TC10 - respectively – [22, 23].

2.2. Neuronal polarity in-vivo

The cerebral cortex is commonly used as a model to examine neuronal polarity *in-vivo* and *in-situ* [24–26]; it is an incredibly complex brain region where most neurons fall within two main populations of excitatory glutamatergic neurons and inhibitory GABAergic neurons [27]. Excitatory cortical neurons originate from the asymmetric division of neuronal progenitors in the ventricular zone (VZ) (Fig. 1B). These neurons then migrate radially - by climbing radial glial (RG) cells - through the subventricular zone (SVZ) and intermediate zone (IZ) toward the cortical plate (CP). During this process, excitatory cortical neurons become multipolar as they exit the lower part of the IZ, to bipolar as they enter the upper part of the IZ - thus forming a leading process (future dendrite) and a trailing process (future axon) [26,27]. Conversely, inhibitory cortical neurons originate in the subpallium and migrate tangentially in the SVZ, IZ or marginal zone (MZ), with a small population of inhibitory cortical neurons descending from the MZ and settling in the CP [28]. During migration, inhibitory cortical neurons also exhibit a bipolar structure with leading and trailing processes, similar to that of bipolar excitatory neurons and eventually undergo an axon-dendrite polarisation [27]. It is still unclear whether neuronal polarisation and migration are interdependent processes or whether one can take place in the absence of the other.

The GTPase Rho family member A (RhoA) plays a central role in neuronal polarisation *in-vivo* [29,30]. RhoA controls axon initiation and extension through the assembly of myosin II arcs at the transitioning zone of the growth cone, leading to rearrangement of the cytoskeleton [29,30]. Interestingly, RhoA does not play a role in axon specification [29]. Of note, the physiological role of many polarity-related factors in neuronal polarity remains unknown. Most studies are largely based on acute overexpression of such factors. This - however – does not describe all physiological functions [31]. To that end, studies using a long-term loss of function of polarity-related factors along with acute loss of function – as in the case of RhoA [29,30] – are required to better understand the functions of specific factors on neuronal polarity.

2.3. The role of extracellular matrix and cell-cell interaction in neuronal polarisation in-vivo

From an experimental perspective, having a homogeneous environment surrounding cultured neurons - from media components to neighbouring cells - has many advantages. However, neuronal polarisation *in-vivo* occurs in a heterogeneous microenvironment characterised by a highly complex extracellular matrix (ECM) [32]. During polarisation, axon orientation *in-vivo* is tightly controlled. For instance, laminin, an extracellular molecule, instructs neurites of Retinal Ganglion cells (RGCs) to become axons and directs their orientation [33]. Laminin was also found to have a role in neuronal progenitor proliferation, differentiation and migration [34]. Other extracellular cues either attract or repel axons during development, guiding them toward their synaptic partners. Such shepherding extracellular molecules are commonly found in the ECM of the CNS. The midline of the spinal cord houses a variety of axonal commissures crossing the midline to connect the contralateral sides of the nervous system [35]. It is also the location of many molecular guidance cues, funnelling axons towards, and through, these complex corridors of the CNS.

Cell-to-cell interactions are key regulators of neuronal polarisation *in-vivo,* with the expression of the cell adhesion molecule transient axonal glycoprotein-1 (TAG-1) by efferent axons guiding those generated by polarising multipolar neurons. Moreover, close physical contact of pioneering axons with the neurite of a multipolar neuron appears to specify the direction of axon growth [26]. Additionally, N-cadherin - another cell adhesion molecule - regulates neurite outgrowth and neuronal migration in the developing cortex [36], whilst Wnt signalling - through protein kinase C (PKC) - and IGF-1 both regulate multipolar to bipolar neuronal transition [37–39].

3. The axonal growth cone

3.1. Axonal growth cones in 2D

Located at the distal tip of developing axons are growth cones, an exquisitely intricate sensory structure responsible for leading axons to synaptic partners [40]. The dynamics that lead to axon advancement are regulated by the growth cone cytoskeleton, made up of actin filaments and MTs [40]. Classically, growth cones are thought to be highly compartmentalised fan-like structures possessing 3 domains: central domain (C-domain), occupying the centre of the growth cone, peripheral domain (P-domain), the outer part of the growth cone and transition zone (T-zone), located between the C and P domains (Fig. 2A). The C-domain consists mainly of stable MT bundles, while the P-domain contains actin-rich filopodia and lamellipodia comprised of filamentous actin (F-actin) as well as protruding dynamic MTs [41]. In the T-zone, myosin II bundles antiparallel actin filaments to form arc structures that prevent MTs from protruding into the periphery of the growth cone [29].

3.2. Axonal growth cones in 3D

In a 3D collagen environment, the morphology of growth cones is different to that observed in classical 2D studies [5]. Unlike those observed in 2D, 3D-cultured growth cones appear smaller in size, lacking the classical T-zone observed in 2D growth cones (Fig. 2B). The growth cone cytoskeletal organisation is also different in 3D cultured growth cones, with less actin and reduced MT volume [5]. Interestingly, MTs in growth cones in 3D collagen matrix protrude further into the leading edge, with the growth cone appearing more dynamic [5]. Such differences between growth cones cultured in 2D and 3D environments are due to the changes in the growth cone cytoskeleton architecture rather than changes in cytoskeleton dynamics [5]. Specifically, actin filaments in growth cones culture on 2D substrate restrain MTs to protrude as actin depolymerisation enhances axon growth. Instead, in growth cones of 3D actin filaments do not generate such a growth restraint on MTs as actin depolymerisation does not further enhance axon growth [5,15].

It is known from *in-vitro* studies that different ECM components elicit different neurite behaviours and growth cone morphologies [5,42]. However, no observations have thus far been made to confirm such differences *in-vivo* or *in-situ*. Growth cones in CNS tissue navigate complex environments and respond to environmental cues; therefore, the morphology of growth cones in a more physiological setting may vary depending on surrounding cues. Further studies are needed to better understand this.

3.3. The growth cone actin mesh

F-actin in the P-domain undergoes a cycle of assembly and disassembly. Actin monomer assembly occurs at the “barbed end” near the leading edge, whereas disassembly occurs at the minus end. An important player in this process is the actin severing protein of the actin depolymerising factor (ADF)/cofilin family. Following actin filaments severing, individual subunits are reused to polymerise actin at the leading edge [43,44]. Thus, this process known as treadmilling is driven by cofilin to a large extent [15]. Myosin II plays an additional role through sliding and compacting actin fibres [45]. In steady-state conditions, actin treadmilling (actin retrograde flow; RF) restricts MTs from protruding further into the P-domain. Changes in this balance result in the disengagement of MTs, allowing them to protrude into the P-domain and drive growth cone advancement [40].

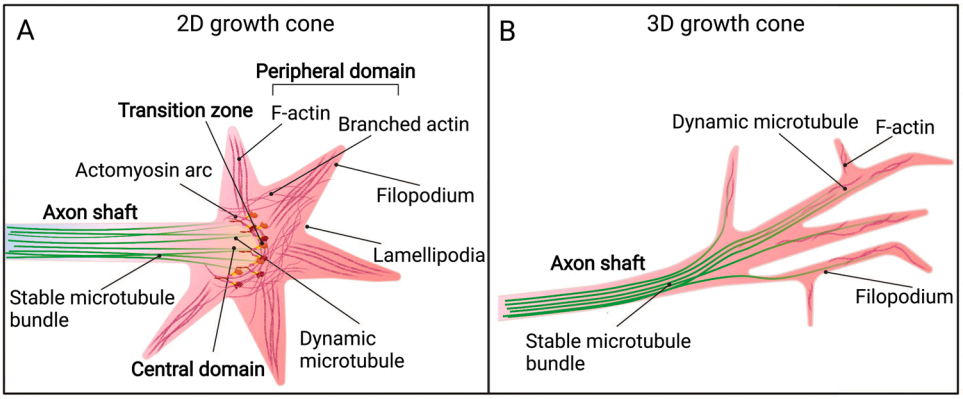

Fig. 2. Structure of axonal growth cones in 2D and 3D. A. An illustration of a growth cone as observed in a 2D environment. F-actin and microtubules make up the three domains of the growth cone: the peripheral (P) domain, the central (C) domain and the transition (T) zone. Actin filaments make up filopodia and lamellipodia, structures present in the P-domain. Stable microtubule filaments characterise the C-domain. Actin arcs are found at the T-zone, the interface of the two domains and consist of actin and myosin II filaments. B. An illustration of a growth cone as observed in a 3D environment. Growth cones appear much smaller in size, lacking lamellipodia. F-actin and microtubules make up the P-domain and C-domain of growth cones in 3D, they also lack a T-zone.

3.4. The growth cone MT network

As well as actin, dynamic MTs play an important role in driving and steering the growth cone, as well as in the transport of cargo along the axon [41,45,46]. MTs are polar structures comprised of dynamic alpha (α) and beta (β) tubulin dimers; these dimers contain the growing plus-end (end exposing β-tubulin) and the unstable minus-end (end exposing α-tubulin) [47]. Orientation of MTs in the axon is conserved across species, with the plus-end facing the periphery of the axon and the minus-end facing the soma [48]. MT nucleation – a process where α and β-tubulin dimers from MT polymer - occurs at particular sites in the cell, known as MT organising centres (MTOCs), and include the centrosome and Golgi [49,50]. During development, MT nucleation shifts from the centrosome to acentrosomal sites [50]. Additionally, minus-ends of axonal MTs are often not attached to the MTOC. This is mediated through MTOC-independent nucleation through gamma-tubulin ring complex (γTuRC) [47,51,52], branching on other MTs through the HAUS/augmin complex [53] and γ-tubulin and katanin-mediated cutting of MTs and subsequent stabilisation of minus-end through calmodulin-regulated spectrin-associated proteins (CAMSAPs) [54,55].

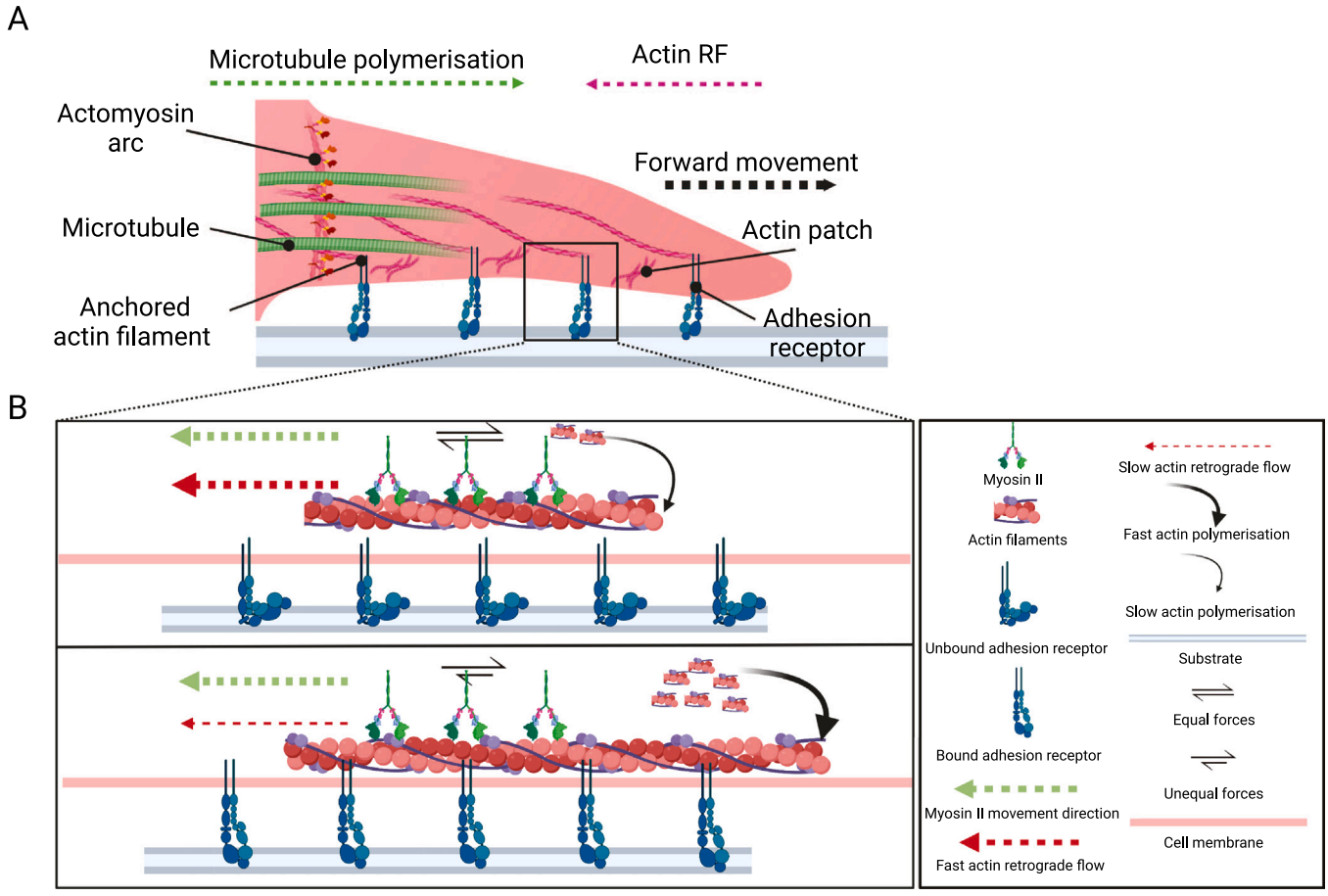

Fig. 3. Modelling of the clutch hypothesis. A. In adhesion-dependent growth, anchored actin causes an increase in actin polymerisation and slowing down of actin RF, and therefore, growth cone forward movement. B. A close-up of the interaction between anchored actin filaments and adhesion receptors. In the absence of interaction between adhesion receptors and actin filaments (B top) forces exerted on the actin cytoskeleton -myosin II pulling actin toward the T-zone and actin polymerisation toward the P-domain- are equal. B. When adhesion receptors engage with the actin cytoskeleton (B bottom), actin filaments are anchored. Actin retrograde flow is slowed down, and polymerisation causes growth cone protrusion.

MTs are also essential in growth cone steering by facilitating growth cone turning and advance. In the P-domain, stable MTs act as a guidance sensor to steer the growth cone attraction. Similarly, dynamic MTs steer the growth cone repulsion [56]. During the engorgement stage of axon extension, stable MTs in the C-domain advance and consolidate the new segment of the axon [57].

3.5. Molecular mechanisms triggering axon growth

Axons navigating the environment are spear-headed by growth cones, which interact with intracellular and extracellular guidance cues to steer axons to their final targets. This growth cone-guidance cue interaction leads to cytoskeletal changes in growth cones, causing either attraction to the cue – and therefore extension - or growth cone collapse and retraction from the cue [40,58]. Cytoskeletal changes leading to axon extension can be characterised by 3 stages: protrusion, engorgement and consolidation. This has been theorised to occur through two pathways: adhesion-dependant [59,60] and adhesion-independent modes [61,62].

3.6. Adhesion-dependant growth

One of the earliest hypotheses on force generation during axon growth has been termed “the clutch hypothesis” [59] (Fig. 3). When no interaction occurs between actin filaments and adhesion complexes (steady-state), actin treadmilling does not produce a resulting force. This involves myosin II-derived rapid actin RF. However, when an interaction between actin filaments and adhesion complexes takes place, the force generated by the treadmilling is transduced to the cell. Actin retrograde flow slows down internally, but part of the force is transduced to move the peripheral part of the growth cone forward.

3.7. Adhesion-independent growth

Many cells migrate in an amoeboid fashion: an adhesion-independent mode of advancement faster than mesenchymal modes [63]. Cells such as human fibroblast cells [64] and pancreatic cancer cells [65] are shown to shift between mesenchymal and amoeboid modes of migration, depending on the environment they are confined within [66,67]. It could be that an amoeboid type of movement prevails in more soft substrates. In fact, the peripheral nervous system (PNS) contains a stiff basal lamina. Hence, pulling on such substate is very effective. By contrast, the CNS is a very soft substrate. Hence, moving in an amoeboid way could be more efficient in such an environment. In the amoeboid type of movement, which is independent of adhesions, actin RF flow is not used for generating pulling force. Instead, the actin RF is a by-product of the cofilin-mediated severing of actin filaments to create space for protruding MTs [15] (Fig. 4). This mode of migration is used to explain axon growth where MTs are found to generate forces in the shaft that can subsequently push axons through the environment [61,62]. This has also been shown in neurons cultured in soft 3D environments [5]. Indeed, axon growth in 3D was not affected following treatment with actin depolymerising agent cytochalasin D. Suggesting a less restrictive role of actomyosin arcs in 3D [5]. Notably, it has been shown that CNS axons do not pull, neither in soft 3D matrix nor on stiff 2D substrates [5,14]. Interestingly, a similar correlation has been suggested by traction force microscopy. Whereby PNS axons pull on the matrix and CNS axons exsert weak pulling forces [68–71]. Since axons are able to extend in the presence of low doses of actin depolymerising agents [72,73], traces of actin patches remain in the growth cone even after using actin depolymerising agents [74]. This suggests residual actin may stabilise MT bundles in the growth cone – and possibly the axon-generating forces necessary for axon extension - [74–76]. Whether this is true, and whether axons *in-vivo* truly grow independent of adhesion, remains to be studied. Furthermore, while growth cones do not pull on the ECM in soft 3D collagen matrices [5] it remains unknown whether the axon shaft itself is still somehow anchored and involved in balancing forces for axon extension.

4. Experimental models to study axon growth

4.1. 2D and 3D in-vitro models

Various *in-vitro* models have been developed to further the understanding of axon growth and growth cone dynamics, employing neurons cultured from different species. (*e.g.*, *Aplysia californica* bag-cell, chick dorsal root ganglion (DRG) neurons and rodent hippocampal/cortical neurons) [41, 77–80]. The gold standard for culturing dissociated neurons has long been 2D cultures. These cultures allow tight control of environmental composition, crucial for understanding the function of

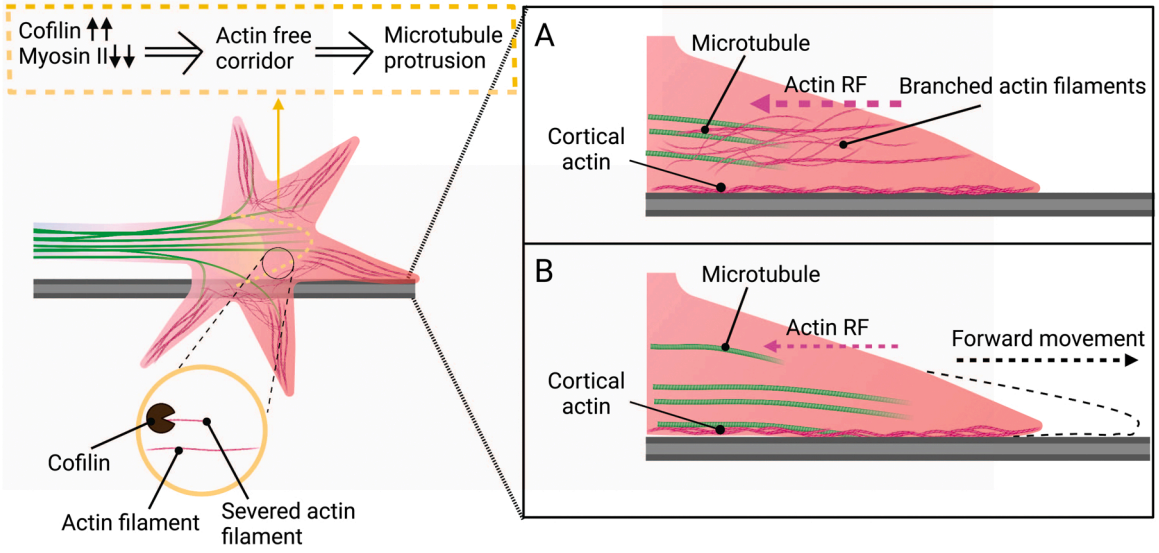

Fig. 4. Modelling of amoeboid growth cone locomotion. Actin-free corridor (dashed yellow arc) caused by decreased myosin II activity and increased cofilin activity (represented in the yellow circle) enables MT protrusion. A. In the absence of ameboid migration, a lack of propelling forces due to branched actin filaments and steady actin RF prevents MTs advancement, and in turn, growth cone movement. B. When adhesion-free ameboid migration is triggered, MTs are able to extend into the P-domain of the growth cone through an actin-free corridor. This results in a forward movement of the growth cone.

single molecular pathways.

Yet despite providing a wealth of information, 2D cultures are far from representing the complexity and multi-dimensionality of the *in-vivo* environment. The effect of multi-dimensionality on cultured cells was first observed in cultured fibroblasts. When embedded in a 3D environment these cells were shown to contain a highly-branched dendritic network; unlike the flat and significantly less-branched fibroblasts on 2D surfaces [81]. Such differences extend to molecular pathways where cells cultured in 3D matrices have diverse cell adhesions, signalling and protein arrangements compared to those cultured in 2D [82–84].

Studies highlighting the effect of dimensionality on cellular properties have ignited the popularity of 3D culturing methods [81–83, 85]. In an effort to produce substrates similar to those found in the ECM, different proteins have been purified and used to embed neurons (*e.g.*, collagen, laminin and fibronectin) [5, 86–88]. When observing axon growth and growth cone dynamics in neurons cultured in 3D collagen gels, significant differences in morphology and polarisation have been identified in comparison to those cultured in 2D. Indeed, axons grow faster within 3D collagen gels and the growth cone exhibits structural differences from those grown on 2D substrates [5,89]. Regardless of the advantage of 3D culture, they remain simplified synthetic matrices with a limited capacity to model the complexity of CNS tissue.

4.2. In-vivo models

Transparent model organisms, such as *Drosophila* larvae, zebrafish and *C. elegans,* have been used to study axon development due to accessibility to growing axons. This characteristic makes them ideal for imaging axon development. During zebrafish embryogenesis, time-lapse imaging of growth cones revealed steady elongation of the axons [90]. This axon growth is guided by environmental cues and target cells [91, 92], as well as signalling pathways, including Neuropilin 1 (Npn1) and bone morphogenetic protein (BMP) [93]. Furthermore, similar to what was recently observed in mouse brain slices, axons in zebrafish embryos show brief pauses during axon extension [29,90]. Zebrafish have also been used to manipulate axon growth. Using the latest developments in optogenetics and imaging, motor neuronal axons expressing photo-activatable Rac1 (PA-Rac1) were manipulated and guided over long ranges as well as through inhibitory environments [94]. Rac1, a member of the Rho GTPase family, plays a conserved role in manipulating cytoskeleton dynamics in response to extracellular signalling cues [95].

As previously mentioned in Section *3.2*, growth cones cultured in 3D environments appear morphologically different to those cultured in 2D environments. Similarly, when looking at TSM1 axons in *Drosophila,* growth cones are dominated by filopodia and lack lamellipodia [96]. This lack of adhesive growth structures - mediated by the Abelson (Abl) tyrosine kinase - suggests that TSM1 axons in *Drosophila* may grow in a non-adhesion dependant manner. Hence, this confirms similar observations of mouse hippocampal neurons in a 3D environment [5].

Despite the number of advantages to studying axon development in such *in-vivo* models, they remain simplistic compared to the mammalian CNS. Access to mammalian neurons during development is more technically challenging. Therefore, alternative models are needed to enable the study of mammalian neurons in their complex environment. One such model is brain organotypic slice cultures, discussed further in the following section.

4.3. Organotypic slice cultures

The term “organotypic cultures” refers to models that enable studying an environment in which physiological events closely replicate those *in-vivo*. These cultures have been successfully applied to modelling neurodegenerative disease [97–100]. Organotypic brain slice cultures were first described in 1947 as a method to study the nervous system in its original environment [101]. Originally, roller-tube cultures were the most common method of performing organotypic slice cultures. In such cultures, tissue slices are placed on glass coverslips and embedded in a mixture of chicken plasma and thrombin, forming a clot. The clot is slowly lysed over time in culture, and the coverslip containing the embedded slice tissue is placed inside a falcon tube containing media to supply the tissue with nutrients. The tube is then placed in a tilted roller to ensure sufficient nutrient and gas exchange in slices [102]. This technique, however, only allows for morphological and electrophysiological characterisation of cell populations when the coverslip is removed from the tube and when the clot is fully lysed. Therefore, the roller-tube technique is often used to perform post-fixation analysis [103].

The organotypic slice culture technique has been further developed to become simpler and more reproducible, allowing morphological and electrophysiological analysis of tissue slices [102,104]. These developments allow for tissue slices to be placed on a thin semi-permeable membrane, where media is placed underneath to enable sufficient gas and nutrient exchange. The use of a transparent membrane facilitates imaging directly through the membrane with high-resolution [105], further facilitating acute electrophysiological recording shortly after culturing [106].

4.4. The potential of organotypic slice cultures in studying axon growth

Studying axon growth in 3D has opened the door for questioning whether data observed in 2D is truly physiologically representative, raising pertinent questions for axon development. How do axons develop surrounded by the complex CNS environment? What role does adhesion play in axon development? How do cell-cell interactions modulate growth cone morphology and cytoskeletal changes? To answer such questions, we believe organotypic slice cultures will be indispensable.

Organotypic slice cultures can be combined with *in-vivo* gene delivery, targeting specific populations of neuronal progenitors [26,29]. Additionally, it is now possible to visualise axon growth and growth cone dynamics in high resolution in such cultures [105]. Using these techniques, it has been possible to measure and quantify axon growth speed *in-situ [*105]. Yet, to image more detailed events - such as growth cone dynamics - it is necessary to also obtain high time resolution. Modern confocal technology makes it possible to achieve such high resolution by employing fast-scanning motors [107]. Using this technology, one can image axonal growth cone dynamics in organotypic slices [105]. Visualising detailed events occurring within the axonal growth cone cytoskeleton is therefore now in reach. This will facilitate an understanding of the dynamics of the growth cone cytoskeleton, and the interaction between the growth cone cytoskeleton and the ECM during development.

Moreover, expression systems are now available, where different neuronal populations within the same brain region can be independently labelled. These systems work by utilising neuron-specific plasmids to label neighbouring neurons [105] (Fig. 5). Such molecular tools can be used to visualise 3D interaction between axons and neighbouring cells during development, in conjunction with growth cone dynamics change during this process. This is a vast improvement in physiological replicability when looking at the interaction between axons and growth cones with neighbouring cells; events that have predominantly been studied in 2D [108].

5. Outlook

Environmental complexity is a crucial component in studying axon guidance and development. How do axons come together to form bundles and synchronously navigate the environment to reach their destination? To answer such questions, *in-vitro* models are unlikely to be the key due to their simplicity. Giant leaps in knowledge have been made over the last decade to advance our understanding of mechanisms of axon growth in more physiological environments. Technological and

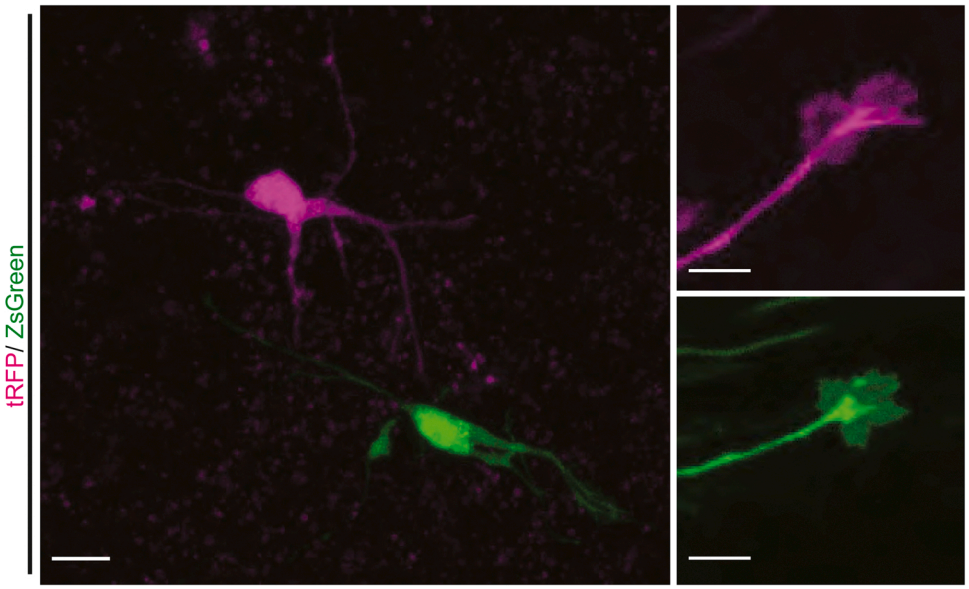

Fig. 5. possibilities of studying axon growth and growth cone dynamics *in-situ*. A view of neighbouring cortical neurons labelled using a dual expression system. Neurons are labelled with turbo red fluorescent protein (tRFP) and zoanthus sp. green fluorescent protein (ZsGreen). Such a system allows studying a different population of neurons side by side in a more physiological context. Scale bar; 5 μm.
Figure adapted from [107].

methodological developments have made it possible to visualise developing axons with high resolution in physiological environments. We envision such models will further advance our understanding of axon growth mechanics by studying interactions with the CNS environment and topography. These models may also shed light on how guidance cues and environmental topography can influence the growth cone cytoskeleton and molecular signalling, as well as how growth cones interact with the environment to trigger axon advancement. Such knowledge of axon development enables a greater understanding of how neural circuits are formed. This knowledge is important to understanding brain wiring. Additionally, it will help us to understand how we can reactivate these processes under pathological conditions, such as a spinal cord injury, to induce axon regeneration [7–9, 30, 109, 110].

Conflict of interest

H. Witte, A. Ertürk, F. Hellal and F. Bradke filed a patent on the use of microtubule-stabilising compounds for the treatment of lesion of CNS axons (European Patent no. 1858498; European patent application EP 11 00 9155.0; U.S. patent application 11/908,118). The authors declare no competing financial interests.

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