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Review

Neuronal filopodia: From stochastic dynamics to robustness of brain morphogenesis

Charlotte B. Wit, P. Robin Hiesinger *

Devision of Neurobiology, Institute of Biology, Freie Universität Berlin, Berlin, Germany

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ABSTRACT

Brain development relies on dynamic morphogenesis and interactions of neurons. Filopodia are thin and highly dynamic membrane protrusions that are critically required for neuronal development and neuronal interactions with the environment. Filopodial interactions are typically characterized by non-deterministic dynamics, yet their involvement in developmental processes leads to stereotypic and robust outcomes. Here, we discuss recent advances in our understanding of how filopodial dynamics contribute to neuronal differentiation, migration, axonal and dendritic growth and synapse formation. Many of these advances are brought about by improved methods of live observation in intact developing brains. Recent findings integrate known and novel roles ranging from exploratory sensors and decision-making agents to pools for selection and mechanical functions. Different types of filopodial dynamics thereby reveal non-deterministic subcellular decision-making processes as part of genetically encoded brain development.

1. Introduction

Neuronal morphologies are elaborate, diverse, and the outcome of morphogenetic processes that critically rely on filopodial dynamics. Filopodia are thin, mostly needle-like, membrane protrusions with an actin-based cytoskeletal core that have historically been referred to as 'fine protoplasmic threads' and 'microspikes' [1,2]. Their distinctive morphology is essential for continuous and fast extension and retraction dynamics compared to the slower dynamics of shallow lamellipodia (reviewed in [3,4]). Filopodia have classically been regarded as sensors that interact with the cellular environment and may serve diverse functions. Such functions include directed movements of a growth cone or cell body (where filopodia are principally transient structures) as well as the morphogenesis of branched tree-like structures (where individual filopodia may stabilize to become permanent parts of a larger structure). Hence, the types of dynamics exhibited by filopodia can define neuronal and brain morphogenesis.

Filopodial dynamics are best studied by live observation. Advances in live imaging of neurons at different developmental stages and in intact brains continue to reveal surprising new roles of filopodia based on their dynamics. Recent observations in particular highlight the diversity of filopodial dynamics in developing brains, provide new answers to old questions that were previously mostly studied in cell culture: are stochastic dynamics essential? do all or only selected filopodia contribute to growth decisions? do filopodia mechanically pull growth cones or cell bodies forward? These questions and new in vivo observations underlying the search for answers are the motivation for a comparative analysis of the different types of filopodial dynamics throughout neuronal and brain morphogenesis in the following sections.

1.1. Filopodial dynamics throughout neuronal development

Filopodia are membrane protrusions, but the regulation of filopodial dynamics is largely based on cytoskeletal mechanisms. Correspondingly, cytoskeletal regulation is a common focus of analyses of filopodial functions and excellent reviews on the topic are available [4–6]. Here we focus on the roles of filopodial dynamics based on the membraneous filopodial structure itself in the context of brain morphogenesis.

Early roles of filopodial interactions between cells already take place during neuronal differentiation [7–9]. However, the arguably best characterized context of filopodial function is directional growth based on sensing environmental cues. In neurons, such directional movement has been studied during migration and axon pathfinding based on directional growth cone dynamics [10–13]. Here, filopodia function as exploratory agents followed by the displacement of the growth cone or cell body they emanate from through signaling or direct pulling forces.

* Corresponding author.
E-mail address: robin.hiesinger@fu-berlin.de (P.R. Hiesinger).

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By contrast, the growth of dendritic trees and axonal branches typically does not involve the displacement of the entire cell (or structure the filopodia emanate from) [14,15]. Here, branching is characterized by rounds of filopodial exploration and selective stabilization of filopodia as new branches, typically aided by the growth of microtubules [14,16,17]. Roles of filopodia that aid directed movements of entire cell bodies or growth cones typically precede the roles of filopodia in the growth of branched structures once part of the neuronal localization and structure have stabilized. Following these morphogenetic processes, filopodia continue to play key roles during synapse formation [18,19]. Finally, filopodial dynamics that underlie dendritic spine formation persist throughout the functional lifetime of neurons [20]. Not surprisingly, these distinct filopodial roles are associated with specialized dynamics during the different developmental stages [18,21,22].

Following the developmental timeline outlined above, we will highlight five developmental processes that critically depend on distinct types of filopodial dynamics (Fig. 1): (1) neuronal differentiation based on filopodia-mediated signaling during early tissue morphogenesis; (2) neuronal migration; (3) neuritogenesis, growth cone motility and axon pathfinding; (4) axonal and dendritic branching; and (5) synapse formation. Our goal is to compare the types of filopodia and their differential utilization of distinct dynamics. For this comparative approach we will highlight specific commonalities and differences, rather than provide a comprehensive review of current literature. Excellent in-depth reviews that focus on specific aspects of each of the five topics are available elsewhere [14,23–26].

1.2. Key questions about the roles of filopodial dynamics in neurons

To facilitate comparative analyses, we will focus on the following three questions for each filopodial type at their distinct developmental stage.

1. 1. To what extent are stochastic dynamics or stabilization of individual filopodia a necessary basis for a growth decision?
2. Do all filopodia contribute to signal integration underlying a growth decision, or do filopodia serve as a pool for selection, with only one or a few selected filopodia contributing to a growth decision?
3. What is the relative contribution of filopodial sensing and signaling versus the exertion of physical forces?

These questions are based on the following considerations: Non-deterministic dynamics are typically observed for most types of filopodia. Probabilistically biased or even completely random filopodial exploration can be a prerequisite for the robustness of morphogenetic outcomes [27]. However, a requirement for stochastic dynamics is compatible with a range of answers to the second and third questions: first, stochastic dynamics can provide a pool of variation where all filopodia, or only selected ones contribute to a growth decision (Fig. 2); second, filopodia may only provide a signal that leads to morphogenetic changes in the cell body or growth cone or, alternatively, directly transduce a force, e.g. by 'pulling' on a growth cone. Combinations and intermediates of these two functional ranges ('all vs selected filopodia' and 'signaling vs direct force', Fig. 2) define different roles during neuronal development.

A first key observation towards answering these questions regards

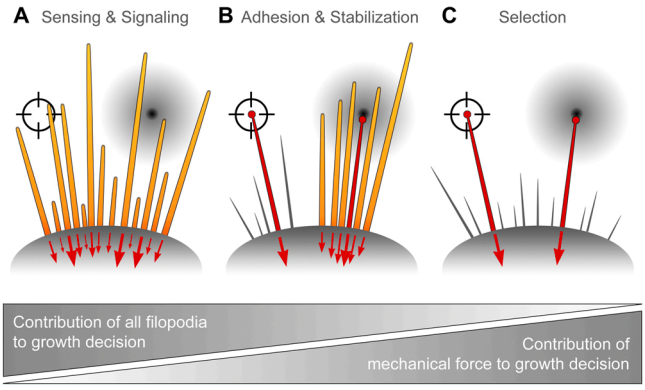

Fig. 2. Modes of filopodial action. Filopodia may sense environmental cues of different types, including spatially localized signals (cross-hairs) or gradients that are distributed over a larger area (grey clouds). A) A pure 'sensing and signaling' function may integrate weighted signals from each individual filopodium to compute a growth choice. In this limiting case all filopodia contribute to a growth decision and none are likely to transduce a mechanical force. B) Individual filopodia may adhere and stabilize upon interaction with the environment. Signaling may be restricted to signal-receiving or stabilized filopodia. In this intermediate case, some but not all filopodia contribute to a growth decision and mechanical forces can be transduced by a stabilized filopodium with an adhesive contact to the substrate. C) Only one or few filopodia may get selected upon stabilization. In this limiting case, none of the non-selected filopodia contribute to the growth choice. Stabilized filopodia can transduce mechanical forces (but may also use mechanosensation for signal transduction).

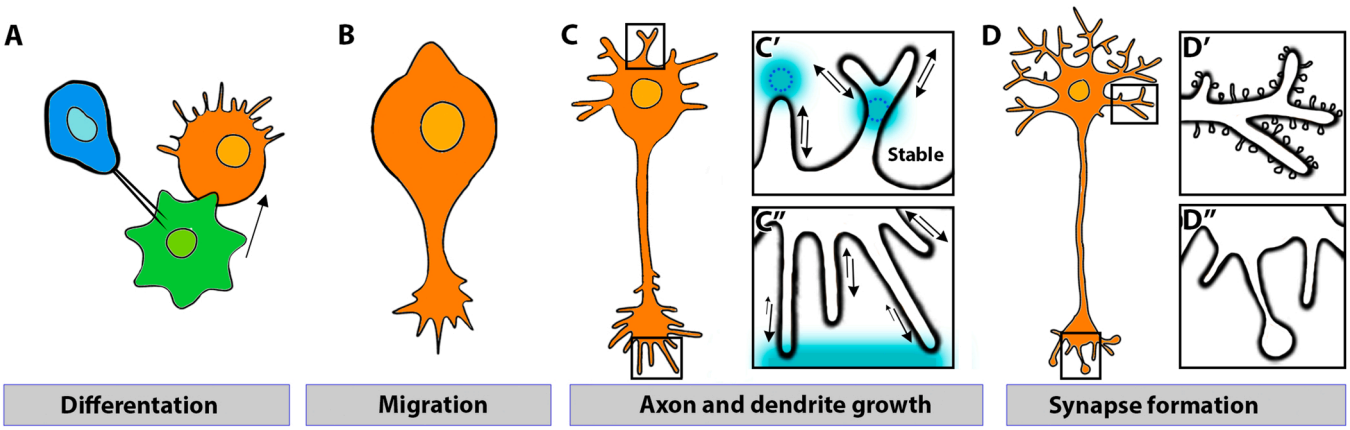

Fig. 1. Neuronal filopodial throughout development, A) Differentiation of a neuronal precursor cell (green) into a neuron (orange) can be mediated by specific filopodial interactions (cytonemes) through other adjacent cells (blue). B) Schematic of a migratory neuron with bipolar morphology and a growth cone-like leading edge. C) Schematic of a neuron with axonal and dendritic growth processes. C') Stabilization of dendritic filopodia at intermediate targets (blue), followed by renewed branching, leads to the development of dendritic trees (see also Fig. 3). C'') Filopodial exploration at the growth cone mediates directional growth choices with or without filopodial stabilization in response to an environmental signal (blue). D) Schematic of synapse formation from the perspective of dendrites (D') and the axon terminal (D''). D') Dendritic filopodia are precursors of dendritic spines. D'') Some axonal filopodia form bulbous tips that stabilize presynaptic contacts.

the effect of the environment on the dynamics of an individual filopodium. If a filopodium never attaches, it is unlikely to transduce a pulling force (question 3). On the other hand, a pure 'sensing and signaling' function is compatible with both a dominant role of one or few selected filopodia as well as an integration of signals from all filopodia (question 2). Indeed, many individually dynamic neuronal filopodia, both of migrating cells or axon terminals, appear to never stabilize and signaling machinery has been found at the tips of neuronal filopodia [3,28,29]. Filopodia of a migrating cell or growth cone may sense and signal based on molecular interactions with the environment, yet the growth decision and actual forces required for movement of the cell or growth cones may be controlled and executed by other cellular contact points with the environment. Corresponding models have been proposed for the integration of spatiotemporal sensing and signaling to compute directional growth of growth cones or migrating cells [12,30,31].

Alternatively, sensing an environmental signal may alter the dynamics of the filopodium itself. Any molecular interaction of a filopodium with its environment implicates some force [32] and adhesion and stabilization of individual filopodia may increase either signaling or a putative role in transducing forces directly (Fig. 2B-C). However, the observation of filopodial stabilization and even attachment alone does not distinguish whether only signaling is affected or the filopodium itself serves as an attachment to pull or push a larger structure like the cell or growth cone [1,33]. Stabilization of individual filopodia is certainly a well-described phenomenon in neurons. In the case of axon pathfinding, the stabilization of a single filopodium has been shown to be sufficient to change the growth direction [34]. In the case of axonal or dendritic branch formation, stabilization of individual filopodia provides the actual substrate to form the larger structure [35–37]. Both cases are suggestive of selection mechanisms: stochastically exploring filopodia serve to sample the environment, while only a few individual filopodia are selected to stabilize. In this scenario, most filopodia only serve as a temporary 'pool of variation' for selection and do not individually contribute to a growth decision. A classic example of this idea is synaptotropic growth, where the non-selected filopodia do not contribute to the developmental outcome, other than having been necessary as part of the selection pool [36,38] (Figs. 2C and 3). These examples highlight a breadth of roles for random filopodial dynamics that range from cases in which each and every filopodia serves a sensing function that contributes to a growth decision, to selection based on a pool of variation where only the selected filopodia contribute to a growth decision (Fig. 2).

1.3. Methods for live observation and quantitative analyses

The analysis of the roles of filopodial dynamics through live observation started with the development of neuronal culture by Ross Harrison in 1910 [2]. Live observation in culture systems has important experimental advantages that are still exploited in quantitative studies of filopodial dynamics and stabilization to this day [39]. In addition, advances in light microscopy techniques increasingly allow for non-invasive live observation of intact developing brains in all major model systems. In the comparably big mouse brain, non-invasive live imaging of dynamic changes of dendritic spines typically relies on time lapse observation through an imaging window [40]. Zebrafish larvae have been instrumental to the non-invasive characterization of dynamic dendritic and axonal morphogenesis [36,41]. Neurons in *Xenopus laevis* tadpoles have been imaged with 2-photon microscopy over multiple hours and repeatedly over sequential days to follow filopodial-to-branch stabilization [42]. For the developing *Drosophila* brain, both completely non-invasive live imaging of brain development at the resolution of individual filopodia as well as *ex vivo* culture systems of entire developing brain preparations have been established based on multiphoton and, more recently, lattice lightsheet microscopy [22,43,44]. These techniques continuously reveal new types of dynamics, new roles and new types of filopodia, but also lead to an analytical bottleneck. Quantitative analyses of live imaging data, especially time lapse of 3D dynamics, are challenging. Recent developments of software tools for the analysis of neurons, growth cones, and individual filopodia are immensely helpful; however, these tools also highlight the difficulties associated with an algorithmic automation of the recognition of dynamics structures that may appear obvious to the human eye [19,45–47]. In the following sections we focus on selected examples of live observations in light of our questions regarding the roles of filopodial stochasticity, stabilization, signaling, and force generation.

2. Cytonemes and signaling in early tissue morphogenesis and neuronal differentiation

Cytonemes are filopodia-like protrusions that have been discovered as a means of long-range communication between cells of developing tissues in *Drosophila* and mouse [48]. Cytonemes provide an alternative or complementary mechanism to diffusion or extracellular vesicle transport for the formation of morphogen gradients and the delivery of signaling molecules [23,49]. Signaling based on morphogens is critical to tissue patterning, including the spatiotemporally organized differentiation of specific cell types in neural tissues. For example, the developing eye-imaginal discs in *Drosophila* give rise to photoreceptor neurons and rely on early EGF (Spitz) signaling provided by specialized cytonemes [7]. In the ventricular zone of the developing brain in mouse embryos, signaling filopodia from blood vessels actively contact dividing neural precursor cells and promote their differentiation [9]. In zebrafish, cytoneme contacts between endothelial cells and neuroblasts suppress proliferation in a time-controlled manner [8]. The underlying filopodial dynamics include extensions and retractions on the time scale

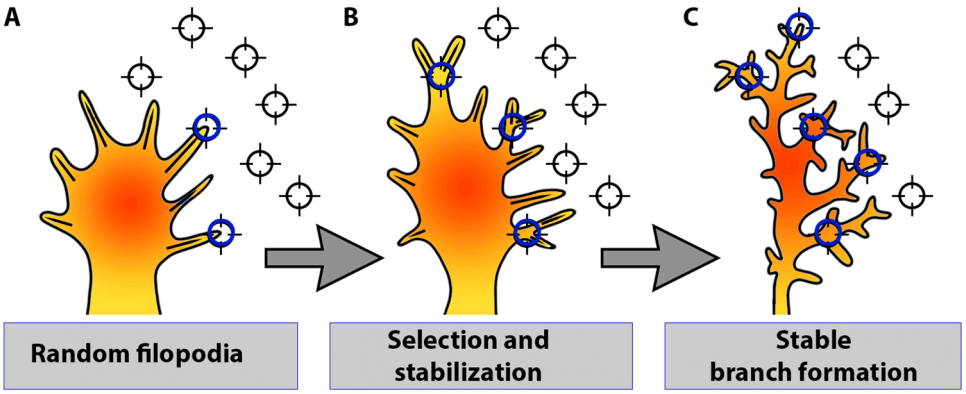

Fig. 3. Synaptotropic growth is based on filopodial exploration and stabilization. A) Random exploration leads to contact with synaptic partners (cross-hairs), branch stabilization and synapse formation (blue circles). B) Synaptic contacts become new origination points for branch formation. C) Iterative application of the first two steps leads to dendritic (or axonal) branch formation in the direction where most synaptic partners can be found.

of seconds as well as stabilization of individual filopodia on the time scale of minutes. These findings suggest a selection mechanism based on stochastic exploration, where only stabilized filopodia contribute to developmental signaling and growth decisions. Similar ideas have been discussed as a 'search-and-capture model' for other cytonemes [50]. However, alternative models for the roles of cytonemes in morphogen gradient formation based on cytoneme dynamics are currently discussed [51] and quantitative studies on cytoneme dynamics are still scarce.

The types of signaling events known to occur at cytoneme contact sites have so far not been suggestive of mechanical forces. The idea of long-range delivery of an otherwise diffusible morphogen indicates a pure 'sensing and signaling' function (Fig. 2A). Similarly, the recent discovery that some cytoneme contacts in non-neuronal tissue utilize the release machinery of the neurotransmitter glutamate is consistent with a signaling role and does not suggest physical forces based on adhesion. [52]. A role for the glutamate receptor was also found in the context of embryonic stem cell self-organization [53]. Hence, current evidence for roles of filopodial dynamics during early differentiation largely support a requirement of stochastic dynamics as a means of exploration, followed by selection of individual filopodia that function as signaling conduits, without a role for force transduction (Figs. 1A and 2A).

3. Neuronal migration

The migration of neurons and their precursors is a prerequisite of nervous system morphogenesis from *C. elegans* to humans [54,55]. In vertebrates, neuronal migration is closely linked to cell type specification, as has been well-characterized for cortical interneurons [12]. Like other migrating cells, migrating neurons form a polarized leading edge that serves both a sensing and motility function. The growth cone-like morphology of this leading edge shares similarities with axonal growth cones as discussed in the next section (4. *Growth cone motility and axon pathfinding*). However, there is a key difference between these two types of growth cones: in migrating cells, the consequence of a growth cone-like leading edge movement is the translocation of the entire cell. Correspondingly, filopodia exhibit shared as well as diverging roles, as highlighted in the following sections.

3.1. Stochastic dynamics, stabilization and signaling of filopodia during neuronal migration

Filopodial dynamics and their manipulation during brain development in vivo have been studied for several migrating neuron types [56,57]. An in vivo study of zebrafish lacking *fascin1a* revealed severe defects in the number, length and dynamics of neural crest cell filopodia. During vertebrate embryonic development, neural crest cells arise from the ectoderm germ layer cells and give rise to neurons as well as other cells. Surprisingly, most of the filopodia-defective cells migrated normally. Hence, there are cell populations that do not rely on 'robust, long and numerous filopodia' for migration (quote from [56]). By contrast, facial branchiomotor neurons extend filopodia in all directions when they initiate their migration and localize the planar cell polarity receptor Vangl2 at their tip, suggesting a sensing function of asymmetrically localized planar cell polarity components in the environment. Different components of the planar cell polarity signaling pathway destabilize these filopodia, with direct consequences for migration [57].

Filopodial sensing and signal transduction most likely originate at the filopodial tip, where receptors and specialized signal transduction machinery localize [3,57,58]. Calcium signals have been observed in filopodia of neuronal growth cones [3,30,59] and are discussed in Section 4; it seems plausible that similar signaling occurs in growth cone-like structures at the leading edge of migrating cells. Similar to other migrating cell types, migrating facial branchiomotor neurons have been proposed to signal via actin regulators and integrate signals from several filopodia [57,60]. Such signal transduction could lead to integration of signals from all filopodia in a more central compartment of the leading edge when making growth choices. However, it is difficult to disentangle the contribution of individual filopodia and dominant roles of one or a few selected filopodia are conceivable in many cases.

3.2. Filopodial force generation during neuronal migration

During neuronal migration, filopodia encounter substrate-specific forces that affect their dynamics [32,61,62]. Forces experienced by filopodia directly depend on myosin-driven actin flow and differ for soft and stiff substrates. Filopodia of cultured neurons from dorsal root ganglia were investigated using optical tweezers to measure the force filopodia apply on their environment. In this study, filopodial tip forces measured between 1 and 2 pN and appeared to modulate their mechanical response by decreasing the duration of collision when encountering a stiffer obstacle, and increasing touching duration at obstacles with lower trap stiffness [62]. For bipolar migrating neurons with a growth cone-like leading edge, a pulling force of the leading process has been experimentally supported using both severing of filopodial tips [63] and inhibition of myosin activity [61]. Continuous addition of actin monomers on F-actin filaments at filopodial tips and deconstruction at their bases result in retrograde flow; clutch proteins–the neuronal equivalent of larger focal adhesions found in other migrating cell types–adhere F-actin filaments to the environment as a basis for retrograde flow to create a traction force that extends the leading process and pulls the soma forward [61]. According to this 'sticky-fingers' theory the growth cone-like structure is the hand that uses sticky filopodia to crawl forward. The clutch protein shootin1a partners with the cell surface protein L1-CAM, thereby mechanically coupling F-actin to laminin at the extracellular matrix [64,65]. Actin retrograde flow and growth speed were thereby found to have a positive correlation with the speed of growth cone advancement during migration [65].

The integration of extracellular adhesion and actin clutches creates a saltatory movement of migrating neurons: leading edge progression based on traction forces and a reduction of adhesion lead to subsequent translocation of the cell body (reviewed by [66]). It remains less clear, however, whether the forces experienced and generated by filopodia themselves contribute to the pulling force, or whether integration of signaling within the leading edge regulates traction of other, larger membrane contact areas in the leading process. Traction force microscopy (TFM) can precisely map location and direction of traction forces, as shown for cultured migrating U2OS cells where all detectable forces exerted on the substrate originate at focal adhesions on filopodia [67]. Traction forces trigger contraction of non-muscular myosin II in the shaft and transmit forces to the soma that lead to translation of the nucleus via interaction of KASH proteins with the cytoskeleton [61]. These findings suggest that filopodia may directly contribute to the pulling force. Consistent with this idea, forces that push and pull on the environment have been measured directly in filopodia [62]. Furthermore, in non-neuronal cells, filopodial pulling forces have been demonstrated between the filopodial tip and an adhesive surface in a myosin-dependent manner [68]. During the termination of migration, bipolar migrating neurons have been shown to extend a transient protrusion called the 'filopodium-like lateral protrusion' that is induced by PlexinD1 downregulation and microtubule polymerization [69]. This stiff cytoskeletal 'brake' may directly pause somal translocation. Based on these studies, filopodial forces that directly contribute to translocation are certainly in place and force-dependent signaling further contributes to the controlled movement of migrating neurons.

4. Neuritogenesis, growth cone motility and axon pathfinding

The formation and movement of axonal growth cones have fascinated biologists through the decades and led to the general idea of filopodia as antennae, or exploring agents, that sense the environment and pass on information required to compute choices for directional

growth [2,3,13,34,70]. Growth cone formation is preceded by neuritogenesis, the first step in neurite generation on the cell body. Importantly, filopodia are a prerequisite for neuritogenesis, i.e. the formation of an axonal or dendritic process on the cell body, based on the stabilization of selected filopodia [71]. By contrast, transient filopodia aid the growth cone during pathfinding. Hence, filopodial dynamics play various roles during the different stages of axonal growth.

4.1. Stochastic dynamics, stabilization and signaling of filopodia during axonal growth

Axonal growth is preceded by neuritogenesis in a process that is entirely dependent on initial filopodial dynamics on the cell body. Loss of filopodia in a mutant for the actin regulators Ena/VASP leads to loss of neurites in mouse cortical neurons; neuritogenesis can be reinitiated in this mutant by expressing an alternative actin nucleating protein that induces filopodia formation [71]. Life observation in this study also showed that a single filopodium out of a pool of probabilistically extending and retracting filopodia is stabilized, dilates and forms the neurite. Hence, axons form through filopodial selection and stabilization (Fig. 2B-C).

The growing axon develops a leading edge that can take various shapes in different neuron types: the growth cone. The 'textbook' growth cone contains a central domain with microtubule plus-ends and a peripheral domain that is characterized by F-actin-induced filopodia. The shape and function of growth cones are closely related to each other. Detailed analyses of growth cone dynamics of neonatal rat superior cervical ganglion cells in culture revealed a growth cone sweeping motion while moving forward, where the oscillation frequency was a good predictor for the growth rate [11]. Likewise, computerized analysis of growth morphology found a strong inverse relationship between neurite outgrowth and filopodial length, as well as a positive relationship between outgrowth and the percentage each filopodium was embedded by a lammelipodial veil [47]. Hence, faster extending growth cones have shorter filopodia and both morphology and dynamics of filopodia correlate with growth cone motility [47].

The role of filopodia in growth cone movements has been investigated in much detail and actin-dependent dynamics have been implicated in directional choices during axon outgrowth for a long time [70]. In the '80 and '90, filopodial studies were based on pharmacological disruption of the cytoskeleton, typically bathing entire preparations in cytochalasin, and suggested a filopodial requirement for correct axon pathfinding [72]. Filopodial dynamics were shown to be required for chemotropic pathfinding, for turning of cultured *Xenopus* spinal neurons [73], for turning of *Xenopus* retinal ganglion cells [72] and for correct pathfinding of *Schistocerca* Ti1 neurons [74]. On the other hand, axons have been shown to be able to extend at normal speed even in the absence of F-actin, at least in cultured hippocampal neurons [75]. A more recent study utilized sequestration of Ena/VASP proteins to mitochondria in retinal ganglion cells, resulting in a 90% reduction of growth cone filopodia. Remarkably, despite the reduction in axon outgrowth speed and increased stalling at decision points, the retinal ganglion cells still grew into the correct target area, the optic tectum [76]. Stalling at decision points and slower arrival are consistent with slower integration of sampling as well as more time for a single filopodium to get selected from the smaller selection pool [21,77]. Hence, filopodia may not be absolutely required for axonal growth per se, but their stochastic exploration has been linked to guidance choices.

Distinct types of filopodia can play different roles in the same growth cone. Jang et al. (2010) identified two filopodial populations on growth cones of a neuron-like neuroblastoma cell line. One pool of randomly distributed, instable filopodia sense the environment, while a different set of more stable filopodia at the tip of the growth cone contribute to directed growth [78]. The amount of probabilistic filopodial exploration may be a balanced 'economic' choice. In a cost vs gain trade-off a limited number of filopodia has a lower cost but a reduced likelihood for target identification, whereas extending long exploring filopodia in all directions has a high energetic cost but is likely to identify all targets [27]. Gowth cones likely employ various strategies with respect to this trade-off, as axons that are intrinsically biased towards a specific direction may need less filopodia, whereas axons that arrive at choice points require more filopodia to sense the environment and 'decide' where to go [77,79].

Midline crossing has been studied extensively to uncover how filopodial dynamics affect growth cone extension and turning in intact tissue. Di1 commissural axons that cross the floor plate–a relatively crowded area–have a streamlined morphology. Formin 2 disruption in Di1 axons led to stalled outgrowth, delayed exiting of the floor plate, and subsequently results in impaired development of the spinal circuit. Investigation at the level of filopodia showed that Formin 2 is necessary to stabilize adhesive filopodial tips via actin bundle assembly [80]. This stabilization is again consistent with either integration of sensory signaling input from each individual filopodium, or an increase of exploratory choices and subsequent selection based on a single filopodium (Fig. 2). Upon exiting the midline, axons enter a softer environment and reach a decision point. Growth cones arriving at such decision points sense the change in stiffness, stall and grow more exploratory and dynamic filopodia [77,79]. Similarly, pioneer axons form larger, more complex morphologies than later-developing axons [81] and 'late midline-crossers' take less time to make a turning decision [79]. These findings suggest that turning requires more sensing filopodia in order to select the correct direction, and that following the trail of early-crossers is easier than forming a trail anew. The extension of longer filopodia and increased dynamics at choice points is consistent with integration of sensory signaling–either by combined input from many filopodia or by increasing exploratory choices–and subsequent selection of single filopodia.

Stabilization of individual filopodia can increase their signaling contribution, or, in the limiting case, lead to the selection of a single filopodium to determine a growth decision (Fig. 2). In cell culture, a single filopodial contact was shown to reorient entire growth cones [82,83]. In vivo, *Xenopus* RCGs were shown to exhibit growth cone turning based on a single filopodium [72]. Moreover, grasshopper Ti neurons extend filopodia in all directions at specific 'choice points' and one filopodium touching a guidepost neuron was shown to be sufficient to stabilize in this direction and dilate the filopodium to form the new growth cone. The original growth cone and filopodia along the shaft disappeared and growth continued in the newly chosen direction [34]. Similarly, wild-type RP2 motor neurons in intact *Drosophila* embryos were shown to extend single filopodia that undergo dilation upon touching an unknown target [84]. In another example, *Drosophila* photoreceptor R8 axons terminate classic growth cone-mediated axon pathfinding in a temporary and superficial layer of the target brain region. Subsequently, a single deeply projecting filopodium stabilizes, thickens, and becomes the terminal extension towards the correct target layer [22,85]. Together, these examples provide evidence across species for the importance of filopodial selection, whereby a single stabilized filopodium, rather than signal integration from many filopodia, can direct growth (Fig. 2B-C).

Many neurons have elaborate axon morphologies with collateral branches and stereotypic branch patterns that spread to target multiple cell layers, and may contact multiple dendritic partners. Collateral axonal branch formation is based on distinct filopodial dynamics that are regulated by specific molecular and cytoskeletal mechanisms. For example, knock-down of the actin-α but not the actin-β isoform impairs collateral branch formation, while knock-down of actin-β reduces the dynamics of growth cone filopodia [86]. Collateral axonal branch formation is mediated by transient actin patches. Increased actin patch formation in SARM1 knock-out dorsal root ganglion neurons leads to increased rates of actin patch formation, an increased probability for filopodium formation, and ultimately increased collateral branch formation [87]. Furthermore, collateral branch formation through local

nanoclusters of PRG2-mediated PTEN inhibition leads to sufficient PI(3,4,5)P3 to initiate branches [39,88]. In another example, *Drosophila* dorsal cluster neurons form an intricate collateral axonal branch network in the adult optic lobes. Here, formation of collateral branches depends on filopodial stabilization based on high-resolution live imaging revealing that less than 10% of filopodia remain stable over a time period of 3 min and filopodial stabilization is under control of branch stabilization based on local EGFR signaling [89]. Hence, random filopodial dynamics create a pool from which only a small selection undergoes filopodia-to-branch stabilization, reminiscent of dendritic growth (see Section 5).

4.2. Filopodial force generation during growth cone movement

The role of filopodia in 'pushing' or 'pulling' growth cones has been a matter of debate for decades. The prominent 'clutch hypothesis' by Mitchison and Kirschner from 1988 first suggested direct actin-mediated force-generation between the growth cone's cytoskeleton and the substrate to propel the growth cone forward [90]. A year earlier, however, Letourneau et al. had found that cytoskeleton-based pulling forces may not be required for neurite elongation – including in liquid medium that offers little opportunity for adhesive contact [91]. More recent nanoscale dynamics measurements of individual growth cone filopodia from embryonic chick forebrain neurons confirmed a variant of the actin-dependent 'clutch' model [32]. Further evidence for the role of mechanical forces in growth cone guidance have been obtained from *Xenopus* retinal ganglion cells [92]. Retinal ganglion axons exhibit slower filopodial dynamics and grow longer on stiff substrate. Conversely, on softer substrate, filopodia are more exploratory and growth is less directed. These results suggest that growth cones are mechanosensitive and regulate growth speed according to the stiffness of the substrate. *In vivo*, the tract where retinal ganglion axons grow shows a stiffness gradient during development and neurons grow straighter on a stiffer surface, while spreading out on softer substrates. This could aid turning when axon bundles grow perpendicular to a stiffness gradient [92]. A measurement of the forces exerted by filopodia and lamellipodia of dorsal root ganglion neurons in 2D culture using optical tweezers found up to 3pN for filopodia and up to 20pN for lamellipodia, both in an actin-dependent manner [62]. However, it has remained unclear whether any of these forces are the actual traction forces that propel a growth cone forward or whether these forces only play an indirect role through signaling or by tranducing forces to the bulk of the growth cone.

Evidence against the role of mechanical pushing or pulling forces for growth cone movement have recently been obtained using mouse embryonic hippocampal neurons in a 3D culture gel [93]. In contrast to numerous studies in 2D culture and findings based on fibroblast motility, the neuronal growth cones in a 3D matrix that offers little to no adhesion were able to move by means of amoeboid forward propulsion. This findings does not exclude mechanical sensing of substrate stiffness for navigational purposes, but it clearly shows that the mechanical forces experienced or tranduced by filopodia are not required for movement per se [93]. The findings also corroborate the earlier evidence of growth cone movement in liquid media [91], the observation of axon extension in culture under conditions of cytoskeletal disruption [94,95] and the in vivo observation of correct axonal targeting in the absence of most filopodia [76]. Taken together, axonal growth cones are mechanosensitive, but filopodial force sensing or generation are more likely to play a navigational role than being required for movement per se.

5. Dendritic growth

Dendrites grow by transforming filopodia into branches through regulatory growth rules that pattern dendritic tree formation. The principal mode of dendritic branch growth is based on stochastic filopodial exploration followed by stabilization of individual filopodia to form new branches. The rules by which individual filopodia stabilize and provide new branch points have been extensively reviewed and lead to a remarkable diversity of dendritic tree shapes [14,26,96,97]. Here we focus on three key rules that control when and where filopodia stabilize: synaptotropic growth [38], self-avoidance [98], and tiling [99]. The stabilization of dendritic filopodia as postsynaptic dendritic spines is discussed in Section 6.

Synaptotropic growth was first described by Vaughn in 1974 for motor neuron dendrites in the mouse spinal cord based on electron micrographs [38]. The basic principle is an iterative two-rule growth process: First, stochastically exploring filopodia only stabilize on contact with a synaptic partner, leading to synapse formation; second, the newly stabilized synapse serves as a branching point for the next round of stochastic exploration and stabilization (Fig. 3). As a result, the dendritic tree branches out towards where most synaptic partners are available. This mechanism ensures flexibility in an unpredictable environment and robustness to developmental perturbation [27]. Synaptotropic growth has been observed live in the zebrafish visual system [36] and has since been described in many systems [100–102]. Live imaging over several days showed that less than 5% of all filopodia that extend from dendritic branch points undergo a filopodia-to-branch transition in *Xenopus* tadpoles [42]. In contrast to axonal growth cone filopodia, the categorisation of these filopodial dynamics is straight-forward: (1) stochastic dynamics are a prerequisite for exploration, (2) individual filopodia stabilize and ultimately contribute to growth while the majority only served as a pool for selection, and (3) 'pulling' and 'pushing' forces are not required, but local forces may play roles in signaling and filopodial stabilization through adhesion.

Self-avoidance of growing dendritic branches has been extensively described in the context of the repulsive function of cell adhesion molecules like Dscam1 in *Drosophila* and clustered protocadherins in vertebrates, which are reviewed in detail elsewhere [97,98,103]. In brief, self-avoidance provides a negative growth signal to exploring filopodia that prevents dendritic extensions from repeatedly growing into the same region or on top of each other (clumping). This signal is exclusive to a single neuron's own dendritic branches through molecular self-recognition. Stochastic filopodial exploration is a prerequisite and stabilization is restricted to individual filopodia that do not receive the self-avoidance signal. Traction forces have not been shown to play a major role. Note that Dscam1 has also been shown to function in dendritic growth processes independent of self-avoidance-induced dendritic spacing, e.g. in promoting the growth of *Drosophila* motoneurons [104].

Finally, dendritic tiling provides a complementary negative growth signal between 'non-self' neurons, resulting in the avoidance of overlap with neighboring dendritic trees [99]. Little is known about the filopodial dynamics underlying this process, but it is likely based on inter-neuronal filopodial interactions and repulsion that could be similar to the intra-neuronal interactions and repulsion underlying self-avoidance. As in synaptotropic growth and self-avoidance, flexibility and robustness are ensured based on initially stochastic exploration through many filopodia and selection of individual filopodia that mature into stable branches.

6. Filopodial dynamics during synapse formation

Following axon pathfinding and dendritic branch formation, dynamic filopodia typically persist throughout the entire brain developmental period of synaptogenesis. In the case of synaptotropic growth, synapse formation follows filopodial contacts and stabilizes filopodia to mature into permanent branches. Other distinct roles of filopodial dynamics during synapse formation are based on transient filopodia at the dendritic or axonal side, leading to filopodial interactions that can directly affect synaptic partner choices.

6.1. Dendritic filopodial dynamics during synapse formation

An active role of dendritic filopodia in the initiation of synaptic contacts was first shown live in hippocampal cell culture [18]. Dendritic filopodia during both development and plastic changes of the functioning brain have been particularly well described for spine formation in mammalian excitatory neurons [20]. Stochastic membrane deformations have been proposed to initiate exploratory dendritic filopodia [37]. Exploratory filopodia underlie the initiation of contacts with potential synaptic partners also in the *Drosophila* visual system [105]. Like the plasticity-related morphogenesis of vertebrate dendritic spines, dendrite dynamics of fly ventral lateral neurons are influenced by neuronal activity. Similarly, dendritic branches of adult *Drosophila* motoneurons develop in an activity-dependent manner. Here, relative dendritic branch extensions are determined by competitive excitatory and inhibitory inputs [101].

Individual dendritic filopodia can stabilize based on a finely tuned molecular cell surface interaction, as shown for EphB2 receptors at the filopodial tips of cortical neurons [28]. Here, small differences in the kinase signaling of the EphB2 receptor are necessary and sufficient for stabilization. Correspondingly, loss of EphBs leads to reduced motility of dendritic filopodia in cultured brain slices [106]. Signaling in dendritic filopodia during synaptic partner selection is further dependent on local calcium [107]. Stabilization of filopodia is more important than the overall density of dynamic filopodia, and may be a rate-limiting step for synapse formation [108]. Similar observations have been made for axonal synaptogenic filopodia, as discussed in Section 6.2. Together, recent live imaging studies support the view that stochastic exploration of dendritic filopodia are an active contributor to synaptic partner selection, followed by selection and stabilization of individual filopodia based on intra-filopodial signaling. Mechanical forces may contribute to this signaling, as changes to dendritic spine structure are accompanied by cytoskeletal forces that have been implicated in the stabilization of synaptic plasticity [109].

6.2. Axonal filopodial dynamics in synapse formation

Axonal filopodia with specialized morphologies for synapse formation were first characterized in live observations for cultured chicken retina neurons in 1985 [110]. These potentially synaptogenic filopodia tips appeared as enlarged bulbous endings that contained 35–40 nm organelles resembling synaptic vesicles. The bulbous morphology is reminiscent of dendritic spines and has since been observed live in other axon terminals, including *Drosophila* photoreceptors [19,22]. Similar to dendritic filopodia, live observations of axonal filopodia support the notion of stochastic exploration as a basis for selection and stabilization [111]. In *Drosophila* pleural muscle motoneurons, early synapse formation at stabilized filopodial tips has been shown to contribute to a synaptotropic growth-like mechanisms of axonal branching independent of synaptic activity [100].

Stochastic axonal filopodial dynamics have also been quantified for axon terminals that do not branch. *Drosophila* R7 photoreceptor axons exhibit stochastic filopodial dynamics in the exact brain layers where synapses are later observed on an enlarged, smooth bouton-like axon terminal in the adult (Fig. 4). These filopodial dynamics have been tracked in 4D, quantitatively analyzed and computationally simulated for the entire brain developmental period of synapse formation [19]. This study supported the general notion of stochastic dynamics underlying exploration, followed by selection and stabilization of individual, synaptogenic filopodia with a bulbous tip, similar to those observed in 1985 [110]. R7 photoreceptor neurons form only one or two synaptogenic filopodia at any given time and have been proposed to determine synapse numbers based on a cell-autonomous serial synapse formation mechanism [19]. Consistent with this model, modulation of the kinetics of synaptogenic filopodia either by altering the destabilizing activation of autophagy in filopodial tips [112], or by stabilizing filopodia using lower developmental temperature [113] changes adult synapse numbers in a predictable fashion. Hence, axon filopodial kinetics alone can determine synapse numbers and exclude synaptic partnerships by restricting how many contacts are stabilized. The pre-synaptically determined number of synapses remains unaltered even after genetic ablation of the main postsynaptic partner of R7 neurons. This led to the insight that R7 axon filopodia recruit a fixed number of synaptic partners even if the available types of synaptic partners have been altered [113]. From the perspective of synaptic specification during brain morphogenesis, filopodial dynamics are therefore one of many contributors to composite instructions that determine adult synaptic partnerships [26,114]. This role of filopodial dynamics blurs the classic distinction of 'instructive' versus 'permissive' mechanisms in brain morphogenesis and wiring specificity; slowing down exploratory filopodia alone is sufficient to recruit synaptic partner neurons that would be excluded by faster filopodial kinetics [26,114]. *Drosophila* development at lower temperature therefore leads to connection differences that can be traced back to filopodial kinetics [113]. Mechanical forces have not been studied in this system, but might contribute to signaling. In sum, the regulation of presynaptic filopodial dynamics during synapse formation plays a quantitative and qualitative role in the recruitment of synaptic partner numbers and cell types.

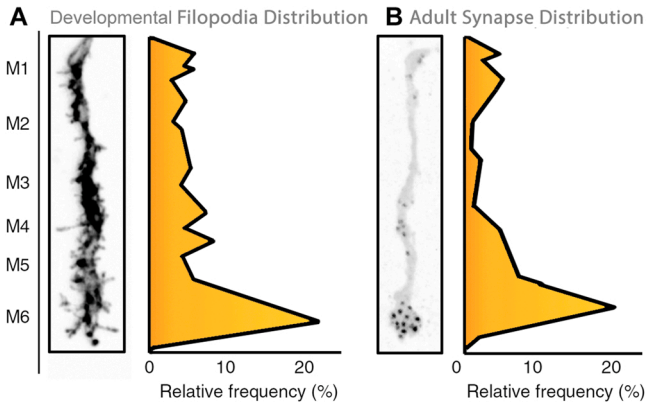

Fig. 4. Filopodial dynamics control synapse numbers and partnerships. A) Live measurements of filopodial dynamics on a *Drosophila* R7 axon terminal during synaptogenesis reveal the distribution of filopodia across the synaptogenic region of the axon terminal and an enrichment at the distal end (medulla layers M5/M6). B) Quantification of adult synapses matches the preceding distribution of developmental filopodia (adapted from [112,113]).

7. Conclusions

Our comparison of filopodial types and their dynamics throughout brain development highlights a prevalent shared principle: stochastic dynamics are an effective means of the morphogenetic program to ensure exploration and thereby flexibility and robustness.

Regarding our first question on the role of stochastic dynamics: Filopodial exploration underlies all developmental processes described here, including differentiation, migration, axonal and dendrite extensions and synaptic partner recruitment. However, the consequences of filopodial exploration for the filopodia themselves and the structures they emanate from differ significantly between developmental processes. While all filopodial roles discussed here implicate environmental sensing, those filopodia helping migration or growth cone guidance are typically transient, while those contributing to axonal or dendritic branched structures transform to become stable branches. Here, exploratory filopodia represent a pool of variation for selection and stabilization of only a small subset of individual filopodia (Fig. 2B-C). This is also particularly evident in the case of filopodial selection preceding synapse formation. Selection as a mode of action is observed for all five developmental stages to various degrees and implies that the

majority of non-stabilized filopodia may not contribute individually to morphogenesis, while only one or a few filopodia can determine outcomes.

Regarding our second question on the contribution of individual filopodia: While the stabilization of individual filopodial can straightforwardly be correlated with an overall morphogenetic outcome, the alternative scenario, a signaling contribution by all filopodia, is more difficult to show and may be considered for morphogenetic processes that function robustly in the absence of filopodial stabilization. Some growth cones of migrating neurons and axons can move in a directed fashion in the absence of any stabilizing filopodia and 'vector integration' of all dynamically extending and retracting filopodia has been suggested to predict a growth direction [43,115]. Here, every single filopodium may contribute as a weighted signal, e.g. based on its length or lifetime. However, even in the absence of any stabilizing filopodia it is possible that only one or a few transient filopodia (e.g. those sensing and transmitting the strongest signal or those with the longest life time) determine a growth decision. Hence, processes purely based on transient filopodial exploration may include input from all or only few dynamic filopodia to compute growth directionality. In the case of migrating neurons the contribution of leading edge filopodia has been shown to be directly required for forward propulsion, at least in 2D culture; by contrast, axonal growth cones can move forward with few or no filopodia based on in vivo studies.

Regarding our third question on the role of physical forces on filopodia and transduced by filopodia: the growth cone-like leading edge of migrating neurons is the only case for which evidence supports a direct contribution of filopodial forces to movement. By contrast, axonal growth cones have been shown to move in an amoeboid fashion in a 3D matrix with little adhesion [93] and growth cones with strongly reduced filopodial numbers still move in vivo [76]. Here, integration of signaling based on mechanosensing contributes to navigational choices rather than movement per se. Importantly, these observations also highlight that different behavior can be observed in 2D versus 3D culture systems or in vivo; hence, only a direct comparison of the same growth process in such different environments can determine to what extent generalized conclusions about movement and navigation can be attributed to the experimental conditions.

The final outcome of all filopodia-driven processes reviewed here is the specificity and robustness of brain connectivity. The types and kinetics of filopodial tip interactions ultimately control synaptic partners. Which filopodial tips get to interact is a consequence of the morphogenetic history based on all preceding types of filopodial dynamics. In this context, the genetically encoded stochastic exploratory behavior of filopodia is a necessary component of the morphogenetic program, and an increased precision (less stochasticity) of such dynamics would lead to a loss of robustness, and in fact precision, of brain wiring.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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