Axon formation: a molecular model for the generation of neuronal polarity

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Summary

Neurons have unique structural and functional polarity. In general, information flows from the short dendrites to the long axon, and each neuron has multiple dendrites but only one axon. A detailed description of the cellular events leading to the establishment of axonal-dendritic polarity has been given from an in vitro hippocampal culture model system. Little is known, however, about the nature of the underlying molecular events. New data strongly suggest that actin depolymerization at a growth cone is crucial for axon fate determination. We hypothesize that an autocatalytic positive feedback loop at all growth cones locally regulates actin dynamics and other cellular events required for axon formation. Meanwhile, a negative feedback signal, produced by the positive feedback loop, propagates from all growth cones throughout the neuron and counteracts the positive feedback loops. Such feedback regulation provides a robust mechanism for spontaneous symmetry breaking and the formation of only one axon, even in a symmetric in vitro environment. Based on data from studies of cell migration, axon guidance, vesicle exocytosis, and the regulation of actin and microtubule polymerization, we propose a molecular scheme for the positive feedback loop and discuss possible negative feedback signals. ***BioEssays*** **22:172–179, 2000.**

Introduction

While we have learned much about the functions of axons and dendrites and how axons are guided, it remains unknown how most neurons in vivo produce only one axon but several dendrites.[1-3] Experiments have also shown that in a symmetric in vitro environment, neurons only form one axon (Fig. 1A).[4,5] Interestingly, the formation of one axon is not an irreversible event but can be reversed by cutting the axon (axotomy). If after axotomy the length of the axon remained a certain fraction longer than the other neurites, the future dendrites,[1] it still regenerated into an axon. If cut below a certain threshold, however, the regeneration of a new axon was a random and slow event that could occur in any of the neuronal processes (Fig. 1B).[5–7] These experiments[4–6] suggested that initially (stage 1-2) all processes have the potential to become an axon, and at some point (stage 2-3) one process becomes (reversibly) committed to an axonal fate. From these observations we conclude that neurons must employ a robust mechanism that guarantees the generation of one and only one axon. Moreover, the process that becomes the axon most likely generates an inhibitory signal preventing other processes from developing into axons.

We are interested in how one process becomes an axon. Some historical events are of relevance in this context. It took approximately 60 years, from the 1830s to the 1890s, to unite the nerve processes and nerve cell bodies into one anatomical unit: the neuron.[8] The law of dynamic polarization ("information flows from dendrites to the cell body to the axon") and the discovery of the growth cone are attributed to Ramón y Cajal[8] but crucial real-time in vitro observations were provided by Ross Harrison in 1907.[9] After observations of processes in real-time (and also after fixation), growing from a piece of medullary tube from a *Xenopus* embryo contained in a (clotted) drop of lymph hanging on a glass slide, he concluded (p 118): "These observations show beyond question that the nerve fiber develops by the outflowing of protoplasm from the central cells [soma]. This protoplasm retains its amoeboid activity at its distal end [growth cone], the result being that it is drawn out into a long thread which becomes the axis cylinder [axon]. No other cells or living structures take part in this process." These observations were also remarkable because of the suggestion that the "amoeboid activity draws out the axon." This coincides with the modern view that an actin-based force-generating system is involved in mechanically moving forward a growth cone.[10]

The aim of this paper is to provide a summary of recent results on axon formation, and on that basis propose a hypothesis for the underlying molecular events. These events involve "feedback loops," which are generally recognized to play a central role in symmetry breaking and morphogenesis[11–14] (as later discussed). It should be noted that the involvement of feedback loops in the context of axon formation, albeit without any molecular details, has been previously suggested.[1] In the next section we discuss how an in vitro system of cultured hippocampal neurons,[4,5] just like Harrison's in vitro system,[9] has significantly advanced

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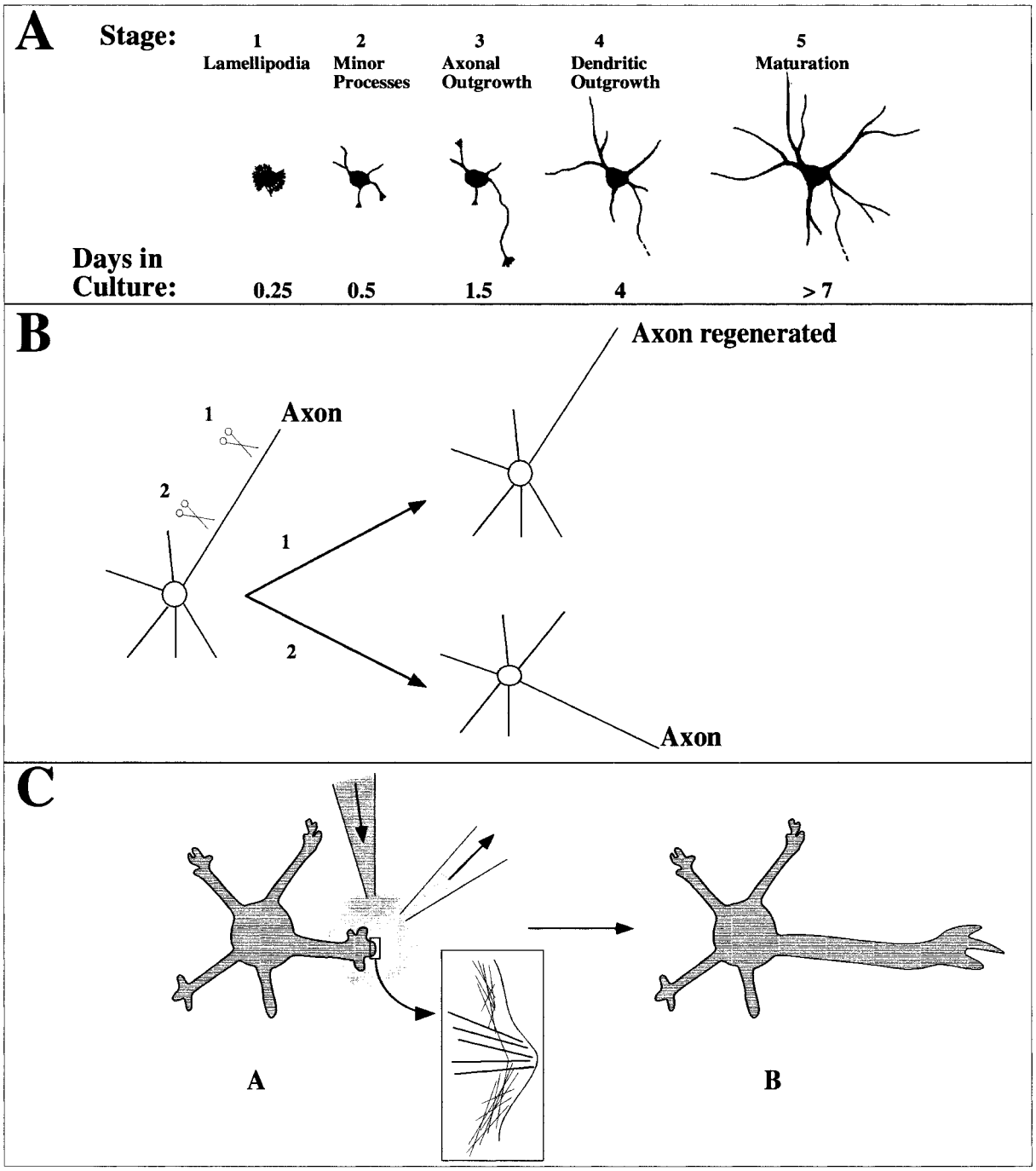

Figure 1. Characteristics of the assay used to show that local actin depolymerization is sufficient for axon fate determination in vitro. **(A)** Stages (1-5) of development of hippocampal neurons in vitro. The approximate times at which cells enter each of the stages are indicated. Axon fate determination occurs between stage 2 and 3 (reproduced from Dotti and Banker, 1988[4] with permission from the Society for Neuroscience). **(B)** The site of axon regeneration after axotomy at a site (1 or 2), is dependent on the relative length of the cut axon compared to the other processes. If more than 10 μm longer than the other processes (1), the axon regenerates again; otherwise (2) any process can after a long latency generate an axon.[5,6] **(C)** *A*, 1 μM of the actin depolymerizing drug Cytochalasin D (gray) was locally perfused (for 15-30 min) onto a randomly chosen process of a stage 2 hippocampal neuron in culture; *box*: local actin depolymerization (thin black lines) probably leads to microtubule (thick black lines) growth and further process extension (see text). **B**: Of 23 locally perfused processes, 18 developed into an axon over a 24 hour period.[19]

our understanding of the intrinsic and extrinsic molecular mechanism leading to axon formation.

Local actin depolymerization is sufficient for axon fate determination

It is known that between stage 2-3 one process starts to grow faster and becomes the axon (Fig. 1A),[1] whereas the other processes simultaneously start to grow slower.[15,16] Detailed analysis using real-time video-microscopy has shown that axon formation is preceded (stage 2) by: an enlargement and increased dynamics of the growth cone, higher intracellular vesicular transport[7] as well as increased microtubule (MT) transport;[17] the other processes remain in a state of undynamic quiescence.[18] Because growth cone dynamics are largely a reflection of actin polymerization and depolymerization,[10] these observations[7] suggested that increased actin dynamics at the growth cone may be important in determining which process becomes the axon. Indeed, a recent paper from Bradke and Dotti strongly supports this idea.[19] Departing from the original cell culture system as used by Dotti and Banker,[4] they studied the morphology of growth cones at early stages (stage 1-3). Using real-time video-microscopy they confirmed that the process with the most dynamic growth cone is most likely to become the axon.[7,19] Addition to the neuronal cultures of low concentrations of the actin depolymerization reagents Cytochalasin D or Latrunculin B, resulted in the formation of multiple axons, indicating that high actin turnover supports axon formation. Moreover, elegant experiments showed that local application of Cytochalasin D for only 12 minutes to a randomly picked process resulted in this process differentiating into an axon over a 24 hour period (Fig. 1C); these results strongly suggest that local actin depolymerization plays a crucial role in axon fate determination.

Why is local actin depolymerization so important? The authors suggest a direct link to microtubule (MT) polymerization. In recent years there has been a growing body of evidence showing that actin and MT polymerization strongly influence each other.[20–24] It is known that MT polymerization is required for axonal and dendritic growth.[17] At the leading edge of migrating cells (equivalent to the growth cone) MTs tend to persist in growth but usually do not grow into the lamellipodia. However, if actin assembly is blocked, neurite extension still continues; under such conditions MTs extend into the lamellipodia and can become bent against the plasma membrane.[20,21,25,26] Thus, the Cytochalasin D-induced depolymerization of actin[19] probably leads to enhanced MT polymerization, resulting in enhanced process outgrowth and, subsequently, axon formation (Fig. 1C and Fig. 2B).

Symmetry breaking by feedback loops

What causes local actin depolymerization at one particular neurite growth cone in the absence of Cytochalasin D, so that this neurite, but not others, becomes an axon? Superficially, this appears to present a paradox, since in culture, the cell is in a uniform external environment, and intrinsically, every initial process apparently has more or less equal potential to become an axon. This situation is analogous to what physicists call "spontaneous symmetry breaking,"[13] where asymmetry emerges from symmetric but unstable initial conditions through internal dynamics. The solution relies on the fact that when a dynamic system reaches a state of instability, some small irregularities that arise from stochastic fluctuations tend to grow. This usually leads to a new and stable state where the symmetry is broken down, and in this case the formation of an axon.[11–14]

In biological systems, similar ideas were enunciated by Alan Turing in his classical work on the theory of morphogenesis.[11] Turing's system was a developing embryo that consisted of many cells. However, the general mathematical framework he used can be readily applied to the problem of a single cell with intracellular signaling factors, (equivalent to different "morphogens") forming both positive and negative feedback loops. In such a scenario, a positive feedback mechanism may exist in all stage 2 growth cones (Fig. 1A): each neurite has the potential to grow further and become an axon. Indeed, global application of actin depolymerizing drugs caused multiple axons to grow from the same cell body,[19] suggesting that there is not a limiting factor for axon formation.[27] Therefore, in order to assure the formation of only one axon, in the absence of drugs, an inhibitory/antagonistic signal(s) (negative feedback) must be generated simultaneously with the positive loops and propagated globally; this notion is supported by the slower growth of non-axonal processes.[15,16] Our hypothesis propose that before stage 3, the positive and the negative feedback factors form an intricate balance. As the feedback gets stronger, this balance will become unstable, and eventually the symmetry is broken: one neurite, the future axon, grows faster while the growth of all others is inhibited (Fig. 2A).

If we look at the physical components that are required for sustained process extension, these must include net cytoskeletal assembly as well as the addition of new membrane. Accumulation of vesicles at the future axonal growth cone appears to be a prelude to axon fate determination, and preferential exocytosis at the axonal growth cone has been observed.[7,28–30] The identity and composition of the vesicles involved in this process are unclear;[31] rather than this membrane insertion serving a purely passive role as a required addition of material to the expanding membrane surface area,[29] it may also play an active signaling role. For example, exocytosis at the growth cone could locally insert more calcium permeable channel proteins and/or release

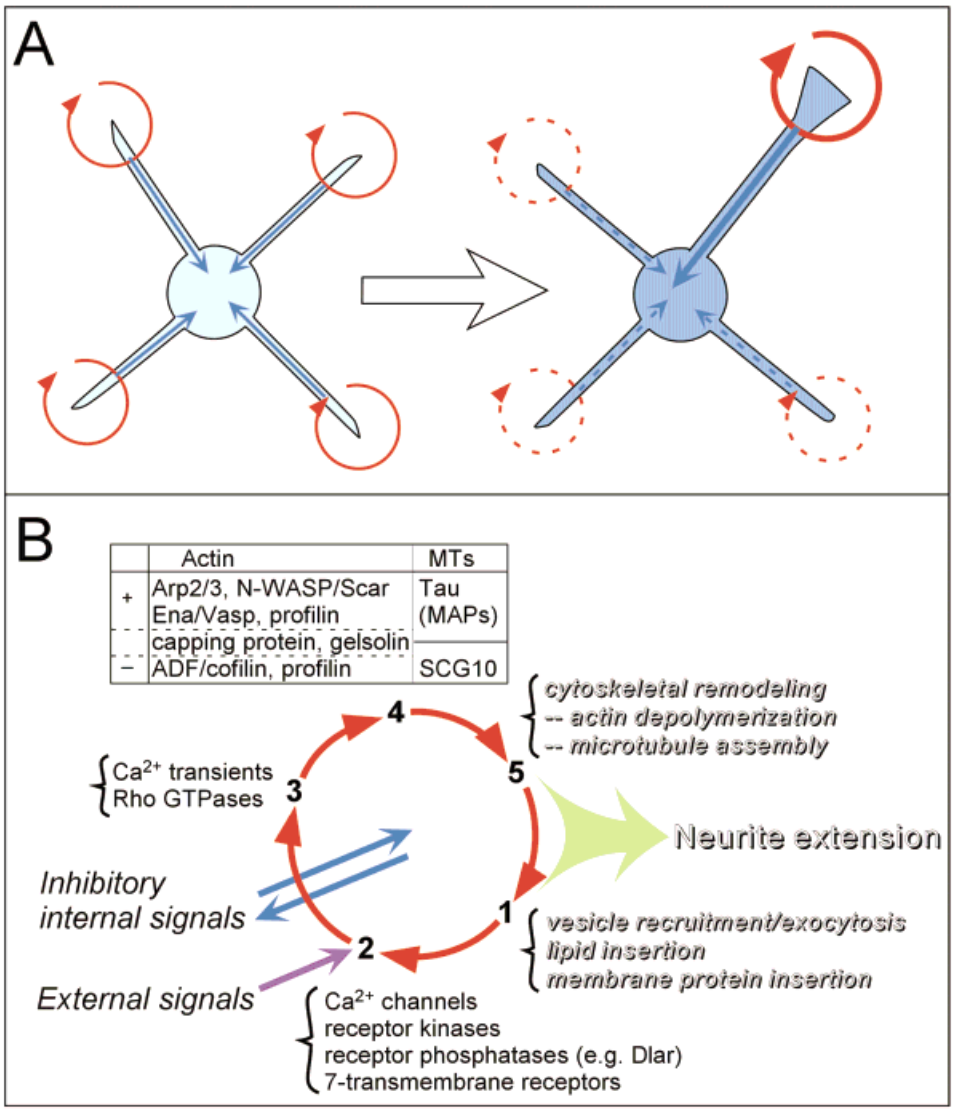

Figure 2. A model for the determination of axon fate by an intrinsic feedback system.

(A) Positive and negative feedback regulation leads to symmetry breaking in a neuron between stage 2 and 3 (Fig. 1A). Red circles with arrows represent molecular signaling cascades that form positive feedback loops in the growth cone of each neurite and promote neurite extension and axon fate determination. Blue arrows represent a long-range negative (inhibitory) factor that is generated at each growth cone, propagates throughout the cell, and counteracts the positive feedback loops in other neurites. After symmetry breaking, one "strong" neurite becomes the axon, and has a strong positive feedback (marked by a thick red circle); it also generates a strong negative signal (marked by a thick blue arrow and dark blue background) to influence the other growth cones, at which both the positive feedback loops (dashed red circles) and the generation of the negative signal (dashed blue arrows) are reduced or blocked. **(B)** Cellular and molecular correlates of the feedback system required for neurite extension (also see text). Red arrows represent signaling among the molecules and cellular events that form the positive feedback loop. The purple arrow represents external signals; depending on the context they can have either positive or negative effects on the positive feedback loop. Blue arrows represent negative feedback signals whose identity is yet to be determined. (**1**) Membrane is added at the growth cone by (regulated) exocytosis; (**2**) among the proteins inserted during exocytosis are Ca^{2+} channels and transmembrane receptors; the purple arrow indicates that external signals such as extracellular matrix proteins are likely to work at this stage in the cycle; (**3**) subsequent to the events in 2, Ca^{2+} transients are generated by local influx through channel proteins; in addition, (auto)-activation of receptor kinases and phosphatases in turn activate downstream signaling molecules (see text); (**4**) these molecules are known to directly affect actin and MT polymerization, and are likely involved in regulating growth cone dynamics; + and − indicate polymerization and depolymerization activities; (**5**) actin is locally depolymerized and unknown signals mediate increased MT polymerization and neurite extension (green arrow); the rearrangement of the cytoskeleton allows more vesicles to be recruited and exocytosed (1). The division of the loop into five steps is arbitrary, and many more interactions among the molecules than those presented here are known. Step 1, vesicle recruitment and exocytosis, and step 5, cytoskeleton remodeling, are directly related to neurite extension.

neurotransmitters.[32] These events could in turn result in auto-activation of receptors at the growth cone and transient Calcium (Ca^{2+}) elevation, which is known to affect filopodial dynamics[33,34] as well as to cause a (transient) breakdown of the local cortical actin and MT networks.[34,35] The latter would allow MTs to protrude into the peripheral region of the growth cone,[20,21,26] resulting in process extension followed by recruitment of more vesicles and more exocytosis.[37] Apart from Ca^{2+} channels, exocytosis may also insert other signaling proteins such as receptor tyrosine kinases and phosphatases. Such receptors may become auto-activated after being incorporated into the plasma membrane, as suggested for the Ca^{2+} channels. These series (or "loops") of events constitute positive feedback loops, the outcome being further growth of that particular growth cone (Fig. 2B).

In vivo, extrinsic factors may play a dominant role for axon formation. It is known that extracellular cues, such as extracellular matrix proteins, by preferential stimulation of unspecified receptors may determine which process becomes the axon.[16] Therefore the events leading to axon formation in vivo may be different from those happening in vitro in that the positive feedback loops may be preferentially activated/enhanced by asymmetrically distributed extracellular cues at some growth cones. In a way, the (supposedly) symmetric environment of the in vitro experiments imposes a more stringent condition upon the system and demonstrates the robustness of the cellular mechanisms underlying axonogenesis.

Molecular composition of the positive feedback loop

This conceptual scheme for how positive and negative feedback loops regulate the formation of only one axon has, so far, little specific molecular foundation. Assuming that an increase in actin dynamics is required, we have pooled together data on actin polymerization, growth cone guidance/dynamics and cell migration, and propose the scheme depicted in Figure 2B. It should be stated that although localized Cytochalasin D treatment can lead to axon formation,[19] whether this treatment activates the same pathway(s) followed in vivo is unknown. For example, axotomy, protease activity or increased intracellular Ca^{2+} concentration all can lead to new growth cone and process formation in vitro.[7,35,36]

As regulation of actin polymerization is a key event for axon formation in vitro, the most plausible candidates include GTPases of the Rho family that are found in all eukaryotic cells and act as switches to control signaling from membrane receptors to the actin cytoskeleton.[38] For example, Cdc42 and Rac, members of the Rho family, are responsible for the formation of filopodia and lamellipodia, and blocking the activity of Rho GTPases by bacterial toxin B caused multiple axon formation.[19] Parallel to these basic signaling pathways, another set of signaling molecules including the Arp2/3 complex, Dlar, Abl, ENA/Vasp, profilin, and WASP/Scar have recently drawn much attention by their roles in axon guidance[39] and actin assembly,[40,41] and may also play important roles in axon formation. For example, the receptor tyrosine phosphatase Dlar is known to bind the cytoplasmic tyrosine kinase Abl as well as ENA; this trimeric complex somehow regulates the activity of the actin monomer binding protein profilin and is required for axon growth and pathfinding.[39] Part of the positive feedback loop (Fig. 2) could consist of auto-activation of the Dlar receptor tyrosine phosphatase with subsequent enhanced actin dynamics through regulation of profilin activity. The heptameric Arp2/3 complex caps the pointed end of actin filaments and, together with WASP/Scar, is involved in the nucleation of new actin filaments, resulting in networks like the ones found at the leading edge of growth cones.[40,42] Thus, receptor-mediated regulation of new actin polymerization at the leading edge could easily be imagined to occur through regulation of the Arp2/3 complex via WASP/Scar and Cdc42.[40,43] In addition, the actin destabilizer ADF/cofilin at the (slow-growing) pointed end, and gelsolin and capping protein at the (fast-growing) barbed end, are also important for regulation of the dynamic state of the actin network at the leading edge.[40,42,44,45] Apart from enhancers of actin dynamics, actin polymerization promoters and stabilizers may also be important.[46,47] One possibility is that the Cytochalasin D treatment[19] only depolymerizes a subpopulation of actin filaments, and that the remaining, unique, filaments serve as an anchor point for the factors involved in axon formation; inspired by work on the cell cycle, one could imagine that a neurite passes an actin-network dependent "morphogenesis checkpoint"[48] that marks its axonal fate. Lacking more data, however, it is at present not possible to incorporate such speculations into the proposed scheme (Fig. 2B).

Precisely how local actin depolymerization leads to MT growth and lamellipodia protrusion is unknown. A causal link has been directly demonstrated in MAP2-transfected tissue culture cells where actin depolymerization subsequently resulted in process outgrowth.[49] Conversely, dynamic MTs have been shown to activate Rac1.[20,24] Generally speaking, in the nascent axonal growth cone, the positive feedback loop must generate a "positive potential for MT polymerization." This is defined as the sum of the activity of MT stabilizing (e.g., microtubule-associated proteins/MAPs) and destabilizing molecules, and a positive potential results in net MT assembly.[50] It is currently unknown which MT stabilizers and destabilizers are the key players for growth cone MT polymerization, and several candidates are possible (Fig.

2B). For example, in the growth cone of the future axon, local dephosphorylation-dependent activation of the MT stabilizer tau, together with local phosphorylation-dependent inactivation of the MT destabilizer SCG10, would lead to a more positive MT polymerization potential and MT polymerization.[50–52] Thus, in addition to the primary effector molecules, spatial regulation of kinases and phosphatases, specific for the MT stabilizer and destabilizer proteins, also is of primary importance.

Negative feedback and hypothesis testing

At present, there is a missing link between the positive and negative feedback loops. Furthermore, nothing is known about the nature of the proposed inhibitory signal or how it may be generated (Fig. 2A). That global actin destabilization with Cytochalasin D causes multiple axon formation[19] suggests: 1) that under these conditions the inhibitory signal is abolished; 2) the positive signal was enhanced more than the inhibitory signal; or 3) Cytochalasin D treatment initiates an alternative pathway for axon formation. Clearly, a more detailed characterization of the Cytochalasin D effect is required. For example, if one locally perfuses two, three, or more stage 2 processes (Fig. 1C), will only one or all of them become axons? If one cuts an axon below the threshold level (Fig. 1B), and then perfuses with Cytochalasin D, will this process develop into the axon? If actin dynamics are locally or globally inhibited (e.g., by Fasplakinolide[53]), will axon formation be prevented or delayed? Such experiments should begin to clarify the properties of the proposed inhibitory signal, and whether the Cytochalasin D treatment disrupts the link between the positive and negative feedback loops. Interestingly, recent experiments[54] involving global application of Cytochalasin E suggest that although more processes grow longer in the presence of this drug, there is not an increased formation of axons based on tau-1 immunostaining (an axonal specific MAP[51]). This contradicts the results reported by Bradke and Dotti, where tau-1 immunostaining was present in multiple long processes from a single cell body following Cytochalasin D treatment.[19] This discrepancy probably reflects experimental differences that need to be elucidated. Regardless, we think that the experiments involving local short application of an actin depolymerizing drug[19] are the most convincing because global cytotoxic non-specific/long-term effects of the drug are minimized. These local application experiments show that local actin dynamics regulation can dictate axon fate determination.[19] A modification of the local Cytochalasin D perfusion experiments, as shown in Figure 1C, could address whether exocytosis or Ca^{2+}-dependent exocytosis (Fig. 2B) is required for axon formation and is part of the suggested positive feedback loop; this could be done by local perfusion of Cytochalasin D dissolved in buffer without Ca^{2+}. In this context it is interesting to note that global Brefeldin A treatment caused axons to retract.[55] This suggests that exo- and endocytosis continuously occur in the axon, and that the Brefeldin A-induced retraction was caused by retrieval of vesicles back into the fused somatic ER-Golgi compartment.

What may be the nature of the inhibitory signal? There are reports that high local cAMP concentrations at a *Xenopus* growth cone resulted in the inhibition of growth of other neurites from that same neuron.[56,57] It is thus possible that cAMP/PKA pathways are involved in the formation of the proposed long-range negative signal during axon formation in hippocampal cultures, but this possibility has to be tested. Ultimately, the propagating negative signal probably causes a decrease in actin dynamics, MT polymerization, and membrane insertion in the processes destined to become dendrites; this could for example be initiated by changing the activity of cytoplasmic phosphatases and kinases in favor of phosphorylation of MAPs. There are, however, many other possibilities.

In addition to these pharmacology-based experiments, it will also be of great interest to characterize the effects on axon formation of expression of different mutated proteins that, for example, regulate actin dynamics (Fig. 2B)[58]. Mutated proteins may be introduced by viruses, microinjection, or through genetics. However, pharmacological experiments, although lacking specificity, will remain important in the elucidation of the nature and interactions between the positive and negative feedback loops because of the spatiotemporal control they offer.

Conclusions

At the molecular level, astonishingly little is known about how neurons regulate the formation of a single axon, but we describe a theoretical model in Figure 2B; we have not included issues such as for example axonal-dendritic protein sorting[7,59,60] or axon hillock formation,[61] as we think these neuronal characteristics probably are established *post* axon fate determination. The feedback system hypothesized here is intrinsically robust—it guarantees the generation of one and only one axon for each neuron. It can maintain the established polarity of the neuron up to stage 4 when dendritic development requires additional mechanisms.[1] The same system can also respond to external cues by steering the direction of growth, as long as the signaling from appropriate membrane receptors engages the feedback loops at a finer local scale.[16] The near future should see an exciting quest for the identification of signals leading to local increased actin dynamics. Without being specific, we think that the field of axon formation will benefit greatly from the lessons learned from axon guidance.[2,3,39,56] With respect to the molecules involved in the negative feedback loop, we are in largely uncharted territory. To find the nature of the

inhibitory signal(s) and the link to the hypothesized positive feedback loop is of outstanding interest.

Acknowledgments

Due to the breadth of the topic discussed we have chosen to mostly cite comprehensive reviews rather than original works. Apologies to those whose observations are not directly cited. We thank J. Endres, T. Esch, T. Gomez, L. Kiss, and two anonymous reviewers for constructive comments on the manuscript, and F. Bradke for a part of Figure 1C.

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