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Neuronal Polarity: Demarcation, growth and commitment

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Abstract

In a biological sense, polarity refers to the extremity of the main axis of an organelle, cell, or organism. In neurons, morphological polarity begins with the appearance of the first neurite from the cell body. In multipolar neurons, a second phase of polarization occurs when a single neurite initiates a phase of rapid growth to become the neuron's axon, while the others later differentiate as dendrites. Finally, during a third phase, axons and dendrites develop an elaborate architecture, acquiring special morphological and molecular features that commit them to their final identities. Mechanistically, each phase must be preceded by spatial restriction of growth activity. We will review recent work on the mechanisms underlying the polarized growth of neurons.

Introduction

Neurons are classical examples of highly polarized cells. They typically have a single long, thin axon and one or several shorter and thicker dendrites. In the canonical multipolar neuron of the mammalian brain, the axon transmits information to the target, while dendrites receive and process the incoming information. How neuronal polarity occurs has been the subject of intense scrutiny for the past 40 years. Early observations in the 1970s and 80s, in fixed and live brain tissue from different species and areas, revealed that migrating neurons are decorated with neurites, implying that breakage of the symmetric shape of a newborn neuron occurs at an early stage of differentiation, before migration. In the 1980s, studies based on the use of embryonic hippocampal neurons in culture defined the morphological steps of polarization [reviewed in 1]. Shortly after plating, these cells extend a motile lamellipodia around the cell body, an event known as stage 1 of polarization. Next, during stage 2, the lamellipodia clusters at particular sites until small cylindrical processes, the “minor” neurites, form. These neurites are highly dynamic, exhibiting periods of extension

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An important review article stressing the importance of the AIS for the maintenance of neuronal polarity.

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and retraction, until one of them initiates a sudden and sustained growth; this neurite becomes the neuron's axon, and this event characterizes stage 3. During stage 4, the remaining minor neurites develop as dendrites, and in stage 5, synaptic specializations and contacts are established.

Early in vitro studies made obvious that neuronal polarization begins, in the strictest sense, with the appearance of the first neurite. Intriguing ulterior studies have revealed that the first neurite is the one with the highest chance of becoming the axon when the choice of one among multiple neurites has to be made [2, 3]. Although the mechanisms involved in this preference have not been extensively explored, these studies stress the notion that axon-dendrite specification tightly correlates to the mechanisms involved in the generation of the first neurite. Studies dealing with this issue will be discussed in the section "First phase of polarity: Generation of the first neurite."

The fact that neurons acquire a strikingly polarized morphology at stage 3 by forming an axon from an array of several almost identical minor neurites has led many researchers to conclude that polarity is established at the transition between stages 2 and 3 [4–6]. This assumption triggered a movement to identify the underlying molecular basis for spatial-growth selectivity. The most recent studies in this regard are reviewed in the section "Second phase of polarity: Generation of the axon and dendrites from minor neurites."

Finally, after axon-dendrite specification, the axon and dendrites of a neuron are committed to distinct developmental paths, though this commitment can be plastic [7–9]. The molecular mechanisms involved in the stabilization/consolidation and further differentiation of axon-dendrite identity are only now beginning to be addressed. Current knowledge will be summarized in the section "Third phase of polarity: Axon-dendrite commitment."

First phase of polarity: Generation of the first neurite

Like any other cell, the architectural polarization of the neuron begins with the appearance of a first deformation, a neurite sprout (Figure 1). Data obtained in cultured hippocampal neurons reveal that this early event may have two major implications: (1) it provides the neurite with a higher chance to undergo rapid (axon-like) growth [2, 3, 10**], and (2) it defines where a second neurite can grow [3, 11*]. This represents, therefore, an initial step in the cells' bipolar organization, crucial for migration and final positioning in the brain [12], and highlights the importance of understanding the mechanisms underlying formation of the first neurite.

Recent studies in mammalian neurons in vivo and in vitro and in *Drosophila* neurons in vivo suggest that the site where the first neurite sprouts is intrinsically defined, by virtue of the polarization of the centrosome/microtubule organizing center (MTOC), the Golgi apparatus (GA), and endosomes [2, 3, 10**]. Although these results agree with early work in neurons in vitro [13] and in situ [14] and with a large body of literature on other polarized cells [reviewed in 15], a number of new studies have challenged this concept [16–19]. For example, fruit flies lacking centrioles, and therefore a compact centrosome, develop a largely normal nervous system [16], and centrosome removal in cultured hippocampal neurons does not interfere with axonal growth [18**]. Moreover, a multicolor in vivo time-lapse imaging study of tegmental hind-brain neurons (THN) from zebrafish has revealed the temporal sequence of subcellular dynamics during axonogenesis, and, in accordance with a previous study of zebrafish retinal ganglion cells [17], excluded an in vivo function of the MTOC in determining the site of axon outgrowth based on proximity to the centrosome [19*].

Along the same line, recent time-lapse studies in *Drosophila* sensory neurons undergoing polarization in situ [20**] or in hippocampal neurons polarizing in vitro [21] have revealed that the MTOC-GA complex moves towards the site of first-neurite formation after this has occurred. These results suggest that cytoplasmic asymmetry, or at least that mediated by MTOC-GA positioning, may not play a pivotal role as the spatial cue mandating where the first neurite will form. However, this may not be true in all cases. More studies of other neuronal types are required; it may well be that centrosomal requirement is cell-type specific. Also, since cells—including neurons—can form an asymmetric cytoplasm independently from centrioles [20, 22, 23], it is still possible to argue that neuronal polarization (or the axonal growth aspect of it) may in fact require cytoplasmic organelle asymmetry close to the place of growth, independent of the existence of a compact centrosome.

If not by intracellular cues, what then defines where the first neurite will sprout? Early work in cultured hippocampal neurons suggested that axon-dendrite growth is critically and selectively influenced by extracellular adhesive cues, such as laminin or tenascin [24, 25]; it was then reasonable to investigate their relationship with first-neurite formation. Three recent publications, on *Drosophila* sensory neurons [20], mammalian hippocampal neurons [21], and zebrafish retinal ganglion cells [26**], have confirmed that adhesive complexes via cadherin (in *Drosophila* and hippocampal neurons) or laminin (in zebrafish neurons) do indeed play a key role in initial polarization. Moreover, the studies of *Drosophila* sensory neurons have revealed that the localized assembly of the cadherin complex follows the same mechanistic principle of adherens junction formation during epithelial polarization. Namely, it is preceded by clustering of the polarity PAR3/Bazooka complex, which is in turn preceded by the localized clustering of PI (4, 5) P2. In addition, this work has shown that the site of assembly of the adhesion complex is coincident with that of the previous mitotic cleavage. This observation suggests that key components of the machinery assembled for cytokinesis are reused to initiate symmetry breakage in the daughter cell, at the same site where cleavage occurred, before its diffusion or degradation. In support of this possibility, it was observed that one of the components of the mitotic furrow, which is inherited and retained at the site where the first neurite will eventually form, is Aurora A, a serine/threonine protein kinase that phosphorylates Par3 [27]. It is also noteworthy that in many cell types, classical cadherins organize nuclear and centrosomal positioning during polarization [28]. Naturally, a number of questions remain unaddressed, including how adhesive complex components are formed at the site of future budding, and for what biological purpose the newly formed neurite signals to the centrosome and associated membrane organelles, which recurrently move to this site. It also seems important to investigate whether and, if so, to what extent—and by which mechanism—being born first helps to confer a final axon or dendritic growth preference later on.

Second phase of polarity: Generation of the axon and dendrites from minor neurites

Studies based on embryonic hippocampal neurons in vitro indicate that the generation of a single axon and multiple dendrites from a rather symmetric array of several morphologically indistinguishable and highly dynamic minor neurites is the hallmark of neuronal polarity. At a microscopic level, the transition from a symmetric to an asymmetric shape (transition stages 2–3) begins with changes in cytoskeletal and membrane organization at the growth cone of the future axon [6, 29]. At a molecular level, the stage 2–3 transition appears to begin with the selective accumulation/activation of several components into the growth cone of the prospective axon, including at least one growth factor receptor [30, 31*], phosphatidylinositol-3 kinase (PI3K) [30, 32–34], the Par polarity complex [32, 33], and Rho GTPase signaling modules [5, 35*]. Downstream effectors of these proteins include

several master regulators of cytoskeletal assembly/dynamics and membrane addition, such as CRMP2 [36], DOCK7-stathmin [37], LKB1/STRAD [38], GSK3 β [33, 39], and the exocyst [40*]. In cultured hippocampal neurons, suppression of any of these proteins halts axon formation, whereas overexpression induces the extension of multiple Tau-1+ axon-like neurites. These observations have identified the proteins crucial for axonal growth and suggest that all minor neurites, independently of their birth timing, have minimal machinery to support fast growth. Yet, as in vivo, hippocampal neurons in culture only extend a single axon, clearly indicating the existence of polarity signals responsible for the accumulation and/or activation—in one growth cone only—of the critical components of the cells' growth machinery. Several mechanisms have been proposed to explain how this occurs, including stochastic fluctuations of intrinsic determinants along with self-reinforcing feedback loops [5, 41], or quantitative ones, because of time-of-birth hierarchy [3], local-activation and global-inhibition mechanisms [42*], selective MT-based transport associated with kinesin or tubulin post-translational modifications [43–45], or microtubule-associated protein (MAP) binding [46]. In addition, the most recent studies have suggested major instructive roles for axon-dendrite polarization through the reciprocal regulation of cAMP/protein kinase A (PKA) and cGMP activities [47, 48**, 49**]. Like other factors that promote axon formation and whose ectopic expression induces multiple axon formation, the global application of PKA agonists promotes single axon formation; FRET analysis has shown that local activation of cAMP in one neurite results in long-range inhibition of cAMP activity in the other minor neurites of the same neuron. The cAMP/PKA-dependent phosphorylation of LKB1 and activation of downstream targets, such as SAD and MARK, mediate the axogenic effect of this cyclic nucleotide [38]. Although this appears to be a genuine polarizing signal, its stringent spatial specificity (PKA acts on one neurite only) implies the existence of a pre-existing growth-favoring background. This has yet to be identified.

Another important aspect in the stage 2–3 transition is whether polarized growth also involves the existence of signals that prevent fast, axon-like growth from the minor neurites. Cumulative evidence suggests the existence of a RhoA inhibitory tone that prevents the transformation of minor neurites into fast growing Tau1+ axon-like neurites [50, 51]. The most recent study favoring this view [52*] shows that reduction of RhoA activity by suppression of Lfc, a RhoA-specific guanine-nucleotide exchange factor, enhances axonal elongation and/or induces the extension of multiple axon-like neurites in both stage 2 and stage 3 hippocampal neurons in vitro; by light microscopy, these neurites look identical, making it difficult to distinguish between bona fide and supernumerary axons. Interestingly, growth cones of neurons treated with inhibitors of RhoA have less filamentous actin [50, 51], consistent with observations suggesting that an increase in actin dynamics is required for axon initiation [52–54]. Profilin IIa and LIMK1, as well as disruption of the self-reinforcing Cdc42-Par3-Par6-Rac-PI3K signaling module, are likely to mediate the effects of RhoA-ROCK on actin dynamics, neuritogenesis, and axon formation [6, 50, 52, 55]. Tctex-1, a dynein light chain required for axon formation and capable of promoting Rac activity and inducing multiple axons [51], physically interacts with Lfc to inhibit its Rho-GEF activity [52]. In other systems, such as rodent enteric neurons, RhoA inhibition also promotes axonal elongation but fails to induce multiple axons [56].

The third phase of polarity: Commitment

Commitment to polarization is the process by which nascent axons and dendrites are transformed into their final architecture, with unique molecular and supramolecular organization that allows proper electrical vectorial function. Immediately after polarization, the morphology and organization of dendrites and axons are similar except for length. This is in sharp contrast to the dramatically distinct dendrite and axon characteristics of mature neurons. Very little is known about the mechanisms underlying this process, but evidence

suggests the involvement of both preexisting differences that arise during the time of axon-dendrite specification and de novo mechanisms. A major factor in the commitment phase is the difference in several aspects of microtubule organization between axons and dendrites [6]. First, the presence of minus-end-distal microtubules in dendrites but not axons [57] may lead to polarized distributions of organelles and proteins that rely on directional transport for subcellular localization. Indeed, when microtubule polarity is compromised in neurons as a result of defective motor proteins, organelle polarity is compromised and dendrite or axon growth is defective [58–60]. The difference in microtubule polarity appears after axon-dendrite morphological specification [61], and is therefore likely to be part of the commitment mechanism if newly polarized axons and dendrites are to develop into their mature forms. Second, differences in MAPs and posttranslational modifications of microtubules. For example, the proximo-to-distal gradients formed by the phosphorylated forms of Tau and MAP1B in axons may regulate the entry, retention, or avoidance of different proteins and lipids, and consequently contribute to the acquisition of mature characteristics [62]. In addition to microtubules, membrane systems, including the secretory and endocytic pathways, also contribute to the transformation of the nascent-to-mature axons and dendrites [63, 64].

Committing dendrites and axons to their distinct developmental paths also requires de novo mechanisms. This notion is supported by the findings of dedicated transcriptional programs that differentiate dendrite and axon development. For example, the SnoN transcription suppressor specifically controls axonal growth in mammalian neurons [65, 66], whereas Dar1, a Krüppel-like transcription factor, is specifically required for dendrite growth in *Drosophila* [67*]. Analyzing the upstream signaling mechanisms and downstream targets of these transcriptional regulators will yield critical insights into how dendrites and axons differentiate into their mature architecture.

A key functional feature of mature axons is the axon initial segment (AIS). The AIS presents barriers for membrane and cytoplasmic proteins to enter the axon, which contributes to the separation of somatodendritic and axonal compartments [68, 69]. Interestingly, silencing of the cytoskeletal scaffolding protein, ankyrin G, dismantles the AIS and causes axons to acquire the molecular characteristics of dendrites, including formation of spines and postsynaptic densities [70]. Another recent study has shown that the plus-end tracking proteins EB-1 and EB-3 participate in the maintenance of AIS and neuronal polarity by coordinating interaction between ankyrin G and the microtubule cytoskeleton [71*]. Together, these observations suggest that ankyrin G— and probably AIS—are required to maintain axon identity [72*]. Nevertheless, there are many open questions about whether and how AIS is linked to the polarization process of axon and dendrites. For example, does the presence of a highly phosphorylated Tau environment around the initial segment contribute to AIS formation? Is AIS formed in the multiple axons induced by ectopic expression of axon-promoting factors, such as CRMP2? Future investigations along this line will not only provide insights into the mechanisms underlying the commitment phase of axon-dendrite polarity, but also provide functional criteria for evaluating this polarity.

Conclusions

Axonal and dendritic differentiation occurs during development. It is now apparent that the polarized shape is the consequence of molecular changes that define the occurrence of two morphologically and temporally different phases: (1) formation of the first neurite and (2) growth of one out of several neurites. The mechanisms involved in the first phase appear to be significantly different from those of the latter. Recent work suggests that the selection of the site where the first neurite will sprout requires the existence of a cluster of cell adhesive cues, which in turn are defined by the plane of the last mitotic cleavage. On the other hand,

recent work suggests that the selection of which neurite develops into the axon depends on the action of particular extracellular ligands. While this can well explain polarized growth in vivo, where extracellular ligands are presented in a spatially organized fashion, in vitro studies have shown that polarization occurs by a cell-autonomous mechanism. A robustness of this type is synonymous with molecular redundancy, which is consistent with numerous reports of axonal growth inhibition on the knockdown of different types of proteins. Whether the cell-autonomous mechanism for polarized growth is the consequence of a universal principle or is cell-specific is a question for future research.

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Highlights

- Neuron polarization consists of three phases: demarcation, growth and commitment.
- Phase I involves the growth of one neurite from a site defined at the precursor stage.
- Regulation of growth-inhibitory and -promoting mechanisms controls axon specification.
- Both preexisting and *de novo* mechanisms determine final axon and dendrite identity.

Figure 1. The three phases of neuron polarization

Phase I first neurite formation. The model is based on studies in *Drosophila* sensory neurons in the notum and hippocampal neurons in culture. In the fly, the newborn neuron “inherits” remnants from the cytokinesis ring (red) with the ability to induce localized cytoskeletal changes (e.g., via RhoA and Aurora kinase) and the recruitment of PI (4,5) P2 (light blue). This, in turn, triggers the clustering and activation of polarity-complex proteins, leading to the formation of a cell-cell adhesive ring (purple). This cascade of events results in the generation of an apical plasma membrane domain from where the first neurite grows. In hippocampal neurons this process is critical to define growth axis *in vivo*. Cytoplasmic organelles, such as Golgi and centrioles, move toward the growing neurite after this has formed. **Phase II**: axon specification. At stage 2, all minor neurites have minimal machinery to support fast growth. Filamentous actin (blue) is assembled at the tip of each neurite, and microtubules are oriented uniformly with the plus-end pointing to the distal (“plus-end-distal” microtubules are shown in orange). A RhoA-inhibitory tone prevents the transformation of minor neurites into fast-growing axon-like neurites. Removal of this inhibitory tone in one neurite enhances the dynamics of actin cytoskeleton and leads to fast growth, which transforms the neurite into an axon (stage 3). This process may be determined through an external cue or occur by a cell-autonomous mechanism. **Phase III**: The nascent axon and dendrites of a neuron are committed to distinct developmental paths to be transformed into their final architecture. During stage 4, the remaining minor neurites are

transformed into dendrites. “Minus-end-distal” microtubules (green) and Golgi outposts appear in the dendrites, but not the axon. Except for length, at this stage the morphology of nascent dendrites and axons is similar. Through both preexisting differences that arise during the time of axon-dendrite specification and de novo mechanisms, axons and dendrites develop the dramatically distinct characteristics of mature neurons (stage 5).