Cellular Mechanisms for the Axonal Pattern Formation: Initiation and Branch Morphogenesis

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(Received June 16, 2014; Accepted September 24, 2014)

The establishment and maintenance of characteristic cellular morphology is especially important in the nervous system, in which neurons make connections with specific targets, thereby enabling the processing of information. The axonal pattern is crucial in determining the target cells, and is controlled by extracellular molecules, called axon guidance molecules. In culture, isolated neurons are capable of extending the axon and establishing mature neuronal morphology without particular cell-extrinsic cues. Molecular systems that control this cell-autonomous process remain to be elucidated. In this paper, we summarize the cellular processes of axonal patterning and some of the intracellular molecular mechanisms that contribute to maintain the axon morphology.
Key words: Neuron, Morphogenesis, Polarity, Axon, Dendrite

1. Introduction

Neurons form a highly polarized morphology by extending molecularly and functionally distinctive processes that emerge from the cell body; the axon, a single thin process that is required to transmit signals to target cells in the form of electrical impulse (action potential), and dendrites, shorter processes that receive signals. The terminal axons synapse with the dendrites of the target neurons. The presynaptic terminals of the axon release neurotransmitters to the postsynaptic site. Axonal length and morphology vary widely depending on the location of their target cells. For example, some interneurons possess only a short (a few hundred micrometers long) axon, whereas the axons of sensory or motor neurons may be over one meter long. In addition to the axon length, their branched morphology is important for information processing. Axons in the hippocampal pyramidal neurons may have hundreds of axonal branches to innervate multiple target cells. Furthermore, the axonal branch pattern of each neuron can change during development. As described in the next section, axonal pattern formation can be divided into several cellular processes.

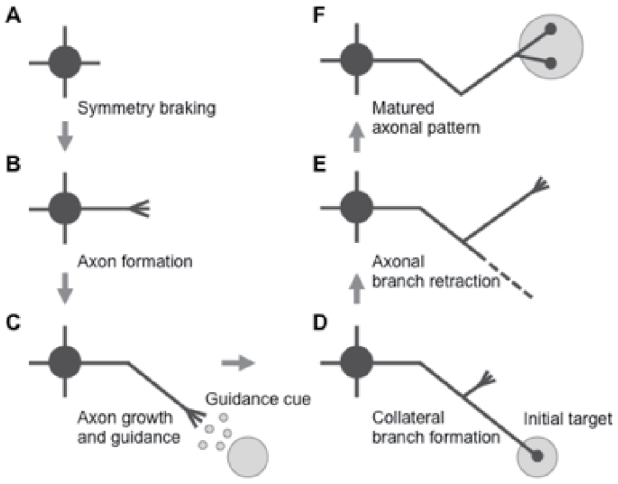

Fig. 1. The processes of axonal morphogenesis during neuronal wiring. There are several cellular processes observed in the developing nervous system that contribute to the establishment of complicated axonal morphology as follows: (A) Unpolarized immature neuron. (B) Formation and growth of the axon. (C) Axon guidance via cell extrinsic cues from target tissue. (D) Innervation of axon to the initial target, and formation of axonal collateral branches. (E) Axonal remodeling by branch retraction and extension. (F) Establishment of final axonal pattern.

2. Axonal Pattering during Development

At particular times during the development of the nervous system, each neuronal cell starts to extend processes (neurites) from the cell body. In many case, a single process continues to grow to become a long extended axonal process. This symmetry braking process is called "neuronal polarization" (Fig. 1) (Dotti *et al.*, 1988). The lengths of axons greatly differ between different types of neurons and are also affected by the cell-extrinsic signals from surrounding tissue. Importantly, surrounding tissues secrete axon guidance molecules that attract axons to or repulse axons from the targets. During development, gradients of axon guidance molecules are generated in the surrounding tissue, and these signals are received by the "growth cone" a specialized dynamic structure consisting of actin and microtubule cytoskeletons that exists at the tip of growing axon (Dickson, 2002). As mentioned above, many neurons extend a

doi:10.5047/forma.2014.008

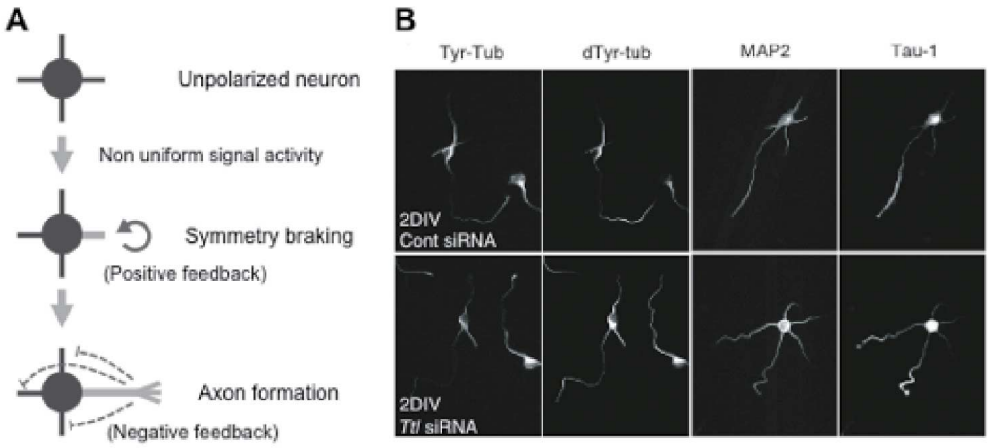

Fig. 2. Cell autonomous mechanisms of neuronal polarization. (A) Process of polarization. Soon after neurogenesis, neurons possess only short neurites, and axons have not been determined (top). Local activation of signaling molecules that determine the axonal fate occurs at a single process (indicated by light gray). To confirm axonal fate, positive feedback mechanisms are thought to exist. This process breaks cell symmetry (middle). The future axon continues to extend. It is thought that it sends inhibitory signals to other minor processes so that only a single neurite becomes the axon. (B) Microtubule dependent mechanisms involved in the neuronal polarization. Distribution of posttranslational modification of microtubules as shown by immunocytochemistry using antibodies against tyrosinated and detyrosinated tubulins. The bottom row shows the neurons in which a tyrosination enzyme, TTL, was inhibited by siRNAs. By inhibiting tyrosination on the microtubules, the neuronal polarization was disrupted (Konishi and Setou, 2009).

single axon from the cell body, but many types of neurons generate branches to connect with multiple synaptic targets. Axonal branches can be generated from either an extending growth cone (growth cone bifurcation) or a shaft of a pre-existing axon (collateral branch). Conversely, some branches are eliminated during development to establish the final axonal pattern. This remodeling process ensures the establishment of proper functional neuronal wiring. For example, layer 5 cortical neurons in different areas extend primary axons toward the spinal cord, followed by extending collateral axonal branches to several brain regions. Later, axons receive selective branch elimination depending on the functional specification of a cortical area, so that neurons in different cortical areas project to different brain regions (O'Leary and Koester, 1993). Another example of axon remodeling can be observed in motor neurons that innervate the muscles. In neonatal mice, motor neuron axons have already reached the muscles and are highly branched. Consequently, each muscle receives inputs from multiple axons. During the first and second postnatal weeks, in which the mice open their eyes and begin to move around, many axonal branches are retracted so that each muscle fiber receives signals from a single axon (Walsh and Lichtman, 2003). This axonal remodeling process is performed in a neuronal activity dependent manner.

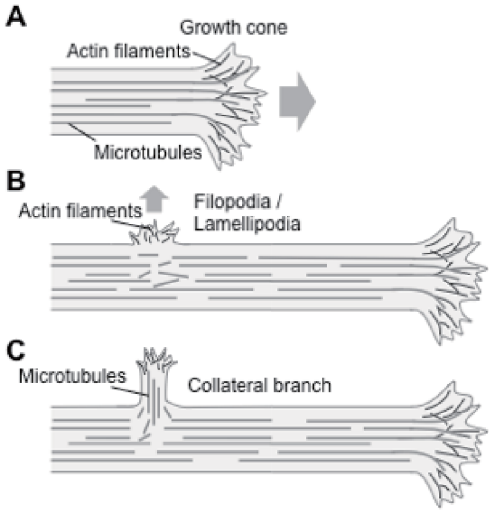

Fig. 3. The process of axonal collateral branch formation. (A) Growth of the initial axons (top). F-actin is enriched in the growth cone, whereas microtubules abundantly exist in the axonal shaft. (B) Accumulation of F-actin induces the formation of filopodia from the preexisting axons. (C) Microtubule innervation to the nascent axonal branch.

3. Mechanisms for the Establishment and Maintenance of Neuronal Polarity

The mechanisms by which neurons establish polarity have been intensively studied using primary cultured hippocampal neurons. Shortly after plating, dissociated hippocampal neurons exhibit a round shape (stage 1). Several hours later, they extend multiple short processes (stage 2), all with similar lengths. By two days after plating, one of the processes extends further, and becomes a morphologically and molecularly distinct process; i.e., axon (Dotti *et al.*, 1988). Axon specification is mediated by cell extrinsic cues and subsequent activation of an intracellular signaling cascades. The partition-defective proteins (PARs) and several kinases such as PI3K, GSK3, LKB1, and SAD as well as regulators of the actin and microtubule cytoskeleton are involved in this process (Shi *et al.*, 2003; Yoshimura *et al.*, 2005; Barnes *et al.*, 2007; Shelly *et al.*, 2007). The factors involved in these pathways are either localized in future axons or excluded from axons and regulate actin and microtubule organization. Intriguingly, even without cell extrinsic directional cues, one of the processes stochastically becomes the axon. It is thought that the future axon sends

both positive feedback to keep itself on the path to becoming an axon and negative signals that suppress other minor processes from becoming the axon (Fig. 2A) (Jacobson *et al.*, 2006).

To maintain axons, molecules required for axonal function need to be delivered to the axon. Kinesin-1 is a plus-end directed microtubule motor and plays a key role in anterograde axonal transport. The cleaved motor domain of Kinesin-1 is preferentially localized to the axon (Nakata and Hirokawa, 2003; Jacobson *et al.*, 2006). The molecular differences (acetylation, tyrosination and GTP/GDP bound state) of tubulins between axons and dendrites could act as the intracellular directional cues to navigate Kinesin-1 into axons (Verhey *et al.*, 2001; Konishi and Setou, 2009; Nakata *et al.*, 2011). For example, axons contain less tyrosinated tubulin compared with dendrites and soma, and when tubulin tyrosine ligase (Ttl) that mediates tyrosination is inhibited in immature hippocampal neurons, neuronal polarization is modulated (Fig. 2B) (Konishi and Setou, 2009). Thus, microtubule mediated polarized transport plays an important role in establishment of polarized neuronal shape. In addition to the microtubule dependent mechanisms, the structure at the proximal axon, called the axonal initial segment (AIS), plays a crucial role in polarized transport. The AIS contains characteristic intracellular structures consisting of F-actin and ankyrin-G that act as diffusion barriers and prevent dendritic molecules entering the axon by an actomyosin-dependent mechanism (Arnold, 2009). The process of neuronal polarization may differ between neuronal subtypes. Indeed, our observations suggest that the contribution of GSK3 in polarization is different between hippocampal pyramidal neurons and cerebellar granule neurons (Kubota *et al.*, 2013). Further studies are required to address this issue.

4. Mechanisms of Axon Branching

Collateral branch formation is a major mode of axonal branching and can be observed both *in vivo* and *in vitro*. The initial step of collateral formation is accumulation of F-actin at the branching site. This patch of F-actin serves as a precursor for the filopodium or lamellipodium that is newly emerging from the axonal shaft (Gallo, 2011). Variable molecules are known to be involved in the proper formation and stabilization of F-actin. Regulators for F-actin possibly play roles in axon branching. Indeed, Arp2/3 and Contactin enhance polymerization of actin and increase the axonal branch number (Strasser *et al.*, 2004). After the emergence of protrusions, they receive innervation of microtubules to become mature axonal branches. Microtubule innervation into immature protrusions can be achieved either by transport of microtubule fragments or capture of the growing microtubule end. Although the localized mechanisms that determine whether microtubules enter the protrusion or not are poorly understood, this step enables delivery of molecules required for further branch elongation and stabilization via the microtubule-dependent axonal transport system.

Kif2a is a member of kinesin family and acts to depolymerize the microtubules. Primary culture neurons prepared from Kif2a deficient mice exhibit increased axonal branch length and lower branch retraction ratio (Homma *et al.*, 2003). Intriguingly, destabilization of microtubules by itself can also enhance axonal branching. Spastin and Katanin can sever microtubules. When these molecules are expressed in neurons, they increase the number of axonal branches (Yu *et al.*, 2008). From these observations, it is assumed that microtubules at the primary axon have to be destabilized for collateral axonal branches to emerge, whereas in the newly generated axonal branch, microtubules have to be stabilized. Thus, localized regulation of microtubule dynamics is important in establishing axonal branch morphology.

Various signaling molecules that act upstream of the actin and microtubule cytoskeleton have been reported to have roles on the axonal branching. However the intracellular mechanisms controlling region specific cytoskeletal dynamics are poorly understood. Intracellular calcium level is transiently increased by neuronal activity and some extrinsic signals, and is reported to be involved in axonal growth, guidance and branching. Interestingly, it is reported that there is spontaneous and signal-dependent calcium transient in axons that occur in a particular branch region and contribute to the growth of the branch (Hutchins and Kalil, 2008). The downstream target molecules including calcium calmodulin dependent kinase II (CAMKII), calpain, and Protein kinase C (PKC) possibly mediate calcium signals to regulate axonal structure.

5. Conclusion

As described above, multiple signals and molecules that play roles in axonal morphogenesis have been reported. However, the systems by which the cytoskeleton is locally altered in each neuron in a particular region to coordinate the axonal pattern remains largely unknown. In addition to the cytoskeleton dependent structural regulation, other cellular functions, not described in this paper, including energy production via mitochondria, and regulation of the plasma membrane via exocytosis and endocytosis may also be critical for axonal patterning. It is important to understand the systems by which these multiple events are locally coordinated to regulate axonal structure in a region specific manner.

Acknowledgments. Y.K. is supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Nanomedicine Molecular Science" (No. 2306) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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