

extracellular and intracellular signaling molecules, and their effectors, on neuronal polarization *in vivo*, using electroporation, and knockout and knock-in mice. It has become clear that both extracellular signaling and intrinsic mechanisms are responsible for the establishment and maintenance of neuronal polarity. Here and in the accompanying poster, we summarize the current understanding of how neuronal polarization generates functional neurons, focusing on the mammalian cerebral cortex and cultured hippocampal neurons as the primary model systems, and discuss the future challenges faced by this field.

Overview of neuronal polarization *in vitro* and *in vivo*

Banker and colleagues established dissociated rodent hippocampal neurons as a basic model system for neuronal polarity (Dotti et al., 1988; Craig and Banker, 1994). The morphological changes of cultured neurons are divided into five stages. Upon isolation, hippocampal neurons retract their processes, so that their development *in vitro* begins with round spheres that spread filopodia (stage 1; shortly after plating). These neurons subsequently form several minor neurites (stage 2; days 0.5–1.5), which show characteristic alternations of growth and retraction. The major polarity event occurs when one of these equivalent minor neurites grows rapidly to become the axon (stage 3; days 1.5–3). The next steps are the morphological development of the remaining short minor neurites into dendrites (stage 4; days 4–7) and the functional polarization of axons and dendrites, including dendritic spine formation (stage 5; >7 days in culture).

In contrast to cultured neurons, the polarization processes of neurons *in vivo* have different properties depending on brain region and developmental stage. For example, vertebrate retinal ganglion cells and retinal bipolar cells inherit their polarity (Barnes and Polleux, 2009). When born, they possess a neuroepithelium-like morphology, with apical and basal processes that eventually develop into a dendrite and an axon, respectively (Barnes and Polleux, 2009). By contrast, cortical and hippocampal pyramidal neurons, and cerebellar granule neurons establish their polarity during differentiation (Noctor et al., 2004; Solecki et al., 2006; Funahashi et al., 2014). Cortical pyramidal neurons are generated in the ventricular zone (VZ) and migrate through the subventricular zone towards the intermediate zone (IZ) (Miyata et al., 2004; Noctor et al., 2004). They extend multiple minor neurites and are called multipolar (MP) cells (Miyata et al., 2004; Noctor et al., 2004). One of the minor neurites grows rapidly to become a trailing process, and another develops into a leading process, which finally develop into an axon and a dendrite, respectively (Miyata et al., 2004; Noctor et al., 2004). The remaining minor neurites are retracted and MP cells subsequently transform into bipolar (BP) cells in the IZ. BP cells are completely polarized and migrate towards the cortical plate (CP). Although neuronal polarization can occur in parallel with neuronal migration, how these two processes are coordinated remains elusive. Polarization of neurons in the cerebral cortex serves as a well-studied model for polarity establishment *in vivo* (Funahashi et al., 2014), and the processes regulating it are discussed in detail below.

Axon versus dendrite initiation *in vivo*

The MP-to-BP transition is a crucial step during neuronal polarization *in vivo*. Time-lapse imaging of cortical slice cultures has revealed variability in the establishment of neuronal polarization in the developing neocortex. Almost 60% of MP cells first extend the trailing process (future axon) and then generate a leading process (future dendrite), whereas ~30% of MP cells first generate a

leading process and then extend the trailing process (Hatanaka and Yamauchi, 2013; Namba et al., 2014). The remaining 10% of MP cells form both processes simultaneously (Namba et al., 2014). We have recently proposed a novel model of axon initiation *in vivo* called the ‘Touch & Go’ model (Namba et al., 2014; Funahashi et al., 2014). According to this model, once a minor neurite of a MP cell ‘touches’ the pioneering axons of early born neurons, it extends rapidly (‘goes’) and develops into an axon. The cell-adhesion molecule transient axonal glycoprotein 1 (TAG1; CNTN2 – Mouse Genome Informatics) is involved in this process through Src family kinase Lyn-induced Rac1 activation. Thus, the TAG1-Lyn-Rac1 signal plays a role in axon specification through cell-to-cell interactions in the developing cortex (Namba et al., 2014).

However, the molecular mechanisms underlying the formation of a leading process in 30% of MP cells are largely unknown. Several reports suggest that the radial glial cell-neuron interaction is involved not only in neuronal migration but also in the formation of a leading process (Kawauchi et al., 2010; Jossin and Cooper, 2011; Gärtner et al., 2012). N-cadherin has been shown to regulate radial glial-guided neuronal migration (Kawauchi et al., 2010). In the developing cortex, the knockdown or expression of a dominant-negative mutant form of N-cadherin disrupts neuronal migration and the MP-to-BP transition (Kawauchi et al., 2010; Jossin and Cooper, 2011). Moreover, inhibition of N-cadherin by a dominant-negative mutant exhibits abnormal morphology of the leading process, thereby impairing neuronal polarization (Gärtner et al., 2012). Based on these results, the N-cadherin-mediated glial cell-neuron interaction may regulate the MP-to-BP transition, particularly formation of the leading process.

Signaling pathways involved in neuronal polarization

Neuronal polarization is precisely regulated by environmental cues in the extracellular matrix such as TAG1 and secreted factors such as neurotrophins [brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3)], transforming growth factor β (TGF β), Wnt5A, insulin-like growth factor 1 (IGF1) and semaphorin 3A (Funahashi et al., 2014). These secreted factors regulate axon specification in cultured neurons (Nakamuta et al., 2011). In the developing cortex, TGF β and neurotrophins have been shown to be involved in neuronal polarization (Yi et al., 2010; Cheng et al., 2011; Nakamuta et al., 2011).

Type II TGF β receptor (T β R2; Tgfr2 – Mouse Genome Informatics) conditional knockout mice are defective in axon formation *in vivo* (Yi et al., 2010). T β R2 phosphorylates partitioning-defective 6 (Par6) at Ser345, contributing to axon formation (Yi et al., 2010). Par6 forms a protein complex with Par3 and atypical protein kinase C (aPKC), leading to axon formation through Rac1 activation (Nishimura et al., 2005; Arimura and Kaibuchi, 2007). The expression pattern of TGF β in the developing cortex is graded, being highest in the VZ, and is thought to reflect the direction of axon initiation (Yi et al., 2010). However, time-lapse imaging of cortical slice cultures shows that MP cells extend their trailing process in any direction and then migrate towards the CP, leaving behind the trailing process and resulting in axon extension towards the VZ (Namba et al., 2014). Therefore, the gradient of secreted factors may be involved in the directional migration and axon elongation, rather than in axon specification *in vivo*.

Neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) are major regulators underlying neuronal polarization *in vivo* (Cheng et al., 2011; Nakamuta et al., 2011). Amplification of BDNF levels in an autocrine or paracrine manner

induces axon specification in cultured neurons (Cheng et al., 2011), whereas inhibition of the neurotrophin receptors TrkB and TrkC (Ntrk2 and Ntrk3) by the expression of a dominant-negative mutant or by knockdown disrupts the MP-to-BP transition (Nakamuta et al., 2011). More recent results show that local exposure to BDNF causes the accumulation of p75^{NTR} (Ngfr – Mouse Genome Informatics) a receptor able to bind all of the neurotrophins, at a minor neurite, thereby leading to axon specification and elongation. Furthermore, knockout or knockdown of p75^{NTR} disrupts axon formation *in vivo* (Zuccaro et al., 2014). These findings suggest that neurotrophin signaling has a vital role in neuronal polarization.

Intracellular signaling in axon specification

The neurotrophic signals that regulate neuronal polarization are transduced through at least four different pathways: the Rac1 activation pathway, the Ras-mediated-pathway, the cyclic adenosine 3',5'-monophosphate (cAMP)-liver kinase B1 (LKB1; Stk11 – Mouse Genome Informatics) pathway and the Ca²⁺/calmodulin-dependent protein kinase kinase (CaMKK; Camkk1 – Mouse Genome Informatics)-calmodulin-dependent protein kinase I (CaMKI; Camk1 – Mouse Genome Informatics) pathway (Tahirovic and Bradke, 2009; Cheng and Poo, 2012; Funahashi et al., 2014). TrkB phosphorylates and activates Tiam1, a Rac1 guanine exchange factor (GEF; positive regulators of Rho family proteins), leading to axon specification through Rac1 activation *in vitro* (Miyamoto et al., 2006).

Ras is known to regulate axon specification in cultured neurons (Arimura and Kaibuchi, 2007). Activation of PI3 kinase by Ras produces phosphatidylinositol 3,4,5-trisphosphate (PIP₃). PIP₃ indirectly activates Akt, thereby inactivating glycogen synthase kinase 3 β (GSK3 β) through its phosphorylation at Ser9. The inactivation of GSK3 β leads to axon specification through the activation of microtubule-associated proteins (MAPs) such as Tau (Mapt – Mouse Genome Informatics) and collapsin response mediator protein 2 (CRMP2; Dpysl2 – Mouse Genome Informatics) (Jiang et al., 2005; Yoshimura et al., 2005). CRMP2 governs a wide variety of cellular events, such as microtubule assembly, the endocytosis of adhesion molecules through Numb, the reorganization of actin filaments through the Sra1/WAVE1 complex and the trafficking of TrkB-containing vesicles during axon formation through interaction with the Slp1/Rab27/kinesin complex (Kawano et al., 2005; Arimura et al., 2009). In culture, expression of CRMP2 induces the formation of multiple axons, whereas the inhibition of CRMP2 impairs axon formation (Inagaki et al., 2001). In the developing cortex, expression of a dominant-negative mutant form or knockdown of CRMP2 impairs neuronal migration and MP-to-BP transition (Sun et al., 2010).

BDNF-induced elevation of cAMP leads to axon specification via protein kinase A (PKA)-dependent phosphorylation of the serine/threonine kinase LKB1 (Guo and Kemphues, 1995). Expression of LKB1 induces the formation of multiple axons, whereas knockdown of LKB1 prevents axon specification *in vitro* (Barnes et al., 2007; Shelly et al., 2007). Moreover, *Lkb1*-knockout mice also exhibit impairment of axon formation, while neuronal migration in the cerebral cortex is unaffected (Barnes et al., 2007). Phosphorylation of LKB1 at Ser431 by PKA induces its local activation in the nascent axon (Shelly et al., 2007). LKB1 then phosphorylates SADA/B kinases (Brsk2/Brsk1 – Mouse Genome Informatics) and microtubule affinity-regulating kinase 2 (MARK2) (Lizcano et al., 2004; Barnes et al., 2007). SADA/B kinase and MARK2 regulate microtubule dynamics through the phosphorylation of Tau. *Brsk1/Brsk2* double knockout neurons have a mixed axon/dendrite identity (Kishi et al.,

2005; Barnes et al., 2007), and the developing cortex of *Brsk1/Brsk2* double knockout mice shows a loss of axons and abnormally orientated dendrites (Kishi et al., 2005). In addition, the downregulation of MARK2 by shRNA also impairs the MP-to-BP transition (Sapir et al., 2008). The cAMP-dependent phosphorylation of LKB1 is also involved in GABA_B receptor-induced axon/dendrite outgrowth and neuronal migration *in vivo* (Bony et al., 2013). However, LKB1^{S431A/S431A} knock-in mice exhibit no overt phenotype and unchanged SADA/B activity (Houde et al., 2014).

NT3 stimulates inositol-1,4,5-trisphosphate (IP₃)-induced Ca²⁺ release and thereby leads to the activation of the CaMKK-CaMKI pathway during neuronal polarization in culture (Nakamuta et al., 2011). A rapid increase in Ca²⁺ level induced by NT3 in an IP₃-dependent manner leads to the local activation of CaMKK at the tip of nascent axons (Nakamuta et al., 2011). The inhibition of CaMKK attenuates NT3-induced axon specification in cultured hippocampal neurons (Nakamuta et al., 2011). *In vivo*, developing cortical neurons expressing a dominant-negative form of CaMKK show impaired axon specification though neuronal migration is unaltered (Nakamuta et al., 2011). CaMKI, which acts downstream of CaMKK, has been shown to regulate axon specification and axon elongation (Wayman et al., 2004; Uboha et al., 2007). Together, these four pathways, perhaps along with others, play crucial roles in defining the future axon, and thus in determining neuronal polarity.

Rho family proteins in neuronal polarity

Members of the Rho family of small GTPases, such as Rac1, Cdc42 and RhoA, are major regulators of cytoskeletal dynamics (Hall et al., 1993; Fukata et al., 2003). Rac1 plays a vital role in axon specification through regulation of actin polymerization. The expression of either the constitutively active or dominant-negative form of Rac1 produces a delay in neuronal migration and a loss of leading and trailing processes (Kawauchi et al., 2003). Moreover, the expression of Rac-specific GEFs, such as STEF/Tiam1 or P-Rex1, also inhibits neuronal migration, suggesting that a balance of Rac1 activity is required for MP-to-BP transition and migration *in vivo* (Kawauchi et al., 2003). Conditional Rac1-deficient cerebellar granule neurons exhibit impaired neuronal migration and axon formation due to impaired localization of the actin regulator WAVE at the plasma membrane of the growth cone (Tahirovic et al., 2010).

The small GTPase Cdc42 is a key regulator of multiple aspects of neuronal development, including filopodial extension in growth cones (Schwamborn and Puschel, 2004; Arimura and Kaibuchi, 2007; Witte and Bradke, 2008). Conditional knockout of Cdc42 in the cortex and hippocampus leads to reduced embryonic cortex size and results in lethality at birth with impairment in axon formation (Garvalov et al., 2007). PI3 kinase regulates activation of Cdc42, and activated Cdc42 interacts with the Par complex (Nishimura et al., 2005). This interaction leads to Rac1 activation through Rac-specific GEFs (STEF/Tiam2 and Tiam1) and Rac1 then further activates PI3 kinase (Nishimura et al., 2005). Therefore, the PI3 kinase/Cdc42/Par complex/Rac1 pathway appears to represent a positive-feedback loop that functions as a driving force for axon formation (Arimura and Kaibuchi, 2007).

The small GTPase RhoA is another polarity regulator and modulates actin cytoskeleton and myosin-based contractility. Several lines of evidence suggest that RhoA is a negative regulator of neuritogenesis, including axon specification (Da Silva et al., 2003; Conde et al., 2010). A constitutively active form of RhoA inhibits the growth of minor neurite processes in hippocampal neurons (Threadgill et al., 1997; Conde et al., 2010),

whereas the inactivation of RhoA enhances neurite extension (Da Silva et al., 2003; Schwamborn and Puschel, 2004). Conditional knockout of RhoA in the midbrain results in the disruption of apical adherens junctions, hyper-proliferation of neural progenitors and massive dysplasia of the brain (Katayama et al., 2011). However, the role of RhoA in neuronal polarization *in vivo* remains to be elucidated. Inhibition of Rho kinase (ROCK), a downstream effector protein of RhoA, also enhances axonal elongation and induces the formation of multiple axons (Da Silva et al., 2003). Rho kinase phosphorylates and activates LIM domain-containing protein kinase (LIMK), which induces the inactivation of cofilin (Aizawa et al., 2001). The Rho GTPase Rnd2 regulates the MP-to-BP transition *in vivo* through the suppression of RhoA (Pacary et al., 2011). Therefore, the activity of RhoA is important for the establishment of neuronal polarity. Interestingly, RhoA activity is higher in the growth cones of minor neurites of polarized neurons than in the growing axon (Gonzalez-Billault et al., 2012). Rho kinase phosphorylates and inactivates p190 RhoGAP, a member of GTPase-activating proteins (GAPs; negative regulators of Rho family proteins), leading to sustained RhoA activation (Mori et al., 2009). Thus, the continuous activation of RhoA/Rho kinase might be required not only for axon specification but also for the maintenance of neuronal polarity by preventing the formation of multiple axons.

Cytoskeletal organization

Neuronal polarization is driven by cytoskeletal organization, primarily through microtubule and actin dynamics (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009). The stabilization of microtubules is crucial for the axon specification *in vitro* (Bradke and Dotti, 1999; Witte and Bradke, 2008). The stabilization of microtubules is regulated by MAPs such as Tau and CRMP2. Tau protects microtubules from the microtubule-serving proteins (Witte and Bradke, 2008). Phosphorylation of Tau by GSK3 β suppresses its ability to bind microtubules and thereby inhibits the stabilization of microtubules (Wagner et al., 1996; Kimura et al., 2014). Phosphorylation of CRMP2 by GSK3 β also inhibits its affinity to $\alpha\beta$ -tubulin heterodimers (Yoshimura et al., 2005).

In contrast to microtubules, actin filaments are more unstable and dynamic in the growing axon than the growth cones of minor neurites *in vitro* (Bradke and Dotti, 1999; Witte and Bradke, 2008). The destabilization of actin filaments by severing proteins such as cofilin allows microtubules to penetrate into the growth cone, thereby leading to axon specification (Bradke and Dotti, 1999; Flynn et al., 2012). Conversely, myosin II and profilin IIa stabilize actin filaments at the minor neurites to prevent the formation of multiple axons through interfering with the penetration of microtubules (Kollins et al., 2009; Da Silva et al., 2003). Shootin 1 regulates axon outgrowth through actin dynamics in the growing axon in a p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1)-dependent manner (Toriyama et al., 2013). Thus, the coordinated regulation of microtubules and actin filaments plays a crucial role in neuronal polarization.

Local activation and global inhibition for neuronal polarization

The involvement of ‘local activation and global inhibition’ models for establishment of neuronal polarity has been extensively proposed (Arimura and Kaibuchi, 2007; Inagaki et al., 2011). ‘Local activation’ is thought to induce axon specification and promote axon elongation, whereas ‘global inhibition’ is thought to prevent the formation of multiple axons, thereby determining

dendrite specification and maintaining polarity. Several molecules, such as Rac1, PI3 kinase, PIP₃, Cdc42, Par complex and Rac-specific GEFs (STEF/Tiam), act as positive regulators of axon specification (Arimura and Kaibuchi, 2007). These positive regulators are continuously activated within one minor neurite and thereby induce axon initiation via multiple downstream routes that impinge on cytoskeletal and intracellular trafficking dynamics (Arimura and Kaibuchi, 2007; Takano et al., 2012).

However, the molecular mechanism of global inhibition remains a mystery. One of the major models proposed is long-range negative-feedback signaling, which is propagated from axon terminal to cell body and/or all minor neurites and thereby inhibits minor neurite outgrowth (Arimura and Kaibuchi, 2007). The continuous activation of RhoA/Rho kinase appears to be involved in this process. Rho kinase phosphorylates Par3 and in turn disrupts the Par complex, thereby abrogating Rac1 activation (Nakayama et al., 2008). Rho kinase also suppresses the STEF-induced Rac1 activation (Takefuji et al., 2007). Thus, RhoA/Rho kinase may serve as a local negative-feedback signal in the cell body and/or all minor neurites, through repression of the positive-feedback loop. However, the long-range negative-feedback signaling from the axon that leads to RhoA/Rho kinase activation remains largely unknown. Local elevation of cAMP in a neurite of the unpolarized neuron also might generate a long-range negative-feedback signaling that results in the reduction of cAMP in all other neurites (Shelly et al., 2010). Recently, a global inhibitory regulatory mechanism has been proposed: because growth-promoting factors are limited, a local accumulation of these factors in the nascent axon could deplete them in other minor neurites without a long-range negative-feedback signal (Inagaki et al., 2011).

According to these paradigms, dendrite specification could be a default pathway in the absence of axon specification in cultured neurons. In the developing cortex, however, one of the minor neurites of a MP cell develops into the leading process. Additional dendrite-specific processes caused by environmental cues might be required for neuronal polarization *in vivo* as described above. The elucidation of the molecular mechanisms inducing global inhibition to determine dendrite specification is a crucial issue in neuronal polarity research.

Conclusion

The establishment of neuronal polarization is essential for establishing proper neuronal circuits and functions. A large number of studies both *in vitro* and *in vivo* have uncovered a complicated signaling network regulating neuronal polarity (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; Tahirovic and Bradke, 2009). However, despite intensive study, it is still unclear how neurons generate only one axon and multiple dendrites. In particular, the molecular mechanisms of global inhibition underlying the maintenance of neuronal polarity remain elusive, and there may be unknown molecular machinery functioning to prevent the formation of multiple axons and in turn to induce dendritic outgrowth. One reason for this major gap in our knowledge is the lack of suitable methodologies for investigating the spatiotemporal regulation of the signaling molecules responsible for negative-feedback signaling. Future challenges will entail exploring these issues using advances in imaging technology, genetic model systems and innovative experimental approaches.

Despite our incomplete understanding, the molecular mechanisms identified thus far seem to be widely used and evolutionarily conserved (Solecki et al., 2006; Doe and Kaibuchi,

2011). Therefore, our understanding of the molecular mechanisms leading to neuronal polarization is likely to provide new insights into the development of brain circuitry.

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Competing interests

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Development at a Glance

A high-resolution version of the poster is available for downloading in the online version of this article at <http://dev.biologists.org/content/142/12/2088/F1.poster.jpg>

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