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Establishment of Axon-Dendrite Polarity in Developing Neurons

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Abstract

Neurons are among the most highly polarized cell type in the body and the polarization of axon and dendrites underlies the ability of neurons to integrate and transmit information in the brain. Significant progress has been made in the identification of the cellular and molecular mechanisms underlying the establishment of neuronal polarity using primarily *in vitro* approaches such as dissociated culture of rodent hippocampal and cortical neurons. This model has led to the predominant view suggesting that neuronal polarization is largely specified by stochastic, asymmetric activation of intracellular signaling pathways. Recent evidence shows that extracellular cues can play an instructive role during neuronal polarization *in vitro* and *in vivo*. In this review, we synthesize the recent data supporting an integrative model whereby extracellular cues orchestrate the intracellular signaling underlying the initial break of neuronal symmetry leading to axon-dendrite polarization.

Keywords

neuronal migration; cortex; signaling; LKB1; Par complex

INTRODUCTION

Cell polarity lies at the center of many biological processes including epithelial morphogenesis, cell migration and chemotaxis. Its disruption is thought to underlie several pathological states including cell transformation and metastasis. Neurons are among the most polarized cell types in our body and are compartmentalized into two molecularly and functionally distinct domains: the axon and the dendrites. Neurons typically form a single axon and multiple dendrites which underlie the flow of information transfer in the central nervous system. Dendrites integrate synaptic inputs triggering the generation of axon potentials at the level of the soma and propagates along the axon making presynaptic contacts onto the dendrite of target neurons. How are the axonal and dendritic compartments generated during development? This question has received a lot of attention both at the cellular and molecular levels over the past three decades. A seminal review published by Craig and Banker fifteen years ago in this journal (Craig & Banker 1994) observed that “we [knew] almost nothing about the cellular mechanisms responsible for the compartmentation in neurons”. Based on existing data, Craig and Banker provided a conceptual framework for the experiments that, over the past decade, have improved our understanding on how neuronal polarity is established mostly using *in vitro* approaches. The current review will provide an updated model synthesizing a recent body of work suggesting that *in vivo*, neuronal polarity is most probably a result of a complex interaction between extracellular cues directing intrinsic cell polarity pathways.

EMERGENCE OF NEURONAL POLARITY *IN VIVO*

Neuronal polarization can be divided in several specific steps: upon cell cycle exit, mammalian neurons usually migrate over a long distance before reaching their final destination. *In vivo*, most neurons undergo axon-dendrite polarization during migration. While migrating, post-mitotic neurons form a leading process and a trailing process which become the axon or the dendrite depending on the cell type (**Figure 1**). Careful examination of the morphological transition between neural progenitors and post-mitotic neurons reveal that neurons can inherit their axon and dendrite polarity directly from the apico-basal polarity of their progenitors. This is the case for retinal ganglion cells and bipolar cells in the developing vertebrate retina (**Figure 1A-B**) (Hinds & Hinds 1978, Morgan et al 2006, Zolessi et al 2006). On the other hand, other neurons undergo extensive stereotypical changes of their morphology leading to polarized outgrowth of their axon and dendrite (**Figure 1C-D**). This is the case for cerebellar granule neurons (CGN) as well as cortical and hippocampal pyramidal neurons (PN), three of the best-studied models of neuronal polarization both *in vitro* and *in vivo* (Gao & Hatten 1993, Hatanaka & Murakami 2002, Komuro et al 2001, Noctor et al 2004, Rakic 1971, Rakic 1972, Shoukimas & Hinds 1978). Importantly, both CGN and PN acquire their axon-dendrite polarity from the polarized emergence of the trailing-leading processes during migration. Therefore, in these two neuronal cell types, there is an important functional relationship between the molecular mechanisms underlying polarized migration and the final axon-dendrite polarity.

COMPARISON OF NEURONAL POLARIZATION *IN VITRO* AND *IN VIVO*

Historically, the advent of *in vitro* dissociated neuronal cultures provided an experimental template for improving our understanding of the cell biology of neuronal polarity. Pioneering work using these cultures established a paradigm where isolated neurons in culture can adopt spatially and functionally distinct dendritic and axonal domains (Craig & Banker 1994, Goslin & Banker 1989). Careful analysis of these cultures led to the observation that cultured hippocampal neurons transition through several stages, from freshly plated stage 1 cells bearing immature neurites to stage 5 cells that exhibit mature axons, dendrites, dendritic spines, and functional synapses (Craig & Banker 1994, Dotti et al 1988) (**Figure 2**). However, it should be noted that in the classical E18 rat hippocampal cultures, most cells plated are post-mitotic neurons upon dissociation and therefore, neuronal polarization using this *in vitro* model corresponds to the 're-polarization' of previously polarized neurons *in vivo*. It is therefore important to keep in mind that molecular manipulations in this *in vitro* model act on previously polarized neurons which can be critical for the interpretation of the results. Recent advances in the techniques allowing the manipulation of gene expression specifically in neural progenitors such as *in utero* or *ex utero* cortical electroporation (Hand et al 2005, Hatanaka & Murakami 2002, Saito & Nakatsuji 2001, Tabata & Nakajima 2001) provide a paradigm to (i) manipulate gene expression in progenitors i.e. before neuronal polarization occurs upon cell cycle exit and (ii) visualize the earliest stages of neuronal polarization in a contextual cellular environment i.e. in organotypic slices or intact embryonic brain (Barnes et al 2007, Calderon de Anda et al 2008, Hand et al 2005).

Most studies reviewed in this article were primarily performed using *in vitro* approaches. The classic paradigm to identify a gene regulating neuronal polarity is to show that down-regulation of its expression using shRNA technology or gene knockout technology is required for axon formation using both staining with axon specific markers and measurement of neurite length since the axon usually grows 5-10 times faster than neurites becoming dendrites. However, this type of evidence is usually not sufficient to distinguish unambiguously an effector of neuronal polarity from a molecule simply regulating axon

growth (Jiang & Rao 2005). Conversely showing that over-expression or over-activation of a candidate molecule leads to the emergence of multiple axons is usually used to suggest that this molecule is sufficient to confer axon identity to immature neurites. However, this approach is limited by the fact that it relies on an over-expression phenotype which could be hampered by ectopic activation of a pathway normally not involved in axon specification or neuronal polarity. Ultimately, validation of the requirement of this signaling molecule *in vivo* for neuronal polarity using gene knockout technology should be the gold standard in the field but rarely is so far.

SIGNALING MECHANISMS UNDERLYING ESTABLISHMENT OF AXON-DENDRITE POLARITY

PI3-Kinase and potential effectors

The lipid kinase phosphatidylinositol 3-kinase (PI3K) lies downstream of Ras during signal transduction, and acts to generate localized sites of the membrane enriched for phosphatidylinositol (3,4,5)-triphosphate (PIP3). Work from several groups has implicated PI3K in axon specification based on the fact that pharmacologic inhibition of PI3K activity using LY294002 or Wortmannin prevents axon formation (Jiang et al 2005, Menager et al 2004, Shi et al 2003, Yoshimura et al 2006). Conversely, over-expression of the constitutively-active catalytic subunit of PI3K (p110 α) leads to the formation of multiple axons (Yoshimura et al 2006) suggesting that PI3K activation is both required and sufficient for axon specification. Interestingly, using the pleckstrin homology (PH) domain of AKT fused to GFP (PH_{AKT}-GFP) as a biosensor for PIP3 formation, Menager et al. have shown that PIP3 accumulates selectively within a single neurite following local application of laminin in a single neurite of stage 2 hippocampal neurons (Menager et al 2004). Future investigations will need to address when and where PI3K activation occurs *in vivo* during neuronal polarization.

Interestingly, two PI3K-interacting proteins, Shootin1 (Toriyama et al 2006) and Singar1/2 (Mori et al 2007), were recently identified as potential regulators of axon formation using a mass-spectrometry approach. Over-expression of Shootin1 leads to the generation of supernumerary axons, and RNAi knockdown inhibits axon formation. Shootin1 is transported via a myosin-dependent mechanism to axonal growth cones and its over-expression leads to aberrant accumulation of PI3K in minor neurites, likely leading to the observed alteration of axon specification. Shootin1 has been shown to colocalize with active pools of PI3K and inhibition of PI3K activity significantly reduces the ability of Shootin1 to induce multiple axons. These data suggest a role for Shootin1 in regulating PI3K activity, and its selective transport to the nascent axon appears critical for establishing axonal identity. Singar exists as at least two splice forms, Singar1 and Singar2 and both are expressed in developing neurons. RNAi against both forms causes cultured neurons to form multiple axons and this effect is prevented when PI3K activity is inhibited. Surprisingly, over-expression does not affect axon formation, unlike the case for Shootin1. When co-expressed, Singar1, but not Singar2, is able to reduce the multiple axon phenotype of Shootin1 over-expression. This result suggests an antagonistic relationship between Shootin1 and Singar 1 and that Singar proteins may inhibit PI3K activity. Both Singar proteins contain a RUN domain, a motif known to be a site of interaction with small GTPases including Rap2. Singar has been shown to interact with Rap2 and not with the closely related Rap1 (Kukimoto-Niino et al 2006). Work from the immune system indicates that Rap2 can inhibit PI3K activity (Christian et al 2003). However, the exact mechanism by which either Shootin1 or Singar1/2 exert their effects on PI3K awaits further elucidation. An additional source of PIP3 may come in the form of PIP3 containing vesicles transported to the forming axon. Guanylate kinase associated kinesin (GAKIN) has been shown to be

important for neuronal polarity through its ability to bind to PIP3-binding protein (Horiguchi et al 2006). In studies using over-expression of wild-type and dominant negative (motor domain deleted) forms of GAKIN, the observed effect was similar to that described for other proteins that modulate PIP3 levels (Horiguchi et al 2006). This result suggests that, in addition to locally derived PIP3, a transported pool may be required for the maintenance of axonal identity.

PTEN

Phosphatase and Tensin Homologue Deleted on Chromosome 10 (PTEN) is a lipid and protein phosphatase that acts in direct opposition to PI3K activity since PTEN dephosphorylates PIP3 into PIP2 and thus serves to limit both spatially and temporally PIP3 signaling. Increasing levels of PTEN expression lead to a loss of axon formation (Jiang et al 2005, Shi et al 2003) whereas reduction of PTEN expression via RNAi-mediated knockdown leads to a multiple axon phenotype (Jiang et al 2005). This effect is consistent with the gain-function mutation of PI3K described above and highlights the critical need to maintain the delicate balance of phospholipid composition at the membrane to insure proper neuronal polarization and axon formation.

The identification of a complex between PTEN and the PAR3/6 polarity complex (see corresponding section below) represents an important convergence between two previously independent pathways (Feng et al 2008). As is true for other signaling cascades, the close proximity of positive (PAR3/6) and negative (PTEN) regulators might allow strict spatial and temporal control of the activation and de-activation of a given pathway.

AKT/Protein kinase B

Several proteins are recruited via PIP3-specific PH domains to the site of PIP3-enriched membrane created by PI3K activity. The protein kinase AKT also called protein kinase B (PKB) undergoes such a translocation to the membrane via its PH-domain, a step required for its dual phosphorylation on T308 and S473 and activation by membrane-targeted protein kinase PKD1 and PKD2 respectively. This activated form of AKT has been shown to be enriched in growth cones of polarized neurons (Shi et al 2003). When a myristoylation site is added to recombinant AKT (myr-AKT), it is constitutively targeted to the membrane, independently of PI3K, and therefore acts as a constitutively active form. When over-expressed in neurons, this form is sufficient to multiple axon formation (Yoshimura et al 2006) consistent with a unified pathway where AKT act downstream of PI3K in regulating axon formation. In addition to PKD, another PI3K regulated kinase, Integrin Linked Kinase (ILK) has been shown to increase AKT activity via S473 phosphorylation (Delcommenne et al 1998). Similar to other regulators of AKT, hyperactivated ILK (S343D) increases axon formation in cultured neurons and RNAi reduction or pharmacologic inhibition leads to failure of axon formation without affecting the adoption of a dendritic fate (Guo et al 2007). These experiments point to an important but not exclusive role of ILK in regulating AKT. A common target protein of both ILK and AKT is Glycogen Synthase Kinase β (GSK3 β) (Delcommenne et al 1998), and phosphorylation by either proteins is capable of inactivating GSK3 kinase activity. In addition, it was recently shown that the small GTPase R-Ras acts upstream to regulate this ILK-GSK pathway (Oinuma et al 2007). The results of several groups point to GSK3 β as the most important target of these proteins in the neuronal polarity cascade. In fact, the co-transfection of a non-phosphorylatable form of GSK3 β (S9A) with ILK-S343D (constitutively active) or a CAAX-box containing subunit of PI3K (constitutively active) and ILK-S343A (dominant negative) is sufficient to at least partially suppress the generation of multiple axons while Myr-AKT is unaffected by ILK-S343A (refs). Conclusion from this work?

Glycogen synthase kinase 3 (GSK3)

GSK3 is a well-studied serine/threonine protein kinase initially identified for its role in regulating glycogen synthesis. They are two genes encoding GSK3 (α and β) that have essentially redundant functions. GSK3 has the fairly unique property of being constitutively active, a state that is reversed following phosphorylation at Ser9 in GSK3 β or Ser21 in GSK3 α by multiple kinases including AKT, ILK and atypical Protein Kinase C (aPKC) (Etienne-Manneville & Hall 2003). Recent work has implicated GSK3 β as a critical regulator of neuronal polarity. Experiments using several types of GSK3 inhibitors indicate that GSK3 α/β act negative regulators of axon formation since they lead to formation of multiple axons (Jiang et al 2005, Yoshimura et al 2005) and can even convert dendritic processes into axonal ones demonstrating that dendrites retain the potential to become axons and that this is normally prevented by dendritic GSK3 activity, a result previously hinted by axon severing experiments in culture (Bradke & Dotti 2000, Dotti & Banker 1987). This effect mimics constitutively active AKT/ILK-mediated phosphorylation and inactivation of GSK3 β and suggests a model for local activation of these kinases that leads to GSK3 inhibition and axon formation. This model is bolstered by experiments showing that GSK3 β S9A mutants are able to suppress multiple axon phenotypes in cases of constitutively active ILK (Guo et al 2007), expression of membrane targeted (myr-)Akt (Jiang et al 2005), or PTEN knock-down (Jiang et al 2005).

Importantly, a recent paper by Gartner et al. suggests that the situation may be somewhat more complex *in vivo*. Using double knockin-mice bearing single point mutations in GSK3 β (S9A) and GSK3 α (S21A), Gartner et al. reported no obvious deficits in neuronal morphogenesis *in vivo* and *in vitro* (Gartner et al 2006). In fact, these mice are viable and do not show any obvious developmental phenotype in the CNS. However, using inhibitors of GSK3 α/β such as Lithium Chloride or more specifically SB-415286, SB-216763 and AR-A014418, Gartner et al. were able to replicate the multiple axon phenotype obtained by others (Garrido et al 2007, Jiang et al 2005). These results indicate that while the exact role of Ser9/Ser21 phosphorylation in GSK3 inactivation remains to be understood or may involve an alternate site (Thornton et al 2008), it is clear that the catalytic activity of GSK3 is a critical regulator of neuronal polarity.

Several downstream targets of GSK3 are potential effectors of neuronal polarity and many involve regulation of the cytoskeleton. Collapsin-response mediator protein-2 (CRMP-2) is one such microtubule-binding protein that is enriched in tips of the nascent axon and is regulated by GSK3 β such that phosphorylated CRMP-2 displays a decreased binding affinity for tubulin heterodimers ((Inagaki et al 2001, Yoshimura et al 2005); reviewed in Arimura et al. (Arimura et al 2004)). As seen for other polarity regulators, over-expression of CRMP-2 is sufficient to induce multiple axons formation, and truncated forms of CRMP-2 can impair axon formation (Inagaki et al 2001). While the ability of CRMP-2 to facilitate microtubule assembly is important in regulating axon formation, it is also known to associate with several other factors including the actin polymerization-regulating Sra-1/WAVE1 complex which might participate to its function in axogenesis. Recently, CRMP-2 was also shown to link the Sra-1/WAVE1 complex with the microtubule-based motor protein Kinesin 1, and Sra1/WAVE expression seems required for CRMP-2's induction of multiple axons (Kawano et al 2005).

Adenomatous polyposis coli (APC) is another well-established effector of GSK3 that is enriched in the neurite that will become the axon early in neuronal polarization (Shi et al 2004). Phosphorylation of APC by GSK3 β blocks its ability to bind the plus-ends of microtubules leading to increased microtubule stability, and inhibition of GSK3 β leads to an accumulation of APC in multiple neurites (Shi et al 2004). Expression of truncated forms of APC is sufficient to inhibit axon formation (Shi et al 2004, Zhou et al 2004). Recent work

suggests that the APC/GSK dyad regulates targeting of another polarity protein PAR3 as over-expression of full-length or truncated APC disrupts neuronal polarization and inhibition of GSK β reduces the pool of APC in the nascent axon (Shi et al 2004, Zhou et al 2004) and growth factor triggered inactivation of GSK3 β by PI3K acts through APC to control axogenesis (Zhou et al 2004). Similar results have been observed for two other GSK targets, the Microtubule Associated Proteins (MAPs) MAP1b (Gonzalez-Billault et al 2004) and Tau (Sperber et al 1995), that when phosphorylated by GSK, alter microtubule dynamics. These results emphasize a key principle that underlies much of what is known about neuronal polarization, namely that the cytoskeleton is a major endpoint for polarity regulators. Interestingly, PTEN was also recently identified as a GSK3 β substrate (Maccario et al 2007). This may represent a negative feedback loop for AKT signaling following activation via stabilization of PTEN.

LKB1 and SAD-A/B and MARK kinases, the mammalian orthologs of Par4 and Par1

A pioneering genetic screen performed by Ken Kemphues and colleagues in the late eighties identified six *Par* genes encoding distinct protein families. A large number of studies have since demonstrated that invertebrate and vertebrates *Par* genes play critical roles in epithelial cell polarity during development as well as in the context of cell transformation and metastasis (Goldstein & Macara 2007, Kemphues et al 1988). While this pathway is critical to polarity in many species, the signaling linking this pathway to extracellular cues has remained elusive. The furthest upstream component known in this cascade is an evolutionarily conserved kinase named LKB1 or PAR4. LKB1 translocates from the nucleus and is activated by hetero-dimerization with one of two related pseudokinases known as Strad α and β (Dorfman & Macara 2008). In addition to Strad binding, LKB1 function in neuronal polarity requires its phosphorylation at S431, a target of both protein kinase A and p90RSK kinases (Collins et al 2000, Sapkota et al 2001) and this phosphorylation can be triggered by extra-cellular cues such as BDNF (Shelly et al 2007) (**Figure 3**). This event might be mediated partly by cues providing chemotactic attraction of radially migrating neurons toward the cortical plate such as Sema3A (Chen et al 2008, Polleux et al 1998) or any other extracellular cues neurotrophins (NTs) such as BDNF/NT4/NT3 (Shelly et al 2007), Netrin (Adler et al 2006), FGFs or any other cues that can activate cAMP-dependent protein kinase (PKA) or p90 RSK (RSK1-3) or another uncharacterized serine/threonine protein kinase (see later section on extracellular cues and **Figures 4 & 5**). Future investigations will have to identify the relevant extracellular cues and the corresponding signaling pathways triggering phosphorylation of LKB1 in position S431 and thereby specifying the axon in developing cortical pyramidal neurons.

Once LKB1 is activated by binding to its necessary co-activator Strad and S431-phosphorylation (which occurs only in the neurite becoming the axon), LKB1 phosphorylates SAD-A/B kinases (and probably microtubule affinity-regulated kinases, MARK1-4) that are required for axon specification partly by phosphorylating microtubule-associated proteins such as Tau. On the basis of the function of SAD-kinases in presynaptic vesicular clustering in *C. elegans* (Crump et al 2001), we can hypothesize that SAD-A/B kinases might also specify axon identity by directing vesicular trafficking in the neurite becoming the axon. Most importantly, genetic deletion of LKB1 in cortical pyramidal neurons prevents axon formation, whereas over-expression of LKB1 and its co-activator Strad in neural progenitors or LKB1 alone in post-mitotic cells is sufficient to lead to the formation of multiple axons (Asada et al 2007, Barnes et al 2007, Shelly et al 2007).

LKB1 has been shown to exist in a complex with two other cell polarity regulators, GSK3 β and aPKC, and experiments in *Xenopus laevis* suggested that LKB1 may regulate aPKC inactivation of GSK3 β (Ossipova et al 2003). LKB1 is also known to phosphorylate and activate a family of thirteen protein kinases related to the *C. elegans* PAR1 protein (Lizcano

et al 2004). To date, three of these have been implicated in regulating axon formation including: SAD-A, SAD-B kinases as well as Microtubule Affinity Regulating Kinase-2 (MARK-2). RNAi knockdown of SAD kinases has been shown to partially abrogate multiple axon formation in neurons over-expressing activated LKB1, indicating that LKB1's function in promoting axogenesis largely (but not completely) derives from activation of SAD-A/B kinases (Barnes et al 2007). Double knockout mice for SAD-A and SAD-B results in neurons that cannot form axons *in vivo* (Kishi et al 2005), but over-expression of SAD-A/B does not induce multiple axon formation (unpublished observations). SAD and MARK kinases target several MAPs including MAP2, MAP4, and Tau by phosphorylating three K-X-G-S motifs within each protein which reduces their microtubule binding affinity, thus destabilizing microtubules (Drewes et al 1997, Illenberger et al 1996). Little is known about SAD kinases regulation, however a recent study suggested that the protein phosphatase PP2 might down-regulate SAD catalytic activity by reversing LKB1-mediated phosphorylation (Bright et al 2008). The microtubule regulatory scheme is the same for the four members of MARK kinase family, but at this point only MARK2 has been implicated in neuronal polarity (Biernat et al 2002, Chen et al 2006b). Since RNAi-mediated knock-down of MARK2 induces supernumerary axons and over-expression of MARK2 inhibits axon formation, it is tempting to hypothesize that MARK2 is a negative regulator of axogenesis (Chen et al 2006b). Intriguingly, MARK2 can interact with the serine/threonine kinase PAK5 and this interaction is thought to inhibit MARK2 kinase activity, while simultaneously destabilizing actin cytoskeleton (Matenia et al 2005). Thus the MARK2/PAK5 dyad might coordinate actin and microtubule cytoskeletal dynamics during the establishment and/or the maintenance of neuronal polarity (discussed later in this review).

Interestingly, recent work has revealed that other potential regulators of neuronal polarity act by regulating MARK2. GSK3 β can inactivate MARK2 catalytic activity through phosphorylation (Timm et al 2008), and similarly aPKC has been shown to inhibit MARK2 activity through phosphorylation of T595 (Timm et al 2008). Interestingly, the planar cell polarity (PCP) signaling molecules Dishvelled1 (Dvl1) and Wnt5a have been added into the MARK2/aPKC pathway of neuronal polarization (Zhang et al 2007). In this scenario, Wnt5a activation of its receptor Frizzled (Fzl) leads to stabilization of aPKC through its direct interaction with Dvl1. This increase in aPKC then leads to increase in the inhibitory phosphorylation of MARK2. Consistent with this model, increased Dvl1 expression leads to multiple axons and RNAi knock-down inhibits axon formation (Zhang et al 2007). Furthermore, the combination of RNAi against MARK2 and Dvl1 actually results in normal axon formation. c-Jun N-terminal kinase (JNK) is another potential target for Dvl1 signaling and plays a role in neuronal polarization (Ciani & Salinas 2007). Inhibition of JNK blocks neuronal polarization in a reversible manner (Oliva et al 2006). The JNK binding partner JIP has also been shown to be required for axon initiation (Dajas-Bailador et al 2008), a function that might require phosphorylation by the c-Abl tyrosine kinase. It is unclear whether this effect is due to mis-localization of JNK, JIP's role in linking vesicle cargos to motor proteins (Verhey et al 2001), its interaction with p190RhoGEF (Meyer et al 1999) or a combination of these factors.

Ras- and Rho-family of small GTPases

Small GTPases are critical regulators of cytoskeletal and membrane dynamics underlying cell motility, cell polarity and cell growth. Small GTPases proteins are molecular switches that constitute a critical component of cellular homeostasis and generally act on downstream effectors when bound to guanosine triphosphate (GTP) and are inactive when this GTP is hydrolyzed to guanosine diphosphate (GDP). Rho-GTPases possess relatively slow intrinsic GTP hydrolysis activity and their catalytic activity is regulated by GTPase-activating proteins (GAP; approximately 50 in the human genome). GAPs therefore act as negative

regulators of GTPase activity by promoting GDP bound (inactive) state. Activation of small GTPases by exchange of GDP to GTP is controlled by GTP-exchange factors (GEF; approximately 69 in the human genome).

There are three members of the Ras-family of small GTPases: H-Ras, K-Ras, N-Ras. Over-expressing either wild-type or a constitutive active mutant (V12 or Q61L) of the H-Ras or the related protein R-Ras (Q87L) leads to the production of multiple axons (Fivaz et al 2008, Oinuma et al 2007, Yoshimura et al 2006). Ras proteins regulate both the MAP kinase and phosphoinositide-3 kinase (PI3K) pathways, and pharmacologic inhibition of either pathway was sufficient to inhibit the production of additional axons, but surprisingly did not impact axon formation in general (Yoshimura et al 2006). Ras activation is coupled to many cell surface receptors including growth factor receptors, and it has been shown that a tyrosine kinase inhibitor, AG1478, can inhibit axon formation (Betschinger et al 2003). Recently, elegant work using a fluorescent reporter of Ras activation demonstrates the restricted nature of Ras signaling and its recruitment during axon determination to contribute to a positive feedback loop with PI3K (Fivaz et al 2008). Additional work remains to identify which upstream activators may act through Ras during neuronal symmetry breaking to fate the nascent axon and we will discuss some potential candidates later in this review.

The best studied of all mammalian Rho-family of small GTPases (22 total) are Cdc42, RhoA and Rac1. Expression of dominant-negative (locked in GDP-bound state) or constitutively-active (locked in GTP bound state) mutants of each of these small GTPases in polarizing neurons, or treatment with the Rho-GTPase inhibitor toxin B (Bradke & Dotti 1999), indicates a critical role for both cdc42 and Rac1 both *in vitro* in rodent neurons (Nishimura et al 2005, Schwamborn & Puschel 2004) and in *Drosophila in vivo* (Luo et al 1994). Specifically, expression of the auto-cycling form of cdc42 leads to multiple axons in rodent neurons (interestingly, a constitutively active mutant leads to a failure of neurite formation (Schwamborn & Puschel 2004)). The loss of cdc42 expression, either through siRNA knock-down (Schwamborn & Puschel 2004) or genetic ablation (Garvalov et al 2007), leads to a strong axon specification defect. In the case of cdc42 conditional knockout mice, the axon phenotype may be due to increased levels of phosphorylated (inactive) cofilin, a regulator of actin dynamics enriched in developing axons (Garvalov et al 2007). This phosphorylation is achieved by LIM kinase, an activity stimulated by a cdc42 effector kinase, Pak1. Paradoxically, Pak1 activity is greatly reduced in the cdc42-null mice, suggesting that the deregulation of another pathway regulating cofilin in the absence of cdc42, most likely the RhoA-regulated kinase ROCK (Maekawa et al 1999). The loss of Pak1 itself has also been shown to inhibit neuronal polarization and conversely constitutively active Pak1 induces multiple tau1-positive processes (Jacobs et al 2007). Interestingly, the latter effect can be partially mitigated by co-expression of either an unphosphorylatable form of cofilin or a GDP-locked Rac1, suggesting that Rac1 may act downstream of Pak1 activation. Taken together, these results demonstrate a role for activated cdc42 in neuronal polarization beyond its association with the PAR3/6 complex described later in this review.

RhoA is another of small GTPase and it is typically associated with destabilization of the actin cytoskeleton and myosin-based contractility. Experiments using a constitutively active form of RhoA show that it inhibits neuritogenesis (Bito et al 2000, Schwamborn & Puschel 2004), while dominant negative form of RhoA enhances neurite outgrowth (Schwamborn & Puschel 2004). This is consistent with the regulatory role proposed above for p190RhoGAP as well as the effect of inhibiting the RhoA-activated kinase, ROCK, on axogenesis (Bito et al 2000). Another pathway implicated in ROCK-inhibition during axon formation potentially involves the localization of ganglioside converting enzyme plasma membrane ganglioside sialidase, PMGS (Da Silva et al 2005). The over-expression of PMGS leads the

generation of multiple axons and inactivation of ROCK observed following PMGS over-expression might involve enhancement of neurotrophic receptor TrkA signaling resulting in signaling that requires both PI3K and Rac1. Much work is required to identify the exact requirement for PGMS signaling for axogenesis *in vivo* as well as to better define the molecular mechanisms underlying the function of RhoA in axon specification versus axon outgrowth.

The examination of Rac1's role in neuronal polarization has led to some confounding results. In *Drosophila*, both dominant activate and negative mutants are able to affect outgrowth but not specifically polarity (Luo et al 1994). In flies expression of either dominant-negative (GDP-locked) (Luo et al 1994) or loss of Rac expression (Hakeda-Suzuki et al 2002, Ng & Luo 2004, Ng et al 2002) affect outgrowth, but not polarity. Similarly, siRNA knockdown of Rac1 typically does not affect axon identity (Gualdoni et al 2007), although some reports detected unpolarized neurons following expression of the dominant negative form of Rac1 (Nishimura et al 2005). In cultured neurons a constitutively-active version of Rac1 does not affect axon specification (Schwamborn & Puschel 2004). These results, while mixed, do hint at a more complex scheme for Rac1 in neuronal polarization. This fact will become clearer later in our review since the only GEF proteins shown to be crucial for axon formation appear to control Rac1. This observation's apparent disjunction with the lack of strong phenotype may reflect the importance of sub-cellular localization of activated pools of Rac1 and compensation by related small GTPases.

Small GTPases have a plethora of effectors within cells and proper activation of these effectors, both spatially and temporally, requires exquisite control of both activation and inactivation by GEFs and GAPs respectively. Apart from p190RhoGAP (discussed later in this review) most studies have so far focused on the function of GEFs in neuronal polarity. This includes the two GEFs Tiam1 and STEF described later and DOCK7 GEF recently reported to be a regulator of axon specification via activation of Rac1 along with phosphorylation of Stathmin/Op18, a microtubule-destabilizing factor critical for axogenesis (Watabe-Uchida et al 2006). Another axonally-enriched, unconventional Rac1 regulatory protein is the cytoplasmic dynein light chain Tctex-1 (Chuang et al 2005). Increased levels of Tctex-1 result in increases in GTP-loaded Rac1 and GTP-Rac1 levels drop following Tctex-1 siRNA treatment. Multiple axons result from over-expression and this effect is preserved using a mutant form (T94E) that cannot bind dynein heavy chain. Consistent with a role in controlling Rac1, the supranumerary axon phenotype is suppressed by constitutively active RhoA or dominant negative Rac1.

Rap1b, a member of the Ras superfamily of GTPases, has also been shown to be required for proper neuronal polarity (Schwamborn & Puschel 2004). It is found at the tip of the nascent axon and its over-expression leads to hippocampal neurons bearing multiple axons. The loss of Rap1b following siRNA knockdown abrogates axon formation and expression of auto-cycling cdc42 can rescue the phenotype. Interestingly, expression of a constitutively active Rap1b fails to reverse the loss of axons observed following loss of cdc42, indicating that Rap1b lies upstream of cdc42 in this pathway of neuron polarization. Similarly, suppressing axogenesis via pharmacological inhibition of PI3-kinase can be reversed by either auto-cycling cdc42 or constitutively active Rap1b, placing both of these small GTPases downstream of PI3K signaling during axon specification. In addition to its place in one of the canonical polarity pathways, studies on Rap1b have explored a novel mechanism for protein localization during neuronal polarity, namely selective protein degradation (Schwamborn et al 2007b). The means of controlling protein activity appears to apply to several polarity regulating proteins and will be expanded upon later in this review.

PAR3-PAR6-aPKC

The other majority of protein in the polarity complex identified in *C. elegans* has its core components the scaffolding proteins PAR-3 and PAR-6. Many binding partners for this complex have been implicated in regulation the polarity of epithelial cells and the neuroepithelial radial glia express the PAR3/6 complex at their analogous apical domain along the ventricular wall (Costa et al 2008, Manabe et al 2002). Proteins reported to exist in complex with PAR3/6 include: atypical forms of protein kinase C (aPKC: PKC λ and ζ) (Joberty et al 2000, Lin et al 2000, Qiu et al 2000), the small GTPase cdc42 (Joberty et al 2000, Lin et al 2000, Qiu et al 2000), the kinesin motor protein KIF3A (Nishimura et al 2004), the guanine exchange factor Tiam1/STEF (Chen & Macara 2005, Nishimura et al 2005), the lipid and protein phosphatase PTEN (Feng et al 2008, von Stein et al 2005), the GTPase activating protein (GAP) p190RhoGAP (Zhang & Macara 2008), the tumor suppressor lethal giant larvae (Lgl) (Plant et al 2003), the scaffold protein inscuteable (Schober et al 1999), the ubiquitin ligases Smurf1 (Ozdamar et al 2005) and Smurf2 (Schwamborn et al 2007a), and the transforming growth factor receptor 1 (TGF β R1) (Ozdamar et al 2005). Each of these proteins has also been implicated in controlling polarity in non-neuronal cells as part of the PAR3/6 complex.

We will consider the potential function of some of these PAR3/6 interacting proteins (Lgl, Inscuteable and Smurf1/2) later in this review in the context of progenitor polarity and protein stability, respectively. PAR3/6 have been shown to be enriched in the nascent axon in stage 3 hippocampal neurons and over-expression of wild-type and truncated forms of either PAR3 or PAR6 perturb the formation of a single axonal process in hippocampal neurons (Shi et al 2003). Intriguingly, in *Drosophila*, orthologs of PAR3 (bazooka), PAR6 or aPKC do not appear to be required for proper axon-dendrite specification (Rolls & Doe 2004). This could mean that PAR3/6 have acquired a function in neuronal polarity late during evolution in the vertebrate radiation. Alternatively, there is so far no genetic loss-of-function evidence in vertebrates (especially in mammals) demonstrating that Par3 and Par6 are required for axon specification. This evidence is clearly more challenging to obtain than in *Drosophila* since there are at least 4 Par6 genes and 2 Par3 like genes in mammalian genomes (Barnes et al 2008, Goldstein & Macara 2007).

The role of aPKC in neuronal polarity is tightly linked to its ability to associate to the PAR3-PAR6 complex. The activity of aPKC is greatly reduced when associated with PAR6 (Yamanaka et al 2001) and this partnership provides a regulatory scheme which requires additional signaling events to produce a very spatially limited pool of activated aPKC as the binding of GTP-bound cdc42 relieves this inhibition. Phosphorylation targets of aPKC include the PAR3/6-binding partner *Lethal giant larvae* (Lgl) and this post-translational modification is thought to play a crucial role in regulating the sub-cellular localization of Lgl during polarization in several cellular contexts (Betschinger et al 2003, Plant et al 2003, Yamanaka et al 2003). As mentioned previously, aPKC can also phosphorylate the PAR1 ortholog MARK2 on Threonine 595 (Hurov et al 2004, Suzuki et al 2004), in this case inhibiting its kinase activity. Global inhibition of aPKC activity in polarizing neurons clearly indicates a role for this kinase in the establishment of neuronal polarity at least *in vitro* (Shi et al 2003), consistent with observed enrichment of activated aPKC in the nascent axon (Schwamborn & Puschel 2004, Zhang et al 2007). PAR3/6 axonal localization likely occurs via PAR3 interaction with the microtubule plus-end directed kinesin KIF3A, since interference with KIF3A function leads to the delocalization of PAR3 and aPKC from the nascent axon tip (Nishimura et al 2004). As discussed previously, inhibition of GSK3, or the perturbation of APC localization, also eliminates PAR3 targeting from the tip of the nascent axon (Shi et al 2004), indicating a potential hierarchical scheme which enriches the Par3/6 complex in the developing axon (**Figure 6**).

The Rac1-GEF Tiam1 also interacts with PAR3/6 which is of particular interest as this protein has been shown to regulate axon formation *in vitro*. Over-expression of Tiam1 leads to multiple tau1 positive processes, and reducing its expression level is sufficient to block neurons in the unpolarized stage 2 (Kunda et al 2001, Nishimura et al 2005). Current models suggest that PAR3 may limit the distribution of Tiam1, thus limiting the activation of the small GTPase Rac1 (Chen & Macara 2005, Nishimura et al 2005, Zhang & Macara 2006). This Rac1 regulatory role for PAR3/Tiam1 requires the association of another GTPase known as cdc42 and its association with PAR6 (Nishimura et al 2005). It is quite likely that the tight regulation of this pathway is required for specificity at early stages of polarization following the cell's encounter with a symmetry breaking cue.

The recent report of an interaction between the RhoA-specific GAP, p190RhoGAP, and aPKC-PAR3/6 pathways (Zhang & Macara 2008) opens the possibility that PAR3/6 may serve as a 'scaffolding hub' for signaling involving the most well studied small GTPases (RhoA, Rac, and cdc42). In this scenario, p190RhoGAP would act to locally inactivate RhoA while the associated Tiam1/STEF would increase levels of active Rac1, thus promoting neurite outgrowth/axon formation. Several observations potentially strengthen this possibility. First, p190RhoGAP activity is reduced by GSK3 β phosphorylation, so that the requisite GSK3 β inactivation in axogenesis would lead to decreased levels of activated RhoA and increased process extension (Jiang et al 2008). Secondly, mice lacking either of the two p190RhoGAP (A and B) genes demonstrate significant decrease in axon tracts (Brouns et al 2000, Brouns et al 2001, Matheson et al 2006) which is compatible with an axon specification or an axon growth defect. Lastly, it has been shown that p190RhoGAP activity can be stimulated by activation of the insulin-like growth factor 1 (IGF-1) signaling (Sordella et al 2003), which has been demonstrated to stimulate axon formation in cultured neurons (Sosa et al 2006) and axon outgrowth *in vivo* (Ozdinler & Macklis 2006). Returning to the theme of balanced antagonism, a very recent study suggests that RhoA activity may inhibit the interaction of PAR3 and PAR6 via activation of Rho-kinase (Nakayama et al 2008), likely disrupting or altering signaling from the PAR3/6 complex. In this way, RhoA activity might be part of a negative feed-back loop negatively regulating PAR3/6 signaling in neurites becoming dendrites. Clearly, much work needs to be done to test this model based mostly on evidence in non-neuronal systems.

GLOBAL CELLULAR MECHANISMS OF NEURONAL MORPHOGENESIS

Local Protein Degradation

The spatial regulation of protein expression by selective degradation has been described in various context during neuronal differentiation including axonal pruning during development (Watts et al 2004) and various aspects of axon guidance (Bloom et al 2007, Campbell & Holt 2001, DiAntonio et al 2001, Lewcock et al 2007), synapse formation (DiAntonio et al 2001, Nakata et al 2005), maintenance (Aravamudan & Broadie 2003, DiAntonio et al 2001, Ehlers 2003, Speese et al 2003) and elimination (Ding et al 2007) (reviewed in (DiAntonio & Hicke 2004)). Interestingly, acute treatment with the proteasome inhibitor lactacystin blocks axogenesis in dorsal root ganglion cells (Klimaschewski et al 2006). Furthermore, more prolonged inhibition of protein degradation with lactacystin leads to formation of multiple axons (Yan et al 2006). The protein kinase AKT that we previously described as critical for neuronal polarity has been shown to undergo selective degradation (Yan et al 2006). In fact, this degradation selectively targets the inactive pool of AKT in neurites resulting in a net enrichment of AKT in a single process that contains that most active AKT, the nascent axon. This phenomenon is consistent with the negative feedback signal model proposed by Kaibuchi and colleagues (Arimura & Kaibuchi 2007) to explain axon specification of a *single* axon during neuronal polarization. The small GTPase Rap1b has recently been shown to undergo a very similar regulatory scheme to that of AKT as the

active form Rap1b is spared from degradation and ultimately enriched in the axon (Schwamborn et al 2007b). In this case, the ubiquitin ligase acting on Rap1b is specifically Smurf-2, whereas the related Smurf1 appears to only affect neurite outgrowth. Additional work has demonstrated an interaction between Smurf2 and the polarity scaffold PAR3 must exist for proper neuron polarization (Schwamborn et al 2007a). This appears to be related to PAR3 targeting of Smurf2 to the axon as perturbation of either the interaction of PAR3-Smurf2 or PAR3-KIF3A results in increase Rap1b in all neurites. The converse situation exists for LIM kinase (LIMK) since levels of the protein must be reduced for axon initiation (Tursun et al 2005). The protein Rnf6 has been shown to catalyze the ubiquitination of LIMK and blocking Rnf6 expression leads increased levels of axonal LIMK similar to those observed following proteasome inhibition. While this phenomenon represents a powerful mechanism to regulate protein enrichment, our complete understanding will require identification of both upstream regulatory signals responsible for its spatially limited activity and the cohort of proteins targeted for degradation.

Cytoskeletal dynamics

Not surprisingly, regulation of the actin and microtubule cytoskeleton has been the focus of intense studies for their role in neuronal polarization. Experiments using the actin destabilizing agents latrunculin B and cytochalasin D indicate that remodeling the actin-based cytoskeleton is an important regulatory step in axon formation (Bradke & Dotti 1999). Specifically, the localized actin-depolymerization to a single neurite in unpolarized Stage 2 hippocampal neurons is sufficient to confer axonal identity. In this way, the loose actin filaments would permit the egress of microtubules and lead to rapid elongation of a given neurite, perhaps outpacing the transport of negative regulators of axonal identity. The idea of cellular asymmetries being reinforced by localized microtubules stabilization and invasion proposed by Kirschner and Mitchison (Kirschner & Mitchison 1986) was elegantly demonstrated via the use of a photoactivatable form of the tubulin-stabilizing compound taxol which is able to direct axonal specification to a single immature neurite (Witte et al 2008). This work directly confirms a hypothesis about the role of tubulin in axon-dendrite polarity that has been developed as result of the observations by many groups regarding post-translation modifications of tubulin and microtubule-binding proteins.

Early experiments showed that severing the putative axon of cultured neuron results in the assignment of an alternate process as the axon (Dotti & Banker 1987) which may also be related to changes in microtubule dynamics. Work from several groups indicated that the distance from the cell body dictates if the axon will regenerate from the same neurite or if a dendrite will adopt the axonal fate upon axotomy (Bradke & Dotti 1997, Bradke & Dotti 2000, Goslin & Banker 1989, Takahashi et al 2007). More recent work indicates that this can occur in relatively mature neurons integrated in established synaptic networks (Gomis-Ruth et al 2008). This work also predicts that the presence of a stable pool of microtubules in the lesioned neurite may dictate if axon regeneration or axonal re-specification will occur. The existence of an intrinsic bipolar polarity axis has also been described in hippocampal neurons with axotomized cells re-growing their axons from an antipodal neurite (Calderon de Anda et al 2008). This work also demonstrates that following axotomy, the microtubule organizing center, the centrosome, does not reorient toward the newly assigned axon, in contrast to a previous suggestion of a role for the centrosome in directing the destiny of the axonal neurite (de Anda et al 2005). This may be reflection of the fact that at later stages of neuronal differentiation, the centrosome is no longer required for axon re-specification. This and other observations (Calderon de Anda et al 2008) suggest that during re-specification of the axonal process, unlike the centrosome, membrane flow does respond by re-orienting. The requirement of centrosome and Golgi positioning in relation to axon initiation still awaits *in vivo* confirmation (see also **Figures 4 and 5**).

Cytoplasmic flow and directed membrane trafficking

It has been observed that subtle changes in membrane trafficking, as indicated by neurite growth cone size and phase-contrast tracking of organelles using time-lapse microscopy, are often predictive of axon specification (Bradke & Dotti 1997). These results indicated that mitochondria, membrane vesicles and other cytoplasmic components are preferentially directed toward the nascent axon and are consistent with earlier analysis of polarizing neurons using electron-microscopy (Deitch & Banker 1993). The components of cytoplasmic flow are likely to include some of the cytoplasmic molecules described earlier as regulators of axogenesis and as indicated the anterograde trafficking of vesicles. It is known that proteins with an established role in synapse function are targeted to the nascent axon (Fletcher et al 1991), however very few of the regulatory molecules controlling this activity have been identified. Early work showed using anti-sense oligonucleotide knock-down of Rab8, a critical regulator of vesicular trafficking can block neuronal polarization (Huber et al 1995). Similarly, a dominant-negative form of the tetanus neurotoxin-insensitive vesicle-associated membrane (TI-VAMP) protein blocks neuronal polarization (Martinez-Arca et al 2001). A corollary to selective axonal transport comes from work exploring the asymmetric genesis of the apical dendrite. In this work, the polarized position of the Golgi network leads to preferential membrane deposition in a single neurite that becomes the apical dendrite (Horton et al 2005), a step likely to follow shortly after axon specification.

A recent *Drosophila* screen for dendritic arbor reduction (*dar*) mutants made the intriguing discovery that perturbing some secretory pathway specifically affects dendrite and not axonal development, a result also observed in mammalian neurons along with the existence of golgi outposts (Ye et al 2007). Polarity mutants have identified that disrupt the polarized sorting of cargoes in the nematode *C. elegans* as well. These genes include: the kinases LRK-1 (Park-8) (Levy-Strumpf & Culotti 2007) and SAD1 (Crump et al 2001, Hung et al 2007) (as described previously, also required for polarity in mammalian neurons), the SAD kinase binding partner Nab-1 (Neurabin) (Hung et al 2007), and the UNC-76 (FEZ1)/UNC-69 complex (Hung et al 2007). The RNAi-mediated knock-down of the mammalian ortholog, FEZ1, has been shown to inhibit axon formation in part due to regulating mitochondrial motility (Ikuta et al 2007), and likely by regulating the engagement of kinesin-1 in cooperation with the JNK-scaffold protein JIP1 (Blasius et al 2007). Taken together, these insights from *C. elegans* buttress the high degree of conservation of some of the pathways regulating neuronal polarity beyond the core group of PAR proteins.

Molecular Motors

A role has long been postulated for molecular motor proteins to play a role in neuronal polarity. Intriguing evidence places a few of the microtubule plus-end directed kinesin proteins as the best candidates. As mentioned above the kinesin-like protein GAKIN appears to have crucial role in transporting PIP3 and likely other cargoes to the developing axon (Horiguchi et al 2006). Similarly, the KIF3A motor has been shown to be important for the localization of the PAR3 protein (Nishimura et al 2004, Schwamborn et al 2007a). Work with Kinesin-1/KIF5C-EGFP fusion protein presents a particularly fascinating picture of motor dynamics during axon specification (Jacobson et al 2006). Time-lapse video-microscopy of isolated hippocampal neurons in dissociated culture at Stage 2 show that KIF5C-EGFP makes transient explorations of single immature neurites but a prolong invasion of the neurite becoming the axon in transition to Stage 3 (Jacobson et al 2006). Obviously more work needs to be done to test (1) if this occurs during neuronal polarization in vivo and (2) test specifically when after cell cycle exit, this preferential kinesin-based motor transport is occurring during axon specification when neurons engage migration. Finally it will be of great interest to use this visualizing tool in order to test how extracellular

cues and their intracellular effectors direct microtubule-based transport activity during axon specification. Given the large number of motor proteins (Hirokawa & Takemura 2004, Hirokawa & Takemura 2005), these data likely represents only the beginning of a complex story regarding the selective transport of proteins, membrane vesicles and organelles during neuronal polarization.

Diffusional Barrier

A straightforward scenario for the subcellular targeting of neuronal proteins envisions polarized microtubule-based transport coupled to docking of specific cargoes to their destination. This situation is likely true for many cytoplasmic and some membrane-bound proteins, but experiments utilizing fluorescent lipids indicate that a barrier to communication between axonal and somatic membranes exists (Kobayashi et al 1992) and would be required to maintain asymmetries established during early neuronal polarization events. A continual refinement of our understanding of the nature of this barrier has led to an appreciation that it is dependent on the actin cytoskeleton and it represents a significant physical barrier to lateral diffusion (Winckler & Mellman 1999). Later experiments have shown that this barrier forms over time in cultured neurons (Nakada et al 2003), suggesting that vectorial trafficking and anchoring/stabilization contribute to the early phase of polarization and the barrier contributes to later maintenance of polarity. The consensus that a “molecular fence” exists within the axon initial segment is fairly solid, but the molecular mechanisms underlying the assembly of the barrier during development remains to be elucidated. It is not clear if the morphological structure that is enriched in components such as sodium channels and ankyrin-G is identical to the diffusion barrier but both are contemporaneous developmentally (Nakada et al 2003). One component of the axon initial segment (AIS) recently identified is the phosphorylated form of I κ B α , an inhibitor of NF κ B (Sanchez-Ponce et al 2008). In this study, inhibition of the I κ B kinases was shown to inhibit axon formation, through a currently unknown mechanism. Two papers disrupting known components of the AIS have shown critical roles for the scaffold protein, ankyrin G, as well as NF-186 and its linkage to the brevican-containing extra-cellular matrix (Hedstrom et al 2007, Yang et al 2007). Clearly more work is required to improve our understanding of the function of the AIS in specification and/or maintenance of neuronal polarity.

ROLE OF EXTRACELLULAR CUES IN ORCHESTRATING INTRACELLULAR SIGNALING DURING NEURONAL POLARIZATION

IN VITRO

Several lines of evidence suggest that extracellular cues can direct the polarized emergence of the axon and the dendrites both *in vitro* and *in vivo*. First, dissociated cortical or hippocampal pyramidal neurons plated on striped substrates coated with two different cell adhesion molecules (Laminin and NgCAM for example) can play an instructive role for axon specification (**Figure 4 A-B**). Interestingly, the first immature neurite of E18 hippocampal neurons contacts the boundary between two stripes systematically becomes the axon. This occurs regardless of the fact that the initial outgrowth of immature neurites occurred on laminin or NgCAM suggesting that immature neurites are able to detect changes in the nature of the extracellular substrate rather than the absolute nature of the novel substrate they are encountering (Esch et al 1999). Using a similar approach, Shelly and colleagues showed that neurites of immature hippocampal growing on a patterned substrate can detect the presence of BDNF which plays an instructive role in axon specification since the first neurite contacting a BDNF-stripe systematically becomes the axon (Shelly et al 2007) (**Fig. 4C-D**). Interestingly, the effect of BDNF on axon specification requires cAMP-dependent protein kinase (PKA) activation and phosphorylation of LKB1 in position 431 by PKA (Shelly et al 2007) (**Fig. 4E**) suggesting that LKB1 phosphorylation on S431 act as a

detector of breaking of neuronal symmetry by extracellular cues such as BDNF in this *in vitro* context.

The overlay assay is an *in vitro* assay developed to detect the existence of putative extracellular cues playing a role in cortical neuron polarization. As depicted in **Figure 4E**, this rather simple assay revolves around plating fluorescently-labeled dissociated cortical neurons onto cortical slices in order to test if polarized axon emergence *in vivo* is mainly the result of asymmetric activation of intracellular effectors (maybe inherited by progenitors) or if extracellular cues can play a role in axon specification (Scenario 2 in **Fig. 4E**). Polleux et al. (Polleux et al 1998) have demonstrated that Scenario 2 is the most likely since only a couple of hours after plating, the vast majority of cortical neurons display a single, short axon directed ventrally towards the ventricle as found *in vivo*. These authors went on to demonstrate that the class 3 secreted semaphorin, Sema3A which is enriched in the most superficial part of the cortical wall (the top of the cortical plate), plays a role in repulsing axon initiation ventrally towards the ventricle (Polleux et al 1998). This work suggests that the polarized emergence of a single axon is controlled at least in part by extracellular cues expressed in a graded manner along their migratory path.

IN VIVO

Is there any *in vivo* evidence for the role of extracellular cues in the specification of neuronal polarity? In *C. elegans*, elegant work has demonstrated that the polarized emergence of a single axon in HSN neurons requires Netrin (UNC6)-induced localization of Netrin-attractive receptor UNC-40 (DCC) as well as a cytoskeletal effector called MIG-10 (lamellipodin) on the ventral part of the neuron at early L2 stage (**Figure 5A**, (Adler et al 2006)).

Careful live imaging experiments of *Xenopus* retinal ganglion cells polarization revealed that polarized axon outgrowth requires some unidentified extracellular cues present in the basal lamina (Zolessi et al 2006) (**Figure 5B**). The axon of developing RGC normally grows on the basal side of the neuron. In a mutant called *Nok* characterized by the absence of retinal pigmented epithelium (RPE), some post-mitotic RGC neurons show a defective polarized outgrowth of their axon on the apical side along the now-exposed basal lamina. Interestingly, in this context, the polarized emergence of the axon on the basal side of the RGC is correlated with the position of the centrosome, Par3 and the apical complex (containing at least aPKC, α -catenin and F-Actin) on the apical side of the cell where the dendrite will emerge (**Figure 5B**). Taken altogether, this work strongly suggests that (i) the basal lamina contains some important extracellular cues playing a role in the polarized emergence of the axon of RGC neurons and (ii) that RGC neurons inherit the intrinsic apico-basal polarity of their progenitor at least with regard to the Par3/aPKC components of the polarity complex. Important questions remain to be addressed: what extracellular component of the basal lamina controls the polarized emergence of the axon? What signaling pathway is involved in this axon specification? How is this pathway linking the extra-cellular cues polarizing axon outgrowth to the apical polarity pathway? Future investigations will address some of these questions using this unique *in vivo* paradigm.

To date, the only signaling cascade linked to extracellular cues is the recently identified LKB1-SAD kinase pathway (Barnes et al 2007). Recent studies using either RNAi knock-down or genetic loss of function have explored this pathway in the developing cerebral cortex. Targeting LKB1 loss to dorsal telencephalic progenitors leads to the selective loss of axon initiation in cortical neurons, but does not impact migration. Structure/function analysis indicates that phosphorylation of LKB1 at Serine 431 is required for its function in axon specification (Barnes et al 2007) and Shelly et al. link this phosphorylation event to the

ability of BDNF application to stimulate PKA-dependent phosphorylation of S431 in the nascent axon (Shelly et al 2007).

POTENTIAL RELATIONSHIP BETWEEN NEUROEPITHELIAL CELL POLARITY AND POST-MITOTIC NEURON POLARITY

One of the important questions emerging from the work reviewed in Figures 1 and 5 revolves around determining if there is a systematic relationship between neural progenitors polarity and post-mitotic neuron polarity in mammals? Clearly, in several cell types such as retinal ganglion cells and bipolar cells, post-mitotic neurons directly inherit the intrinsic apico-basal polarity of progenitors which is transformed into axon-dendrite polarity upon cell cycle exit (refs Zolessi et al. ND 2006; Hinds and Hinds 1978, Morgan et al. NN 2006). However, this relationship between progenitors polarity and post-mitotic neuron polarity is not so clear at first glance for other neuronal sub-populations such as cerebellar granule cells and pyramidal cortical and hippocampal neurons.

Dividing neuroepithelial progenitors in the neocortex and elsewhere in the nervous system present a strong apico-basal polarity where the apical and baso-lateral membranes are separated by cadherin-based adherens junctions (AJ) but not by tight junction as observed in epithelial cells (**Figure 6A**; reviewed by Gotz and Huttner, (Gotz & Huttner 2005)). Recent results have allowed to determine the molecular composition of the apical membrane of cortical neuroepithelial progenitors which revealed that it contains the core components of the apical polarity complex (aPKC/Par3/Par6) as well as cdc42. Interference with Par6 or cdc42 expression in cortical progenitors alters the balance between asymmetric versus symmetric cell divisions (Cappello et al 2006, Chen et al 2006a, Costa et al 2008). The apical domain of cortical progenitors is also closely associated with the cadherin-based AJ and the centrosome (Microtubule Organizing Center or MTOC) ((Xie et al 2007), Chenn and McConnell MCN 1995; (Rasin et al 2007)). It is not clear at this point whether any of the proteins or organelles present in the apical polarity complex of progenitors are inherited by post-mitotic neurons upon cell-cycle exit (**Figure 6B**). Recent analysis suggested that the centrosome position upon the terminal mitosis predicted the axon initiation site in post-mitotic neurons at least *in vitro* (de Anda et al 2005). Although this work awaits *in vivo* confirmation, it is tempting to hypothesize that there is a functional relationship between (1) the apical position of the Par3/Par6/aPKC/cdc42 polarity complex, (2) the centrosome position and (3) the axon initiation site during the multipolar to bipolar transition (**Figure 6B**). Based on the fact that cdc42 has been shown to be required for axon specification *in vivo* and that interference with Par3/Par6 and aPKC signaling also has an impact on neuronal polarization at least *in vitro*, this hypothesis of a functional relationship between the apico-basal polarity of neural progenitors and the axon-dendrite polarity of post-mitotic neurons represents an exciting avenue for future investigations.

SPECIFICATION OF DENDRITIC IDENTITY

Axon specification is for many neurons, both *in vitro* and *in vivo*, the earliest symmetry breaking event. Our review has focused on this event because the majority of recent studies have identified molecular regulators of axon specification but very few studies have identified genes involved in specification of dendritic identity. On the other hand, the molecular mechanisms involved in dendritic growth, guidance and branching are intensely studied but we will refer to recent, exhaustive reviews on this topic (Grueber et al 2005, Scott & Luo 2001, Whitford et al 2002a).

As described earlier, motor proteins can affect neurite identity and CHOP/MKLP1 is required for dendrite initiation (Sharp et al 1997) and maintenance (Yu et al 2000). As

mentioned earlier, the polarized orientation of the Golgi appears to be another event during dendrite outgrowth (Horton et al 2005). Work in cells from the superior cervical ganglion, that do not form dendrites when cultured at low density, demonstrates an astounding dendritogenesis in response to the soluble ligand bone morphogenetic protein 7 (BMP7/OP-1) (Lein et al 1995). This effect requires Smad1 signaling and inhibition of proteasome function can disrupt this activity (Guo et al 2001). In addition to Smad1 signaling in dendrite formation, the activated BMP receptor 2 has also been shown to bind and facilitate the activation of the kinase LIMK1 (Lee-Hoeflich et al 2004). This would lead to increased cofilin phosphorylation, a situation previously shown to inhibit axon formation (Garvalov et al 2007) and may bias neurites to become dendrites. The small GTPase Rit has proved to antagonistic to BMP stimulated dendrite formation by stimulating ERK1/2 activation (Lein et al 2007). The related ligand TGF- β has a similar affect on dendrite initiation in *Xenopus* retinal ganglion cells (Hocking et al 2008).

These results present a picture of dendrite formation utilizing the same or related pathway as those seen previously for axogenesis. This is consistent with observations made for a secreted cue, Semaphorin 3A, that can regulate the site of axonal outgrowth (Polleux et al 1998) and simultaneously the orientation and outgrowth of dendrites by activating differentially localized transduction machinery (Chen et al 2008, Polleux et al 2000). Interestingly, the loss of the axogenic kinase LKB1/Par4, affected neither migration nor dendrite formation. Given the identity of the leading processes of migrating neuron in the cortex, this may suggest that regulators of migration may also control dendrite formation as seen for Sema3A (Chen et al 2008). The outgrowth of *Drosophila* neurons also provides strong evidence for extracellular cues in regulating dendritogenesis. Two cues implicated in dendrite formation and orientation are the Slit-Robo (Furrer et al 2007, Godenschwege et al 2002, Whitford et al 2002b) and netrin-frazzled (DCC ortholog) (Furrer et al 2003), (reviewed in (Kim & Chiba 2004)). Similar mechanisms are described below with regard to axon formation in the nematode.

IN VIVO IDENTIFICATION OF NEURONAL POLARITY REGULATORS IN MODEL ORGANISMS

While many of the molecules and phenomena described to this point have been established using *in vitro* approaches using mammalian neurons, important progress has been made using model organisms. In particular, this approach has resulted in significant progress in the area of extracellular cues that instruct axon specification *in vivo*. The neurons of the nematode have a stereotyped morphology, e.g., specific projections along the anterior-posterior body axis. Two studies have identified the diffusible signal Wnt and its receptor as critical regulators of neuronal polarity (Hilliard & Bargmann 2006, Prasad & Clark 2006). In addition to identifying loss of function for Lin-44 (Wnt) and its receptor Lin-17 (Frizzled), one of the screens identified VPS-35, a component of retromer complex which regulates vesicular traffic and is required for proper Wnt secretion (Prasad & Clark 2006). Further experiments have identified another extra-cellular cue, UNC-6 (netrin) along with its receptor UNC-40 (DCC), as a critical gene orchestrating axon specification *in vivo* (Adler et al 2006) (**Figure 4**). This work also identified down-stream proteins in this pathway, including (mammalian orthologs are shown in parenthesis when known): AGE-1 (PI3K), DAF-18 (PTEN), UNC-34 (Enabled), CED-10 (Rac), UNC-115/AbLIM, and MIG-10/Lamellipodin. The current model for the relationship of these genes to the signaling of UNC-6 involves DAF-18 limiting the activity of AGE-1 following UNC-40 stimulation and the asymmetric recruitment of MIG-10 to the plasma membrane (see Figure 4). Very recent work has shown that this recruitment requires activated CED-10 directly binding to MIG-10 and the involvement of the PAK-like kinase, Pak-1 (Adler et al 2006). The involvement of a kinase involved in cytoskeletal rearrangement is consistent with the similar role of MIG-10

and the likely mechanism it operates under once recruited to the plasma membrane to stimulate directed neurite outgrowth. Another regulator thought to act in concert to drive filopodial formation with MIG-10 is the Enabled homolog mentioned above, UNC-34 (Chang et al 2006). SLT-1 (Slit) is another extracellular cue that appears to act through MIG-10 recruitment (Chang et al 2006) to control neuronal polarity. Interestingly, recent work using mammalian neuronal precursors links repolarization of migrating neurons to Slit signaling mediated by GSK3 β and aPKC (Higginbotham et al 2006). Subsequent genetic screens to understand these neuronal defects have identified more genes that underlie this phenotype. These include a component of the AP-2 adaptor complex (*dpy-23*) and in the same study it is shown the both *dpy-23* and the VPS-35 mutants act by perturbing MIG-14 (Wntless) and its role in UNC-6 secretion (Pan et al 2008). Four other genes have shown to be involved in this signaling, the SLT-1 receptor Sax-3 (robo), the kinesin-like protein VAB-8, and the RacGEF UNC-73 (trio), and the small GTPase MIG-2 (Levy-Strumpf & Culotti 2007, Watari-Goshima et al 2007). Analysis of these genes reinforces the roles of UNC-6 and SLT-1 and somewhat surprisingly given their expected role as receptor effectors, VAB-8, UNC-73 and MIG-2 act upstream of Sax-3 and UNC-40 to control the localization of these receptors. This observation suggests a positive reinforcement mechanism to enrich receptors in the locale of previously activated ones. Genetic evidence from Vab-7 (even-skipped) mutants also indicates that stereotyped responses to cues can result as part of a larger transcription factor controlled program of cell fate. It will be important to test if the mammalian orthologs of any of these genes play a role in the specification of neuronal polarity in higher vertebrates.

CONCLUSION

The polarization of axon and dendrites underlies the ability of neurons to integrate and transmit information in the brain. Important progress has been made toward the identification of the molecular mechanisms regulating neuronal polarization using primarily *in vitro* approaches such as dissociated culture of rodent hippocampal neurons. The predominant view emerging from this paradigm is that neuronal polarization is initiated by intrinsic activation of signaling pathways underlying the initial break in neuronal symmetry that precedes the future asymmetric growth of the axon. Recent evidence shows that (i) axon–dendrite polarization is specified when neurons engage migration *in vivo*, (ii) that a kinase pathway defined by LKB1 and SAD-kinases (Par4/Par1 dyad) is required for proper neuronal polarization *in vivo* and that (iii) extracellular cues can play an instructive role during neuronal polarization both *in vitro* and *in vivo*. Here, we reviewed some of these recent results and highlight future challenges in the field including the determination of how extracellular cues control intracellular responses underlying neuronal polarization *in vivo*.

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Figure 1. Cell-type specific patterns of neuronal polarization *in vivo*

Examples of the sequence of events leading to the polarized emergence of axon and dendrites in four distinct vertebrate neuronal cell types *in vivo*. Throughout these figures, the nascent axon is depicted in red and the somatodendritic domain in purple. **(A)** *In vivo* polarization of retinal ganglion cells in zebrafish (*Danio rerio*) and mouse (*Mus musculus*). Neuroepithelial progenitors characterized by an apical and a basal attachment undergo asymmetrical cell division at the apical surface (A1-A3). Upon cell cycle exit, the nucleus undergoes basal translocation (A4) and specifically loses its apical attachment while its basal process starts growing along the basal membrane (A5). The axon (red) therefore emerges from the basal process and the dendrite emerges from the apical process (A6). **(B)** Polarization of mouse bipolar cells in the mouse retina. Neuroepithelial progenitors (B1) transform into bipolar cells by first losing their basal attachment which starts branching in the inner plexiform layer (IPL) while the apical process starts branching in the prospective outer plexiform layer (OPL) before (B2) losing its apical attachment (B3). The axon emerges from the basal process (red) and the dendrite emerges from the apical process (B4). **(C)** Polarization of granule cells in the mammalian cerebellum. Granule cell progenitors divide rapidly in the external plexiform layer (EGL; C1) and upon cell cycle exit start adopt a bipolar morphology (C2) before migrating tangentially with a leading and a trailing process (C3). Another process emerges from the cell body in the tangential direction (C4) and becomes the leading process, leading its migration towards the inner granule layer (IGL; C5). The trailing processes form a characteristic T-shaped axon (red in C6) whereas the leading process gives rise to the dendritic domain. **(D)** Polarization of radially migrating pyramidal neurons in the mammalian neocortex. Neurons are generated between E11 and E17 by radial glial progenitors in the ventricular zone of the mouse neocortex. These cells have a long basal (radial) process attached to the basal membrane at the pial surface and a short apical process on the ventricle side (D1; see detail in Figure 5). Upon cell cycle exit through asymmetric cell division (D2), the post-mitotic neuron (blue) goes through a multipolar transition where multiple neurites emerges rapidly from the cell body (D3) before one major process forms in the radial direction (D4) and becomes the leading process (LP). At this point, the neuron initiates radial translocation along a radial glial process (D5) and leaves behind a trailing process which elongates tangentially in the intermediate zone (red). The cell body continues to translocate to towards its final destination (the top of the cortical plate; CP) while the axon rapidly elongates (D6). The leading process gives rise to the apical dendrite (purple in D7) which initiates local branching in the marginal zone while over the first postnatal week (until radial migration ends) the cell body will translocate ventrally (D8-D9) as neurons born at later stages (in orange D10) bypass their predecessors (inside-out accumulation pattern).

Adapted from: (a.) Hinds & Hinds (1978) and Zolessi et al (2006); (b.) Morgan et al (2006); (c.) Rakic (1971), Komuro and Rakic (2001) and Gao and Hatten (1993); (d.) Shoukimas and Hinds (1978), Rakic (1972), Noctor et al. (2003), Hatanaka and Murakami (2002)

Figure 2. Parallel between neuronal polarization *in vitro* and *in vivo*

Comparison of the sequence of events leading to the polarization of cortical pyramidal neurons *in vivo* and *in vitro*. **(A)** As depicted in Figure 1D, the axon-dendrite polarity of pyramidal neurons is derived from the polarized emergence of the trailing (TP) and leading processes (LP) respectively. *In vivo*, pyramidal neurons acquire other key features of their terminal polarity such as the axon initiation segment (AIS; yellow cartridge) and dendritic spines (pink protrusions) during the first postnatal weeks of development. **(B)** In dissociated cultures, postmitotic cortical neurons display specific transitions as classically described for hippocampal neurons by (Dotti et al 1988). At Stage 1, immature post-mitotic neurons display intense lamellipodial and filopodial protrusive activity which leads to the emergence of single immature neurites, Stage 2. Stage 3 represents a critical step where neuronal symmetry breaks and a single neurite grows rapidly to become the axon (red) while other neurites acquire dendritic identity. Stage 4 is characterized by rapid axon and dendritic outgrowth. Finally, Stage 5 neurons are terminally differentiated pyramidal neurons harboring dendritic spines and the AIS.

Figure 3. Signaling pathways involved in mammalian axon specification during neuron polarization

See text for details. G-protein coupled receptor (GPCR). Extra-cellular matrix (ECM). Red arrows indicate negative regulation, whereas blue arrows indicate activation.

Figure 4. Experimental evidence for the instructive effects of extracellular cues on neuronal polarization *in vitro*

(A-B) When hippocampal neurons are plated on stripes coated with alternating substrates such as the cell-adhesion molecule NgCAM (blue) and Poly-L-Lysine (PLL) plus laminin (yellow), the first neurite contacting the a new substrate (arrowhead in A and B) is specified to become the axon (red). Note that this axon specification event can occur regardless of the type of substrate interface (see A and B) suggesting that in these conditions, axon specification can occur when unspecified neurites detect a relative change in the substrate composition rather than a specific substrate. Adapted from (Esch et al 1999) (C-E) Hippocampal neurons plated on control stripes coated with PLL (purple) or PLL plus Bovine Serum Albumin (BSA) show no trend for axon specification when neurites encounter a stripe boundary (C), whereas an immature neurite encountering a stripe containing Brain Derived Neurotrophic Factor (BDNF) become an axon (D). This effect is abrogated if neurons express a non-phosphorylatable form of LKB1 (LKB1^{S431A}; E). Adapted from (Shelly et al 2007). (F-G) The slice overlay assay was developed to test if the cortical wall contains extracellular cues that could polarize axon emergence towards the ventricle. In this assay, immature E18 rat cortical neurons are dissociated and fluorescently labeled with DiI before being plated onto isochronic E18 or heterochronic (P3) cortical slices. Only three hours after plating most neurons have a short neurite becoming the axon allowing to test between two hypothesis: in scenario 1, polarized axon emergence is the sole result of intrinsic polarization inherited, for example, from neuroepithelial cell progenitors (see Figures 5 and 6). In this case, the plated neurons should show a randomized direction of axon emergence. According to scenario 2, graded extracellular cues are able to polarize the direction of axon emergence and therefore, neurons plated on the slice should show a directed axon outgrowth towards the ventricle. Polleux et al. (1998) demonstrated that scenario 2 is the most likely one since the overwhelming majority of pyramidal neurons present in the cortical plate show directed axon outgrowth in this assay, only few hours after plating (arrowheads in **Fig. 4G**, (Polleux et al 1998)).

Figure 5. Experimental evidence for instructive effects of extracellular cues on neuronal polarization *in vivo*

(A) In *C. elegans*, the immature HSN neurons undergo a series of morphological changes leading to the polarized outgrowth of their axon ventrally at larval stage 4 (L4) (A1). Most noticeably, the polarized lamellipodial outgrowth observed at stage early L2 correlates with a polarized distribution of the attractive Netrin receptor (Unc40) and the intracellular cytoskeletal effector Lamellipodin (MIG-10) (A2) and also requires the presence of Netrin (UNC-6) secreted from the ventral part of the embryo since the *Unc6* mutant shows non-directional process outgrowth at stage early L2 accompanied by non-asymmetrical distribution of UNC-40 (A3). Adapted from (Adler et al 2006) (B) Axon polarization during

the migration of *Xenopus* retinal ganglion cells. As described in Figure 1A, retinal ganglion cells inherit their axon-dendrite polarity as their cell body translocate basally to the ganglion cell layer. In these cells, the basal process of the dividing progenitor gives rise to the leading process of the migrating RGC which becomes the axon (red). Using live cell imaging Zolessi et al. demonstrated that the centrosome and the polarity complex protein PAR3 are localized to the apical side of the RGC during translocation. The apical membrane containing atypical Protein Kinase C (aPKC), α -catenin and F-actin is also localized apically in the translocating RGC in the trailing process. Therefore, in RGC, the PAR3/apical polarity complex is localized in the trailing process which becomes the dendritic domain whereas on the basal side, the leading process becomes the axon which grows rapidly along the basal membrane. Adapted from (Zolessi et al 2006). (C) A cellular and molecular model of the function of LKB1 and SAD kinases in the polarization of pyramidal cortical neurons. (C1) Upon asymmetric cell division of radial glial progenitors, early unpolarized post-mitotic neurons show a transient phase of non-directed neurite outgrowth in the subventricular zone before adopting a bipolar morphology in the intermediate zone where they engage radial migration with a leading process directed toward the pial surface and a trailing process directed toward the ventricle. (C2) On the basis of recent reports (Barnes et al 2007, Shelly et al 2007), we propose that **in vivo**, the trailing process is specified to become the axon in response to putative extracellular cues that preferentially induce phosphorylation of LKB1 on Serine 431. Modified from (Barnes et al 2008).

Figure 6. Relationship between epithelial cell polarity, neuroepithelial progenitor polarity and post-mitotic neuron polarity

(A) Parallel between epithelial cell polarity and neuroepithelial cell polarity. Epithelial cells have two main cell membrane compartments: the apical membrane and the baso-lateral membrane separated by tight junctions (TJ) and adherens junctions (AJ) which act both as cell adhesion sites and strong diffusion barriers preventing direct exchange between the apical and the baso-lateral domains. Neuroepithelial progenitors form a pseudo-epithelium *in vivo* which is characterized by a basal attachment through their radial process to the pial surface and an apical domain separated by Cadherin-based adherens junctions. (B) Potential model for the molecular control of apical polarity in cortical neuroepithelial progenitors and its potential relationship with post-mitotic neuron polarity. Several protein complexes recently involved in apical polarity of cortical progenitors include (1) the centrosomal proteins Cep120-TACC1-3 and microtubules, (2) Numb-regulated adherens junctions composed of cadherins and catenins, and (3) atypical protein kinase C (aPKC)-cdc42-Par3/6 (see text for details). Question marks point to unresolved issues regarding the functional interactions between specific molecular components of the apical polarity complex. During cell cycle exit, the centrosome might play a role in axon specification (de Anda et al 2005) by leaving an ‘apical trace’ transiently localizing to the base of multipolar neurons which might play a role in specifying the position of the trailing process (future axon) before translocating to the base of the leading process (future apical dendrite) when neurons initiates radial translocation (Tsai et al 2007).