The Oriented Emergence of Axons from Retinal Ganglion Cells Is Directed by Laminin Contact In Vivo

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INTRODUCTION

The extension of axons and dendrites from the cell body marks the dramatic morphological polarization of the typical neuron. This morphology is critical because it is tightly coupled to neuronal network function, where electrical information is picked up in the dendrites and transmitted down the axon. The first step in the generation of neuronal networks, therefore, is the efficient coordination of neuronal polarization. This can also be thought of as the first step in axonal/dendritic guidance. Thus, how this orientation decision is regulated, and how the appropriate axis is selected from the myriad of possibilities offered by a 3D tissue, is an important question in developmental neurobiology, yet little is known about how this happens within the embryonic nervous system.

In the late 1980s it was discovered that isolated hippocampal neurons plated on homogeneous substrates first undergo a period of randomly oriented explorations, but then project a single axon and multiple dendrites in the absence of any polarizing extracellular cues (Dotti et al., 1988). These neurons progress through a staged series of behaviors, including a prolonged multipolar phase, known as Stage 2, where dynamic neurites are extended and retracted in various orientations from the cell body. Morphological symmetry is broken in Stage 3, when one neurite grows much longer than the others and develops into the axon (Craig and Banker, 1994). This remarkable cellular behavior of neurons in tissue culture has allowed investigators to identify many factors involved in the extension of a single axon from a set of more or less equivalent neurites (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; Tahirovic and Bradke, 2009), and to formulate a general model of cytoskeletal regulation, which lies at the heart of neuronal polarization. In this model, localized actin destabilization in the preaxonal neurite leads to increased microtubule penetration and stabilization in this neurite, which as a result, makes it grow faster than the others neurites and so become the axon (Bradke and Dotti, 1999; Witte et al., 2008). Once selected, the axon upregulates cAMP. This directs the other neurites to become dendrites, which upregulate cGMP. This reciprocal cAMP/cGMP regulation acts as a symmetry-breaking positive feedback loop, ensuring that only a single axon is formed (Sheily et al., 2010).

Strikingly, classes of neurons develop along preferred aligned orientations in vivo, rather than at the random orientations chosen in culture. For example, all retinal ganglion cells (RGCs) send out their axons from the basal part of the cell body (Hinds and Hinds, 1974). What orients axon emergence in vivo? Neurons in the brain derive from highly polarized neuroepithelial cells, with distinct apical and basolateral domains (Randlett et al., 2010). Many cell cycles in advance of any neuronal differentiation, these cells already exhibit polarized behaviors, such as cell divisions only at the apical surface and apically directed movements of the nucleus prior to mitosis during interkinetic nuclear migration (Norden et al., 2009). Also, at the division preceding the neuron’s birth, intracellular factors such as the apical complex, the centrosome, and the Golgi apparatus may become localized to one pole of the cell, leading to an intrinsic cellular polarity (Calderon de Anda et al., 2005, 2008).

The model of intrinsically regulated axon emergence meshes well with some studies in cultured hippocampal neurons, where...
the position of the apical complex and centrosome seems capable of influencing the nearest neurite to become an axon (Calderon de Anda et al., 2005; Shi et al., 2003). Whether the position of apical complex components and the centrosome actually specifies the position of the axon in vivo is, however, controversial. In mice, the initiation of the apically directed axon in cortical pyramidal neurons correlates with the reorientation of the centrosome to a position apical to the nucleus (Calderon de Anda et al., 2010). In Drosophila, however, apical complex components and the centrosome appear to be completely dispensable for normally oriented neuronal polarization (Basto et al., 2006; Rolls and Doe, 2004). In zebrafish RGCs, the apical complex and the centrosome are localized apically while the axon emerges basally (Zolessi et al., 2006). Therefore, a clear consensus on the role of intrinsic factors in the generation of oriented axon emergence has not yet been reached.

This led us to examine the polarized environment in which the differentiating neurons reside. Developing neurons in vivo live in an environment that is far from homogeneous because there are extracellular biases along the apico-basal axis. This polarity, we previously argued (Zolessi et al., 2006), could serve to direct the site of axon genesis in vivo. Is it the case that external cues acting directly upon polarizing neurons result in axon emergence toward or away from the stimulus? In support of this idea, neuron polarization in vitro can be directed by asymmetric presentation of Netrin 1, BDNF, TGF-β, cAMP/cGFP, or Sema3a, or by contact with cell adhesion or extracellular matrix molecules (Esc et al., 1999; Gupta et al., 2010; Mai et al., 2009; Ménager et al., 2004; Polleux et al., 1998; Shelly et al., 2007). There is also some in vivo evidence for the importance of extracellular cues directing neuronal polarization in C. elegans, where HSN neurons require Netrin/Unc-6 signaling to orient axon extension, and disruptions in Wnt signaling result in inversions in the polarity of PLM and ALM neurons (Adler et al., 2006; Hilliard and Bargmann, 2006; Prasad and Clark, 2006).

Evidence for the importance of extracellular cues in vertebrate neuronal polarization has been more challenging to establish. Recent studies combining in vitro experiments and in vivo electroporation techniques in mice found that the type II TGF-β receptor and LKB1 are required for neuronal polarization in the cortex, and localized BDNF can direct neuronal polarization in vitro through LKB1 phosphorylation. This led to the hypothesis that gradients of TGF-β and/or BDNF could be orienting neuronal polarization in the cortex (Shelly et al., 2007; Yi et al., 2010). However, neurons with disruptions in these genes and elsewhere often fail to put out axons at all, leaving the question of the initial orientation of axons unresolved (Barnes et al., 2007; Calderon de Anda et al., 2010; de la Torre-Ubieta et al., 2010; Kishi et al., 2005; Shelly et al., 2007; Yi et al., 2010).

To investigate whether an extracellular cue does influence the orientation of axonogenesis in vivo, we make use of RGCs in the zebrafish retina. We can image these cells at short time intervals at subcellular resolution from genesis through polarization and axon extension, within a living embryo (Poggi et al., 2005; Zolessi et al., 2006). RGCs are born at the apical surface of the retina, and translocate their cell body toward the basal surface, where the ganglion cell layer will develop. As the apical process detaches from the apical surface of the retina, the axon extends directly from the basal surface of the RGC, showing no prolonged, multipolar, Stage 2 behavior. In nok zebrafish mutants, in which the transmembrane protein Pais-1/Stardust is defective, the pigment epithelium is disrupted and newly generated RGCs sometimes come in contact with the basal lamina of the pigment epithelium (Bruch’s membrane) instead of the basal lamina of the neural retina. When they do so, they polarize upside-down, suggesting that the basal lamina is responsible for RGC polarity (Zolessi et al., 2006). Here we demonstrate that RGC polarization toward the basal lamina requires the presence of an extracellular cue, Laminin 1 (Lam1). In the absence of Laminin α1 (Lamα1), RGCs exhibit Stage 2 behavior and mispolarization. Contact of newborn RGC processes with Lam1 either in vitro or in vivo is sufficient to cause the specific accumulation of Kif5c560-YFP, a marker of axonal microtubules, followed by axon extension. Thus, in the normal retina, basally localized Lam1 directs the normal orientation of RGC axon extension in vivo.

RESULTS

RGC Axon Extension Is Preceded by Directed Kif5c560-YFP Accumulation in the Tip of the Basal Process In Vivo

Live imaging in zebrafish demonstrated that axons extend directly from the most basal portions of RGCs in vivo (Zolessi et al., 2006). This previous study, however, was limited by the unavailability of an intracellular marker of axonogenesis. The earliest reported marker for definitive axon commitment during hippocampal neuron polarization is the constitutively active motor domain of Kinesin 1 fused to YFP, Kif5c560-YFP. This construct selectively accumulates in axons, directed by biochemical differences in axonal microtubules; perhaps reflecting stabilized microtubules (Hammond et al., 2010; Jacobson et al., 2006; Konishi and Setou, 2009). During Stage 2, Kif5c560-YFP displays a remarkably dynamic behavior, where YFP signal accumulates in just one (or sometimes a few) neurites, but this accumulation is only transient, passing from one neurite through the cell body to another neurite (Jacobson et al., 2006). As the neuron progresses to Stage 3 and the axon is selected, Kif5c560-YFP accumulates specifically and permanently in the tip of the preaxon process, and remains there during axon extension (Jacobson et al., 2006). Thus, the oscillatory behavior provides a visual readout of the uncommitted Stage 2 phase, and the cessation of this oscillation and stable Kif5c560 accumulation in one neurite marks axonal commitment.

To see whether this marker of axonogenesis behaves in the same manner in zebrafish RGCs, we performed time-lapse imaging of ath5:GAP-RFP transgenic embryos injected with Kif5c560-YFP RNA at the one-cell stage to label RGCs (Poggi et al., 2005; Zolessi et al., 2006). At 30 hours post-fertilization (hpf), the approximate onset of RGC genesis, eyes were dissected and dissociated to obtain isolated cells. After a 12–15 hr incubation at 28.5°C, many RGCs had extended long axons, with bright Kif5c560-YFP signal accumulation within their growth cones (Figure 1A). This confirms that the rat construct maintains its abilities to recognize axonal microtubules and to accumulate in the axonal growth cones of zebrafish RGCs. To test the dynamics of Kif5c560-YFP during
polarization, ath5:GAP-RFP-positive neurons were imaged for 12–20 hr beginning at 1–2 hr after plating. Consistent with what was reported for cultured hippocampal neurons, the YFP signal in multipolar RGCs demonstrated an oscillatory behavior, where signal accumulation was seen traveling between different areas of the cell body and individual neurites, typical of Stage 2 behavior (arrows, cyan phase). The construct eventually stabilizes in a single neurite (white arrows, yellow phase), and this neurite extends to form the axon (green phase). Figure is related to Movie S1. Time is shown in hr:min; scale bars = 10 μM.

We next analyzed how this construct behaves in RGCs polarizing in vivo. Injection of in vitro synthesized Kif5c560-YFP RNA resulted in homogeneous expression in all cells. It was immediately evident that Kif5c560-YFP accumulates basally in the retinal neuroepithelium, even before neurogenesis begins, resulting in a ring of YFP signal surrounding the lens (Figure 2A). To assess the localization and dynamics of Kif5c560-YFP at the single-cell level, we used a transplantation approach to create mosaic embryos (Figure 2B). ath5:GAP-RFP transgenic embryos were used because all RGCs are brightly labeled through ath5-regulated fluorescent protein expression, and RGCs can be imaged from before their birth through polarization and axon extension (Poggi et al., 2005). These embryos were injected with Kif5c560-YFP RNA and P53 morpholino at the one-cell stage, and blastomeres were transplanted into unlabeled host embryos, generating mosaic embryos, where individual cell behaviors could be tracked by time-lapse confocal microscopy. Using this strategy it was apparent that the Kif5c560-YFP construct accumulates basally in all neuroepithelial cells during interphase, being mostly confined to the basal processes. During mitosis and cytokinesis, however, diffuse labeling in the cell body was seen (Figure 2C). The lack of spindle microtubule labeling during mitosis is consistent with the idea that Kif5c560 recognizes stabilized microtubules, and will not label the dynamic spindle microtubules.

Consistent with the in vitro data, imaging of RGC axons extending within the eye demonstrated that Kif5c560-YFP accumulation is highly directed in polarizing RGCs in vivo. At the end of the final mitosis marking the birth of RGCs, when RFP signal begins to increase in neonatal RGCs, Kif5c560-YFP is still mainly in the cell body (Figure 2D). Very soon after this, however, a Kif5c560-YFP-positive basal process extends from the cell body. The YFP signal spans a large portion of the re-extending basal process at this time (red arrowheads, Figures 2D and 2F, Movie S2). At the point when the basal process appears to contact the basal surface of the retina, the YFP signal spans a large portion of the re-extending basal process at this time (red arrowheads, Figures 2D and 2F, Movie S2). The length decreases from 10.6 ± 0.6 μm (mean ± SEM) before contact, to 6.1 ± 0.4 μm after contact.
Normalizing the measured lengths to the longest length observed for each cell, and centering the data on basal surface contact (t = 0), the trend of decreasing Kif5c560-YFP signal length immediately following basal surface contact is apparent (Figure 2I). This specific accumulation remains until a

Frames are taken from Movie S2. Time is shown in hr:min; scale bars = 10 μM.
Kif5c560-YFP-positive growth cone sprouts from the cell and extends toward the optic nerve head. The YFP signal remains accumulated in the growth cone throughout this extension, and is not visible in the remainder of the cell.

**Laminin at the ILM Is Required for Directed Axon Extension**

Because the specific accumulation of Kif5c560-YFP at the tip of the basal process correlates, both in time and in space, with RGCs contacting the basal surface of the retina, we hypothesized that an extracellular cue localized to this region plays a role in this event. The extracellular matrix component Lam1 is a heterotrimer consisting of three subunits (α1, β1, and γ1), and contact with Lam1 is known to be able to polarize neurons in vitro and promote axon growth in RGCs (Esch et al., 1999; Menager et al., 2004). Moreover, it has been shown that zebrafish embryos lacking the Lamα1 subunit display severe axon guidance defects in multiple neuronal types, including RGCs (Paulus and Halloran, 2006). Using a polyclonal antibody raised against Lam1, strong staining is seen at the basal lamina lining the surfaces of the zebrafish retina (Figure 3Ai) (Lee and Gross, 2007), making it a strong candidate for directing RGC polarization.

**Figure 3. Lamα1 Is Necessary for Directed RGC Polarization**

(Ai) Immunofluorescent staining of aths5:GAP-GFP embryos with polyclonal rabbit anti-Lam1 antibody reveals strong staining at the basal lamina lining the basal surface of the retina, or ILM (B, arrow), as well as at the basal lamina of the RPE, or Bruch’s membrane (A, arrowhead). (Aii) Injection of an antisense morpholino targeted against Lamα1 results in efficient loss of Lam1 staining at the basal surface (arrow), while Lam1 staining at Bruch’s membrane remains. Images are of a single confocal slice. (B) Confocal reconstruction from a WT 3 dpf retina (Bi) demonstrating the highly ordered nature of the ganglion cell layer (GCL) and the RGC axon fascicles (†) collecting to form the optic nerve (ON). (Bii) After lamα1 morpholino injection, the GCL is disorganized, as are the axon fascicles, which meander through the retina before collecting to form the ON. (C) Mosaic embryos with WT aths5:GAP-GFP-labeled RGCs in a lamα1 morphant environment were analyzed by time-lapse confocal microscopy beginning at approximately 35 hpf. (Cii) RGCs in this environment progress through a transient multipolar phase (marked by †, cyan phase) before projecting an axon (arrow, green phase). (D) Mosaic embryos with lamα1 morphant, aths5:GAP-GFP-labeled RGCs in a WT environment were analyzed by time-lapse confocal microscopy. (Dii) Morphant RGCs behave normally in this environment, and axons project directly from the basal surface of the cell (marked by arrowheads). (E) Mosaic embryos with WT aths5:GAP-GFP-expressing, Kif5c560-YFP-labeled RGCs in a lamα1 morphant environment were analyzed by time-lapse confocal microscopy. (Ei) In this context, Kif5c560-YFP signal accumulation (marked by cyan arrows) oscillates between the cell body and transient neurites (cyan phase) before stably accumulating in a single neurite (marked by white arrows, yellow phase) that extends to form the axon (green phase). Note that the individual confocal z-slices were cropped to remove signal not associated with the cell. A reconstruction of the uncropped frames is shown in Movie S5. Frames are taken from Movies S3, S4, and S5. Time is shown in hr:min; scale bars = 10 μM.
To test the necessity for Lam1 in the normal polarization of RGCs in vivo, we injected a previously described lam1 morpholino (Pollard et al., 2006) into ath5:GAP-GFP transgenic embryos. Morpholino injections generally resulted in a complete loss of the Lam1 staining at the ILM (Figure 3Aii). Strong Lam1 staining remained at Bruch’s membrane at the apical retinal surface, indicating that other α chains could be compensating for the loss of Lam1 in this region. However, because the RPE acts as a physical barrier between Bruch’s membrane and retinal neurons, for our purposes we can assume that the Lam1-deficient retina is devoid of any accessible Lam1. When fixed at 3 dpf and imaged by confocal microscopy (Figure 3B), morphants showed an apically displaced RGC layer, and instead of the highly ordered axon fascicles, RGC axons in Lam1 morphants demonstrated severe intra-retinal disorganization. Axons bundles in morphant retinas often took meandering paths through the retinal neuroepithelium prior to collecting at the optic disk to exit the eye.

This axonal disorganization phenotype could be due to axon misguidance, or to problems with neuronal polarization. To differentiate between these two possibilities, we performed time-lapse imaging experiments. Blastomeres were transplanted from ath5:GAP-GFP transgenic embryos to Lam1 morpholino-injected host embryos, resulting in mosaically labeled WT RGCs in a Lam1-deficient retina (Figure 3C, Movie S3). In this environment, RGCs were observed to progress through a prolonged multipolar phase, where many short neurites were extended from the cell prior to axon extension. Axon extension was often misoriented, projecting from regions of the cell body other than the most basal point. In contrast, when blastomeres were transplanted from ath5:GAP-GFP transgenic embryos injected with Lam1 morpholino into WT host retinas, RGCs polarized normally (Figure 3D, Movie S4). The multipolar stage was not seen, and axons extended directly from the basal surface of the RGC, confirming that the Lam1 morphant phenotype is non-cell-autonomous. To quantitively this effect, we measured the time spent in a multipolar state. The time elapsed between the extension of the first observable dynamic/unstable neurite and the extension of the stable process that became the axon was measured. If the first process that extended became the axon, then Δt = 0 min. When transplanted into a Lam1 morphant environment, RGCs spent 169 ± 4 min in a multipolar state (mean ± SEM, n = 10 cells from six embryos, where one cell had Δt = 0), while Lam1 morphant cells transplanted into the WT environment extended a stable axon after a significantly shorter time, 37 ± 2 min (n = 14 cells from seven embryos, where five cells had Δt = 0; p = 0.0028, Mann-Whitney test). Therefore, in the absence of environmental Laminin, RGCs lose their directed polarization behavior, and progress through a multipolar stage, where multiple short neurites are extended from the cell body.

We next used Kif5c560-YFP to visualize the intracellular polarization behavior in vivo when RGCs lack environmental Lam1. We transplanted cells from ath5:GAP-RFP transgenic embryos coinjected with Kif5c560-YFP mRNA into lam1 morphant host embryos (Figure 3E, Movie S5). Time-lapse microscopy demonstrated that, similar to RGCs in WT retinas, Kif5c560-YFP is localized to the cell body as ath5:GAP-RFP expression begins to increase. As RGCs progressed through the multipolar phase, Kif5c560-YFP accumulated in some transient neurites, but this accumulation was not stable and the signal moved back into the cell body upon process retraction. This led to an oscillation of YFP signal accumulation between the cell body and different short neurites typical of Stage 2 neurons. Kif5c560-YFP eventually stably accumulated in a single neurite, which did not retract, and extended as a growth-cone-tipped axon. Therefore, with respect to the Kif5c560-YFP marker, RGCs polarizing in retinas lacking Lam1 behave more similarly to cultured neurons than they do to RGCs polarizing in WT retinas.

**RGCs Polarizing In Vitro and in Lam1 Morphant Retinas Show Dynamic Centrosomal Movement**

Centrosomal localization has been suggested to be important for neuronal polarization in some neurons (Calderon de Anda et al., 2008, 2010; Zmuda and Rivas, 1998), but not in others (Basto et al., 2006; Seetapun and Odde, 2010). In zebrafish retinal neuroepithelial cells, the centrosome is localized to the tip of the apical process. Live imaging in zebrafish demonstrated that this apical centrosome localization is maintained during RGC axon extension in vivo (Zolesi et al., 2006).

To examine the role of the centrosome in RGC polarization further, we first dissociated RGCs from ath5:GAP-RFP/Centrin-GFP transgenic embryos and imaged them during axon extension (Figures 4A and 4B, Movies S9 and S10). Although centrosomes were reported to be stably positioned within the cell body in cultured neurons in other systems (Calderon de Anda et al., 2005, 2008), centrosomes in cultured RGCs exhibited remarkably dynamic behavior. They mainly scooted around the cell body, and could also be seen darting into neurites in some instances (Figure 4B, t = 04:00). The dynamic centrosome behavior was evident both in multipolar Stage 2 RGCs and in Stage 3/4 RGCs that had extended long axons. To test for a spatial relationship between extended axons and centrosome position, we performed centroid analysis by dividing the cell body of RGCs that had extended long axons into four quadrants relative to the base of the axon. This demonstrated that centrosome positioning is not significantly biased to any of these quadrants (Figure 4C, p = 0.9536, Chi square test, n = 33 cells).

Therefore, a simple correlation between centrosome position and neuronal polarity is not apparent in cultured RGCs, suggesting that its position is not important in this context. However, imaging of the centrosome provided a second intracellular marker that behaves differently in the in vivo and in vitro (Stage 2) context. For this reason, we looked at centrosome behavior within RGCs in vivo, both in WT and Lam1-deficient retinas. Blastomeres were transplanted from ath5:GAP-RFP/Centrin-GFP into either WT or lam1 morpholino-injected embryos, respectively. Consistent with previous observations (Zolesi et al., 2006), RGCs within a WT environment demonstrated static and apical centrosomal localization which persisted in maturing RGCs until the formation of the inner plexiform layer (IPL) was clear. However, in Lam1-deficient retinas, the centrosome is localized to the apical process. This demonstrated that centrosome positioning is not significantly biased to any of these quadrants (Figure 4D, Movie S7). When developing within a Lam1-deficient environment, the centrosomes of RGCs were initially observed in the proper position apically, at the tip of the apical process.
This was seen in neuroepithelial progenitor cells and immature RGCs as ath5:GAP-RFP signal began to increase (Figure 4E, Movie S7). However, as RGCs matured and began to polarize within Lamα1-deficient retinas, the centrosomes of such cells often “fell” out of the apical process and moved into the cell body. Once mislocalized from the apical process, centrosomes moved dynamically within the cell body of the RGC, traveling, for example, to the basal side of the cell body and then back up apically. Therefore, Lam1 at the basal lamina in vivo is essential for the normal polarized behavior of the centrosomes, and in the absence of this extrinsic cue, polarizing RGCs behave more similarly to RGCs in vitro.

**Lam1 Is Sufficient to Orient RGC Polarization In Vivo**

Having established that Lam1 is necessary for directed RGC axon extension, we wanted to know whether Lam1 is playing an indirect role, such as maintaining general retinal organization, or if Lam1 alone is capable of instructing this process. To 

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This text continues with additional experimental details and figures illustrating the dynamic behavior of centrosomes in both WT and Lamα1-deficient conditions. The figures and accompanying text highlight the differences in centrosome behavior between in vivo and in vitro conditions, emphasizing the role of Lam1 in guiding RGC polarization.

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address this sufficiency question, we first tested if Lam1 can directly axon extension in vitro. Polarizing RGCs plated on poly-L-lysine were presented with polystyrene beads coated with Lam1 and analyzed by time-lapse microscopy. (A and B) When a Stage 2 neurite contacts a Lam1 bead (contact point marked by arrow), this quickly (within one 30 min time point) induces a dramatic transformation from a thin neurite to one with an elaborate growth cone typical of an RGC axon. (C) When presented with BSA-coated control beads (pseudocolored cyan), neurite contact (cyan arrows) does not have an observable effect. (D) Imaging of cultured ath5:GAP-RFP/Centrin-GFP RGCs contacting a Lam1 bead (pseudocolored yellow, arrow indicates contact point) demonstrates that along with axon induction, neurite contact causes the centrosome to orient toward the site of Lam1 contact, and induces a transient migration toward the bead. Frames are taken from Movies S8 and S9. Time is shown in hr:min; scale bars = 10 μM.

cerebellar granule neurons (Gupta et al., 2010). We found that when a Stage 2 neurite of a cultured ath5:GAP-RFP/Centrin-GFP cell contacts a Lam1 bead, this quickly (within 1 hr) causes the centrosome to reorient to the Lam1 contact point (Figure 5D, Movie S9). This result is quite surprising given that RGCs extend basal axons with apical centrosomes in vivo, and that centrosome positioning does not correlate with the position of axon extension in vitro. However, we also observed that Lam1 contact can induce a subtle migration or translocation of the RGC cell body toward the bead, where the cell body appears to be tugged toward the bead. It could be that Lam1 can actually induce two separate behaviors in vitro: converting a Stage 2 neurite into an axon, and inducing cell migration/translocation, where perhaps only the latter depends on centrosome reorientation or stable centrosome positioning. In support of this, proximal centrosome localization was sometimes unstable, and the centrosome could reorient during Stage 3. This was especially obvious in cases where the bead became dislodged from its original position after neurite contact and was pulled onto the surface of the cell body. The neurite that had originally contacted the bead remained committed to form the axon, while the centrosome tracked the bead as it moved around the cell body. This indicates that Laminin-dependent axon commitment is an early event that only transiently depends on localized Laminin contact, and is separable from the persistent effect of Laminin on centrosome localization.

Although we have established that Lam1 is sufficient to direct axon commitment in vitro, we wanted to know if this was also the case in vivo. To answer this question we developed a system to implant polystyrene beads into the retina of 24 hpf zebrafish embryos using a sharp glass needle. This system allowed us to reintroduce Lam1 into a Lamα1-deficient embryo,
to unambiguously identify where the ectopic Lam1 was located, and to assess its influence on polarizing RGCs. The bead implantation procedure did not have a dramatic effect on the structure of the retina, which had no noticeable structural defects, and appeared normal with a bead, or a clump of beads, suspended within it (data not shown).

Lam1-coated beads were implanted into 24–28 hpf lamα1 morphant embryos (Figure 6A). Embryos were grown until 3 dpf, and we imaged them by confocal microscopy to look for an interaction between the beads and RGC axons. In many cases an interaction between the beads and RGC axons was obvious, where large axon bundles were observed in contact with the beads/bead clumps. Axons hugged the surface of the beads, often causing them to lie within the axon fascicle (Figure 6B). Beads were generally positioned at the base of the RGC axon bundles, close to RGC cell bodies, consistent with the hypothesis that Lam1 is acting to direct polarization and RGC axon sprouting.

Axon growth can be directed by the physical nature of a substrate. Therefore, it is possible that the physical presence of a polystyrene bead, rather than the Lam1 coating, is able to influence RGC polarization and axon extension. To control for this possibility, we implanted BSA-coated beads into Lamα1 morphant embryos. BSA-coated beads very rarely showed an association with RGC axons. To quantify this observation, confocal stacks from retinas implanted with either Lam1 or BSA-coated beads were blinded and classified as either showing a clear and dramatic interaction with RGC axons, where many RGC axons were seen in contact with the surface of the bead (visible growth cones marked by arrowheads) and bead engulfment by the axon fascicle (arrows).

Figure 6. Lam1 Contact Is Sufficient to Transform a Neurite into an Axon In Vivo

(A) ath5:GAP-GFP transgenic, lamα1 morphant embryos were grown to ~24 hpf, and Lam1-coated polystyrene beads were implanted into the retina of the right eye.

(B) Confocal reconstruction of an embryo that was implanted with a clump of 1 μM Lam1 beads and grown until RGC axons had extended (~3 dpf), immunostained with polyclonal anti-Lam1 antibody. This demonstrates the intimate association between the highly stained Lam1-coated beads and the extended axons. Axons hug the surface of the bead clump (arrow), causing the beads to lie within the axon fascicle.

(C) Time-lapse confocal imaging during axon extension demonstrates the dramatic interaction between polarizing RGC axons and the Lam1-coated beads (pseudocolored yellow), resulting in axon extension along the surface of the beads (visible growth cones marked by arrowheads) and bead engulfment by the axon fascicle (arrows).

(D) Highlighting an individual RGC by pseudocoloring it green demonstrates that after Lam1 bead contact (white arrows), the contacting neurite transforms into a process tipped with an elaborate growth cone that extends to form the axon, while the axon shaft remains associated with the bead (blue arrows).

Frames are taken from Movies S10 and S12. Time is shown in hr:min; scale bars = 10 μM.
Laminin Contact Directs RGC Axon Emergence

A requisite step in axon selection is the differential rearrangement of microtubules in the preaxonal neurite (Witte et al., 2008). This is likely what is visualized using the Kif5c560-YFP microtubule motor construct. Based on the coincident timing of Kif5c560-YFP signal accumulation with when the tip of the RGC basal process contacts the basal surface of the retina, as well as the lack of directed Kif5c560-YFP accumulation when RGCs lack this Lam1 cue, we hypothesized that the role of Lam1 may be to direct the localized microtubule modifications leading to the specific Kif5c560 accumulation preceding definitive axon extension. To test this, we wanted to see if we could recapitulate the normal directed behavior using our Lam1 bead assays.

Kif5c560-YFP-expressing RGCs were cultured in the vicinity of Lam1-coated polystyrene beads (Figure 7A, Movie S13). Consistent with our model, when a Stage 2 neurite contacted a Lam1 bead, this induced the translocation of the Kif5c560-YFP signal to the contact point, demonstrating that Laminin contact catalyzes this specific accumulation. Interestingly, when two or more neurites contacted Lam1, Kif5c560-YFP accumulated in specifically these contacting neurites, but often only in one neurite at a time, and oscillated between these (but rarely other) neurites (Figure 7B, Movie S14). RGCs were also cultured along borders of poly-L-lysine and Lam1 by plating on coverslips with islands of Lam1 within a homogenous poly-L-lysine coating. Similar to when RGCs contacted multiple Lam1 beads, an RGC polarizing along a Lam1 border demonstrated a clear bias in Kif5c560-YFP accumulations, where the signal oscillated between different Lam1-contacting neurites before stabilizing in one, which extended to form the axon (Figure 7C, Movie S15).

Having established that this was the case in vitro, we moved to the in vivo assay. Lam1 beads were implanted into mosaic embryos created by transplantation of blastomeres from ath5:GAP-RFP, Kif5c560-YFP RNA-injected embryos into lam1 morphant host embryos (Figures 7D and 7E, Movie S16). As described above, RGCs in the Lam1-deficient environment exhibit oscillatory Kif5c560-YFP accumulations. However, when one of the neurites contacted the Lam1-coated bead, Kifc560-YFP accumulated specifically at the contact point. The YFP signal accumulation was stable, with only very transient and weak signal visible within the basal process, and the Lam1 contacting process did not retract. Subsequently this neurite transformed into the axon and extended away from the bead. Therefore, contact with Lam1 caused the cessation of the Kif5c560-YFP oscillations within Stage 2 RGCs in vivo, and recapitulated the normal behavior of RGCs when they come in contact with the basal surface of the WT retina, where Lam1 contact results in specific and stable Kif5c560-YFP accumulation preceding axon extension.

DISCUSSION

Lam1 Orient the Polarization of RGCs In Vivo

Imaging experiments in the vertebrate retina have demonstrated that bipolar cell polarization occurs through the directed sprouting of axons and dendrites from basal and apical processes, respectively (Morgan et al., 2006). Similarly, RGC polarization occurs through directed sprouting of axons from the most basal point of the cell. In contrast to behavior in cultured neurons, no multipolar Stage 2 behavior is seen prior to RGC axon extension in vivo. We have confirmed and extended these findings, and have shown that RGC polarization is highly directed intracellularly, where Kif5c560-YFP, a marker of axonal microtubules, is directed to accumulate at the tip of the basal process prior to axon extension. Therefore, it is clear that these retinal neurons are endowed with polarity information that is not present in cultured neurons and allows them to extend axons directly from the relevant part of the cell body. There are two potential sources of information that neurons could exploit. Neurons derive from the terminal divisions of highly polarized neuroepithelial cells, and this inherited polarity could be instructing neuronal polarization. Alternatively (or additionally) neurons are born into highly heterogeneous environments, in which multiple
potential extracellular cues exist that could provide polarizing information.

Here we demonstrate that a major determinant for the orientation of RGC polarization is in fact an extracellular cue acting upon the neuron. Just prior to axon extension, the RGC contacts the basal surface of the retina. Lining the basal surface of the retina is a basal lamina, which contains Lam1. For five reasons we conclude that Lam1 contact is the major cue instructing the RGCs to extend their axons at this precise point. First, contact with the retinal basal surface correlates with the specific and stable accumulation of the axonal marker Kif5c560-YFP. Second, in a retina devoid of Lam1 at its basal surface, RGCs show ectopic polarization behaviors and progress through a Stage 2 phase before extending an axon. Third, in a Lam1-deficient retina, centrosomes were localized appropriately and apically in very young RGCs, but mislocalized and wandering centrosomes are visible within Stage 2 RGCs. This suggests that Lam1 is most crucial to direct neuronal (rather than neuroepithelial) polarization, at least with respect to this marker in these specific cells. Fourth, when cultured RGCs contact a Lam1-coated bead, they will extend their axons from the contact point. Fifth, and most importantly, when Lam1-coated beads are implanted into a Lam1-deficient retina, RGCs that contact the bead consistently extend their axon along the Lam1 surface.

Figure 7. Lam1 Contact Directs Kif5c560-YFP to the Contacting Neurite In Vitro and In Vivo

(A–C) RGCs were dissociated from ath5:GAP-RFP embryos injected with Kif5sc560-YFP mRNA at the one-cell stage, plated on poly-L-lysine with scattered 1 μM Lam1-coated beads (A and B, pseudocolored yellow), or on islands of Lam1 stained with Texas red dye (C, magenta substrate). When a single neurite contacted Lam1 (A), this quickly caused the YFP signal to accumulate specifically in that neurite (arrow). When multiple neurites contacted Lam1 (B and C), this caused the YFP signal to accumulate specifically in Lam1-contacting neurites, and to oscillate preferentially between these neurites (arrow). (Di) Blastomeres were transplanted from ath5:GAP-RFP transgenic embryos that were injected with Kif5sc560-YFP mRNA into lam1 morphant embryos or mosaic embryos grown to 24 hpf, and 6 μM Lam1-coated beads were implanted into the right eye. (Dii) The highlighted RGC exhibits typical Kif5sc560-YFP oscillations during Stage 2 (cyan phase, marked by red arrowheads). Upon contact with the Lam1 bead (white arrowhead; bead is pseudocolored yellow), the contacting process is stabilized and does not retract, and YFP signal is concentrated at the contact point. A reconstruction of the uncropped frames is shown in Movie S16. (E) Kymograph of the cell shown in (D), demonstrating the specific Kif5sc560-YFP signal accumulation upon neurite contact with the Lam1-coated bead (contact marked by white arrow; bead pseudocolored yellow). Frames are taken from Movies S13, S14, S15, and S16. Time is shown in hr:min; scale bar = 10 μM.
Laminin Contact Directs RGC Polarization through the Stabilization of the Basal Process and Localized Changes in Microtubules

Although they are clearly demonstrating disrupted polarization behaviors, RGCs within a Lam1-deficient retina rarely invert, and Kim5c560-YFP only ever localizes to basally directed neurites within Stage 2 RGCs. Therefore, while these cells do exhibit a polarization behavior more similar to that of cultured neurons, complete intracellular (or morphological) inversions rarely occur. Thus, other extracellular cues that prevent apical Kim5c560-YFP accumulations and RGC inversions may be present in the retina (Bauch et al., 1998; Zolesii et al., 2006). Alternatively, there may be an intrinsic polarity to the RGCs, which is independent of Lam1 and acts to prevent RGC inversions. At present we are unable to differentiate between these two possibilities. However, since Kim5c560-YFP accumulates basally in neuroepithelial cells, this seems to lend support to the latter possibility. We propose that there are likely to be multiple factors that are directing the polarization of RGCs, acting independently of Laminin to prime RGCs to polarize toward the basal surface. Lam1 then acts as the final cue, defining the precise point where the axon will emerge, and committing the axon to sprout at the contact point (Figure 8). Laminin contact occurs so soon after RGC birth that it directs the maturation into Stage 3 before the cell has a chance to enter Stage 2. Our in vitro and in vivo Lam1 bead assays indicate that this occurs through the capture and stabilization of the contacting process (normally the re-extending basal process), and the direction or reinforcement of the localized changes in microtubules, resulting in Kim5c560 accumulation and axon extension. In the absence of Lam1, this rapid transition to Stage 3 does not occur, and RGCs revert to Stage 2.

Exactly how Laminin contact influences microtubules in this context is not clear. Perhaps telling is the observation that when multiple neurites of cultured RGCs are contacting Lam1, Kim5c560-YFP oscillates only between these neurites. This demonstrates that whether or not a neurite contacts Laminin somehow differentially influences the microtubules of that neurite, so that its capacity to accumulate Kim5c560-YFP is altered. This could occur through the formation of a more ordered array of microtubule plus ends aligned at the tip of the neurite, resulting in more localized accumulations of the plus-end-directed Kinesin 1 motor. Alternatively, the specific recruitment of MAPs or tubulin-modifying enzymes to the Lam1 contact point could direct biochemical changes thought to direct Kim5c560 accumulation in mature axons (Hammond et al., 2010; Konishi and Setou, 2009).

Laminin contact can also direct axon extension in cultured hippocampal neurons, and perhaps cerebellar granule neurons (Esch et al., 1999; Gupta et al., 2010; Ménager et al., 2004). This Laminin signal is generally received through Integrin receptors on the cell surface. We designed and tested morpholinos against the candidate Integrin Laminin receptors: Itgβ1a, β1b, and α6β1. These morphors showed phenotypes consistent with β1a/β6 Integrins being the relevant receptor on the RGCs, with disorganized axon bundles within the retina, and Stage 2 RGCs during polarization (data not shown). However, because Integrins are required for many developmental events, these morphors exhibited a dramatic amount of retinal disorganization, and we were unable to specifically attribute these...
phenotypes to a cell-autonomous lack of Laminin responsiveness. However, previous studies have demonstrated that laminin-dependent neurite extension occurs through Integrin receptors (Gupton and Gertler, 2010), and expression of dominant-negative β1 Integrin constructs prevents axon extension in Xenopus RGCs (Lilienbaum et al., 1995), providing a strong indication that this is the relevant receptor for RGCs to respond to Lamin1. In the future it will be important to determine precisely how the localized cytoskeletal rearrangements leading to Kif5c560 accumulation and axon commitment are directed by Lam1 contact, because an analogous mechanism is likely to have occurred for every polarized neuron in the brain.

A Localized Lam1 Cue in the Retina Causes the Discrepancy between RGC Polarization Behavior In Vivo and In Vitro

Determining how neurons polarize in vivo is challenging due to the requirement for precise genetic manipulation of generally pleiotropic genes, and the detailed analysis of intricate cell behaviors within their often-prohibitive location deep within the developing embryonic brain. For these reasons, most of the research on neuronal polarization has been done on neurons polarizing in culture. Unlike RGCs, cortical neurons polarizing in vivo do appear to progress through a multipolar stage (Noctor et al., 2004), which has been likened to the multipolar Stage 2 of cultured hippocampal neurons (Barnes and Polleux, 2009; Calderon de Anda et al., 2010). One interpretation of this contradiction could be that the mechanism of RGC polarization is intrinsically different than that of mammalian cortical neurons. However, cultured RGCs exhibit the classical staged series of behaviors typical of cultured hippocampal neurons (Zolesi et al., 2006). We have further demonstrated that Kif5c560-YFP exhibits the transient oscillations in different areas of the cell body and Stage 2 neurites before stably accumulating in the axon just prior to extension, as was previously shown in cultured hippocampal neurons (Jacobson et al., 2006). Therefore, these two types of neurons are actually behaving identically in culture, at least at the fundamental level of the microtubule cytoskeleton, indicating that they are actually quite similar intrinsically. The major difference between these two systems appears to be one of environmental context, rather than intrinsic cellular differences, because removal of the endogenous cue, Laminin, causes polarizing RGCs within morphant retinas to switch their behavior to that of cultured neurons. In vitro polarization appears to represent the behavior of cells without their relevant cues, and there may be striking differences between this behavior and what actually occurs within the developing brain. Therefore, despite the experimental challenges, determining the molecular mechanisms governing the polarization of diverse neuron cell types in vivo will be critical to understanding how this process is actually regulated within the embryonic brain.

**EXPERIMENTAL PROCEDURES**

**Animals**

Zebrafish were maintained and bred at 26.5°C, and embryos were raised at 28°C–32°C and staged based on hpf. Embryos were treated with 0.003% phenylthiourea (Sigma) from 10 hpf to prevent pigmentation. All animal work was approved by Local Ethical Review Committee at the University of Cambridge and performed according to the protocols of project license PPL 80/2198.

**Transgenic Lines and Constructs**

Transgenic lines Tg(ath5:gapr3-EGFP)10mfinal and Tg(ath5:gapr3-mRFP)10mfinal have been previously described (Zolesi et al., 2006), and are abbreviated here as ath5:GAP-GFP and ath5:GAP-RFP. The Tg(Centrin-GFP) line was created using the pCJ126 plasmid, where the 5′-actin promoter drives expression of zebratfish centrin fused to GFP, all flanked by ISc-e-1 sites (Zolesi et al., 2006). This construct was injected along with ISce1 enzyme into one-cell stage ath5:GAP-RFP embryos to obtain a double transgenic line with ubiquitous Centrin-GFP expression. The coding sequence of Kif5c560-YFP was subcloned into the BamHI and EcoRI sites of PCS2+ by PCR amplification of the coding region from pBa-Kif5c560-YFP (Jacobson et al., 2006), using the following primer pairs: 5′-GGGGGATCCATGGCAGATTGCAAGAATTG-3′ (frw) and 5′-GGGGGATCCATGGCAGATTGCAAGAATTG-3′ (rev). RNA was created by linearizing with Not1 enzyme and synthesizing capped RNA from the Sp6 promoter using mMessage mMachine SP6 Kit (Ambion).

**Embryo Manipulations**

RNA and morpholinos were injected into the yolk of one- to two-cell stage embryos. One-half to one nanogram of Lam1 morpholin (5′-TCATCCT CATTCCCATGATCTGCTCA-3′, Gene Tools) was injected. For blastomere transplantations, high- to oblong-stage embryos were dechorionated by pronase digestion (Sigma) and placed in agarose molds, and between 5 and 30 blastomeres were transferred between embryos using a glass capillary connected to a 2 ml syringe. In most transplantation experiments, the p53 morpholin (5′-GGGCGATTGCTTTGCAAGAATTG-3′, Gene Tools) was injected into donor embryos to prevent apoptosis of donor cells and increase the success rate of transplanted cell survival. This was especially important for transplantation from Kif5c560-YFP RNA-injected donors because this construct exhibited a mild degree of cellular toxicity. H2B-RFP/GFP RNA was generally injected as a lineage tracer to screen embryos for successful transplantations.

For bead implantation experiments, 6 μm fluoresbrite polychromatic red dyed beads, or 1 μM polystyrene beads (Polysciences), were coated by incubation with murine Lam1 (L2020, Sigma) or BSA (A2153, Sigma) for 1 h at room temperature, and washed three times for 5 min in PBS. Efficient Lam1 coating was obtained as the Lam1-coated beads clumped together and formed aggregates, which was not seen for BSA or uncoated beads, and confirmed by strong Lam1 staining by immunofluorescence. Bead implantations were performed by mounting 24 hpf embryos in 2%–4% methylcellulose (Sigma), containing 0.4 mg/ml MS222 (Sigma) as anesthetic. Beads were suspended in the methylcellulose, sucked into a sharp glass capillary connected to a mineral-oil filled Hamilton syringe, and injected into the retina of the embryo. Embryos were then transferred to clean Petri dishes containing embryo medium and penicillin/streptomycin/fungicide to recover. The polychromatic red dye showed extremely bright fluorescence, and the signal bleedthrough into the green channel was strong enough for bead visualization in most experiments. When imaging in red channel was also necessary, beads were photobleached by being placed on the windowsill for 2–4 weeks.

Dissociated retinal cell culture was performed as previously described (Zolesi et al., 2006). For the creation of a substrate with Laminin islands, coverslips were coated overnight with poly-L-lysine (Sigma, 10 μg/ml), and then sprayed with an atomizer creating a fine mist of Lam1 (Sigma, 20 μg/ml) mixed with Texas-red-conjugated Dextran (D-1863, Invitrogen) in order to stain the Laminin deposits.

**Confocal Imaging and Immunostaining**

Imaging of live and fixed embryos was performed as described previously (Poggii et al., 2005), using a Perkin Elmer spinning Disk UltraView ERS, Olympus IX81 Inverted microscope and 60× (1.2 NA) water immersion objective, and a motorized XY stage (H117, Prior) to allow for simultaneous imaging of multiple embryos. A confocal laser scanning microscope (Leica) and 63× (1.2 NA) water immersion objective (Leica) were also used for experiments.
shown in 3A–3C and 6B. Optical sections at 0.75–1 μm separation were taken to cover the majority of the retina (between 40 and 100 μm) at the relevant time intervals. Whole-mount immunostaining was performed using standard methods, using rabbit polyclonal anti-Lam1 (L9393, Sigma, 1:100) and anti-rabbit Alexa-594 (Invitrogen, 1:1000).

Data Analysis
Confocal data was analyzed using Velocity (Improvement). Deconvolution was generally performed on data acquired by spinning disk confocal microscopy using the iterative Restoration tool at 25 iterations and 99.99% confidence levels. Unless otherwise stated, the confocal z-slices were cropped to a rectangular region containing the cells of interest in XYZ and reconstructed using 3D Opacity. Brightness, contrast, and gamma were adjusted for maximal visibility of cellular morphology and fluorescent signal using Velocity, Photoshop (Adobe), and ImageJ (NIH), and the RFP channel was converted to magenta using the channels tool in ImageJ. Pseudocoloring and cell tracing was done in Photoshop, and the outline of the cell was determined by comparing it to the original confocal z-slices. For data presented in Figures 3Ei and 7Dii, each confocal z-slice was cropped tightly to the cell surface in all three spatial dimensions using Photoshop, carefully removing signal from the images not associated with that cell. The stack was then flattened into a maximum z-projection using ImageJ. For quantifications presented in Figure 2, lengths were measured within the original confocal z-slices using the line tool in Velocity. Statistical tests were performed using InStat (GraphPad).

SUPPLEMENTAL INFORMATION
Supplemental Information for this article includes 16 movies and can be found with this article online at doi:10.1016/j.neuron.2011.03.013.

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