Extrinsic cues orient the cell division axis in Drosophila embryonic neuroblasts

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INTRODUCTION

The proper morphogenesis of most tissues requires establishing cell polarity and then organizing these polarized cells relative to each other and to their environment. In Drosophila, epithelial tissue polarity is governed by apicobasal cell polarity proteins and planar polarity genes, which organize tissue polarity in three dimensions (Klein and Mlodzik, 2005; Nelson, 2003; Schweisguth, 2005). However, it is less clear how non-epithelial tissues, such as the CNS, achieve proper tissue polarity.

The Drosophila embryonic CNS develops through a series of asymmetric cell divisions of neural progenitors called neuroblasts (Wodarz, 2005). Neuroblasts delaminate from an apicobasal polarized neural ectoderm and rapidly begin a series of asymmetric cell divisions to ‘bud off’ smaller daughter cells (called ganglion mother cells; GMCs). Neuroblasts are polarized cells, with molecularly distinct apical and basal cortical domains. The neuroblast mitotic spindle invariably orient along the cortical polarity axis, to segregate apical proteins into the regenerated neuroblast and basal cortical proteins into the smaller GMC. The GMC divides once, giving rise to two daughters that differentiate into neurons or glia.

The Drosophila CNS has a well-defined tissue polarity. Neuroblasts are most apical, positioned adjacent to the neural ectoderm from which they derive, while the GMCs and their neural progeny are positioned more basally, with the earliest-born neurons occupying the deepest (most basal) layer and the most recently born neurons lying more superficially (Schmid et al., 1999). It is not clear how this level of organization is achieved, but one factor is neuroblast spindle orientation, which is tightly regulated such that GMCs are always deposited towards the interior of the CNS.

Neuroblast spindle orientation is controlled, in part, by the localized activity of apical cortical proteins. These apical proteins include the evolutionary conserved Par complex [which consists of Bazooka (Baz; Par3 in mammals), atypical protein kinase C (aPKC; aPKCa/ε in mammals) and Par6; Inscuteable (Insc); a hetero-trimeric G protein alpha subunit (Gox) and its associated proteins Partner of Inscuteable (Pins) and Locomotion defective (Loco); and the tumor suppressor proteins Discs-large (Dlg), Lethal giant larvae (Lgl) and Scribble (Scrib) (Wodarz, 2005; Yu et al., 2005). One simple model is that Par complex proteins, initially inherited from the polarized epithelium, remain polarized at the neuroblast apical cortex where they recruit Insc, Pins, Gox and Dlg. These proteins capture astral microtubules to control spindle orientation. The precise and reproducible alignment of the mitotic spindle relative to surrounding tissues is determined by the initial position of Par complex polarization. Although it is likely that the initial spindle orientation cue is inherited from the polarized ectoderm, it is not clear how neuroblasts repeatedly orient themselves along the same apicobasal axis from one division to the next. Is there an intrinsic cortical mark that persists from division to division, similar to budding yeast? Or is there an extrinsic cue that is responsible for spindle orientation at each division, similar to Drosophila germ line stem cells?

Here, we show that extrinsic cues are required for orienting the neuroblast division axis in the same direction from division to division. We find that these cues emanate from epithelial cells and reproducibly position the mitotic spindle during consecutive divisions through two means. First, these cues act on the neuroblast to maintain a constant centrosome position within the neuroblast closest to the epithelia-neuroblast contact site. Second, during a single cell division, these cues act to orient one pole of the mitotic
spindle towards the cortical position previously occupied by the G2 centrosomes, which also corresponds to the epithelia-neuroblast contact site. In addition, we find that extrinsic cues are required for the correct temporal and spatial polarization of Par proteins to the cortex. Our work suggests that extrinsic cues are important for CNS tissue polarity through regulating spindle position and apical protein localization.

**MATERIALS AND METHODS**

Fly stocks

We used G147-GFP, generated in a GFP protein trap screen (Morin et al., 2001), for analysis of centrosome and spindle dynamics. We used v32a-GAL4 (D. St Johnston) to drive the ubiquitous embryonic expression of UAS-Dlg:eGFP (Koh et al., 1999), which was used in live cell imaging, and we used worniu-GAL4 (Albertson et al., 2004) to drive the neuroblast-specific expression of UAS-Dlg:eGFP and positively identify neuroblasts in fixed cultures.

In vitro neuroblast culture

Primary cell cultures were made from wild-type and transgenic embryos aged 4-5 hours as previously described (Grosskortenhaus et al., 2005). They were then prepared for either live imaging or fixed for immunofluorescence.

**Immunofluorescent staining and antibodies**

Cultures were fixed in 4% paraformaldehyde for 15 minutes, rinsed several times in 1×PBS supplemented with 10 mM glycine, placed in 0.5% TritonX-100/1×PBS for 4 minutes, and then blocked for 15 minutes in 1×PBS/1% BSA. Primary antibodies used for these studies include: rabbit phospho-Histone H3 (1:1000; Upstate), mouse α-tubulin (1:2000; Sigma-Aldrich), rat α-tubulin (1:100; Serotec), rabbit GFP (1:1000; Torrey Pines), mouse GFP (1:500; Roche), rat Pins (1:500), mouse Dlg 4F3E2 (Parnas et al., 2001), rabbit Insc (1:500; W. Chia), rabbit Baz (1:500; A. Wodarz), rabbit αPKC (1:500; Santa Cruz) and rat Worni (Lee et al., 2005).

We used fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch or Molecular Probes. Images were collected using a BioRad Radiance scanning confocal mounted on a Nikon Eclipse E800 equipped with a 60×/1.4NA oil immersion objective: 3-5 μm steps were collected at 1.5 μm intervals every 30 seconds using LaserSharp software. All data were processed and reconstructed into Quicktime movies using Metamorph and ImageJ software.

**RESULTS**

**Epithelial-neuroblast contact correlates with the neuroblast division axis in vivo**

During wild-type CNS development, neuroblasts are initially in contact with the overlying ectoderm, but towards the end of embryogenesis the epithelium separates from the CNS. If intrinsic cues regulate spindle orientation, we would expect no difference in spindle orientation in early versus late stage neuroblasts. By contrast, if epithelial signals influence neuroblast spindle orientation, we may find a correlation between epithelial-neuroblast contact and spindle orientation. We found that the neuroblast division axis in early and late stage embryos showed a clear correlation between epithelial-neuroblast contact and spindle orientation (Fig. 1). During early stages (e.g. stage 9-11), the neuroblast apical cortex (marked with Baz) always directly abutted the basal surface of the epithelium, whereas the neuroblast basal cortex (marked by Mira) was always furthest away from the epithelium; the spindle axis bisecting these two cortical domains was close to perpendicular to the overlying epithelium (Fig. 1A). During later stages, after separation of epithelial and CNS tissues (e.g. stage 15), we found that embryonic neuroblasts failed to maintain their apicobasal orientation in a fixed position relative to the overlying ectoderm or the surface of the CNS (Fig. 1B). These neuroblasts could have their apical domain positioned 180° away from the epithelial surface, and took on a wider variation in orientation with respect to one another (Fig. 1B and quantified in Figs 1A,B). These results suggest that the epithelial cell layer could provide a cue to orient embryonic neuroblasts relative to each other and neighboring tissue.

**Extrinsic cues orient the neuroblast division axis from division to division**

To test the role of extrinsic cues in directing embryonic neuroblast spindle orientation, we dissociated and cultured neuroblasts from four- to five-hour-old embryos. This corresponds to a developmental time represented in Fig. 1A. Using differential interference contrast (DIC) microscopy, we imaged consecutive cell divisions from two classes of neuroblasts – those in cell clusters versus those in isolation – and followed the positions of newly born
GMCS as a readout for neuroblast spindle orientation (Fig. 2). Clustered neuroblasts were defined as neuroblasts maintaining direct cell-cell contact with several epithelial cells in addition to other cell types. Isolated neuroblasts were defined as neuroblasts cultured in the absence of any neighboring cell, except their GMC daughters. Neuroblasts were positively identified based on their large cell size, mitotic potential and ability to produce smaller daughter cells (GMCS) by asymmetric cell division; epithelial cells were identified based on their smaller cell size, tight contact between each other, limited mitotic potential and ability to produce equally sized daughter cells during cell division. Both clustered and isolated neuroblasts proceeded through mitosis at the same rate (Table 1).

We found that clustered neuroblasts repeatedly orient themselves to divide along the same environmental axis from one division to the next (Fig. 2A). This resulted in all GMC daughter cells being tightly clustered on one side of the neuroblast (see cartoon summary in Fig. 2A and quantified in 2C). We found that a neuroblast must be in contact with at least two epithelial cells in order to orient the division axis (Fig. 2D and data not shown). By contrast, isolated neuroblasts failed to align their spindle axis from one division to the next, resulting in the random placement of newly born GMCS around the neuroblast cortex (Fig. 2B and quantified in 2C). As a control, we followed the movement of Concavalin A-coated beads stably bound to surface receptors and found that these beads mostly remained restricted to a single neuroblast quadrant from division to division and that their position did not correlate with the position of newly born GMCS (see Fig. S1 in the supplementary material), confirming that isolated neuroblasts remain firmly attached to the glass substrate. In addition, we plated our dissociated cells on Sea-Tak coated coverslips, a very sticky cell adherent support, and observed Table 1. Mitotic timing of embryonic neuroblasts in culture

<table>
<thead>
<tr>
<th>Mitotic stage</th>
<th>Clustered neuroblasts</th>
<th>Isolated neuroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB to Ana B</td>
<td>4:54 (n=19)</td>
<td>4:42 (n=20)</td>
</tr>
<tr>
<td>NEB to NEF</td>
<td>10:06 (n=19)</td>
<td>9:06 (n=20)</td>
</tr>
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*Neuroblasts identified on the basis of size, asymmetric division pattern and mitotic potential.

1NEB (nuclear envelop breakdown); Ana B (anaphase B); NEF (nuclear envelop reformation after mitosis).

2Mean time in minutes:seconds.
the same behaviors between clustered and isolated neuroblasts as in the previous experiments (data not shown). We conclude that epithelial cells provide a contact-mediated extrinsic cue that can maintain reproducible neuroblast spindle orientation over multiple cell divisions.

**Neuroblasts contacting epithelial cells maintain centrosome position at the site of contact from one cell cycle to the next**

To better understand the extrinsic cue-induced spindle orientation mechanism, we imaged the behavior of GFP-tagged centrosomes and mitotic spindles in clustered and isolated neuroblasts. We used *G147-GFP*, a gene trap line expressing eGFP fused in frame to a microtubule-associated protein (Morin et al., 2001), which allows for live imaging of the microtubule cytoskeleton in embryonic neuroblasts throughout the cell cycle. In clustered neuroblasts, we found that the centrosome was positioned apically within the neuroblast, closest to the site of epithelial cell contact, throughout the cell cycle (Fig. 3; see Movie 1 in the supplementary material). We conclude that in the presence of epithelial cell contact, centrosome position is stably maintained at the apical cortex where it accurately predicts orientation of the division axis and position of newly born GMCs.

The only exception to the fixed apical position of the centrosome/spindle pole was observed during prophase, where centrosome duplication and rotation could transiently displace the centrosomes from the apical cortex (Fig. 4). The two centrosomes exhibited one of two behaviors with equal frequency. Either both centrosomes left their apical position to move basally until reaching full separation, in a lateral orientation (Fig. 4A,B; n=17) or one centrosome remained stationary while the other migrated 180° away, reaching full separation (Fig. 4D,E; n=17). In the first case, one
centrosome always rotated back to the original G2 centrosome position by the start of metaphase (Fig. 4A,C). We conclude that despite transient displacement of the centrosomes at prophase, G2 centrosome position accurately predicts spindle orientation from metaphase through telophase in neuroblasts with epithelial cell contact.

**Neuroblasts in isolation fail to maintain centrosome position from one cell cycle to the next**

To test whether centrosome/spindle pole anchoring was dependent on epithelial-neuroblast contact, we imaged the behavior of G147-GFP labeled microtubule cytoskeleton in neuroblasts dividing in isolation. We found that isolated neuroblasts exhibited several differences in centrosome and spindle behavior. First, the orientation of the mitotic spindle at metaphase often failed to align with the G2 centrosome position (Fig. 5A and quantified in 5D; see Movie 2 in the supplementary material). However, like clustered neuroblasts, the spindle at metaphase and telophase were always closely aligned to each other (Fig. 5A and quantified in 5E). Finally, we found that the apical centrosome at telophase was not stably maintained at a single position throughout interphase, such that prior to mitotic reentry, it could be up to 180° away on the opposite side of the neuroblast (Fig. 5B and quantified in 5F). Together, failure to orient the spindle towards the G2 position during mitosis and failure to stably maintain centrosome position during interphase can result in a large variability of G2 centrosome position from one division to the next and results in the observed random placement of GMCs during consecutive divisions (Fig. 5C and quantified in 5G). We conclude that extrinsic cues are required to maintain centrosome/spindle pole position within the neuroblast. The only exception is the metaphase-telophase interval, where neuroblast-intrinsic cues have the ability to maintain spindle position.

**Extrinsic cues promote polarization of the neuroblast apical cortex at prophase**

To address the mechanism by which extrinsic cues anchor centrosome/spindle pole position within the neuroblast, we assayed cortical polarity markers in neuroblasts dividing with or without epithelial contact. In neuroblasts with epithelial contact, the apical Par proteins Baz and aPKC formed a cortical crescent at or just prior to prophase (82%, n=38; Fig. 6A), similar to the in vivo situation (Schober et al., 1999; Wodarz et al., 2000; Wodarz et al., 1999). By
contrast, isolated neuroblasts typically did not have Baz/aPKC crescents at prophase, and the crescents that formed were weaker than normal (Fig. 6E; quantified in Table 2). We found that lack of cortical polarity correlated well with aberrant spindle pole movement in isolated neuroblasts: Baz/aPKC crescents were reduced from late interphase to metaphase (Fig. 6), which was the interval where we previously showed failure of the mitotic spindle to properly align relative to the position of the G2 centrosomes (Fig. 5A,D). However, we found that isolated neuroblasts recovered cortical Baz/aPKC crescents by metaphase and that this cortical polarity persisted through telophase (Fig. 6B-D,F-H; quantified in Table 2), precisely the interval during which we previously showed that the mitotic spindle remained anchored (Fig. 5A,E). In addition, we also found that spindle morphology was indistinguishable between isolated and clustered neuroblasts, and that spindle orientation relative to cortical polarity was normal in both classes (Fig. 6, and data not shown). We conclude that extrinsic cues are required for establishing Par protein asymmetry at prophase, and suggest that this contributes to maintaining the axis of spindle orientation over multiple cell divisions.

We next followed the establishment of cortical polarity in living neuroblasts, with or without epithelial contact, using Dlg:eGFP. This protein forms robust apical cortical crescents co-localizing with Baz/aPKC in wild-type metaphase neuroblasts in vivo (Peng et al., 2000) (S.E.S. and C.Q.D., unpublished). We found that in clustered neuroblasts, Dlg:eGFP crescents formed at the same cortical position, closest to the G2 centrosome/epithelial cell contact site, over consecutive cell cycles (Fig. 7A). In isolated neuroblasts, however, Dlg:eGFP crescents occurred at seemingly random positions at each cell division, although once a Dlg:eGFP crescent formed at metaphase it maintained its position (Fig. 7B). This supports our previous findings that isolated neuroblasts lack a mechanism for maintaining centrosome/spindle pole position from telophase of one division to prophase of the next division, but that a neuroblast-intrinsic mechanism functions from metaphase to telophase to maintain an invariant association between spindle orientation and cortical polarity. We conclude that extrinsic cues influence the timing and position of apical cortical crescent formation, but not the maintenance or function of cortical polarity from metaphase through telophase.

**DISCUSSION**

**Extrinsic signaling and neuroblast orientation**

We have shown that embryonic neuroblasts require an extrinsic signal from the overlying epithelium to anchor their centrosome(s) at the apical side of the cell, induce Par cortical polarity at prophase, and position Par cortical crescents at the apical cortex (Fig. 8).

How does the extrinsic cue stabilize centrosome position throughout multiple rounds of cell division? It is likely to stabilize centrosome-cortex interactions, perhaps by regulating association of microtubule plus-ends with the apical neuroblast cortex. During mitosis, the apical cortex is enriched with several proteins with the potential to interact with microtubules directly and indirectly, such as Pins, Gai, Dlg and Insc (Brenman et al., 1998; Bulgheresi et al., 2001; Du et al., 2001; Wang et al., 1990), but it remains unknown whether one or more of these are involved in transducing the extrinsic cue that promotes centrosome anchoring. During interphase, none of these proteins shows apical enrichment, although several have uniform cortical localization (e.g. Dlg, Gai) and could help stabilize the neuroblast centrosome following the completion of telophase.

The epithelial extrinsic signal is also required for the timing and position of Par cortical polarity in embryonic neuroblasts. In the presence of the extrinsic cue, Par polarity is established around the G2/prophase transition; without the extrinsic cue, Par polarization is delayed until prometaphase/metaphase. Because adjacent neuroblasts divide asynchronously, it is likely that the epithelial cue is always present, but the neuroblast only becomes competent to form the Par crescent at the G2/prophase transition. The best candidates would be mitotic kinases or phosphatases that change levels at the G2/prophase transition.

The position of the Par cortical crescent is also determined by the epithelial cue. In isolated neuroblasts, the Par cortical crescent forms at random positions during subsequent cell cycles, correlating with randomization of the cell division axis. It is not known how Par protein crescents are formed in wild-type embryonic neuroblasts exposed to the epithelial cue or in isolated neuroblasts that lack extrinsic signals. In wild-type neuroblasts, the initial events in Par protein polarization are likely to involve polarization of Baz or Insc, the two most upstream components in the Par cortical polarity

![Fig. 6. In the absence of extrinsic cues, neuroblast cortical polarity does not form until metaphase. (A-D) Double-labeled clustered and (E-H) isolated neuroblasts in culture, fixed and stained for aPKC and PH3 (top row), and for α-tubulin (bottom row). Mitotic stages are listed above each panel. White arrowheads indicate the center of the aPKC crescent. Scale bar: 5 μm.](image-url)
What is the nature of the extrinsic cue?

One of the best candidate pathways for regulating orientation of the neuroblast division axis by extrinsic cues is the non-canonical Wnt signaling pathway, because it is known to orient cell divisions in Danio rerio, C. elegans and Drosophila (Bellaiche et al., 2004; Gho and Schweiguth, 1998; Gong et al., 2004; Schlesinger et al., 1999).

This pathway uses the Frizzled (Fz) receptor and the cytoplasmic Disheveled (Dsh) and Gsk3 proteins from the Wnt pathway, but does not use a Wnt ligand (Strutt, 2003). In addition, these three components are joined by the two transmembrane proteins Strabismus (Stm) and Flamingo (Fmi) during planar cell polarity signaling in Drosophila (Fanto and McNell, 2004). However, we could find no evidence to support a role for this pathway in orienting embryonic neuroblast divisions. RNAi of each of the four Drosophila Fz receptors, individually and in combination, had little effect on neuroblast spindle orientation or cortical polarity (data not shown). Nor did we observe spindle orientation defects following expression of a dominant-negative Fz1 lacking the cytoplasmic domain, expression of the Wnt pathway antagonist Axin, or in dsh maternal zygotic mutants, fmi zygotic mutants, stm maternal zygotic mutants or fz1 fz2 double mutants (data not shown). The non-canonical Wnt pathway may still be involved in the ectodermal signal that regulates neuroblast orientation, but its role may be masked by genetic redundancy.

A second candidate pathway for regulating epithelial-to-neuroblast signaling is an extracellular matrix (ECM)-integrin pathway (Martin et al., 2002). ECM is deposited by the basal surface of epithelia, which is where neuroblasts contact the overlying embryonic epithelia. However, we do not detect a major integrin ligand, Laminin, at the basal surface of the embryonic ectoderm during stages 9-11, nor do we detect the core β-integrin protein in neuroblasts. In addition, maternal zygotic nms mutants lacking β-integrin show normal embryonic neuroblast spindle orientation (data not shown). It is unlikely that the ECM-integrin signaling regulates embryonic neuroblast spindle orientation.

Interestingly, neuroblasts located in the procephalic neural ectoderm are reported to undergo asymmetric cell divisions within the plane of the epithelium and reproducibly orient along the apicobasal embryonic axis to bud GMCs towards the interior of the embryo. Similar to during adult PNS development, the pIIb cell lies within the imaginal disc epithelium yet divides along the apicobasal axis. In both cases, the reproducibly apical spatial pattern of cell divisions occurs independent of an overlying polarized epithelium. It remains unknown whether the oriented pattern of these cell divisions is regulated by intrinsic cues or extrinsic cues (e.g. more internal cells). Unlike ventral cord embryonic neuroblasts, neuroblasts in the brain and in the PNS contain several cell-cell
junctions, including cadherin-containing adherence junctions and septate junctions. These signaling rich sites could provide spatial information for spindle orientation as seen in other cell types (Le Borgne et al., 2002; Ligon et al., 2001; McCartney et al., 2001).

Although the nature of the cue required to orient embryonic neuroblasts is not clear, there are several approaches to identify potential genes required for this process. As extrinsic cues are required for early localization of Par proteins and because baz and insc mutants have mis-oriented spindles relative to the epithelium, identifying binding partners for either Insc or Baz could be informative. In addition, we have identified a small genetic deficiency that, when homozygous, results in embryonic neuroblast spindle orientation defects relative to the overlying ectoderm without affecting epithelial morphology; one or more genes within this genetic interval would be excellent candidates for components of the extrinsic signaling pathway.

Finally, does neuroblast cell behavior in culture accurately reflect neuroblast behavior in vivo? It has previously been shown that in vivo embryonic neuroblasts establish apicobasal spindle orientation through one of two behaviors. Either the mitotic spindle first forms parallel to the overlying epithelium and then rotates 90° to align orthogonal to the overlying epithelium or the spindle forms as it rotates into its proper orientation (Kaltenschmidt et al., 2000). Centrosome separation and rotation behavior were not described. We also observed both behaviors in cultured neuroblasts, however, with several differences. First, we only observed rotations of fully formed spindles at a very low frequency and this behavior usually correlated with an unhealthy culture. Second, if both centrosomes moved basally or away from the epithelium contact site after separation, we frequently observed initial spindle formation coinciding with rotation into a position orthogonal to epithelial cells, similar to some of the reported in vivo cases. One additional difference in the analysis between these two systems involves the Drosophila stocks used for live imaging. We relied on following microtubule behavior from cells expressing endogenous levels of a microtubule-associated protein fused in frame to GFP, rather than upon overexpression of a tagged GFP fusion protein. This difference alone could account for the observed differences between the two studies.

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Supplementary material
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