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Extrinsic cues orient the cell division axis in *Drosophila* embryonic neuroblasts

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Cell polarity must be integrated with tissue polarity for proper development. The *Drosophila* embryonic central nervous system (CNS) is a highly polarized tissue; neuroblasts occupy the most apical layer of cells within the CNS, and lie just basal to the neural epithelium. Neuroblasts are the CNS progenitor cells and undergo multiple rounds of asymmetric cell division, 'budding off' smaller daughter cells (GMCs) from the side opposite the epithelium, thereby positioning neuronal/glial progeny towards the embryo interior. It is unknown whether this highly stereotypical orientation of neuroblast divisions is controlled by an intrinsic cue (e.g. cortical mark) or an extrinsic cue (e.g. cell-cell signal). Using live imaging and in vitro culture, we find that neuroblasts in contact with epithelial cells always 'bud off' GMCs in the same direction, opposite from the epithelia-neuroblast contact site, identical to what is observed in vivo. By contrast, isolated neuroblasts 'bud off' GMCs at random positions. Imaging of centrosome/spindle dynamics and cortical polarity shows that in neuroblasts contacting epithelial cells, centrosomes remained anchored and cortical polarity proteins localize at the same epithelia-neuroblast contact site over subsequent cell cycles. In isolated neuroblasts, centrosomes drifted between cell cycles and cortical polarity proteins showed a delay in polarization and random positioning. We conclude that embryonic neuroblasts require an extrinsic signal from the overlying epithelium to anchor the centrosome/centrosome pair at the site of epithelial-neuroblast contact and for proper temporal and spatial localization of cortical Par proteins. This ensures the proper coordination between neuroblast cell polarity and CNS tissue polarity.

KEY WORDS: Tissue polarity, Par proteins, Extrinsic, Intrinsic, Cortical polarity, Spindle orientation, *Drosophila*

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 orients 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; aPKCλ/ζ in mammals) and Par6]; Inscuteable (Insc); a heterotrimeric G protein alpha subunit (Gαi) 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, Gαi and Dlg, which probably 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* germline 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

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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 (1:1,000; 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 aPKC (1:500; Santa Cruz) and rat Worniu (Lee et al., 2005). We used fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch or Molecular Probes. Images were collected using a BioRad Radiance confocal using a 60×/1.4NA objective or a Leica TCS SP2 confocal using a 63×/1.4NA objective. Biorad LaserSharp, Image J, Adobe Photoshop and Illustrator software were used for data analysis and figure formatting.

Time-lapse analysis of neuroblast cell divisions in culture

For analysis of GMC daughter cell positions and cell cycle timing, cultured neuroblasts were imaged using DIC microscopy and frames were collected every 5 to 20 seconds using Scion Image software. For following localization of GFP tagged proteins, cultured neuroblasts were imaged using either one of the following confocal microscopes. A Perkin Elmer spinning disk confocal, mounted on a Nikon Eclipse TE2000-U inverted microscope, equipped with a 60×/1.4NA oil immersion objective: 7-10 *z* steps were collected at 1 μm intervals every 30 seconds using Metamorph software. A BioRad Radiance scanning confocal mounted on a Nikon Eclipse E800 equipped with a 60×/1.4NA oil immersion objective: 3-5 *z* 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

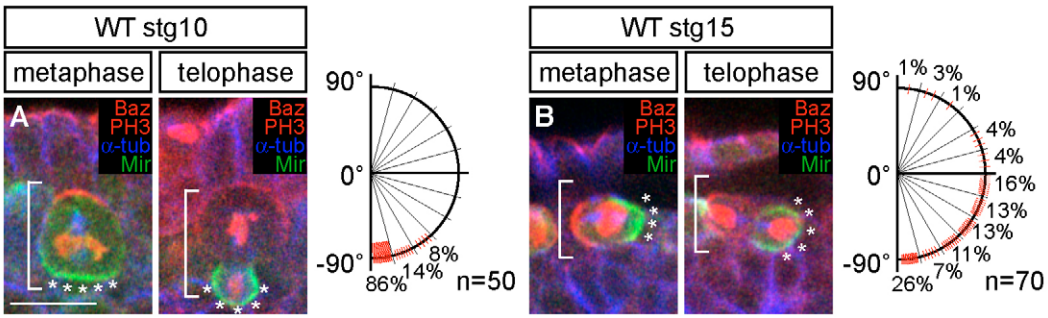

Fig. 1. Epithelial cell contact correlates with the embryonic neuroblast division axis. (**A**) Lateral view of stage 10 and (**B**) stage 15 metaphase and telophase neuroblasts. Markers listed within each panel. To the right are summaries of neuroblast orientation relative to the epithelium. Each red mark corresponds to the angle between a straight line connecting the apicobasal neuroblast axis at telophase and a straight line through the epithelial cell center. Brackets indicate neuroblasts; asterisks indicate Mir-positive basal cortex. Apical is upwards. Scale bar: 10 μm.

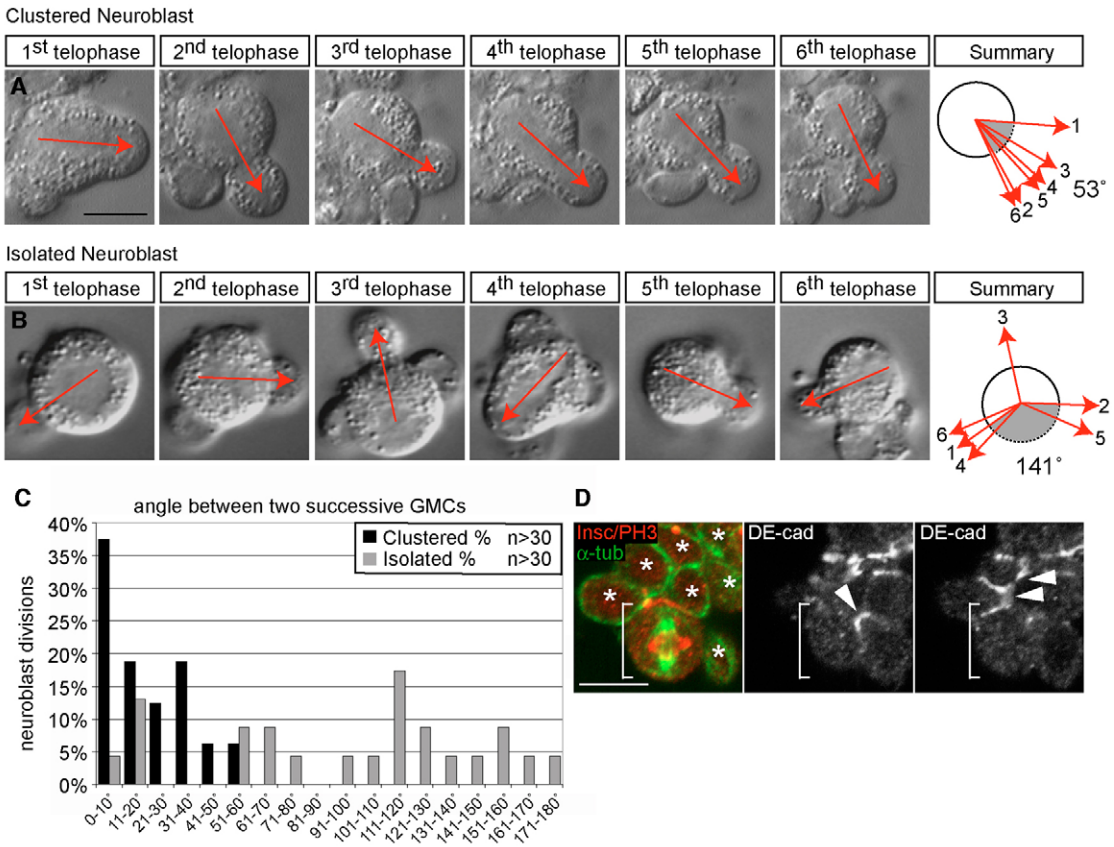

Fig. 2. Extrinsic cues orient the neuroblast division axis over multiple cell cycles. (**A**) Series of still images from a time-lapse DIC movie showing six consecutive telophase figures from a single clustered or (**B**) isolated neuroblast in primary cell culture. The red arrow points towards the developing GMC along the neuroblast intrinsic apicobasal polarity axis. (A,B) Diagrams on the right summarize GMC position at telophase from all six consecutive divisions, showing tight grouping of GMCs in clustered neuroblasts versus dispersal of GMCs in isolated neuroblasts. Maximum distance between two sister GMCs is uncovered by a 53° angle in clustered neuroblasts and 141° angle in isolated neuroblasts. (**C**) Quantitation of GMC position at telophase during successive divisions in clustered and isolated neuroblasts. Only neuroblasts dividing more than three times were scored. (**D**) Three serial confocal sections of a cell cluster fixed and stained for neuroblast and epithelial markers (as indicated). The Insc-positive neuroblast cortex directly contacts several DE-cadherin-positive epithelial cells. Brackets indicate the same neuroblast in the three sections, asterisks indicate the DE-cad positive epithelium only in the left panel and arrows indicate localized DE-cadherin in the middle and right panels. Scale bar: 10 μm.

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

	Clustered neuroblasts*	Isolated neuroblasts*
Mitotic stage†	Time‡	Time‡
NEB to Ana B	4:54 (*n*=19)	4:42 (*n*=20)
NEB to NEF	10:06 (*n*=19)	9:06 (*n*=20)

*Neuroblasts identified on the basis of size, asymmetric division pattern and mitotic potential.
†NEB (nuclear envelop breakdown); Ana B (anaphase B); NEF (nuclear envelop reformation after mitosis).
‡Mean time in minutes:seconds.

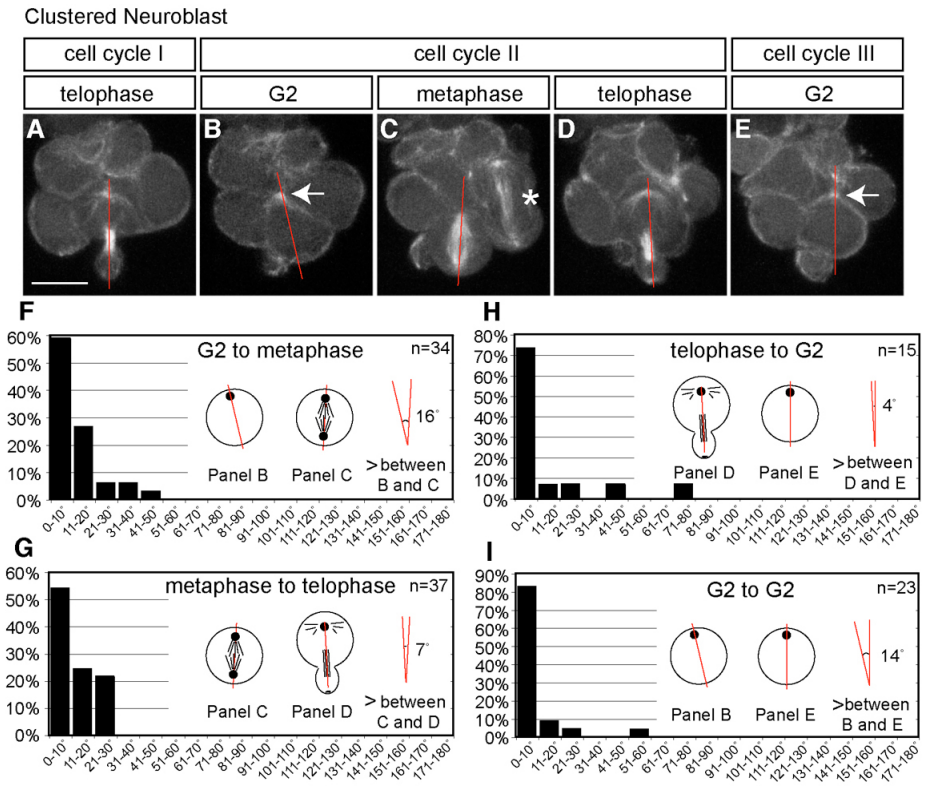

Fig. 3. In the presence of extrinsic cues, G2 centrosome position remains constant and correlates with a reproducible division axis. (**A-E**) Series of still images from a time-lapse confocal movie of a clustered neuroblast dissociated from G147-GFP embryos, during three consecutive cell cycles. Mitotic stages and cell cycles are as indicated. A red line connects both apical and basal centrosomes during mitosis through the spindle axis or the G2 centrosome and approximate cell center during interphase. The white arrows indicate the two centrosomes that remain closely apposed at G2. The asterisk indicates an epithelial cell dividing symmetrically. (**F-I**) Quantitation of the angle between the following two lines: (F) a line connecting the G2 centrosomes to cell center and a line through the metaphase spindle; (G) a line through the metaphase spindle and a line through the telophase spindle; (H) a line through the telophase spindle and a line connecting the G2 centrosomes to cell center in the subsequent division; and (I) a line connecting the G2 centrosomes to cell center and a line connecting the G2 centrosomes to cell center in the following division. Black bars indicate neuroblast percent. Scale bar: 10 μm.

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

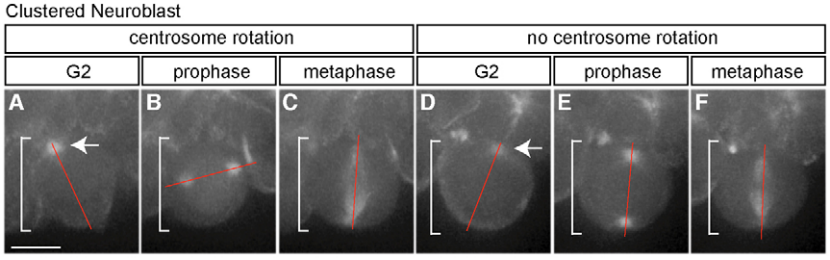

Fig. 4. In the presence of extrinsic cues, spindle orientation correlates with G2 centrosome position despite centrosome rotation at prophase. (A-F) Series of still images from two different confocal time-lapse movies from clustered neuroblasts from a single division, dissociated from G147-GFP embryos. Mitotic stages labeled above each panel. (**A-C**) An example of a neuroblast showing centrosome rotation prior to spindle formation and (**D-F**) an example of a neuroblast showing no centrosome rotation prior to spindle formation. A red line connects both apical and basal centrosomes during mitosis or the G2 centrosomes and approximate cell center during interphase. Brackets indicate neuroblasts. The white arrows indicate the two centrosomes that remain closely apposed at G2. Scale bar: 10 μm.

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

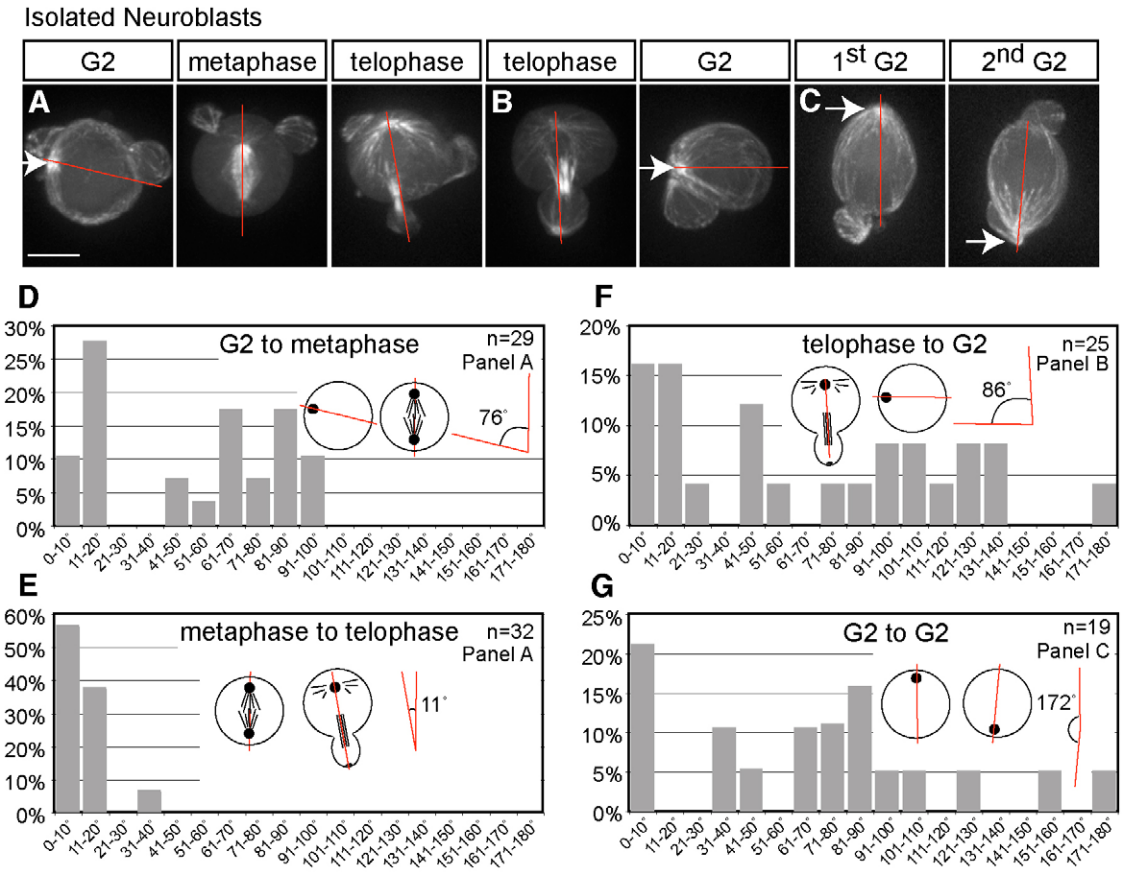

Fig. 5. In the absence of extrinsic cues, centrosome position and the cell division axis drift over multiple cell cycles. (**A-C**) Series of still images from three different time-lapse confocal movies of isolated neuroblasts dissociated from G147-GFP embryos. Mitotic stages are labeled above each panel. A red line connects both apical and basal centrosomes during mitosis through the spindle axis or the G2 centrosomes and approximate cell center during interphase. The white arrow indicates the two centrosomes that remain closely apposed at G2. (**D-G**) Quantitation of the angle between the following two lines: (D) a line connecting the G2 centrosomes to cell center and a line through the metaphase spindle as seen in A; (E) a line through the metaphase spindle and a line through the telophase spindle as seen in A; (F) a line through the telophase spindle and a line connecting the G2 centrosomes to cell center in the subsequent division as seen in B; (G) a line connecting the G2 centrosomes to cell center and a line connecting the G2 centrosomes to cell center in the following division as seen in C. Gray bars indicate neuroblast percent. Scale bar: 10 μm.

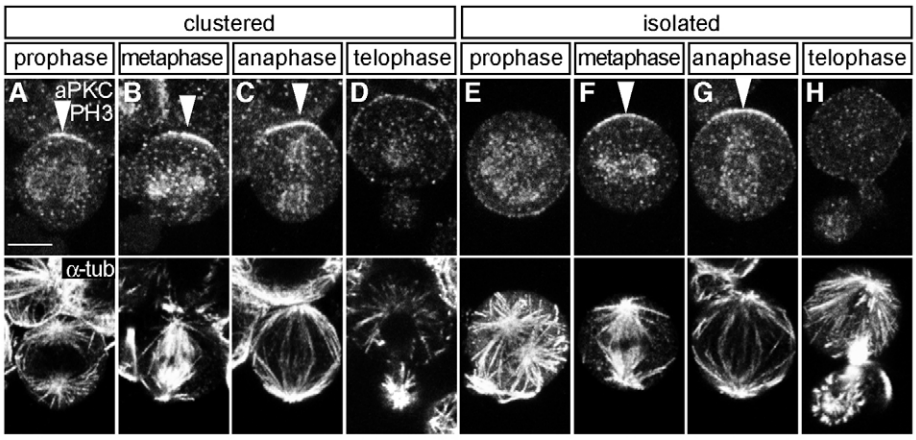

Fig. 6. In the absence of extrinsic cues, neuroblast cortical polarity does not form until metaphase. (**A-D**) Double-labeled clustered and (**E-H**) isolated neuroblasts in culture, fixed and stained for aPKC and PH3 (top row), and for α-tubulin (bottom row). Mitotic stages are listed above each panel.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

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.

Table 2. Asymmetric localization of Par proteins in clustered and isolated mitotic neuroblasts

	Par crescents* in clustered neuroblasts (%)†	Par crescents* in isolated neuroblasts (%)†
Prophase	82 (*n*=38)	25 (*n*=55)
Metaphase	100 (*n*=77)	100 (*n*=64)
Anaphase	100 (*n*=17)	100 (*n*=10)
Telophase‡	100 (*n*=21)	94 (*n*=18)

*Includes Baz and aPKC crescents.
†Only Worniu or GFP (from embryos expressing Dlg:eGFP driven by *worniu-GAL4*)-positive neuroblasts were scored.
‡Par proteins restricted to neuroblast cortical domain.

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, Gαi, 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, Gαi) 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

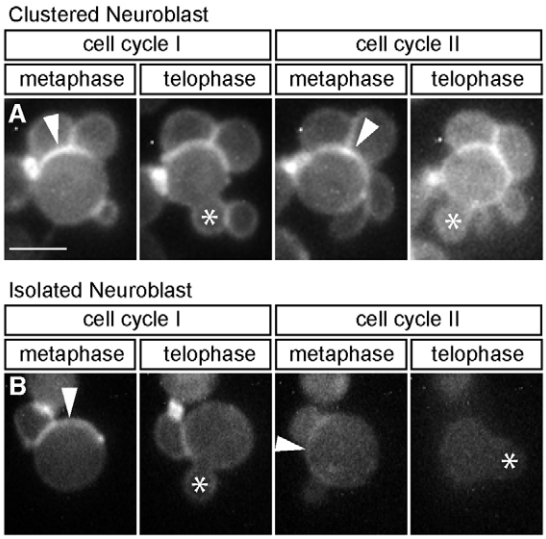

Fig. 7. In the absence of extrinsic cues, neuroblast cortical polarity does not form at the same position over multiple cell cycles. Series of still images from time-lapse confocal movies from a clustered (**A**) and an isolated (**B**) neuroblast dissociated from embryos ubiquitously expressing Dlg:eGFP. Mitotic stages and cell cycles are listed above each panel. White arrowheads indicate the center of the Dlg:eGFP crescent, most prominent at metaphase. White asterisks indicate the position of the newly born GMCs. Scale bar: 10 μm.

pathway (Rolls et al., 2003; Schober et al., 1999; Wodarz et al., 1999). In isolated neuroblasts, Par crescents form over one pole of a randomly oriented mitotic spindle, raising the possibility that astral microtubules may induce Par crescents, similar to their ability to trigger Pins/Gαi/Dlg crescents (Siegrist and Doe, 2005). Although Par crescents can still form in the absence of both microtubules and extrinsic cues (such as in Colcemid-treated isolated neuroblasts; data not shown), astral microtubules may be necessary to direct the position of Par crescents in isolated neuroblasts.

In the future, it will be important to determine the relationship between centrosome position and position of cortical polarized Par proteins. Both require an extrinsic signal from the overlying epithelium, but they could be independently regulated by two different signals, independently regulated by the same signal, or they could act in a linear pathway. For example, a single extrinsic cue could anchor the G2 centrosome pair, and then the centrosome pair could induce apical cortical polarity at the G2/prophase transition, similar to centrosome-induced cortical polarity in the *C. elegans* zygote (Cowan and Hyman, 2004).

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 Schweisguth, 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 McNeill, 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 *mys* 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. Similarly, during adult PNS development, the pIIb cell lies within the imaginal disc epithelium yet divides along the apicobasal axis. In both cases, the reproducibly apicobasal spatial pattern of cell divisions occurs independent of an overlaying 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

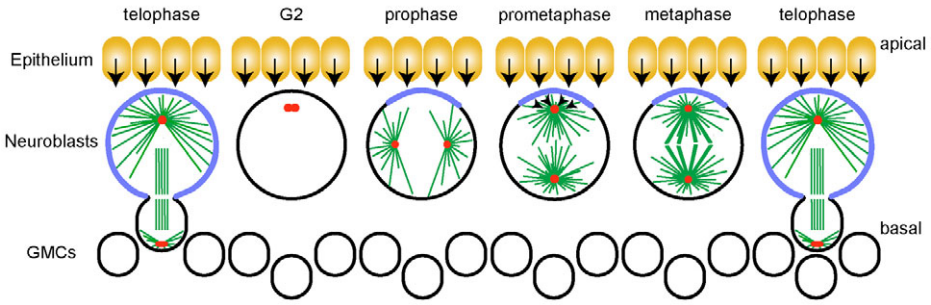

Fig. 8. Model for extrinsic signaling. During the cell cycle, the neuroblast receives positional cues from the epithelium, to polarize along the apicobasal embryonic axis, and during mitosis this allows for the basal placement of all GMC daughters and their progeny after cytokinesis. At late G2, extrinsic signaling indicates the position on the neuroblast cortex to establish Par apical polarity. During prophase, apical Par crescents are forming at this pre-selected position, independently of centrosome behavior. The position of the apical crescents determines spindle orientation at prometaphase and spindle position is maintained throughout mitosis, ensuring the reproducible basal placement of all GMCs. Extrinsic cues also maintain centrosome(s) position at the epithelial/neuroblast contact site throughout the cell cycle. See Discussion for further details.

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 overlaying epithelium and then rotates 90° to align orthogonal to the overlaying epithelium or the spindle forms as it rotates into its proper orientation (Kaltschmidt 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 epithelial 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 tau:GFP fusion protein. This difference alone could account for the observed differences between the two studies.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/3/529/DC1

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