

Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts

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SUMMARY

Stem cells and neuroblasts derived from mouse embryos undergo repeated asymmetric cell divisions, generating neural lineage trees similar to those of invertebrates. In *Drosophila*, unequal distribution of Numb protein during mitosis produces asymmetric cell divisions and consequently diverse neural cell fates. We investigated whether a mouse homologue *m-numb* had a similar role during mouse cortical development.

Progenitor cells isolated from the embryonic mouse cortex were followed as they underwent their next cell division *in vitro*. Numb distribution was predominantly asymmetric during asymmetric cell divisions yielding a β -tubulin III⁻ progenitor and a β -tubulin III⁺ neuronal cell (P/N divisions) and predominantly symmetric during divisions producing two neurons (N/N divisions). Cells from the *numb* knockout mouse underwent significantly fewer asymmetric P/N divisions compared to wild type, indicating a causal role for Numb.

When progenitor cells derived from early (E10) cortex undergo P/N divisions, both daughters express the progenitor marker Nestin, indicating their immature state, and Numb segregates into the P or N daughter with similar frequency. In contrast, when progenitor cells derived from

later E13 cortex (during active neurogenesis *in vivo*) undergo P/N divisions they produce a Nestin⁺ progenitor and a Nestin⁻ neuronal daughter, and Numb segregates preferentially into the neuronal daughter. Thus during mouse cortical neurogenesis, as in *Drosophila* neurogenesis, asymmetric segregation of Numb could inhibit Notch activity in one daughter to induce neuronal differentiation.

At terminal divisions generating two neurons, Numb was symmetrically distributed in approximately 80% of pairs and asymmetrically in 20%. We found a significant association between Numb distribution and morphology: most sisters of neuron pairs with symmetric Numb were similar and most with asymmetric Numb were different. Developing cortical neurons with Numb had longer processes than those without.

Numb is expressed by neuroblasts and stem cells and can be asymmetrically segregated by both. These data indicate Numb has an important role in generating asymmetric cell divisions and diverse cell fates during mouse cortical development.

Key words: Stem cells, Progenitor cells, Asymmetric cell division, Fate determination, Mouse, Cerebral cortex

INTRODUCTION

A rich variety of central nervous system (CNS) cells arise from a small number of neuroepithelial progenitor cells in the early embryo. It is important to understand how this cell diversification occurs, as it is fundamental to normal neural development, and has implications for possible nervous system repair therapies.

At embryonic day 10 (E10) in the mouse, the primordial cerebral cortex consists of a single layer of proliferating neuroepithelial cells. Clonal studies indicate that approximately 15% of these are multipotent stem cells that generate both neurons and glia, while the remainder are restricted neuroblasts that generate solely neurons (Qian et al., 1998; Qian et al., 2000). Both these progenitor cell types can

undergo repeated asymmetric cell divisions, as observed by time-lapse microscopy of clonal development *in vitro*. At first, both neuroblasts and stem cells undergo asymmetric cell divisions generating neurons. Later, stem cells undergo a specific type of asymmetric cell division at which point they start to produce glia (Qian et al., 1998; Qian et al., 2000). Interestingly, the largely asymmetric, neural lineage trees of mouse cortical progenitor cells are similar to those of invertebrates (Sulston and Horvitz, 1977; Sulston et al., 1983; Doe and Technau, 1993; Shen et al., 1998), suggesting that this is a fundamental, evolutionarily conserved feature of neural development.

There is mounting evidence that repeated asymmetric divisions of cortical progenitor cells observed *in vitro* also occur *in vivo*. The slow increase in cortical cell number and

the proliferation characteristics of progenitor cells during neurogenesis suggest predominantly asymmetric division modes (Rakic, 1995; Mione et al., 1997; Reid et al., 1997). Clones that span multiple cortical layers are observed both after retroviral labeling and in chimeric embryos, consistent with repeated asymmetric division of ventricular zone cells (Tan and Breen, 1993; Kornack and Rakic, 1995; Reid et al., 1995; Mione et al., 1997). More recently, observation of embryonic cortical slices has shown that radial glia, now recognized as major neuronal progenitors, divide asymmetrically to produce a neuron and another radial progenitor (Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2002). Hence it is likely that asymmetric cell divisions play an important role in generating diverse neural cells in vertebrates, as they do in invertebrates.

Molecular mechanisms underlying asymmetric cell divisions have been elucidated in invertebrate systems. In *Drosophila*, Numb is a key factor in asymmetric neural progenitor cell divisions. In PNS sensory organ precursor cells (SOPs) Numb segregates in a crescent to one side of the SOP during metaphase and is then found in the IIB rather than the IIA daughter. Subsequently Numb directs asymmetric division of both IIA and IIB cells. CNS neuroblasts divide asymmetrically repeatedly, giving another neuroblast and a ganglion mother cell (GMC) at each division. Numb protein becomes localized to a crescent in the neuroblast, and as division ensues it segregates into the GMC. Numb can direct further asymmetric cell division of GMCs (Rhyu et al., 1994; Knoblich et al., 1995; Spana et al., 1995; Spana and Doe, 1996; Buescher et al., 1998).

Numb acts at multiple points in these lineages: at early stages to determine progenitor cell types and at terminal cell divisions to distinguish final fates. Loss of Numb function in CNS and PNS lineages equalizes cells, producing two identical daughters (Uemura et al., 1989; Rhyu et al., 1994; Spana et al., 1995). However, Numb acquisition does not confer one particular cell fate: Numb can be segregated into progenitor cells, neurons, or glia (Rhyu et al., 1994; Spana et al., 1995; Gho et al., 1999; Van de Bor et al., 2000; Roegiers et al., 2001).

Vertebrate homologues of *Drosophila numb* have been identified in mouse, rat, chicken and human (Zhong et al., 1996; Dho et al., 1999; Verdi et al., 1996; Wakamatsu et al., 1999; Salcini et al., 1998; Verdi et al., 1999), and are structurally similar: for example mouse Numb can rescue the fly Numb loss-of-function phenotype (Zhong et al., 1996; Verdi et al., 1996). The role of Numb in vertebrates is not yet clear, and there are some apparently contradictory findings. There are indications for a role of Numb in progenitor cells. For example, in mouse cortical ventricular zone cells, Numb may be localized at the apical membrane so that a horizontal division would distribute Numb into the apical daughter, believed to be the progenitor based on its migratory behavior (Chenn and McConnell, 1995; Zhong et al., 1996). Over-expression of Numb in vivo in the chick CNS enhances progenitor proliferation (Wakamatsu et al., 1999) and knockout of *numb* in mice results in premature expression of neuronal markers, again indicating a role in neural progenitor cells (Zhong et al., 2000). In contrast, there are also indications for a role of Numb in neuronal differentiation. Over-expression of Numb in vitro leads to greater neuron production, and *numb* mutant mice have impaired neuronal differentiation in selected

CNS and PNS lineages (Verdi et al., 1996; Verdi et al., 1999; Zilian et al., 2001).

It is quite possible that Numb has multiple roles during neural development, in progenitor maintenance and differentiation. Still, an important question regarding Numb function remains unresolved: no study to date has determined whether Numb plays a similar role in generating asymmetric cell divisions in the vertebrate as it does in *Drosophila*. Asymmetric Numb distribution has been observed in mouse cortical ventricular zone cells (Zhong et al., 1996), in chick neuroepithelial cells and neural crest lineages (Wakamatsu et al., 1999; Wakamatsu et al., 2000), and in rat retinal neuroepithelial cells (Cayouette et al., 2001), but it has not been shown that this leads to production of different cell fates. Moreover, for cortical progenitor cells it is not known whether Numb is indeed asymmetrically segregated during mitosis. Resolution of these issues requires following Numb distribution during progenitor cell divisions, and correlating this with daughter cell fates. Because this is currently impossible to accomplish in vivo, we investigated this question using clonal cultures of cortical progenitor cells as a model system.

Numb expression was examined in dissociated cells from normal and *numb* knockout E10-E14 mouse embryos as they divided and generated differentiated progeny. We provide direct evidence that Numb is asymmetrically distributed during cortical progenitor cell divisions, and that this is linked to asymmetry in cell fate in both early progenitor cells and at terminal cell divisions generating two neurons.

MATERIALS AND METHODS

Tissue dissociation

Timed pregnant Swiss Webster mouse embryos (Taconic farms) at E10-E14 (plug date is designated day 0) were used. Cerebral cortices were dissected and enzymatically dissociated using papain (Worthington) as described previously (Qian et al., 1998). Briefly, cortical tissue was incubated in 10-12 Units/ml activated papain solution plus 32 µg/ml DNase in DMEM with rocking for 30 minutes at room temperature. The tissue was rinsed 3 times with DMEM (Gibco) and triturated with a fire-polished glass Pasteur pipette to generate a single-cell suspension.

Cell culture

Single cells were plated at clonal density into 12 µl of culture medium in poly-L-lysine coated Terasaki wells in serum-free culture medium: DMEM with L-glutamine, sodium pyruvate, B-27, N-2 (Gibco), 1 mM N-acetyl-cysteine (Sigma) and 10 ng/ml bFGF (Gibco) added as a mitogen. Plated cells were incubated at 35°C with 6% CO₂ and 100% humidity.

Immunohistochemistry

Sections

E10.5 embryos were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) (pH 7.4) at 4°C overnight and cryoprotected in 30% sucrose in 0.1 M PB. 10 µm cryostat sections were blocked in 0.1% Triton X-100 and 1% normal goat serum in PBS for 15 minutes. Primary antibody diluted in blocking solution was added overnight at 4°C. For Numb staining, affinity-purified rabbit polyclonal antibody (1:500) was added overnight at room temperature.

Acutely isolated and cultured cells

Dissociated cells were plated for 2-4 hours for acute staining, or cultured for 1-7 days. Cells were fixed in ice-cold 4% PFA at room

temperature for 30 minutes. Affinity-purified rabbit anti-Numb antibody (1:500 with 10% normal goat serum) was added overnight at 4°C, and visualized with Alexa 546 goat anti-rabbit IgG (1:200; Molecular Probes) or biotinylated goat anti-rabbit IgG (1:200; Vector Labs) followed by streptavidin Alexa 546 (1:200; Molecular Probes) or ABC-VIP kit (Vector Labs). For monoclonal anti- β -tubulin III (1:400; Sigma), and Nestin (1:4; Developmental Studies Hybridoma Bank), fixed cells were permeabilized with 100% methanol at -20°C for 5 minutes, and incubated with primary antibody overnight at 4°C. Staining was visualized using Alexa 488 or 546 goat anti-mouse IgG (1:200; Molecular Probes).

Time-lapse video microscopy

Cells were placed under an inverted microscope, and the image captured with a CCD camera connected to a Panasonic time-lapse video recorder. Cultures were monitored for up to 7 days, then fixed and stained for Numb and β -tubulin III expression.

BrdU incorporation assay

E10-12 cortical cells were cultured in Terasaki microwells and pairs were identified at 16 hours. 10 μ g/ml BrdU was added to the wells for 8-10 hours. Cells were washed, fixed in 4% PFA and stained using an anti-BrdU antibody directly conjugated to fluorescein (1:10; Becton-Dickinson) and β -tubulin III.

Morphological measurements of neurons

Comparison of neurite length

Low density cortical cultures were fixed at 7 days and stained for Numb and β -tubulin III. Images of all neurons were digitized and total neurite length was measured (Scion software) and compared between Numb⁺ and Numb⁻ cells by Student's *t*-test.

Comparison of sister neuron morphology

Sister neurons were scored for number of primary processes, average process length, and branch points (Scion software). Differences in these values between sister neurons were calculated. The differences obtained for Numb symmetric neuron pairs and Numb asymmetric neuron pairs were compared by Student's *t*-test.

RESULTS

Numb is expressed by cortical stem cells, neuroblasts and immature neurons

Using a rabbit polyclonal antibody specifically recognizing Numb and not the related protein Numb-like (Zhong et al., 1996), we examined Numb expression in the early cortical neuroepithelium at E10.5. As shown in Fig. 1A, Numb was detectable in most cells at this stage. There are some patches of more intense labeling, and occasional cells at the ventricular surface appear to have more Numb at their apical border, as described previously for later stages (Zhong et al., 1996; Zhong et al., 1997).

To quantify Numb expression in different cortical progenitor cell populations, E10-14 mouse cortical cells were dissociated, fixed acutely and stained for cell-type markers and Numb (Fig. 1). At E10.5, almost all cells stained for the progenitor marker Nestin (Lendahl et al., 1990) and approximately 90% of these also expressed Numb. Numb was detected in 83% of cells expressing LeX, a neural stem cell marker (Capela and Temple, 2002). Numb was also found in newborn, β -tubulin-III⁺ neuronal cells, but at a lower level (65%) (Fig. 1). The broad expression of Numb in cortical stem cells, neuroblasts, and differentiating neurons is similar to its broad expression in

Drosophila CNS where Numb is present in stem-like neuroblasts, restricted GMC progenitor cells and their neuronal and glial progeny (Jan and Jan, 2001).

Numb is symmetrically and asymmetrically distributed during embryonic mouse cortical progenitor cell divisions

Acutely isolated cells from E10-E13 cerebral cortex were sometimes observed to have a crescent of Numb staining, as shown in Fig. 2F, similar to Numb crescents described in insects (Rhyu et al., 1994; Knoblich et al., 1995; Spana et al., 1995). The low frequency of crescents (7% of total cells) suggests they form in a narrow time window, possibly during metaphase, as they do in insects.

To examine how Numb protein is distributed when cells divide, we developed the 'cell pair assay' (Fig. 2A). Single E10-13 cortical cells were plated at low density (20-30 cells/well) in Terasaki plates. Cells in each well were mapped 2 hours after plating to record their location. Most of the first rounds of divisions were complete by 24 hours, and cultures were mapped again to identify daughter cell pairs. The cells were fixed and examined for Numb distribution. In some pairs Numb was detectable in only one daughter cell (asymmetric) and in others it was detectable in both daughters (symmetric), (Fig. 2G-I). Of 225 pairs of E10 cortical cells examined, 60.4% had symmetric Numb distribution and 31.1% had asymmetric Numb; the remaining 8.4% of pairs had no detectable Numb (Table 1). Similarly, of 206 pairs of E13 cortical cells examined, 63.1% had symmetric Numb and 31% had asymmetric Numb. In cases where both cells of a pair had detectable Numb (symmetric), the amount present appeared similar in each cell.

The observed Numb distribution at 24 hours could be the result of three different processes: segregation of Numb during mitosis, de novo synthesis, and degradation. To determine if Numb could actually be segregated asymmetrically during a division, we examined cortical progenitor cell pairs right after mitosis. Low-density E10-E11 cortical progenitor cells on a Terasaki plate were examined by time-lapse video microscopy. Every 10 minutes the field was checked for a cell division, and when one was observed the cells were fixed and stained for Numb. The time-lapse recordings were used to precisely identify and time each cell division. Of 24 cells that divided within 10 minutes before fixation, 50% produced pairs with symmetric Numb distribution and 46% pairs with asymmetric Numb. Pairs generated during the 2-hour period from 22-24 hours after plating were also examined. Of 134 pairs, 43% showed symmetric Numb and 44% showed asymmetric Numb

Table 1. Numb distribution during E10 cortical cell divisions

Numb staining	Time after division when pairs were fixed		
	0-10 minutes (24 pairs)	0-2 hours (134 pairs)	0-24 hours (225 pairs)
●●	50%	43.3%	60.4%
●○	45.8%	44.0%	31.1%
○○	4.2%	12.7%	8.5%

●●/○-○ symmetric Numb distribution.
●○ asymmetric Numb distribution.

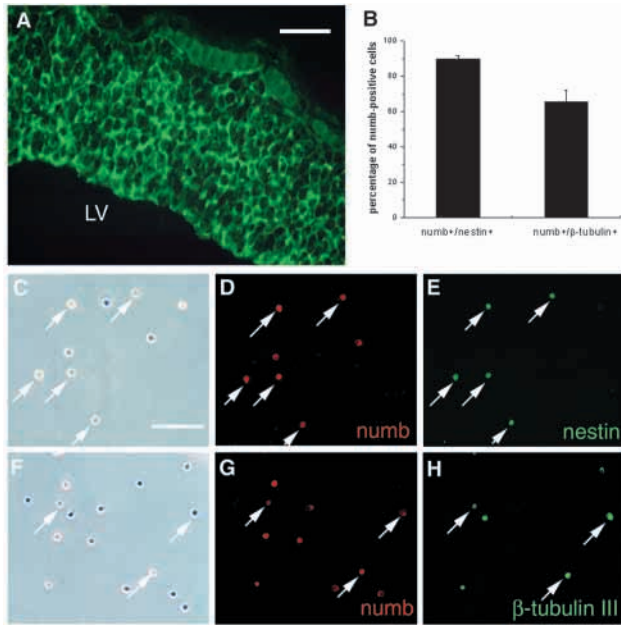


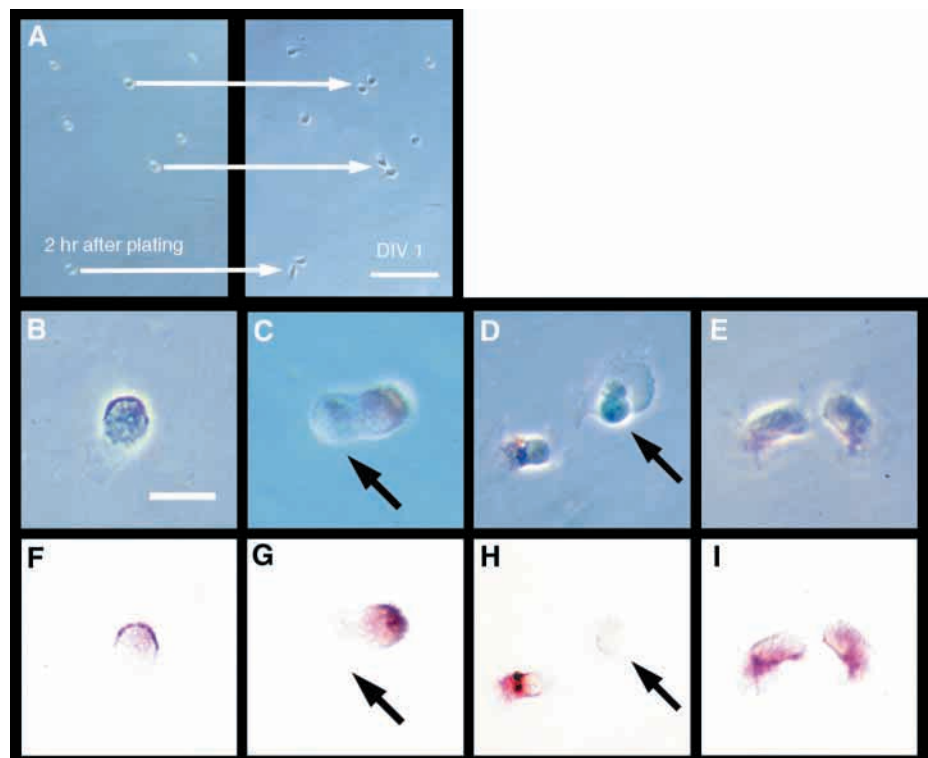
Fig. 1. Numb expression in the E10.5 mouse cortex in vivo and in acutely isolated cells. (A) Numb staining is seen throughout the E10.5 mouse cerebral cortex. Some ventricular zone cells show strong staining at their apical border. LV, lateral ventricle. (B-H) E10.5 cortical cells were isolated, fixed acutely, stained with anti-Numb antibody and double labeled with an antibody to Nestin or β -tubulin III. (B) Quantification of Numb⁺ cells in the progenitor cell population (Nestin⁺) and immature neuronal population (β -tubulin III⁺). (C,F) Phase-contrast images of the fields stained for Numb (D and G, in red), Nestin (E, in green), or β -tubulin III (H, in green). Arrows indicate cells double labeled for Numb and Nestin (C-E) and Numb and β -tubulin III (F-H). Scale bars: 100 μ m.

distribution, indicating that the initial Numb distribution after mitosis was maintained at the 2-hour time-point (Table 1). These data demonstrate that Numb can indeed be asymmetrically segregated during mouse CNS progenitor cell divisions. In fact, the percentage of pairs with asymmetric Numb distribution was slightly, although not significantly, greater at 10 minutes compared to 24 hours, showing that some initially asymmetric pairs had become obscured due to Numb synthesis and/or degradation.

Asymmetric distribution of Numb is associated with asymmetric cortical cell divisions

Given the significant incidence of asymmetric Numb distribution by

Fig. 2. Symmetric and asymmetric Numb distribution is observed during cortical cell divisions. (A) Illustration of the pair assay. The left panel shows a phase image of a field of single cortical cells 2 hours after plating into Terasaki microwells. The cells were monitored under the microscope and cells that divided were identified: arrows point to pairs of sister cells after 1 day in vitro (DIV). (B-I) Numb staining in cortical progenitor cells. E10-E12 cortical cells were dissociated, plated into Terasaki plates, fixed at different time-points, and stained for Numb. (B-E) Phase-contrast images of the fields shown in F-I. (B,F) A cortical progenitor cell with Numb distributed in a crescent on one side of the cell cortex. (C,G) A pair of sister cells fixed within 10 minutes after mitosis that was in the process of separating. Numb is seen in only one of the daughter cells, demonstrating that it can be asymmetrically segregated during mitosis. Arrow indicates the Numb-negative cell. (D-I) Pairs of sister cells identified 24 hours after plating. Numb may be asymmetrically (H) or symmetrically (I) distributed into the two daughter cells. Scale bars: (A) 100 μ m; (B-H) 20 μ m.



cortical progenitor cells, we investigated whether this is related to asymmetric cell division, i.e. production of two different daughter cell fates.

During the early embryonic period, most differentiated cells generated within the cerebral cortex are neurons, while most glia are produced postnatally. Hence, using the neuronal marker β -tubulin III we could theoretically detect the following types of daughter cell pairs from embryonic cortical progenitor cells: two progenitor cells (P/P), a progenitor and a neuron (P/N) and two neurons (N/N). Without other distinguishing markers, we do not know whether the two cells in a P/P or an N/N pair are the same or different. However, a P/N division is clearly asymmetric, hence we focused on this type of pair.

Single E10-E14 cortical progenitor cells were plated and allowed to undergo their next cell division in vitro. Pairs of cortical cells were identified, fixed 24 hours after plating and stained for both Numb and β -tubulin III; P/N (β -tub⁻/ β -tub⁺) and N/N pairs (both β -tub⁺) (Fig. 3) were identified. As shown in Table 2, the vast majority of P/N pairs (81% at E10 and 72% at E13) showed asymmetry in Numb distribution. Similarly, the vast majority of N/N pairs (79% at E10 and 88% at E13) showed symmetric Numb distribution. A Chi-squared test

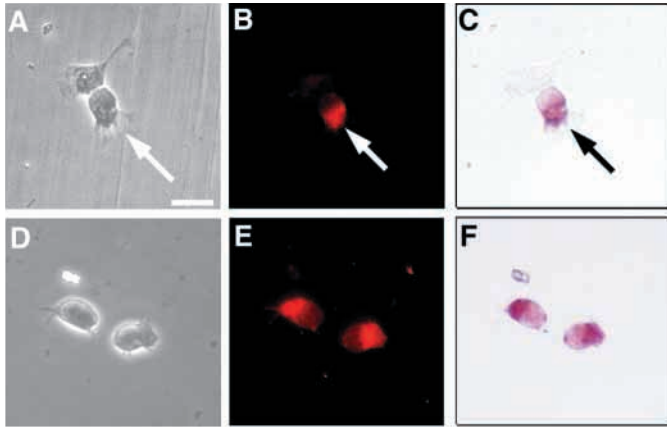


Fig. 3. Asymmetric Numb distribution is correlated with asymmetric cell divisions generating a cortical progenitor cell and a neuron. (A-C) An E10 cell pair consisting of a neuron and a progenitor cell that has asymmetric Numb expression. (A) Phase-contrast image; (B) β -tubulin III staining is present in one daughter; (C) Numb is present only in the neuronal daughter (arrow). In other pairs, Numb was found in the progenitor and not the neuronal daughter (not shown). (D-F) An E10 cell pair consisting of two neurons that have symmetric Numb expression. (D) Phase-contrast image; (E) β -tubulin III staining is present in both daughter cells; (F) symmetric Numb staining. Scale bars: 20 μ m.

shows a highly significant association between asymmetric Numb distribution and the asymmetric P/N fate, and between symmetric Numb distribution and the N/N fate.

Asymmetric P/N cell divisions are decreased in the Numb knockout mouse

We examined whether the association between asymmetric Numb distribution and asymmetric cell division was causally related. Mice homozygous for a loss-of-function mutant allele of *numb* exhibit defects in cranial neural tube closure and die around embryonic day 11.5 (Zhong et al., 2000; Zilian et al., 2001). To investigate whether asymmetric divisions are affected when *numb* function is disrupted, we compared the divisions of cortical progenitor cells from E10.5 mutant embryos with those from wild-type littermates.

E10.5 mutant embryos were identified by their distinct phenotype. Dorsal telencephalic tissue was dissected, and progenitor cells isolated from mutant and wild type littermates were cultured separately. 24 hours after plating, pairs of daughter cells from single progenitor cells were identified, fixed, and stained for β -tubulin III.

Table 2. Numb distribution in cortical pairs is associated with cell fate

	E10*		E13*	
	P/N pairs	N/N pairs	P/N pairs	N/N pairs
Asymmetric Numb	80.6% (25)	20.7% (25)	72.2% (26)	12.3% (10)
Symmetric Numb	19.4% (6)	79.3% (96)	27.8% (10)	87.7% (71)

E10 and E13 cell pairs were stained for Numb and β -tubulin III (number of cell pairs in parentheses). There is a significant association between the asymmetric P/N fate and asymmetric Numb distribution and between the N/N fate and symmetric Numb distribution.

* $P < 0.05$, χ^2 test.

There was a significant reduction, approximately two fold, in asymmetric P/N cell divisions in the mutant mice compared to wild-type littermates (Table 3), and production of P/P divisions increased by an equivalent amount. The percentage of N/N pairs was unchanged in the mutant compared to wild type. This suggests that without Numb, progenitor cells are impaired in their ability to undergo an asymmetric division producing a neuronal daughter and another progenitor cell, while their production of two neurons remains unhindered. Long-term analysis of Numb mutant progenitor pairs was not possible because the cells died by 3 days in vitro, indicating Numb may be essential for neural cell survival.

Developmental change in Numb distribution during asymmetric cortical divisions

Having shown that asymmetric Numb distribution is strongly associated with, and critical for, asymmetric P/N divisions, we investigated whether Numb segregates into the progenitor or neuronal daughter. In E10 P/N pairs stained at 24 hours, Numb was found in the neuronal daughter in 60% of cases and in the progenitor daughter in 40% ($n=25$). A similar distribution was seen in E10 P/N pairs stained at 2 hours: Numb was found in the neuronal daughter in 56% of cases and in the progenitor in 44%. Hence, at this early age, which is prior to the period of active neurogenesis in vivo, Numb segregates into the progenitor or the neuronal daughter of isolated progenitor cells at approximately the same frequency.

The major period of cortical neurogenesis begins around E12 in the mouse, accompanied by an increase in asymmetric cell divisions (Takahashi et al., 1996). We examined the direction of Numb segregation in asymmetric P/N pairs derived from this period. Surprisingly, by E13, the direction of Numb movement was strongly towards the neuronal daughter: of 26 P/N pairs, Numb segregated into the neuronal daughter in 77% of cases. By E14, Numb was even more inclined to be segregated into the neuronal daughter: in 82% of P/N pairs (Fig. 4A).

The differences in Numb distribution observed between E10 and E13 may reflect differences in the types of asymmetric division occurring at these stages. Supporting this, we found that at E10 nearly all progeny expressed Nestin: even in asymmetric P/N divisions both daughter cells are Nestin⁺ (Fig. 4J-L), indicating they may have progenitor properties: β -tubulin III⁺ progenitors have been described previously (Memberg and Hall, 1995; Menezes et al., 1995). Indeed, we found some E10 β -tubulin III⁺ cells could incorporate BrdU and undergo mitosis (data not shown). Thus at E10 asymmetric P/N divisions may generate two different types of progenitor

Table 3. m-numb mutation reduces the number of cortical asymmetric P/N divisions

	Asymmetric P/N pairs		Symmetric	
	⊖-⊖	⊖-⊕	⊕-⊕	⊕-⊖
Wild-type (839 pairs)	15.73%	37.55%	46.72%	
Mutant (624 pairs)	8.17%**	37.98%	53.85%*	

⊕ β -tubulin III⁺ daughter cell.
** $P < 0.001$, * $P < 0.01$, χ^2 test.

cells, one in the neuronal lineage and one not, which is likely to occur in the pre-neurogenic period. In contrast, progenitors at E13-14 are undergoing active neurogenesis *in vivo* and many asymmetric divisions at this stage are destined to produce differentiated neurons. We found E13 asymmetric P/N divisions generate a Nestin⁺β-tub⁻ progenitor cell and a Nestin⁻β-tub⁺ neuron (Fig. 4): the lack of Nestin indicating that the neuronal daughter is a mature, terminal cell. To test this, we examined whether the Nestin⁻ neuronal daughters derived from older progenitors were postmitotic. Single E13 cortical cell pairs were identified at 16 hours. 10 μg/ml BrdU was added to the culture medium for the next 8-10 hours. The cells were then fixed and stained for β-tubulin III and BrdU. In 80% of pairs identified as asymmetric using β-tubulin III labeling, the β-tubulin III⁻ daughter cell had incorporated BrdU, indicating that it was indeed a proliferating progenitor cell, while its β-tubulin III⁺ sister was BrdU⁻, confirming its postmitotic status (Fig. 4H,I). As expected, Numb moves predominantly into the Nestin⁻ neuronal daughter at E13 (Fig. 4M-O).

Numb level influences neuronal morphology

Previously we showed, using long-term time-lapse recording, that both neuroblasts and stem cells from the cerebral cortex had asymmetric lineage trees (Qian et al., 1998; Qian et al., 2000). By staining time-lapse recorded clones, we found that Numb was expressed throughout the lineages in both stem cell and neuroblast clones (Fig. 5). Moreover, at terminal divisions generating two neurons, Numb could be symmetrically expressed in both daughters (Fig. 5B) or asymmetrically expressed in only one daughter (Fig. 5A) (because of the small number of lineages examined, the frequency was not quantified). The fact that Numb could be asymmetrically distributed at terminal neuroblast divisions was interesting given that in *Drosophila* asymmetric Numb distribution in the GMC can generate two different neurons (Spana and Doe, 1996; Buescher et al., 1998). In an earlier study we found that 80% of terminal neuron pairs from E14 mouse cortex cultured for 14 days had similar morphologies, while 20% were different (Qian et al., 1998). Could Numb distribution at the

final division of mouse neuroblasts determine whether the final neuronal fates were the same or different, as it does in *Drosophila*? To investigate this question, we examined Numb expression and neuronal phenotype in sister pairs.

E14 cortical cells were cultured at clonal density in Terasaki wells for 24 hours and pairs of daughter neurons were identified. 78% were found to have symmetric Numb and 18% asymmetric ($n=166$), the remainder lacked Numb staining. We noted that the frequency of pairs with

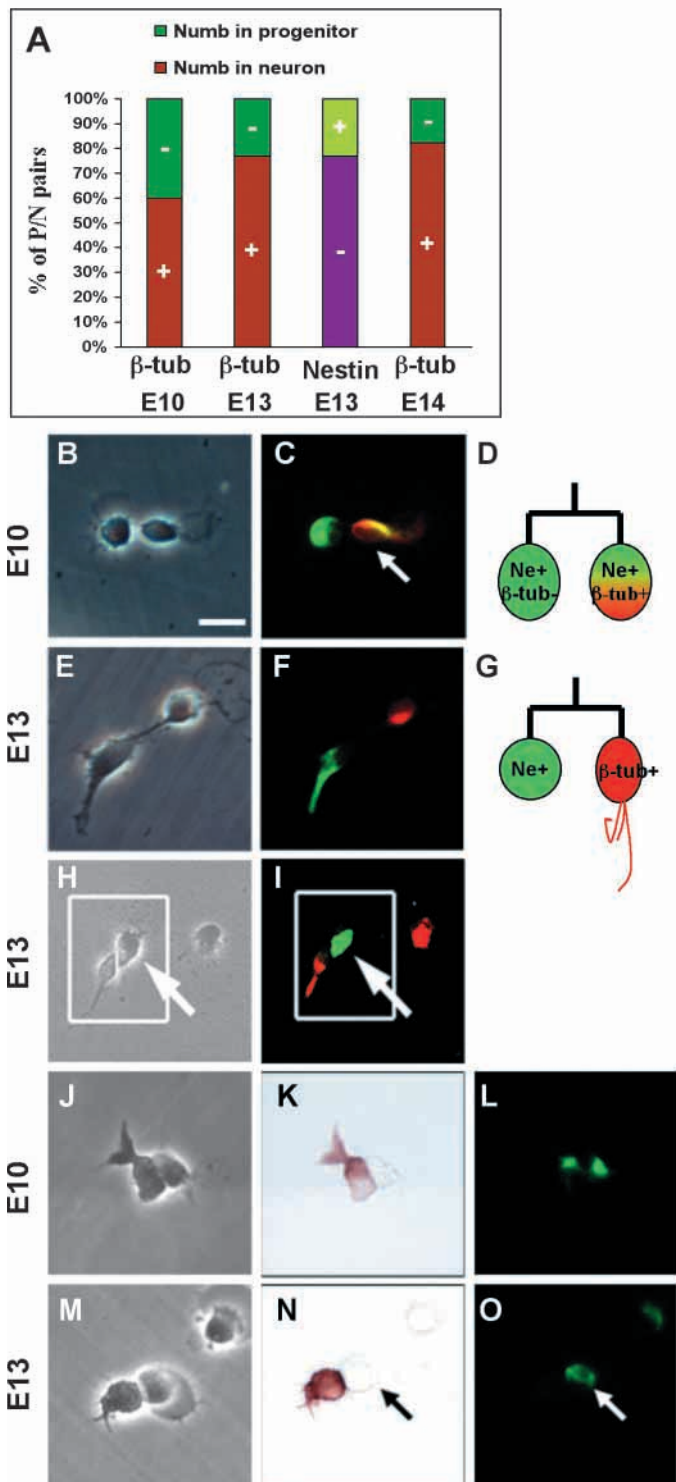
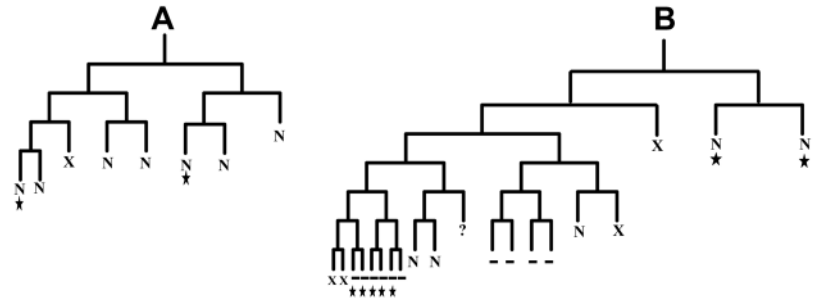


Fig. 4. Developmental change in the direction of Numb segregation at P/N divisions. (A) E10-E14 cortical cells were plated at clonal density fixed 24 hours after plating and stained for Numb and β-tubulin III or Nestin. Progenitor cell/neuron (P/N) pairs were analyzed for Numb distribution. Numb segregates into the neuronal daughter less frequently at E10 compared to cells isolated from the active neurogenic period, E13-14. (B-D) An E10 cell pair stained for Nestin and β-tubulin III. (B) Phase-contrast image. (C) Both daughter cells are Nestin⁺ (green), but only one is β-tubulin III⁺ (red). Arrow indicates the cell with double staining (yellow). (D) Diagram illustrating how P/N divisions at E10 generate a Nestin⁺β-tub⁻ cell and a Nestin⁺β-tub⁺ cell. (E-G) An E13 cell pair stained for Nestin and β-tubulin III. (E) Phase-contrast image. (F) One daughter cell is Nestin⁺ (green), the other one is β-tubulin III⁺ (red). (G) Diagram illustrating how P/N divisions at E13 generate a Nestin⁺β-tub⁻ cell and a Nestin⁻β-tub⁺ cell. (H-I) BrdU incorporation into E13 P/N pairs. A pair of sister cells (boxed) was stained for β-tubulin III (red) and BrdU (green). Only the β-tubulin III⁻ cell had incorporated BrdU (arrow), consistent with it being a proliferating progenitor cell, while the neuron was BrdU⁻. (J-L) A pair of E10 cortical sister cells stained for Numb and Nestin. (J) Phase-contrast image. (K) Numb staining is asymmetric. (L) Both daughter cells are Nestin⁺. (M-O) A pair of E13 cortical sister cells stained for Numb and Nestin. (M) Phase-contrast image. (N) Numb staining is asymmetric. (O) The Nestin⁻ daughter is Numb⁺. Arrows point to the Nestin⁺ Numb⁻ daughter cell. Scale bars: 20 μm.

Fig. 5. Lineage trees constructed from time-lapse recordings reveal that Numb is expressed at multiple stages in cortical stem cells and neuroblasts. E10-E12 cortical cells were followed with time-lapse video microscopy for up to 7 days then fixed and stained for Numb and β -tubulin III. The lineages were constructed from the recorded images, and Numb⁺ cells within the trees were identified (indicated by star). (A) A neuroblast clone. Numb was expressed in neuronal progeny generated at various stages within the lineage trees. (B) A stem cell clone. Numb was seen in neurons and also in the dividing β -tubulin III⁻ glial progenitor cells found at the end of the lineage. In terminal neuroblast divisions that generated two post-mitotic neurons, Numb could be distributed asymmetrically or symmetrically. Examples are seen in A and B respectively, although both types of distribution occurred in neuroblast and stem cell lineages. N, β tubulin III⁺ neuron; -, non-neuronal cell; X, cell died; ?, cell migrated out of field.



symmetric and asymmetric Numb was similar to the frequency of pairs with similar versus different morphologies (80% and 20% respectively) (Qian et al., 1998). Of these newly generated neuron pairs, 62 pairs had noticeable neurite outgrowth at 24 hours, and these were analyzed for the relationship between Numb distribution and sister neuron morphology (Fig. 6A).

By eye, sister cells with symmetric Numb usually appeared similar while those with asymmetric Numb had obvious differences in morphology. To quantify this, measurements were taken of total process length, average primary process length, number of primary processes and number of branch points in neuron pairs with asymmetric or symmetric Numb staining. As shown in Fig. 6C, sister cells with asymmetric Numb had significantly larger differences in all these criteria compared to sister pairs with symmetric Numb. Hence, there is a strong association between Numb distribution and early sister neuron morphology.

To examine whether a similar relationship between Numb distribution and sister neuron morphology existed over the longer term, we plated E10.5 cortical cells, identified cell pairs, and followed terminal neuron pairs for 4 days as they elaborated processes. The pairs were then fixed and stained for β -tubulin III and Numb (Fig. 6B). 72% of these 4-day-old pairs had symmetric Numb and 19% had asymmetric Numb, which is very similar to the percentages seen at 24 hours for E14 pairs (78% and 18%) noted earlier. As in the short-term studies, visual assessment showed an obvious relationship between asymmetry and symmetry in Numb distribution and overall morphology of the cells. In particular, we noticed that when Numb was expressed asymmetrically, the Numb⁺ daughter usually had longer processes: the only exception we observed is shown in Fig. 6Bc. Numb⁺ neurons also had fewer, less branched processes, including dendrites, than their Numb⁻ sisters (Fig. 6B). In Fig. 6Bd for example, a pair of neurons has unequal Numb and the difference in process number and branching is noticeable, while a similar pair of neurons in panel 6Bh has equal Numb and does not exhibit this difference.

To quantify the relationship between Numb expression and process formation, we measured total process length for E10-derived cortical neurons cultured for 7 days. Numb⁺ neurons elaborated significantly longer processes than Numb⁻ neurons over this time-period, as shown in Fig. 7. These data indicate a close relationship between Numb distribution at terminal neuroblast divisions and the generation of similar or different sister neuron types.

DISCUSSION

Asymmetric cell division is a prominent feature in the development of a wide range of species from bacteria and yeast to worms, flies and mammals. Construction of mouse cortical progenitor cell lineage trees has shown that they share common asymmetric division patterns with neural progenitor cells from *Caenorhabditis* and *Drosophila* (Shen et al., 1998). Do they also share the same molecular mechanisms of lineage construction? We have examined possible parallels in Numb function by following the development of progenitor cells from normal and *numb* knockout mice in a simple model system. Our data show that Numb protein can be either asymmetrically or symmetrically segregated into two daughter cells during a cortical progenitor cell division, that Numb distribution is correlated with final cell fates, and that Numb function is critical for asymmetric P/N cell divisions to occur.

Numb can be concentrated at the apical border of dividing cortical ventricular zone cells in vivo (Zhong et al., 1996; Zhong et al., 1997). We showed Numb can be seen in crescents on acutely isolated cortical progenitor cells, and that it can be differentially distributed into daughter cells during a cell division. Even within 10 minutes after mitosis, 46% of E10-E12 cortical progenitor cell pairs have Numb protein in one daughter and not the other, demonstrating a high frequency of asymmetric Numb segregation. Recently, asymmetric Numb distribution has been described during cell division in the retina (Cayouette et al., 2001).

In this study, we found a significant association between asymmetric Numb distribution and asymmetric cell fate. The fact that asymmetric Numb localization is visible in daughter cells as they are exiting mitosis, (Fig. 2, Table 1), strongly suggests it is an early determinant of asymmetric fate. A causal role for Numb in generating asymmetric divisions is also indicated by the observation that P/N divisions are markedly decreased in the *numb* knockout mouse compared to the wild type. The fact that some asymmetric cell divisions still occurred in the mutant implies the existence of other asymmetric cell determinants operating at E10. However, given that the mutant dies so early, it is still unknown how lack of Numb might influence asymmetric cell divisions during the later neurogenic period.

At E13-14, which is the peak neurogenic period in vivo, during asymmetric P/N divisions, Numb moves preferentially into the neuronal daughter. Recent studies have demonstrated that radial glia are major progenitors for neurons during this

period and that they divide asymmetrically to produce another radial glia and a neuron (Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2002). Radial glia express Nestin and RC2 (Lendahl et al., 1990; Misson et al., 1988), and we found that in P/N pairs at E13-E14, the progenitor is Nestin⁺ and RC2⁺ (Fig. 4, data not shown). Hence, these cell divisions might correspond to those of radial glia. Notch is expressed in radial glia and overexpression of Notch in the embryonic cerebral cortex promotes the radial glial fate and subsequently astrocyte formation (Gaiano et al., 2000; Tanigaki et al., 2001). In *Drosophila*, Numb acts by inhibiting Notch activity (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Campos-Ortega, 1996; Van de Bor and Glangrande, 2001). For example, when *Drosophila* CNS neuroblasts divide, Numb segregates into the GMC and inhibits Notch to stimulate neuronal differentiation. In vertebrates, Numb also appears to inhibit Notch function (Verdi et al., 1996; Berezovska et al., 1999; Sestan et al., 1999; Wakamatsu et al., 1999; Redmond et al., 2000). Thus, during cortical neurogenesis, Numb segregation into one daughter of a radial glial progenitor could inhibit Notch, allowing the cell to differentiate down the neuronal pathway (Fig. 8).

Although during the neurogenic period Numb segregates preferentially into the neuronal daughter, this was not observed at earlier stages. At E10, while asymmetric distribution of Numb is highly correlated with asymmetric cell division, Numb moves into the progenitor or the neuronal daughter at

approximately the same frequency. In *Drosophila*, the direction of Numb movement also varies during development. At the CNS neuroblast division, Numb segregates into the GMC progenitor, but at other neural cell divisions, it segregates into different progenitor cells or into differentiated cell types. The primary function of Numb is not to specify a particular cell fate; rather differential segregation of Numb creates two daughter cells that can respond differently to environmental cues. Thus, inhibition of the Notch pathway by Numb can produce a progenitor, a neuron

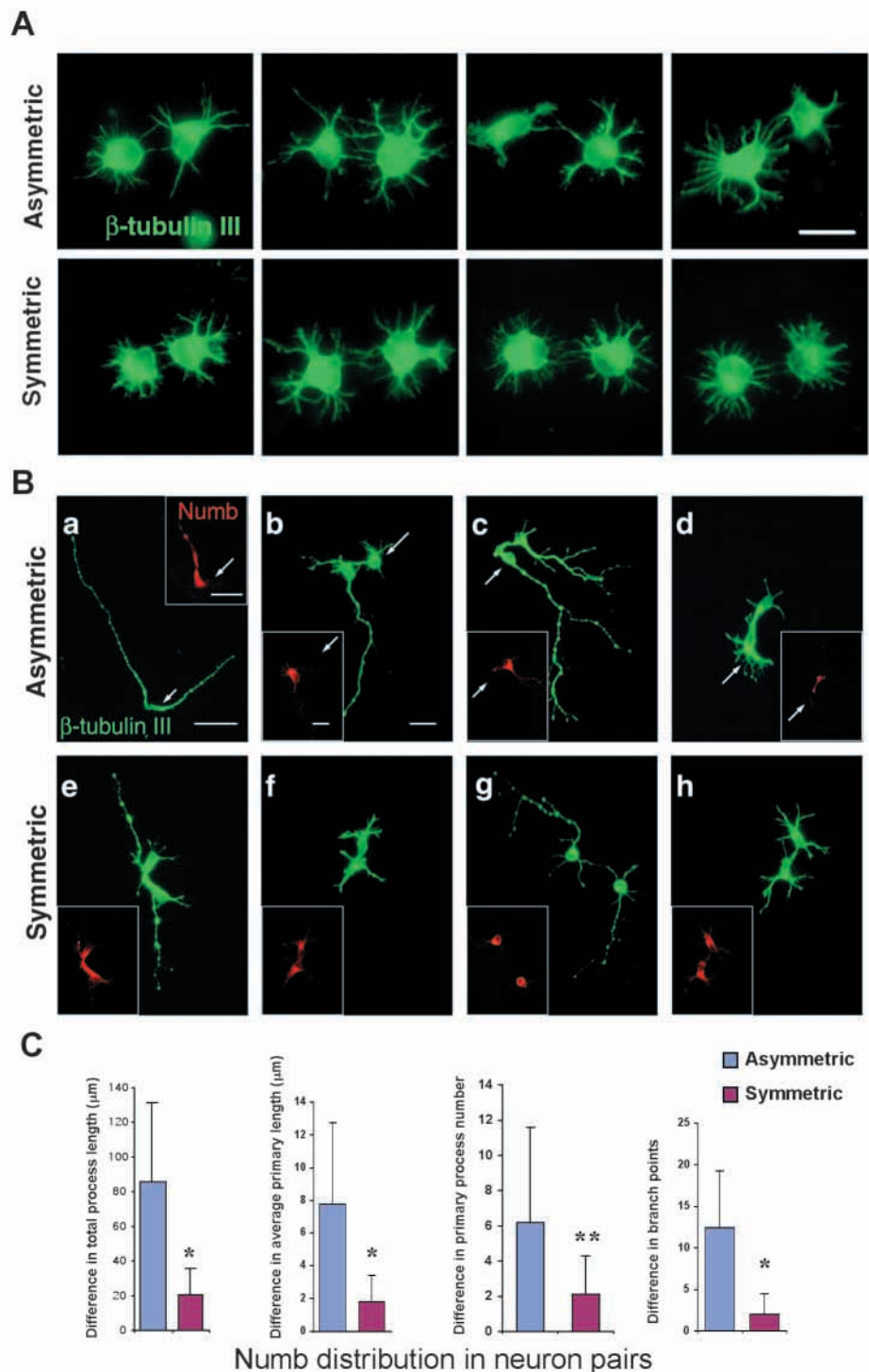


Fig. 6. Numb expression in pairs of sister neurons is related to their morphology. (A) E14 cortical pairs were identified after 24 hours in culture, fixed and stained for Numb (not shown) and β -tubulin III (green). Examples of neuron pairs with asymmetric Numb are shown in the upper panel, and with symmetric Numb in the lower panel. Note that upper panel pairs usually have different morphologies, while those in the lower panel have similar morphologies. (B) E10.5 cortical cells were cultured in serum-free medium with 10 ng/ml bFGF. In some experiments, C6 membrane homogenate was added to enhance neurite outgrowth and neuron survival (Temple and Davis, 1994). Pairs of sister cells were identified after 1 day in vitro, cultured for a further 3 days, and then stained for Numb (red) and β -tubulin III (green). Arrows indicate Numb⁻ neurons. (a-d) Neuron pairs with asymmetric Numb distribution usually had different morphologies. (e-h) Neuron pairs with symmetric Numb distribution usually had similar morphologies. (C) Differences between sister neurons in total process length, average primary process length, number of primary processes and branch points (mean \pm standard deviation) were plotted for Numb asymmetric pairs and Numb symmetric pairs. * $P < 0.05$, ** $P < 0.001$, Student's *t*-test. Scale bars: (A) 20 μ m; (B) a: 100 μ m; b-h: 20 μ m.

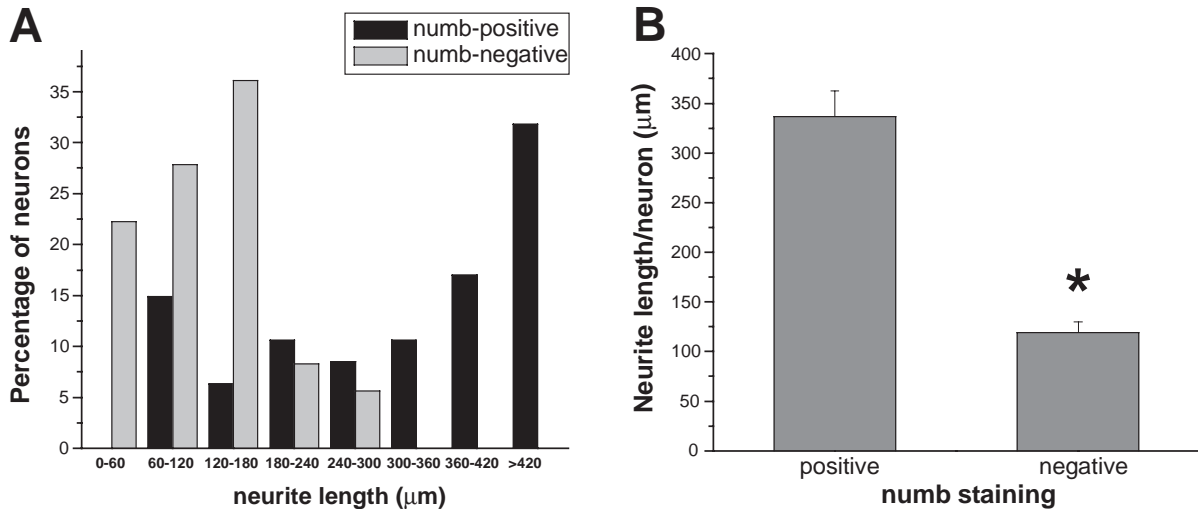


Fig. 7. Neurons with Numb have longer neurites than those without Numb. E10.5 cortical cells were cultured at low density for 7 days, then fixed and stained for Numb and β-tubulin III. The total neurite length of Numb⁺ neurons was compared with that of Numb⁻ neurons. (A) The percentage of neurons within each neurite length category is shown. Generally, Numb⁺ neurons had longer processes than Numb⁻ neurons. (B) The mean neurite length per neuron was calculated; there was a significant difference, approximately three fold, for Numb⁺ versus Numb⁻ neurons. **P*<0.001. Bars represent standard errors.

or a glial cell depending on the state of the starting cell and its environment.

A similar phenomenon might occur in vertebrates. Progenitor cells isolated from different stages are diverse, and their responses to Numb acquisition might vary. At early stages differential Numb movement could create differences between sister progenitor cells, with the direction of Numb movement (into the neuroblast or not) being related to the specific types of progenitors being formed. As more markers are discovered for sub-populations of neural progenitor cells, we might correlate the direction of Numb movement with the formation of different progenitor cell types. At later stages during active neurogenesis Numb moves more consistently into the cell that undergoes neuronal differentiation (Fig. 8). This could explain the apparently contradictory findings from overexpression and

knockout studies that Numb has a role in both progenitor and neuronal populations.

Previously we observed that most (80%) terminal E14 cortical neuroblasts cultured for 14 days produce two neurons that are essentially identical, with many being mirror images, while around 20% produce two morphologically different neurons (Qian et al., 1998). In the present study conducted on pairs grown for 1 or 4 days, we found that approximately 80% of newborn neuron pairs had symmetric Numb and approximately 20% asymmetric, an intriguingly similar ratio that spurred us to investigate the relationship between Numb distribution and morphology. We found Numb distribution was indeed highly associated with the similar or dissimilar morphology of neuronal pairs. This was found as early as 1 day after plating, and also by 4 days after plating when more

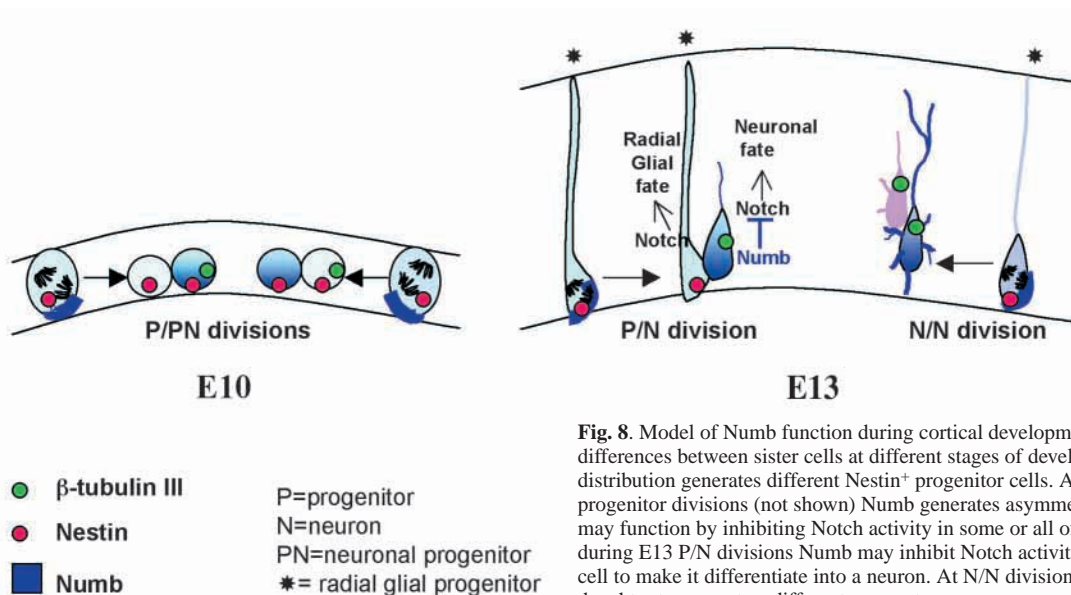


Fig. 8. Model of Numb function during cortical development. Numb asymmetry generates differences between sister cells at different stages of development. At E10, asymmetric Numb distribution generates different Nestin⁺ progenitor cells. At E13, besides asymmetric progenitor divisions (not shown) Numb generates asymmetric P/N and N/N divisions. Numb may function by inhibiting Notch activity in some or all of these divisions. For example, during E13 P/N divisions Numb may inhibit Notch activity in one daughter of a radial glial cell to make it differentiate into a neuron. At N/N divisions, Numb may inhibit Notch in one daughter to generate a different neuron type.

complex phenotypes had developed. It is unlikely that most of the morphological differences between sister cells at 4 days simply reflect differences in maturation: they often included multiple features such as soma shape, process length and direction, making it difficult to envision how a similar morphology might eventually be attained. Rather, it seems likely that many morphologically different pairs consist of two different cortical neuron types, which have distinct morphologies in culture (Kriegstein and Dichter, 1983). Thus differential Numb segregation may increase cell diversity at the final stages of neuron production.

We do not know whether the asymmetric distribution of Numb at the terminal mitosis is maintained during subsequent neuron differentiation, however the fact that a similar percentage of pairs had asymmetric Numb at 24 hours and at 4 days suggests this might be the case. In the future, live visualization of fluorescently tagged Numb, as accomplished recently in *Drosophila* (Lu et al., 1999; Roegiers et al., 2001), should show directly whether segregation of Numb at the terminal division of neuroblasts is related to subsequent long-term development of cell morphology and type.

Recent studies have demonstrated that over-expression of Notch inhibits axon and dendrite growth and that antagonizing Notch activity by Numb over-expression in cortical neurons promotes neurite growth (Sestan et al., 1999; Berezovska et al., 1999; Redmond et al., 2000). Our results further indicate that endogenous differences in Numb level can exert a profound effect on neurite development and overall cell morphology. If the action of Numb on cell morphology is mediated through Notch signaling, it is interesting that this can occur at the extremely low cell densities used in this study, in which cell-cell contact is minimal and largely limited to sister cells. How Notch activation might be translated into cell morphology is not yet clear. *Sanpodo*, a component of the Numb-Notch pathway in *Drosophila*, encodes an actin-associated protein that might regulate the cytoskeletal network and hence alter cell shape (Dye et al., 1998; Skeath and Doe, 1998).

In conclusion, this study indicates that Numb plays a critical role in asymmetric cell divisions in CNS cortical lineages, suggesting evolutionary conservation of essential mechanisms underlying asymmetric cell divisions from flies to mammals. However, it also highlights differences in the vertebrate, including the complexity of progenitor cell types, different Numb isoforms, the possibility of other asymmetric determinants and multiple downstream pathways for Numb signaling, that remain to be explored.

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