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Neural cell fate in *rca1* and *cycA* mutants: the roles of intrinsic and extrinsic factors in asymmetric division in the *Drosophila* central nervous system

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Abstract

In the central nervous system (CNS) of *Drosophila* embryos lacking *regulator of cyclin A* (*rca1*) or *cyclin A*, we observe that several ganglion mother cells (GMCs) fail to divide. Whereas GMCs normally produce two sibling neurons that acquire different fates ('A/B'), non-dividing GMCs differentiate exclusively in the manner of one of their progeny ('B'). In zygotic *numb* mutants, sibling neuron fate alterations ('A/B' to 'A/A') occur infrequently or do not occur in some sibling pairs; we have determined that depletion of both maternal and zygotic *numb* causes sibling neurons to acquire equalized fates ('A/A') with near-complete expressivity. In *rca1, numb* mutant embryos, we observe binary cell fate changes ('B' to 'A') in several GMCs as well. Finally, we have demonstrated that expression of *Delta* in the mesoderm is sufficient to attain both sibling fates. Our results indicate that the intrinsic determinant Numb is absolutely required to attain differential sibling neuron fates. While the extrinsic factors Notch and Delta are also required to attain both fates, our results indicate that Delta signal can be received from outside the sibling pair.

Keywords: Asymmetric division; Cell fate; Central nervous system; *numb*; Cell cycle; Ganglion mother cell

1. Introduction

A fundamental question in developmental biology is how a single cellular division can generate two cells that acquire different fates. Such asymmetric divisions occur frequently in the insect embryonic central nervous system (CNS). The neural precursors of the insect CNS, known as neuroblasts, divide several times to produce a series of ganglion mother cells (GMCs). Each GMC then divides once to produce two post-mitotic sibling neurons, which often acquire different fates (reviewed by Goodman and Doe, 1993). A striking example of asymmetric division occurs when GMC 1-1a divides to produce the siblings aCC and pCC. aCC is a motoneuron that projects its axon laterally to innervate a dorsal muscle, whereas pCC is an interneuron that projects its axon anteriorly toward the brain. This cell fate decision was originally examined using cell ablation experiments in the grasshopper, *Schistocerca americana*. When one of the two sibling neurons was ablated shortly after GMC division, the remaining cell nearly always differentiated into pCC ($n = 8/9$). However, when one sibling was ablated at a slightly later time point, the remaining cell took the fate of aCC ($n = 4$) or pCC ($n = 4$) equally (Kuwada and Goodman, 1985). These experiments suggested a model whereby siblings start as equivalent cells upon GMC division; cell fates are then specified through sibling-sibling interactions (Fig. 1A). When only a single cell is present, this cell takes the 'primary' fate, in this case pCC.

In recent years, the peripheral nervous system (PNS) and CNS of *Drosophila melanogaster* have been useful models for studying the molecular components that control asymmetric division. The gene *numb (nb)* was initially recognized for its function in cell fate determination in the PNS; in *numb* mutants, cells normally fated as neurons differentiate as support cells (Uemura et al., 1989). It has been shown that Numb protein is asymmetrically localized during PNS cell divisions into neuronal precursors; Numb then acts as a fate determinant in the cell into which it has segregated (Rhyu et al., 1994; Knoblich et al., 1995). Mutations in the gene *Notch (N)* were found to have opposing fate

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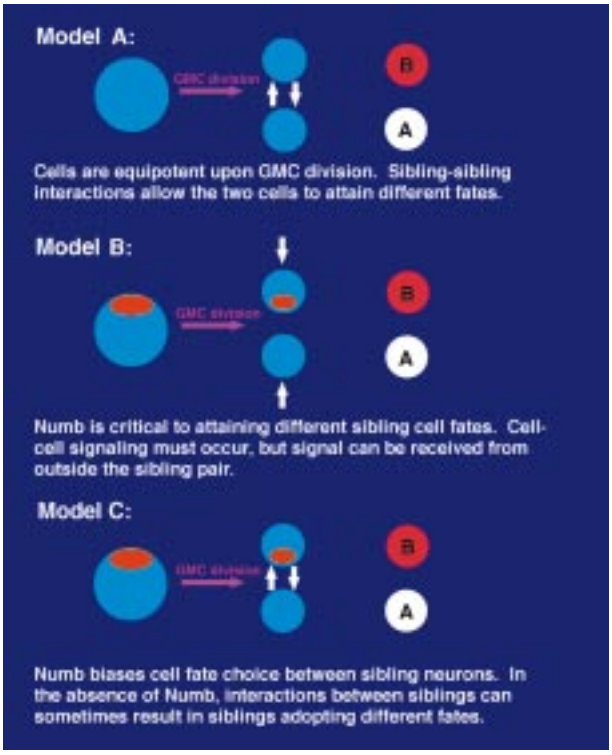

Fig. 1. Models for asymmetric division of ganglion mother cells in the insect CNS. (A) In this model, sibling neurons are initially equipotent upon GMC division; interactions between siblings allow these cells to acquire different fates. Ablation experiments in the grasshopper, *Schistocerca americana*, initially suggested that such a mechanism was utilized in sibling neuron fate choice (Kuwada and Goodman, 1985). (B,C) Two possible models for attaining differential sibling fates in *Drosophila* based on knowledge of mutations that affect sibling fate choice. (B) In this model, asymmetric localization of Numb into one sibling is critical for allowing sibling neurons to attain differential fates. Cell–cell signaling (i.e. Notch–ligand interactions) must occur; however, signal from the Notch ligand may be received from outside the lineage. (modified from Spana and Doe, 1996) (C) This model proposes that interactions between siblings are important in neuronal fate choice. Asymmetric localization of Numb into one cell biases sibling fate decisions; however, differential sibling fates can still be attained in the absence of an intrinsic determinant through sibling–sibling interactions involving Notch and its ligand (modified from Jan and Jan, 1995).

changes to those of *numb* mutants in the PNS (Hartenstein and Posakony, 1990). *Notch* encodes a transmembrane receptor and is involved in cell fate decisions at many levels of development; extracellular interactions between Notch and its ligand (Delta or Serrate), which is expressed on another cell, activate a signal transduction pathway within the Notch-expressing cell (reviewed by Artavanis-Tsakonas et al., 1995). It has been shown that *Notch* is genetically epistatic to *numb*, and Numb is thought to act through inhibition of the Notch signaling cascade (Guo et al., 1996; Spana and Doe, 1996). The involvement of Numb and Notch in the *Drosophila* PNS indicates that an interplay between intrinsic and extrinsic factors acts to mediate PNS asymmetric divisions.

In the CNS, Numb is also asymmetrically localized during the division of a neuroblast into its GMC progeny (Rhyu et al., 1994). However, the role of this localization is not clear, as Numb has no known function in neuroblast division or in GMC identity. It has been demonstrated that loss of *numb* does have an effect on sibling neuron fate in the CNS. At varying expressivity, several sibling pairs show equalized ('A/A') neuronal fate in the absence of zygotic *numb*; these same pairs exhibit the opposite fate changes ('B/B') in the absence of *Delta, Notch* or members of the Notch pathway (Spana and Doe 1996; Skeath and Doe, 1998) (Table 1). It has also recently been observed that Numb protein is asymmetrically localized during the division of specific GMCs into one of the sibling neuron progeny (Buescher et al., 1998). These data suggest that both intrinsic and extrinsic factors are often utilized in the CNS to mediate differential fates between sibling neurons. The partial expressivity observed in the CNS upon loss of zygotic *numb* can be accounted for in two different models of asymmetric division. In one model (Fig. 1B), Numb is a critical factor for achieving differential sibling fates; variable expressivity in zygotic *numb* mutants is a result of maternal contribution of *numb*. In this model, interactions between siblings would not be necessary, and signaling could be received from outside the lineage (modified from

Table 1
Proposed GMC lineage relationships and observed cell fate phenotypes among neurons that express Even-skipped (Eve) (Skeath and Doe, 1998)

GMC lineages	GMC 1-1a[a]	GMC 4-2a[a]	U or CQ GMCs[b]	EL GMCs[b]
Eve expression in GMC	EVE+	EVE+	some EVE+	EVE−
Wild-type GMC progeny	pCC/aCC	RP/RP2 sib	U-CQ/U-CQsib	EL/EL
Eve expression	EVE + /EVE+	EVE+/EVE−	EVE+/EVE−	EVE+/EVE+
WT cell fates (defined below)	A/B	A/B	A/B	B/B
Notch mutant phenotype (defines 'B/B')	aCC/aCC	RP2/RP2	U-CQsib	EL/EL
numb mutant phenotype ('A/A')[c]	pCC/aCC	RPsib/RP2sib	U-CQ/U-CQ	?/? (EVE−)

[a] The lineages of these GMCs have been well-characterized using multiple markers (see Table 2 and Section 2).

[b] Proposed relationships based on Eve expression. For U/CQ lineages, note that the 3 U and 2 CQ neurons per hemisegment can be distinguished from each other in stage 16 embryos. There are at least 3 Eve+ U/CQ GMCs but U GMCs/progeny in stage 11/12 embryos cannot be distinguished from CQ GMCs/progeny. Our data supports the notion that all 5 U/CQ neurons have EVE-siblings (see Section 2), but it is possible that 1–2 of the EVE+ U/CQs turn on Eve through a different mechanism.

[c] Zygotic mutation only; expressivity of phenotype varied among lineages and no aCC/pCC phenotype detected.

Spana and Doe, 1996). An alternative model suggests that Numb may provide a bias for asymmetric division, but that sibling cells can sometimes attain different fates in the absence of Numb through interactions with each other (Fig. 1C) (modified from Jan and Jan, 1995). Interestingly, one asymmetric division that is not affected at all in zygotic *numb* mutants is that of GMC 1-1a into aCC and pCC (Table 1; Skeath and Doe, 1998). One interpretation of this result is that, while *Notch* signaling is involved in this particular sibling fate choice, *numb* plays no role in this lineage. Alternatively, the role of *numb* in this lineage may be completely masked by the maternal contribution of *numb* product. Resolution of this issue is important for comparing the process of sibling fate choice between *Drosophila* and grasshopper because this is the only lineage for which experimental data exists in the grasshopper.

To examine sibling neuron fate decisions in more detail, we have analyzed cell fate decisions in the CNS of embryos lacking zygotic *cyclin A* (*cycA*) or *regulator of cyclin A* (*rca1*). We demonstrate here that, in the CNS of *cycA* or *rca1* mutants, GMCs often fail to divide into sibling neurons. In the absence of this terminal division, these GMCs differentiate as neurons, and they always acquire the 'B' fate normally assumed by one of their sibling progeny. To further analyze the mechanisms of this fate decision, we combined the *rca1* mutation with either a zygotic *numb* mutation or with an activated form of *Notch*. In both cases, GMCs that acquire the 'B' sibling fate in *rca1* mutants alone now frequently adopt the fate of the 'A' sibling. This result suggests that Numb promotes the 'B' fate in non-dividing GMCs through inhibiting activation of the Notch pathway; it also suggests that either the 'A' or 'B' fate can be acquired in the absence of sibling-sibling interactions. Additionally, we have determined that expression of *Delta* (*Dl*) in the embryonic mesoderm of a *Dl* mutant embryo can rescue sibling neuron fate specification, suggesting that the mesoderm may be a source of Dl signal in wild-type embryos. Finally, we have shown that the phenotypic variation in zygotic *numb* mutants is due to maternal contribution of *numb*; sibling neurons acquire equalized fates ('A/A') with near-complete expressivity when both maternal and zygotic *numb* are depleted. These data support a model whereby Numb is a critical factor in achieving differential sibling fates in the CNS; cell-cell interactions appear to be insufficient to mediate fate differences in the absence of intrinsic determinants. Finally, for GMC 1-1a, a comparison of our data in *Drosophila* with the previous data in grasshopper suggests that these two insects may deploy different mechanisms for sibling fate choice during neurogenesis.

2. Results

2.1. GMCs often fail to divide in cycA or rca1 mutants

In both *cycA* and *rca1* mutant embryos, epidermal cells are blocked in the G_2 phase of their final mitotic cycle, cycle 16 (Lehner and O'Farrell, 1989; Dong et al., 1997). We initially identified and characterized the *rca1* mutation from a screen for aberrant expression patterns of Even-skipped (Eve) protein in the embryonic CNS (I. Orlov, R. Saint, N. Patel, unpublished results). Eve is normally expressed in the nuclei of several cells in the CNS; these include GMC 1-1a and its progeny, aCC and pCC (Fig. 2C, arrowheads), GMC 4-2a and one of its progeny, RP2 (Fig. 2C, arrow), as well as the EL, U, and CQ neurons (see Table 1; Patel et al., 1989, 1994). In *cycA* and *rca1* mutants, Eve is expressed in fewer cells per hemisegment than wild-type. In the position where the siblings aCC and pCC normally sit,

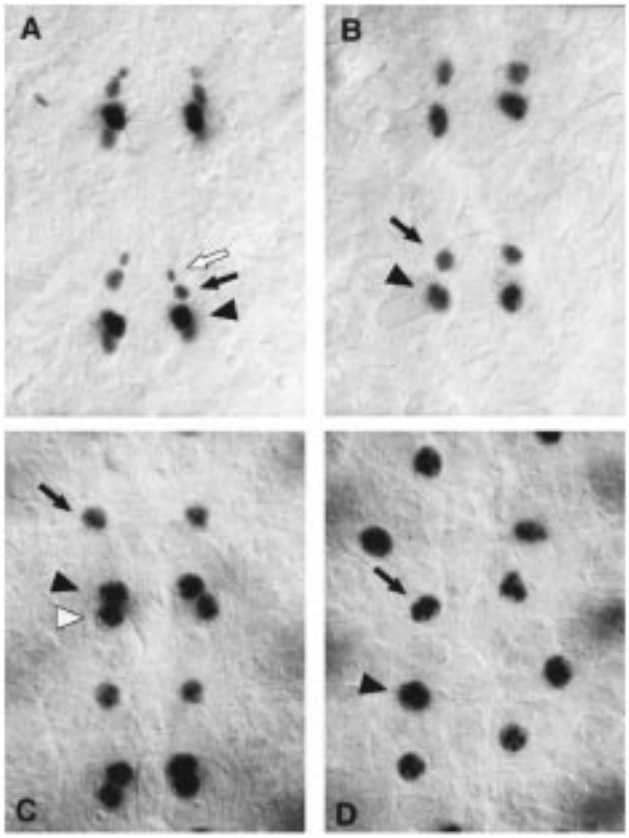

Fig. 2. Even-skipped expression in the CNS of rca1 and cycA embryos. (A,B) Early stage 12 *Drosophila* embryos immunolabeled for Even-skipped (Eve). (A) Wild-type. Eve is expressed in the nuclei of the GMC 4-2a progeny, RP2 (black arrow) and the smaller RP2 sibling (white arrow). Eve is also expressed in a cluster of cells posterior to RP2 including aCC, pCC, and the U/CQ neurons (black arrowhead). (B) $cycA^5$ embryo. GMC 4-2a has not divided, and there remains a single, large, Eve-positive nucleus (arrow). GMC 1-1a has also not divided into aCC and pCC (arrowhead). A similar phenotype is observed in *rca1* mutants (Table 2). (C,D) Stage 15 embryos labeled for Eve protein; photographs are at the same magnification. (C) Wild-type. RP2 maintains Eve expression (black arrow), while RP2 sibling does not. The progeny of GMC 1-1a, aCC (black arrowhead) and pCC (white arrowhead), both express Eve. (D) $rca1^{P1}$. GMC 4-2 expresses Eve (arrow) and often appears larger than the WT RP2; the non-dividing GMC 1-1a (arrowhead) sits in the position where aCC and pCC normally reside. A similar phenotype is observed in *cycA* mutants (Table 2).

Table 2
Frequency of GMC divisions in real and cycA mutants[a]

	GMC 1-1a progeny (aCC/pCC)	% WT	GMC 4-2a progeny (RP2/RP2 sibling)	% WT	EL GMC progeny	% WT
Wild-type	1.99 (153/77)		1.95 (240/123)		8.43 (506/60)	
rca1[33X16]	1.18 (161/137)	59	1.13 (99/88)	58	7.61 (761/100)	90
rca1[1]	1.08 (91/84)	55	1.05 (106/101)	54	7.50 (645/86)	89
cycA[5]	1.00 (77/77)	50	1.02 (130/127)	52	6.83 (546/80)	81

[a] For each genotype and lineage, the number of Eve-positive cells per hemisegment is indicated; parentheses show the total number of EVE + cells over the total number of hemisegments scored. For mutant genotypes, the percent formation of EVE + cells relative to wild-type is indicated. In wild-type embryos, an average of 1.99 GMC 1-1a progeny, 1.95 GMC 4-2a progeny, and 8.43 EL neurons is produced per hemisegment (see Section 4.2). To better identify GMC 1-1a progeny, embryos scored for this lineage were double-labeled with mAb 22C10. The rca1[33X16] allele is considered a moderate hypomorph, while rca1[1] and cycA[5] are considered to be strong alleles.

we observe a single Eve-positive nucleus that is larger than the wild-type aCC or pCC (Fig. 2D, arrowhead). In the position of RP2, there is still one Eve-positive nucleus, but again it often appears larger than normal (Fig. 2D, arrow). We also observe a loss of Eve expression where the U and CQ neurons normally sit and a decrease in the number of Eve-positive EL neurons (Table 2).

We have focused most closely on the GMC 4-2a and GMC 1-1a lineages because of their well-characterized development and because various molecular markers exist that label these GMCs and their progeny (Table 3). In wild-type embryos, GMC 4-2a divides early in stage 11, and two Eve-expressing nuclei are initially observed upon this division (Fig. 2A, arrow); Eve expression is quickly shut off in the smaller RP2 sibling nucleus but remains on in RP2. In *cycA* or *rca1* mutants, Eve expression turns on normally in GMC 4-2a; however, we rarely observe two nuclei during stage 12 (Fig. 2B, arrow; Table 2), and the single Eve-expressing nucleus remains large. Likewise, GMC 1-1a normally divides during stage 10 in wild-type embryos to generate the Eve-positive neurons aCC and pCC (Fig. 2A, arrowhead). In *cycA* or *rca1* mutants, GMC 1-1a expresses Eve as in wild-type but rarely divides (Fig. 2B, arrowhead; Table 2). Instead, this GMC comes to reside in the same dorsal plane and posterior position where aCC and pCC sit in wild-type embryos (Fig. 2D, arrowhead). Other Eve-expressing lineages, including the U/CQ neurons and the EL neurons (see Table 2), appear to be affected as well in *cycA* and *rca1* mutants. Notably, even the most severe alleles of *cycA* and *rca1* examined do not show complete expressivity of CNS phenotypes in all lineages.

Table 3
Expression of various molecular markers in GMC 1-la and GMC 4-2a progeny

	GMC 1-1a		GMC 4-2a	
	aCC	pCC	RP2	RP2sib
Even skipped (Eve)	X	X	X	
mAb 22C10	X		X	
latebloomer	X		X	
Y72		X		
Vnd		X		
eve–*lacZ*	X	X	X	X
MZ465-GAL4	X	X	X	

2.2. GMC 4-2a and GMC 1-1a differentiate as neural fate 'B' in cycA and rca1 mutants

In addition to the observed failure of GMCs to divide in *cycA* and *rca1* mutants, we find that these GMCs differentiate in the manner of neurons. Using various markers, we have analyzed the cell fates acquired by these undivided GMCs. Focusing primarily on both GMC 1-1a and 4-2a, we attempted to learn if these GMCs took on the fate of one or the other of their normal progeny, or some type of hybrid fate. Given the suggestion from grasshopper ablation experiments that pCC is the primary fate of the GMC 1-1a progeny (see Section 1; Kuwada and Goodman, 1985), we were especially interested to compare this grasshopper result to our *Drosophila* data.

We analyzed the axon projection patterns of GMCs in *cycA* and *rca1* mutants using monoclonal antibody (mAb) 22C10. In wild-type embryos, mAb 22C10 labels the cell body and axons of both RP2 and aCC, but not pCC or RP2 sibling (Fig. 3A). In *cycA* and *rca1* mutants, mAb 22C10 recognizes both GMC 1-1a ($n = 81/81$) and GMC 4-2a ($n = 73/73$) in all hemisegments examined (Fig. 3D). We frequently observe the axon of GMC 4-2a to project anteriorly and laterally, just as RP2 does (compare Figs. 3A and D, blue arrows), while the axon of GMC 1-1a projects posteriorly and laterally in the manner of aCC (Figs. 3A and D, red arrows).

We used additional molecular markers to examine this fate decision as well (see Table 3). In wild-type embryos, the gene *latebloomer* (*lbl*) is expressed in the motoneurons RP2 and aCC but not in RP2 sibling or pCC (Fig. 3B; Kopczynski et al., 1996). In *cycA* or *rca1* mutants, *latebloomer* RNA is expressed in both GMC 4-2a and GMC 1-1a (Fig. 3E). We also utilized the Y72 enhancer-trap line, which promotes expression of β-galactosidase (β-gal) in the Eve-positive pCC neuron but not in aCC, RP2, or RP2

sibling (Fig. 3C; Duman-Scheel et al., 1997). In *rca1* mutants in a Y72 background, β-gal is not expressed in GMC 1-1a or GMC 4-2a (Fig. 3F). Thus, through an analysis of both axon projection patterns and the expression patterns of several molecular markers, we conclude that GMC 1-1a adopts the aCC fate and GMC 4-2a adopts the RP2 fate in the absence of division. The finding that GMC 1-1a acquires the fate of aCC was of particular interest, as it would not necessarily be predicted from the grasshopper ablation experiments discussed above. Significantly, the neuronal fates acquired in *cycA* and *rca1* mutant embryos correspond to the 'B' fates observed in Notch-pathway mutants (Table 1).

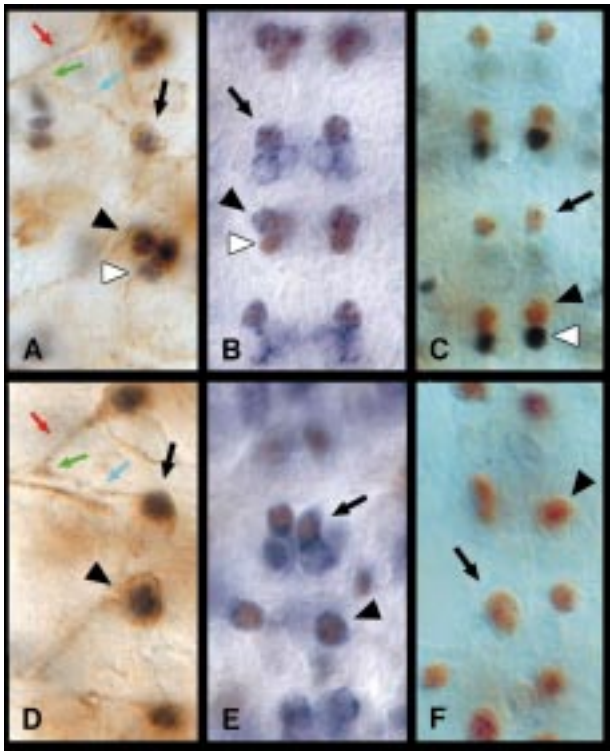

Fig. 3. GMC 4-2a and GMC 1-1a differentiate in *rca1* and *cycA* mutants. (A,D) Stage 14 *Drosophila* embryos are labeled with mAb 22C10 (brown) and Eve antibody (black). (A) Wild-type. mAb 22C10 labels the cell bodies of RP2 (black arrow) and aCC (black arrowhead) but not pCC (white arrowhead) or RP2 sibling (not shown). mAb 22C10 also labels the anterior- and laterally-projecting axon of RP2 (blue arrow) and the posterior- and laterally-projecting axon of aCC (red arrow); these axons fasciculate (green arrow) to form part of the intersegmental nerve. (D) $cycA^5$. GMC 4-2a (black arrow) is recognized by mAb 22C10 and projects an axon like RP2 (blue arrow); GMC 1-1a (arrowhead) is also labeled by mAb 22C10 and it projects its axon like aCC (red arrow). These axons often fasciculate in the manner of RP2 and aCC (green arrow). We observe the same phenotype in *rca1* mutants. (B,E) Antibody to Eve (brown) and RNA expression of *latebloomer* (*lbl*) (purple), as labeled by in situ hybridization. (B) Wild-type. *lbl* is expressed in RP2 (arrow) and aCC (black arrowhead) but not RP2 sib (not shown) or pCC (white arrowhead). (E) In *cycA* (and *rca1*; not shown) embryos, *lbl* is expressed in both GMC 4-2a (arrow) and GMC 1-1a (arrowhead). (C,F) Using the Y72 enhancer trap line, β-gal is expressed in pCC, but not aCC, RP2, or RP2 sibling (Duman-Scheel et al., 1997); stage 14 embryos in the Y72 background are labeled for Eve (brown) and β-gal (black). (C) Y72. RP2 (black arrow) and aCC (black arrowhead) express Eve; pCC (white arrowhead) expresses both Eve and β-gal. (F) $rca1^{33X16}$ in a Y72 background. β-gal is not expressed in either GMC 4-2a (arrow) or GMC 1-1a (arrowhead).

2.3. Loss of zygotic numb or activation of the Notch pathway can induce fate changes in GMCs

Having observed that GMCs acquire the fate of the 'B' sibling neuron in *cycA* or *rca1* mutants, we wanted to determine whether GMCs could acquire the 'A' fate through activation of the Notch pathway. If Delta signal must be provided from a sibling neuron, then GMCs, which lack a true 'sibling', may not have the potential to acquire the 'A' fate through extracellular signaling. We combined the *rca1* mutation with either a zygotic *numb* mutation or an activated form of *Notch*, N^{intra} (Struhl et al., 1993), in order to examine this question.

To analyze RP2 and sibling fates in *numb* or N^{intra} backgrounds, we utilized a *Drosophila* line in which *lacZ* is driven by a portion of the *eve* enhancer. This eve–*lacZ* line acts as a marker for both RP2 and its sibling through perdurance of β-gal (see Section 4 and Table 3; Sackerson et al., 1999). Using this marker, we observe that RP2 sibling sits somewhat ventral to and, oftentimes, posterior to the position of RP2. When eve–*lacZ* is present in the *numb* background, two β-gal-expressing cells that lack Eve expression are often observed ventral of the normal RP2 position (Fig. 4A, arrow); this is consistent with both GMC 4-2a progeny adopting the RP2 sibling fate ('A/A') (Table 4). We have observed the same fate alterations between RP2 and sibling when hs-N^{intra} embryos were heat-shocked at the appropriate times (see Section 4). We also examined GMC 4-2a fate in *rca1*,*numb* double mutants, as well as in hs-N^{intra},*rca1* embryos. In these embryos, GMC 4-2a often lacks Eve expression and sits ventral and/or posterior to its position in *rca1* mutants alone (Fig. 4B, arrow; Table 4). These observations indicate that GMC 4-2a frequently adopts the 'A' fate of RP2 sibling in *rca1*,*numb* or hs-N^{intra},*rca1* embryos. In contrast, GMC 4-2a always acquires the 'B' fate of RP2 in *rca1* mutants alone ($n = 73$). Notably, we have observed that the 'B' to 'A' fate change (RP2 → RP2 sibling) occurs with greater frequency in *rca1*,*numb* double mutants than the RP2/sib ('A/B') to sib/sib ('A/A') fate change occurs in *numb* mutants alone (Table 4).

We examined the fates of GMC 1-1a and its progeny in N^{intra} or *numb* embryos using the Y72 enhancer trap line (see Table 3). In zygotic *numb* mutants or wild-type embryos in the Y72 background, we observe one aCC and one β-gal-positive pCC neuron (Fig. 4G, arrow). However, using the hs-N^{intra} construct in a Y72 background, we have observed embryos in which both GMC 1-1a progeny express β-gal (Fig. 4H, arrow). This is consistent with both siblings adopting the 'A' fate of pCC upon activation of the Notch pathway. We analyzed GMC 1-1a fate by examining *rca1*,*numb* or hs-N^{intra},*rca1* embryos in a Y72 background. In these

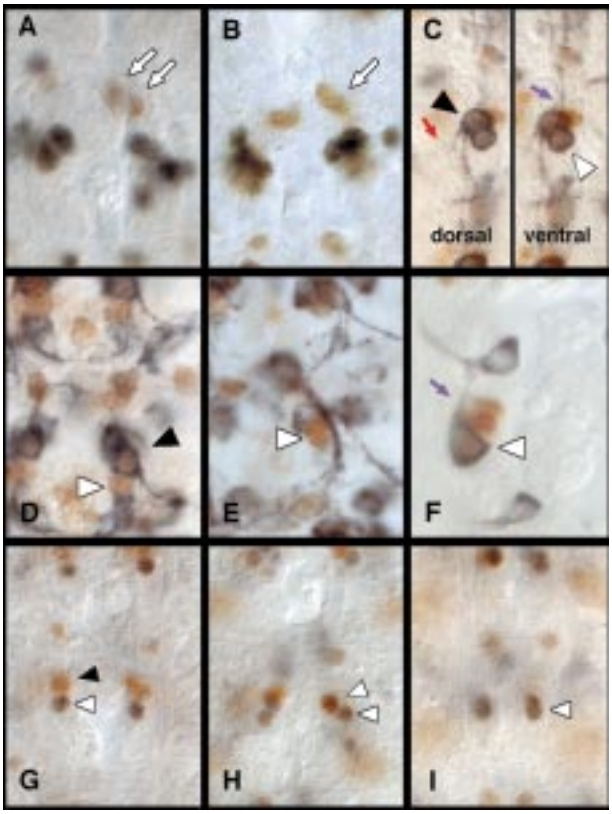

Fig. 4. GMC fate decisions are mediated by *numb* and the Notch pathway. See Table 3 for specificity of marker expression. (A,B) Stage 14 embryos in the eve–*lacZ* background are labeled for β-gal (brown) and Eve (black). (A) In eve–*lacZ*, nb^2 (or eve–*lacZ*, hs-N^{intra}) embryos, two β-gal-positive cells that do not express Eve (white arrows) are often observed in a position somewhat ventral to that of RP2. (B) In eve–*lacZ*, $rca1^{33X16}$; nb^2 embryos (or eve–*lacZ*, hs-N^{intra}; $rca1^{33X16}$), a single β-gal-positive, Eve-negative cell (white arrow) is often observed; the position of the cell is again characteristic of RP2 sibling, and the cell morphology different from that of RP2. (D,E) Stage 14 embryos in the eve–*lacZ* background are labeled with anti-β-gal (brown) and mAb 22C10 (black). (D) In eve–*lacZ*, nb^2 embryos, the axon and cell body of the β-gal-positive aCC (black arrowhead) are labeled by mAb 22C10; β-gal-positive pCC is not labeled by mAb 22C10. (E) In eve–*lacZ*, $rca1^{33X16}$; nb^2 embryos, the β-gal-positive GMC 1-1a (arrowhead) is often not recognized by mAb 22C10. (G,H,I) Y72 background; β-gal is expressed in pCC. Embryos are labeled for β-gal (black) and Eve (brown). (G) $numb^2$, Y72 embryo. β-gal is expressed only in a single pCC (white arrowhead), just as in WT (Fig 2C). (H) hs-N^{intra}, Y72 embryo. Upon heat-induced expression of activated Notch (see Section 4), two β-gal-positive, Eve-positive cells (arrowheads) in the position of aCC and pCC are often observed. (I) $rca1^{33X16}$,nb^2, Y72 (or hs-N^{intra}; $rca1^{33X16}$, Y72). GMC 1-1a expresses both β-gal and Eve (arrow) in the manner of pCC. (C, F) MZ465-GAL4; UAS-tau–*lacZ* background; tau–*lacZ* is expressed in the cell bodies and axons of RP2, aCC, and pCC (see Table 3; Hidalgo and Brand, 1997). Embryos are labeled for β-gal (black) and Eve (brown). (C) UAS-tau–*lacZ*, MZ465-GAL4 embryo. β-gal is observed in the cell body (black arrowhead) and axon (red arrow) of aCC; in a more ventral view of the same embryo (right side), β-gal is observed in the cell body (white arrowhead) and axon (purple arrow) of pCC. (F) UAS-tau–*lacZ*, $rca1^{33X16}$; nb^2, MZ465-GAL4. In GMC 1-1a (arrowhead), β-gal is expressed in the cell body (white arrowhead) and axon (purple arrow) in the manner of pCC.

embryos, we often observe β-gal expression in GMC 1-1a (Fig. 4H, arrowhead), suggesting that the GMC adopts the pCC fate. This result was somewhat unexpected in *rca1*,*numb* mutants given the lack of phenotype upon loss of zygotic *numb* (normal aCC/pCC). Therefore, we examined *rca1*,*numb* mutants using other markers. mAb 22C10, a marker of the aCC cell body and axon, often fails to label GMC 1-1a in *rca1*,*numb* embryos (Fig. 4E, arrowhead; Table 4). Additionally, using GAL4-driven expression of tau–*lacZ* (Callahan and Thomas, 1994), we observe that GMC 1-1a frequently projects an axon in the manner of pCC in *rca1*,*numb* embryos (compare Figs. 4C and F). We conclude that GMC 1-1a often adopts the pCC fate in these mutants.

Table 4
Frequency of B → A vs. A/B → A/A fate change[a]

	GMC 1-1a progeny	GMC 4-2a progeny
A/B[b] → A/A *numb[2]* (zygotic only)	pCC/aCC → pCC/pCC 0% (0/97)	RP2sib/RP2 → sib/sib 25% (54/214)
B[b] → A *rca1[33X16], nb[2]* (zygotic)	aCC → pCC 71% (55/78)	RP2 → RP2 sib 46% (94/205)

[a] The percentage of hemisegments exhibiting the binary fate change in indicated (no. altered/total examined). Embryos in an eve–*lacZ* background were labeled with anti-β-gal and either anti-Eve (for GMC 4-2a progeny) or mAb 22C10 (GMC 1-1a progeny) to distinguish between the 'A' and 'B' fates (see Table 3).

[b] A/B is WT fate for these lineages; the B fate (i.e. aCC, RP2) is always acquired in rca1 mutants alone (see Section 2).

Other Eve-expressing lineages are affected in *rca1*,*numb* mutants as well. In *rca1* or *Notch* mutants, Eve expression is lost in the position of the U and CQ neurons; in *Notch* mutants this is interpreted as both progeny of the U/CQ GMCs taking the fate of the Eve-negative U/CQ sibling ('B/B'). In *numb* mutants, there are extra Eve-positive U/CQs, described as the 'A/A' fate (Table 1; Skeath and Doe, 1998). In *rca1*,*numb* mutant embryos, we frequently observe Eve-expressing cells in the position of the U/CQ neurons, often with large nuclei; using mAb 22C10, we observe that some of these Eve-positive cells project axons in the manner of the U neurons (data not shown). These data suggest that some or all of the GMCs that normally produce the U/CQ neurons and their siblings adopt the 'B' fate of the Eve-minus U/CQ siblings in *rca1* or *cycA* mutants; in *rca1*,*numb* mutants, the GMCs now often acquire the U/CQ fate ('A').

2.4. Loss of both maternal and zygotic numb results in near-complete expressivity of numb phenotypes in the CNS

The analysis of GMC 1-1a and GMC 4-2a fate specification in *rca1*,*numb* mutants suggests that the addition of the *rca1* mutation enhances the phenotype of the *numb* mutation (see Table 4). This result could be consistent with a model in which interactions between sibling neurons compensate in *numb* mutants in order to insure that both the 'A' and 'B' fates are attained (Fig. 1C); when the GMC no longer

Table 5
Maternal effect of numb

			Maternal loss	% WT	Zygotic loss	% WT	Mat. and zyg. loss	% WT
		numb[2]						
		RP2[a]	1.00 (114/114)	100	0.55 (97/176)	55	0.02 (5/230)	2.2
		U/CQs[a]	4.94 (84/17)	100	794 (500/63)	737	8.91 (517/58)	180
Wild-type		Els[a]	8.96 (242/27)	98	0.60 (58/97)	6.5	0.11 (12/106)	1.2
RP2[a]	1.00 (233/234)	pCC[b]	1.02 (234/230)	101	1.01 (201/199)	100	1.95 (265/136)	194
U/CQs[a]	4.94 (494/100)							
Els[a]	9.17 (917/100)	*numb[4]*						
pCC[b]	1.01 (141/140)	RP2[a]	1.00 (110/110)	100	0.65 (139/214)	65	0.03 (7/210)	3.3
		U/Cqs[a]	4.67 (42/9)	95	7.52 (481/64)	152	8.56 (334/39)	173
		Els[a]	8.74 (201/23)	95	1.11 (117/105)	11	0.20 (24/120)	2.2
		pCC[b]	1.00 (64/64)	99	1.01 (183/182)	100	1.94 (269/139)	192

[a] The number of Eve-positive cells per hemisegment is indicated; the total number of EVE + cells over the number of hemisegments scored is shown in parentheses. In wild-type embryos, an average of 1.00 RP2, 4.94 U/CQ and 9.17 EL neurons form per hemisegment.

[b] The number of Vnd-positive pCC neurons per hemisegment is shown here; the number of VND + cells over the number of hemisegments scored is shown in parentheses. In wild-type embryos one Vnd-positive pCC forms in each hemisegment.

divides, as in the *rca1* mutation, the cell is sensitized to the loss of *numb*. Alternatively, the variation in phenotypic expressivity could involve maternal contribution of *numb*; differences among neuronal lineages, as well as changes in the genetic background, could result in a variable response to maternal product.

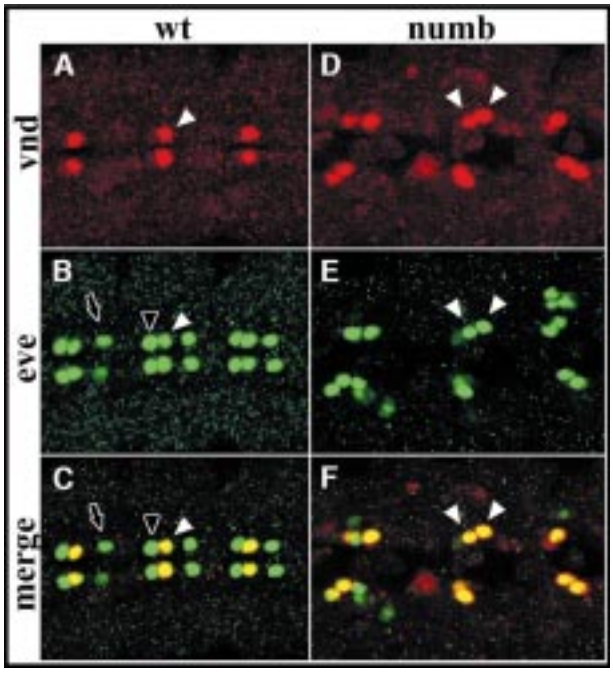

Fig. 5. Embryos lacking both maternal and zygotic *numb* show aCC/pCC → pCC/pCC fate changes. Stage 16 embryos were fluorescently labeled and visualized using confocal microscopy. (A–C) Wild-type embryo immunolabeled for Vnd (A, red) and Eve (B, green). (C) Merged image indicates that both Eve and Vnd are expressed in pCC (white arrowhead), while RP2 (arrow) and aCC (black arrowhead) do not express Vnd. (D–F) *numb* germline mutant in which both maternal and zygotic *numb* are depleted. Embryo is immunolabeled for Vnd (D, red) and Eve (E, green). (F) Merged image indicates that Eve and Vnd are now co-expressed in both cells in the aCC/pCC position (white arrowheads), suggesting that both cells take the pCC fate. Note also the absence of Eve expression in the RP2 position.

While there is some evidence that the level of maternal contribution of *numb* affects phenotypic expressivity (Skeath and Doe, 1998), we have now assayed this effect directly by generating germline clones of two strong *numb* alleles, nb^2 and nb^4. Removal of both maternal and zygotic *numb* function using these alleles results in near-complete expressivity of *numb* phenotypes. In *numb* germline embryos, GMC 4-2a initiates Eve expression and divides to produce two Eve-positive cells normally (94% [278/296] of stage 11 hemisegments examined). However, by stage 16, Eve is absent in both GMC 4-2a progeny in 97–98% of hemisegments observed (Table 5); thus, in the absence of *numb*, both GMC 4-2a progeny almost always acquire the RP2 sibling fate. We also examined the GMC 1-1a progeny in these mutants by following the expression of Vnd (Ventral nervous system defective) protein. In stage 15 embryos, Vnd is expressed in pCC, but not in aCC or RP2 (Fig. 5A–C) (McDonald et al., 1998). In embryos depleted of both maternal and zygotic *numb*, we observe two Vnd-expressing cells in the aCC/pCC position in 94–96% of hemisegments examined (Table 5). Co-expression of Eve and Vnd is observed in all hemisegments scored ($n = 38$; Fig. 5D–F), indicating that these cells correspond to the GMC 1-1a progeny. Thus, the loss of both maternal and zygotic contribution of *numb* equalizes the cell fates of the GMC 1-1a progeny to pCC/pCC. Additionally, near-complete expressivity of *numb* phenotypes ('A/B' or 'B/B' → 'A/A') is observed in all other lineages examined (Table 5). This verifies that *numb* plays a critical role in sibling neuron fate determination in the CNS, and that the observed phenotypic variation in zygotic *numb* mutants occurs as a result of maternal contribution of *numb*.

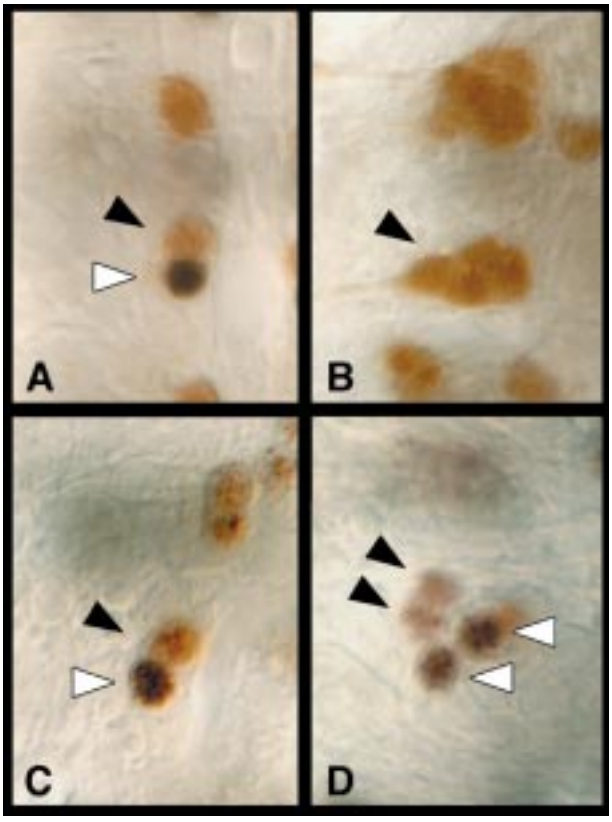

Fig. 6. Expression of *Delta* in the mesoderm rescues aCC/pCC fate specification. (A,B) Stage 15 eve–*lacZ* embryos are immunolabeled for Vnd (black) and β-gal (brown). (A) eve–*lacZ.* pCC is labeled by Vnd (white arrowhead), while aCC is not (black arrowhead). (B) eve–*lacZ*; Dl^3. There are several β-gal-expressing GMC 1-1a progeny (black arrowhead); none of these cells are labeled by Vnd (black staining). (C,D) Stage 15 embryos were immunolabeled for Eve (brown), Vnd (black), and Delta (red; not shown). Embryos of the genotype twi-GAL4; UAS-Dl; Dl^3 were identified by their expression of ectopic Delta protein in combination with a weak neurogenic phenotype. (C) In most hemisegments of these embryos, a single Vnd-expressing pCC (white arrowhead) and an aCC sibling (black arrowhead) are observed. (D) We have observed hemisegments that appear to have two Vnd-positive pCC neurons (white arrowheads) and two aCCs (black arrowheads).

2.5. Delta expression in the mesoderm rescues the aCC/pCC cell fate decision

Our observation that GMCs can acquire the 'A' fate in *rca1,numb* mutants suggests that Delta (Dl) signal received from outside the normal sibling pairs can activate the Notch pathway. *Delta* is normally expressed at high levels in the embryonic mesoderm during stages 10/11 (Kopczynski and Muskavitch, 1989); this corresponds to the time when GMC 1-1a progeny fates are specified. An analysis of MP2 lineage in *Drosophila* also had suggested that the source of Dl for patterning this fate choice came from somewhere outside of the CNS (Spana and Doe, 1996), and again the expression of Delta in the mesoderm occurs at the appropriate time to be involved in this fate determination. Therefore, we have examined whether expression of *Delta* in the mesoderm of a *Delta* mutant embryo allows for correct fate specification of the aCC/pCC sibling neurons.

In Dl^3 mutant embryos, misspecification of neuroectodermal cells results in an excess of neuroblasts and their resulting neuronal progeny (reviewed by Artavanis-Tsakonas et al., 1995). Additionally, binary cell fate alterations are observed at the sibling neuron level (Skeath and Doe, 1998). In wild-type embryos, Vnd protein is expressed in pCC but not aCC (Fig 6A, white arrowhead); in Dl^3 mutants, the numerous GMC 1-1a progeny all lack Vnd expression (Fig. 6B, black arrowhead), indicating that all of these neurons acquire the aCC fate. We utilized the twist-GAL4 line in order to drive *Dl* expression in the embryonic mesoderm (Baylies and Bate, 1996). In a wild-type background, expression of ectopic *Dl* using the twist-GAL4 line appears to have little effect on the embryo; significantly, we detect no effect in CNS cell fate specification (data not shown). When we express *Dl* in the embryonic mesoderm of a Dl^3 homozygous mutant using twist-GAL4, we observe that many sibling neuron pairs now attain differential fates ('A/B'). Specifically, we observe at least one aCC (Fig 6C, black arrowhead) and one Vnd-expressing pCC (Fig. 6C, white arrowhead) in each thoracic/abdominal hemisegment of these embryos. Thus, cell fate specification of the aCC/pCC sibling pair is rescued by expression of *Dl* in the mesoderm. The observation of a single aCC/pCC pair in some hemisegments also suggests that a single NB 1-1 progenitor was produced, indicative of rescue of the neuroblast phenotype. However, we do observe multiple aCC/pCC pairs in some hemisegments (Fig. 6D). Moreover, the use of an alternate mesodermal GAL4 driver (G4.W381) in a UAS-Dl, Dl^3 embryo rescues sibling neuron fate decisions but does not rescue the neurogenic phenotype (data not shown; see Section 4). Nonetheless, the use of both GAL4 drivers demonstrates that mesodermal expression of *Dl* clearly does rescue sibling neuron cell fate decisions in a *Dl* mutant background.

3. Discussion

We have analyzed asymmetric divisions in the *Drosophila* CNS through consideration of both the cell cycle and the molecular components involved in cell fate determination. This approach has allowed us to examine various models of the way in which sibling cells acquire different fates. We have determined that GMCs can differentiate in the manner of either of their sibling progeny in the absence of cell division; this fate decision depends on the presence of Numb and the activation state of the Notch pathway. This suggests that neither cell cycle progression itself nor interactions between siblings are required to attain either sibling fate. We have demonstrated that *Delta* expressed in the mesoderm can rescue sibling neuron fate specification in a *Delta* mutant background; this suggests that the mesoderm may act as a source of Delta signal in wild-type embryos.

Additionally, our data further strengthens the notion that *numb* is a critical factor in attaining differential sibling fates in the embryonic CNS; cell–cell interactions cannot compensate for the loss of this intrinsic determinant. We also point out that the removal of both maternal and zygotic *numb* contribution leads to defects in lineages which had appeared to be normal when only zygotic *numb* was removed. Finally, our analysis has served to characterize the effects of the cell cycle mutations *cyclin A* and *regulator of cyclin A* on the *Drosophila* embryonic CNS.

3.1. cycA and rca1 phenotypes in the embryonic CNS

In the CNS of embryos lacking zygotic contribution of *cycA* or *rca1*, we have demonstrated that GMC 1-1a and GMC 4-2a rarely divide into sibling neurons. Instead, these GMCs continue to differentiate and exclusively adopt the fate of one of their sibling progeny. Our data also suggest that at least some of the GMC divisions that produce the U/CQ and EL neurons are often blocked in these mutants, and that these GMCs may also differentiate in the manner of one of their sibling progeny. In the epidermis of *cycA* mutants, it has been suggested that cells reach cycle 16 through residual amounts of maternally provided *cycA* and the presence of *cyclin B*, but that these factors are insufficient to complete mitosis 16 (Lehner and O'Farrell, 1989; Knoblich and Lehner, 1993). Our results in the CNS of *cycA* or *rca1* mutants are not consistent with mitosis 16 always being prevented, supporting the idea that there are differences in cell cycle regulation between the CNS and the epidermis. The division of GMC 1-1a into aCC and pCC corresponds to mitosis 15, while the GMC divisions that produce most of the EL neurons are likely to be later than cycle 16, based on neuroblast lineage data (Schmidt et al., 1997). Our results from zygotic *cycA* and *rca1* mutants also suggest that maternal products are not depleted at a specific developmental time in the CNS. EL neuron production is less affected in *rca1* and *cycA* mutants than either the GMC 4-2a or GMC 1-1a lineages (Table 2), and the GMC divisions producing EL neurons occur later than the divisions of GMCs 1-1a and 4-2a (Skeath and Doe, 1998). The phenotypic variation among lineages in *cycA* and *rca1* mutants may thus reflect the action of cyclins other than *cycA*.

3.2. Neuronal fate choice in cycA and rca1 mutants

We have determined that GMCs in *cycA* and *rca1* mutants differentiate as neurons, assuming the 'B' fate normally taken by one of their sibling progeny. These GMC fate decisions correspond to Notch pathway mutants ('B/B'), and they oppose the fate changes observed in embryos lacking *numb* ('A/A') (Skeath and Doe, 1998). We have observed that the loss of zygotic *numb* or constitutive activation of *Notch* in a *rca1* background allows for a binary fate switch in GMCs: GMCs often differentiate as the 'A' sibling in the context of these mutations. Our results indicate that activation of the Notch pathway causes GMCs to adopt the 'A' neuronal fate. Thus, fate choice in non-dividing GMCs appears to occur in much the same way that binary fate decisions occur in sibling neurons. Although the binary cell fate changes in *rca1,numb* mutants show partial expressivity (Table 4), the differentiating GMCs do not appear to adopt an obscure or mixed fate. Our results regarding GMC 4-2a cell fate concur with a recent, independent report (Buescher et al., 1998). We note, however, that the putative and uncharacterized cell cycle mutant (*GA339*) used by Buescher et al. (1998) had no documented effect on the GMC 1-1a division, and the effect on the GMC 4-2a division appears to be much weaker than what occurs in any of the characterized cell cycle mutants. Because only the GMC 4-2a lineage was examined, and markers for the RP2 sibling neuron were not used, it is difficult to judge whether GMC divisions were indeed blocked and if these GMCs acquired a specific or mixed fate.

3.3. Models of asymmetric division

Our results involving fate specification in GMCs have provided insight into the mechanism by which asymmetric cell fates are generated in the *Drosophila* CNS. In some models of asymmetric division, a specific factor required to attain one of the sibling fates is produced only upon progression of the cell cycle. Our observation that GMCs can attain the fate of either sibling neuron indicates that gene products dependent upon GMC division are not required in this fate decision.

Other models of asymmetric division require that interactions between sibling cells occur to attain one of the cell fates; the primary fate would be taken in the absence of a sibling. As discussed previously (see Section 1), ablation experiments in the grasshopper involving aCC and pCC originally suggested that such a model is utilized in sibling neuron fate choice (Fig. 1A; Kuwada and Goodman, 1985). We observe in *Drosophila* that GMCs can acquire the fate of either of their sibling progeny in the absence of a true 'sibling'. This suggests that differential fates can be attained without sibling-sibling interactions. If this idea is viewed in the context of interactions between Notch and Delta (Dl), it implies the Dl signaling can be received from outside the GMC lineage. We have demonstrated that expression of *Dl* in the embryonic mesoderm can rescue aCC/pCC fate specification in a *Dl* mutant background. Given that *Dl* is normally expressed at high levels in the embryonic mesoderm at the time of aCC/pCC specification (Kopczynski and Muskavitch, 1989), this result suggests that the mesoderm may provide a source of Dl signal in wild-type embryos. While we cannot rule out the possibility that interactions between siblings occur in the wild-type setting or in specific lineages that we have not focused on, our data is consistent with a model in which sibling-sibling interactions are not required to attain differential fates. Such a model for the embryonic CNS agrees with the conclusions of Spana and Doe (1996) for the MP2 progeny. In the adult PNS of *Droso-*

phila, however, evidence has recently been uncovered indicating that Notch-ligand interactions between sibling cells are required to attain certain fates (Zeng et al., 1998). One possible explanation for these different results involves the access of Notch-expressing cells to cells that express Notch ligands. As discussed above, *Dl* transcript and protein are highly expressed in the embryonic mesoderm and ectoderm surrounding the CNS at the time that many neuronal fates are specified (Kopczynski and Muskavitch, 1989; Spana and Doe, 1996). In the adult PNS, perhaps other Delta- or Serrate-expressing cells are not in close enough contact with the PNS daughter cells to allow for proper fate specification in the absence of ligand in a sibling cell. It could be relevant to examine what occurs in the embryonic PNS, where fate determination processes are quite similar to those of the adult PNS.

We have demonstrated that GMCs can acquire the 'A' or 'B' neuronal fate in the absence of a true sibling, but we have further considered the potential relevance of sibling-sibling interactions in CNS binary fate choice. One interesting observation from our *rca1,numb* mutant analysis was that the lack of a final division appears to sensitize the GMCs to the loss of *numb*. In both the GMC 4-2a and GMC 1-1a lineages, the frequency of binary fate changes ('B' → 'A') in *rca1,numb* double mutants was greater than the frequency of fate changes ('A/B' → 'A/A') in *numb* mutations alone (Table 4). These results could be consistent with a model in which interactions between siblings partially compensate for lack of an intrinsic determinant (Fig. 1C). However, upon depletion of both maternal and zygotic *numb*, we observe near-complete expressivity of *numb* phenotypes ('A/B' or 'B/B' → 'A/A') in all CNS lineages examined. Thus, our results support a model in which the intrinsic determinant Numb is a critical factor in attaining differential sibling neuron fates (Fig. 1B). In such a model, cell–cell interactions would be insufficient to mediate sibling neuron fate differences in the absence of intrinsic determinants. As discussed above, our results also suggest that interactions between siblings are not necessary to mediate the observed fate differences of these cells. These conclusions agree with other models regarding binary cell fate determination in the *Drosophila* CNS (Spana and Doe, 1996; Buescher et al., 1998).

Our experiments in *Drosophila* involving GMC 1-1a and its sibling progeny, aCC and pCC, also allow for the first time a direct comparison to the ablation experiments in the grasshopper, *Schistocerca americana*. In *Schistocerca,* when one of these two siblings is ablated immediately after GMC division, the remaining sibling adopts the pCC fate (Kuwada and Goodman, 1985). Thus, we had initially expected that in our *cycA* and *rca1* mutants that the undivided GMC 1-1a would take on the fate of pCC. To our surprise, however, we have determined that in *Drosophila*, GMC 1-1a adopts the aCC fate in the absence of division. It has also been shown that, in the absence of Notch pathway activation in *Drosophila*, both siblings adopt the aCC fate (Skeath and Doe, 1998). Based on other systems studied, these contrasting results between the two insects would not necessarily be expected if *Drosophila* and *Schistocerca* make these fate choices in similar ways. In *C. elegans* vulval development, for example, vulval precursor cells (VPCs) acquire a primary fate upon sibling ablation; this same fate is acquired by VPCs in *lin-12* (*Notch* homologue) mutants (reviewed by Hill and Sternberg, 1993). If the *Schistocerca* aCC/pCC pair acquire differential fates through the proposed mechanism in *Drosophila* (Fig. 1B), then one might have expected an ablation result in which the remaining sibling cell acquired the fates of aCC and pCC equally no matter how soon after the division the ablation was done. Alternatively, if Notch signaling must be received from a sibling cell in *Schistocerca*, then one might have expected that the non-ablated siblings would always acquire the aCC fate (as in *Notch* or *Delta* mutants in *Drosophila*). Either way, the adoption of the pCC fate when ablations are done early in *Schistocerca* is clearly inconsistent with the data from *Drosophila*. Thus, it is possible that *Schistocerca* utilizes intrinsic determinants and/or Notch signaling in a different manner than *Drosophila* does in the GMC 1-1a lineage. One way to test this possibility would be to attempt cell cycle perturbation experiments in the grasshopper that are comparable to these genetic manipulations performed in *Drosophila*. If GMC divisions can be prevented in the grasshopper and differentiation occurs, we can then analyze the fate of these GMCs.

3.4. The role of numb in the Drosophila CNS

Beyond dissecting the GMC division and its relationship to fate determination, the results we have presented serve to clarify the role that *numb* plays in the embryonic CNS. We have examined embryos in which both maternal and zygotic contributions of *numb* are depleted, and our results support the notion that *numb* is not involved in establishing GMC identity. The GMCs that we have examined express Eve normally in the absence of both maternal and zygotic *numb*. Embryos depleted of both maternal and zygotic *numb* do show near-complete expressivity of sibling fate alterations (A/B → A/A). These results indicate that the presence of Numb in the GMC is necessary to allow its pair of neuronal progeny to take on different fates. It has recently been shown that Numb is asymmetrically localized in the division of GMCs 4-2a and 1-1a into one of the sibling neurons (Buescher et al., 1998), but the previous genetic data had not indicted a function of *numb* in the GMC 1-1a lineage. The data presented here clearly shows that *numb* is involved in the fate decisions made by the progeny of GMC 1-1a and that *numb* promotes acquisition of the aCC fate. Our data would also predict that expression of Numb in a sibling neuron immediately upon GMC division could rescue it to the 'B' fate even in an embryo that lacks Numb in both neuroblasts and GMCs. This appears to be

a somewhat different role for Numb than in the PNS, where Numb is required in multiple levels of division.

In conclusion, we have examined sibling neuron fate determination in the *Drosophila* embryonic CNS through an analysis of both molecular fate determinants and GMC division. We observe that several GMCs fail to divide yet continue to differentiate in the absence of zygotic contribution of *cycA* or *rca1*, and we have also shown that neuronal fate decisions in these GMCs are mediated through *numb* and activation state of the Notch pathway. Our analysis of the GMC 1-1a lineage has allowed for a direct comparison to previous cell ablation experiments in the grasshopper (Kuwada and Goodman, 1985). Additionally, the germline removal of *numb* confirms the absolute requirement for *numb* in several sibling lineages; extracellular interactions cannot compensate for the loss of *numb* in any lineage examined. We have also demonstrated that Notch–Delta signaling between sibling neurons is not a requirement for the cells to attain differential fates in *Drosophila*; *Dl* expression in the mesoderm is sufficient to mediate these fate difference. Finally, our characterization of CNS phenotypes in *cycA* and *rca1* mutants further reveals the differences in cell cycle regulation between the CNS and other tissues.

4. Experimental procedures

4.1. Drosophila strains

The P-element-lethal stock l (2) 03300 was isolated from the BDGP collection in a screen for mutations affecting neural development (I. Orlov, N. Patel, unpublished results) and corresponds to $rca1^{P1}$ (Dong et al., 1997). The line $rca1^{33X16}$ was created through imprecise excision of the P-element (I. Orlov, N. Patel, unpublished results); β-galactosidase is no longer expressed in this line, but it retains the *rca1* phenotype. nb^4 corresponds to the stock l (2) 06740 from the BDGP collection (Skeath and Doe, 1998). The eve–*lacZ* line used here was obtained as a gift from C. Sackerson and consists of the *lacZ* gene driven by a specific region of *eve* enhancer (+4.8–+12.0 kb) (Sackerson et al., 1999). $rca1^1$ was obtained from L. Zipursky (Dong et al., 1997), nb^2 from Y.N. Jan (Uemura et al., 1989), $cycA^5$ ($cycA^{neo114}$) from A. Spradling (Cooley et al., 1988), MZ465-GAL4 from G. Technau (Hidalgo and Brand, 1997), G4.W381 from F. Jimenez (Carmena et al., 1995), and UAS-Dl from Marc Muskavitch (Huppert et al., 1997). Fly crosses were performed using standard procedures. Balancer chromosomes and genetic markers were used as described by Lindsley and Zimm (1992).

4.2. Embryo collections and phenotypic quantitations

Embryo collections were performed using standard procedures. Note that comparisons of the quantitated data are only reliable within each table and not between tables, primarily because of variation in genetic backgrounds (as noted in tables and below); variation among tables also occurred in the conditions in which embryos were raised, and, in some cases, the developmental stages examined. Embryos analyzed for germline loss of *numb* (Table 5) were raised at 25°C; all quantitations in this table were made using stage 16 embryos. Embryos used for quantitations in Tables 2 and 4 were raised at 23°C. Embryos examined in Table 4 and those analyzed for GMC 1-1a progeny (Table 2) were quantitated at stage 14; GMC 4-2a progeny (Table 2) were quantitated at early stage 12, while EL neurons (Table 2) were counted at stage 15.

4.3. Immunohistochemistry and in situ hybridization

Fixation and immunostaining of embryos were carried out using a standard protocol (Patel, 1994). Monoclonal antibody to Eve (mAb 2B8) (Patel et al., 1994) and mAb 22C10 (Fujita et al., 1982) have been previously described. Antibody to Vnd protein was obtained as a gift from D. Mellerick-Dressler (McDonald et al., 1998). Polyclonal antibody to β-galactosidase was obtained from Cappell. Secondary antibodies (goat anti-mouse, goat anti-rabbit conjugated to HRP, flourescein, or rhodamine) were obtained from Jackson Labs. In situ hybridization was performed with an RNA probe, as discussed by Patel, 1996; digoxigenin labeling materials and alkaline phosphatase conjugated antibody to digoxigenin were obtained from Boehringer-Mannheim. *latebloomer* RNA expression has been described previously (Kopczynski et al., 1996).

4.4. Heat shock experiments

Heat shock experiments were performed with some modifications to Brand et al. (1994). A series of 1-h collections were made prior to heat shock treatment, and embryos were raised at varying temperatures between 18°C and 26°C to synchronize collections. For analysis of RP2 and RP2 sibling phenotypes, embryos that ranged from approximately 5.0 to 7.0 h (based on 25°C development) were heat-shocked for 10–13 min. For analysis of aCC/pCC phenotypes, embryo collections were aged to approximately 4.0 to 6.0 h old (25°C development) and heat-shocked for 10–13 min. Heat shocks were performed by directly submerging sieves of embryos into 37°C phosphate-buffered saline or distilled water. Embryos were then transferred to molasses plates and allowed to continue development for 4.5–6.5 h (based on 25°C development) before fixation.

4.5. Generation of numb germline clones

Standard genetic crosses were performed to generate fly stocks that contained either the nb^2 or the nb^4 allele together with FRT 40a in the same chromosome. To generate *numb* germline clones we crossed male w hsflp/Y; P{w[+mC] = ovoD1-18}2L1P{w[+mC]=ovoD1-18}2L2P{ry[+ t7.2] = neoFRT}40A/CyO flies to virgin female flies carrying either

the nb^2 FRT40a or nb^4 FRT40a chromosome balanced over CyO. We collected embryos at 1-day intervals, allowed the embryos to develop to the wandering late third instar stage and then subjected the larvae to a 2-h heat pulse at 37°C followed by a 30-min recovery in a 16°C waterbath. At all other times flies were reared at 25°C. Upon eclosion of these larvae, we collected virgin w hsflp/+; nb FRT40a/ P{w[+mC] = ovoD1-18}2L1 P{w[+mC] = ovoD1-18}2L2 P{ry[+t7.2] = neoFRT}40A flies and mated these to either nb^2/CyO-ftzlacz or nb^4/CyO-ftzlacz males. Note that the ftzlacZ promoter construct is inserted into a chromosome that contains a functional copy of the *numb* gene and allows unambiguous identification of embryos that carry this chromosome. We then collected, fixed and stained 0–20 h embryos raised at 25°C for β-gal and either eve or Vnd protein expression. Approximately 50% of embryos were positive for β-gal: these embryos lack maternal *numb* product but contain zygotic *numb* product inherited paternally. The other 50% of embryos fail to stain for β-gal: these embryos lack both maternal and zygotic *numb* function.

4.6. GAL4-UAS mediated expression of Delta

twist-GAL4/+; UAS-Dl/+; Dl^3 embryos were generated by crossing twist-GAL4/Y; Dl^3/+ males X UAS-Dl/+; Dl^3/+ females. Ectopic Delta protein was detected using monoclonal antibody 9B (Rebay, 1993; Klueg et al., 1998). Dl^3 homozygous embryos were identified by the neurogenic phenotype; in the context of ectopic Dl, the neurogenic phenotype is mild but detectable. twist-GAL4/+; UAS-Dl/+ embryos were independently generated to examine the phenotypic consequences of ectopic Dl expression in a $Dl^{+/+}$ background. Additionally, twist-GAL4/+; UAS–*lacZ*/+ embryos were labeled for β-gal and analyzed in order to confirm that the *twist* promoter does not cause ectopic gene expression in the CNS between stages 10 and 12. Like twist-GAL4, the line G4.W381 also drives expression of GAL4 in the embryonic mesoderm, although this expression starts slightly later (Carmena et al., 1995). We used a G4.W381 stock in the same manner as twist-GAL4 in order to generate mesodermal expression of Delta in Dl^3 mutant embryos. In this case, however, UAS-Dl/+; Dl^3/+ males were crossed to G4.W381/Dl^3 females in order to allow for a necessary recombination event between G4.W381 and Dl^3, which are both on the third chromosome.

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