INTRODUCTION

In both insects and vertebrates, midline cells play a key role in the formation of the axon commissures that interconnect the left and the right sides of the developing CNS (Crews et al., 1988; Thomas et al., 1988; Nambu et al., 1990; Nambu et al., 1991; Klambt et al., 1991). These midline cells also serve as a source of signaling molecules that repulse axons away from the midline, attract them towards it, provide guidance to axons, regulate axon growth, and promote axon scaffold formation (reviewed by van Vactor and Flanagan, 1999; Harris and Holt, 1999; Seeger and Beattie, 1999). The down regulation of these proteins allows the asymmetric localization of Inscuteable, leading to the asymmetric division of GMC-1. Consistent with this, over-expression of these POU genes in a late GMC-1 causes mis-localization of Insc and symmetric division of GMC-1 to generate two RP2s. Similarly, increasing the dosage of the two POU genes in sli mutant background enhances the penetrance of the RP2 lineage defects whereas reducing the dosage of the two genes reduces the penetrance of the phenotype. These results tie a cell-non-autonomous signaling pathway to the asymmetric division of precursor cells during neurogenesis.

SUMMARY

The bipotential Ganglion Mother Cells, or GMCs, in the Drosophila CNS asymmetrically divide to generate two distinct post-mitotic neurons. Here, we show that the midline repellent Slit (Sli), via its receptor Roundabout (Robo), promotes the terminal asymmetric division of GMCs. In GMC-1 of the RP2/sib lineage, Slit promotes asymmetric division by down regulating two POU proteins, Nubbin and Mitimere. The down regulation of these proteins allows the asymmetric localization of Inscuteable, leading to the asymmetric division of GMC-1. Consistent with this, over-expression of these POU genes in a late GMC-1 causes mis-localization of Insc and symmetric division of GMC-1 to generate two RP2s. Similarly, increasing the dosage of the two POU genes in sli mutant background enhances the penetrance of the RP2 lineage defects whereas reducing the dosage of the two genes reduces the penetrance of the phenotype. These results tie a cell-non-autonomous signaling pathway to the asymmetric division of precursor cells during neurogenesis.

Key words: Neurogenesis, Slit, Roundabout, Cell signaling, Ganglion mother cells, Asymmetric division, Drosophila melanogaster

INTRODUCTION

In both insects and vertebrates, midline cells play a key role in the formation of the axon commissures that interconnect the left and the right sides of the developing CNS (Crews et al., 1988; Thomas et al., 1988; Nambu et al., 1990; Nambu et al., 1991; Klambt et al., 1991). These midline cells also serve as a source of signaling molecules that repulse axons away from the midline, attract them towards it, provide guidance to axons, regulate axon growth, and promote axon scaffold formation (reviewed by Van Vactor and Flanagan, 1999; Harris and Holt, 1999; Seeger and Beattie, 1999). Axon repulsion and axon attraction towards and away from the midline play a principal role in the formation of commissures and longitudinal connectives. Recently, efforts have been directed to elucidating axon repulsion mediated by the Slit (Sli)-Roundabout (Robo) signaling pathway (Rothberg et al., 1990; Kidd et al., 1999; Brose et al., 1999; Li et al., 1999; Wang et al., 1999; Ba-Charvet et al., 1999; Simpson et al., 2000; Rajagopalan et al., 2000). These studies reveal that growth cones that express Robo, a cell surface receptor, will not enter and cross the midline where the Sli ligand is expressed at high levels owing to a repellent Sli-Robo interaction. An environment that has high levels of Sli appears to be inhospitable for those growth cones that express Robo. In robo mutants, therefore, axons freely cross and re-cross the midline. In sli mutants, however, axons that normally do not enter the midline now freely do so (Kidd et al., 1999). These studies have also attributed the pathfinding or axon projection defects in sli mutants to this lack of axon repulsion mechanism. Although there have been several studies that reveal the repellent action of Sli-Robo in axon guidance, whether or not Sli signaling is also required for neuronal lineage elaboration has never been addressed.

In the Drosophila ventral nervous system, hundreds of different cell types are generated outside of the midline from a relatively few primary precursor cells called neuroblasts (Bate, 1976; Goodman and Doe, 1993). During neurogenesis, about 30 neuroblast (NB) cells in each hemisegment delaminate in a stereotyped and spatiotemporal pattern. Following formation, each neuroblast functions as a stem cell and divides asymmetrically renewing itself and producing a chain of secondary neuronal precursor cells called ganglion mother cells (GMCs). A GMC is always committed to differentiate and does not self-renew; it divides asymmetrically to generate two distinct neurons (Bate, 1976; Thomas et al., 1984; Skeath and Doe, 1998; Dye et al., 1998; Buescher et al., 1998; Wai et al., 1999).

Recently, several genes such as inscuteable (insc), miranda (mira), numb (nb) and Notch (N) have been shown to be required for the asymmetric division of neural precursor cells (Buescher et al., 1998; Wai et al., 1999; Schober et al., 1999; Lu et al., 1999). The asymmetric divisions mediated by these proteins appear to be tied to their asymmetric segregation into one of the two daughter cells during division. For instance, during the division of GMC-1 of the RP2/sib lineage, Insc localizes to the apical end of GMC-1 while Nb segregates to the basal end. The cell that inherits Nb is specified as RP2 owing to the ability of Nb to block Notch signaling from...
specifying sib fate, whereas the cell that inherits Insc is specified as sib by Notch. Thus, in insc mutants, Nb is distributed to both cells and both the progeny of GMC-1 adopt RP2 fate; whereas in nb mutants, they assume sib fate (Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999).

In a screen for mutations that affect the development of NB4-2→GMC-1→RP2/sib, a typical neuroblast lineage, we discovered that loss of function of sim and its upstream activator single-minded (sim) affects the elaboration of GMC-1 and GMC-1-1a of the aCC/pCC lineage in a partially penetrant manner. In this study, we show that Sli signaling promotes the terminal asymmetric division of GCMs of the RP2/sib and aCC/pCC lineages. In the RP2/sib lineage, in embryos mutant for sim, robo or sim, the GMC-1 symmetrically divides to generate two RP2 neurons. Analysis of the expression of insc in sim mutant embryos and analysis of double mutants between sim and nb indicates that Sli signaling promotes the asymmetric division of GCMs by regulating the asymmetric localization of Insc. Our results also indicate that the disruption of the asymmetric localization of Insc by loss of sim in at least GMC-1 of the RP2/sib lineage is due to the up-regulation of the two POU proteins, Nubbin (Nub; also known as Pdm1) and Mitimere (Miti; also known as Pdm2). Consistent with this, up-regulation of miti or nub in a late GMC-1 by over-expression leads to mis-localization of Insc and symmetric division of GMC-1 to generate 2 RP2s. Moreover, while the penetrance of the symmetrical division phenotype is not high in both sim and sim mutant background, for instance, significantly enhances the penetrance; similarly, halving the dosage of these POU genes suppresses the symmetrical division phenotype.

### MATERIALS AND METHODS

#### Mutant strains, genetics

For the analysis of sim function, the null mutations slit and slitGA20 and several deficiencies that remove sim and the combination of slit, slitGA20 and slit deficiencies were used. For the analysis of sim, the point mutations, sim2 (which is a null mutation), two hypomorphic sim alleles, simE120 and simB062, and a sim deficiency [Df (3R) ry85] were used. In sim hypomorphic alleles, ~2.3% of the hemisegments showed the GMC-1 symmetrical division phenotype. For insc, insc22, for nb, nb796 and nb4 were used. For robo, robo4 (null allele), robo5 (hypomorphic allele) and a robo deficiency were used. The duplication chromosome for miti and nab, Dp (2; 2) GYL is described by Lindsley and Zimm (Lindsley and Zimm, 1992). The deficiency for miti and nab, Df (2L) OR4 is described by Yeo et al. (Yeo et al., 1995). To determine the effect of ectopic expression of miti and nab, transgenic lines carrying these genes under the control of the heat shock protein 70 gene promoter were used (see Bhat et al., 1995). Mutant embryos were identified using blue balancers, marker phenotypes or immunostaining (lack of positive staining). The various mutant and genetic combinations were generated by standard genetics.

#### Miti and Nub over-expression experiments

To determine the effect of over-expression of miti and nab, transgenic lines carrying these genes under the control of hsp70 promoter were used. To determine if the GMC-1 undergoes a symmetrical mitosis at elevated levels of Miti, Hs-miti or Hs-nab transgenic embryos were collected for 2 hours, aged for 7 hours and subjected to a heat shock at 37°C for 20 minutes. These embryos were allowed to develop until they reached stage 12 and 14 before being fixed. To determine if the localization of Insc is affected in embryos ectopically expressing Miti or Nub at high levels, embryos collected and aged as above were heat shocked for 25 minutes. These embryos were allowed to recover for 20 minutes before being fixed and double stained for Eve and Insc. As control, non-heat shocked transgenic embryos and heat shocked wild-type embryos were used.

#### Antibodies and immunostaining

Embryos were fixed and stained with the following antibodies: Eve (polyclonal, 1:2000 dilution; monoclonal, 1:5), Zfh-1 (1:400), 22C10 (1:4), Ins(1:500), Nub (1:50), Miti (1:10), Sim (1:500), Robo (1:10), b-gal (1:3000 or 1:400), Fitz (1:50), Sli (1:200) and alpha-spectrin II (1:10). For confocal microscopy, cyt3 and FITC-conjugated secondary antibodies were used. For light microscopy, alkaline phosphatase or DAB-conjugated secondary antibodies were used. Mutant embryos were identified using blue balancers, marker phenotypes or immunostaining (lack of positive staining). To examine Huckebein expression, a lacZ enhancer-trap line, hkb5653 in the hkb locus was used.

### RESULTS

**Slit promotes the terminal asymmetric division of GCMs**

To determine the requirement for Sli signaling in precursor cell division, we focussed on two GMC lineages in the ventral nerve cord of the Drosophila embryo: the GMC-1→RP/sib lineage generated by NB4-2 and the GMC1-1a→aCC/pCC lineage, generated by NB1-1 (Thomas et al., 1984). The GMC-1 asymmetrically divides at ~7.5-7.45 hours of development to generate the RP2 motoneuron and its sibling (sib) cell whereas GMC1-1a asymmetrically divides at ~7.5-7.45 hours of development to generate aCC and pCC neurons. All these cells can be reliably identified by several ways (see below).

Initially, embryos mutant for slit were examined with anti-Even-skipped (Eve) antibody. Eve is first expressed in GMC-1 of the RP2/sib lineage (Fig. 1A); it is also expressed in a newly formed RP2 and sib (Fig. 1B). The sib eventually loses Eve expression whereas RP2 maintains Eve (Fig. 1C). Eve staining of slit mutant embryos (slit2 or slitGA20) revealed that the GMC-1 in slit mutants frequently divides symmetrically to generate two RP2s instead of an RP2 and a sib. Thus, while approx. 7-hour old slit embryos had only one GMC-1 as in wild type, in 10% of the hemisegments (number of hemisegments examined, n=1092; the maximum penetrance that we observed in a slit mutant embryo was approx. 20%), approx. 8.5-hour old mutant embryos had two cells of equal sizes, both expressing Eve (Fig. 1D and G). This is in contrast to the wild type where a larger RP2 and a smaller sib are faithfully observed by 7.5-8 hours of age (Fig. 1A). It must be pointed out that there is no de novo synthesis of Eve in sib; thus the entire stock of Eve protein in a sib is inherited from GMC-1 and thus, the immunoreactivity for Eve in sib is ancestry dependent/indicator. Similarly, in wild type the difference in the size of the nuclei between RP2 and sib is generated prior to cytokinesis, and thus inherent to the lineage (compare Buescher et al., 1998). When approx. 10-hour old slit embryos were examined with Eve, 7% of the hemisegments (n=780) had two RP2s (Fig. 1E,H). In 13-hour old slit embryos, two RP2s of equal sizes were observed (Fig. 1F,I) in 11% of the hemisegments (n=776). Moreover, as shown in Fig. 1G-I, symmetric division of GMC-1 to generate two RP2s was also...
observed in single-minded (sim) embryos in 9% of the hemisegments (n=210). Sim is a transcription factor and is the upstream activator of sli (Thomas et al., 1988; Crews et al., 1988; Nambu et al., 1990; Klambt et al., 1991). We note that RP2 and aCC neurons are occasionally misplaced within a hemisegment in the CNS of 14-hour or older sli mutant embryos, however, in younger embryos (approx. 10 hours or less) these cells are not misplaced and do not cross the midline or segmental boundary.

We sought to obtain more direct evidence for the symmetric mitosis of GMC-1. If the GMC-1 in a sli mutant embryo divides symmetrically to generate two RP2s, the cytokinesis and nuclear division of GMC-1 must also be symmetrical as opposed to the non-symmetrical nuclear and cytokinesis of GMC-1 in wild type. Thus, it must be possible to observe symmetrical versus non-symmetrical division by examining GMC-1s that are undergoing cytokinesis with cell cortex markers in combination with nuclear markers. Therefore, sli mutant embryos were stained with the cell cortex marker spectrin (Byers et al., 1987; Prokopenko et al., 1999) and the lineage specific nuclear marker Eve. As shown in Fig. 2A and C, the asymmetric cytokinesis of GMC-1 (and the unequal nuclear sizes of daughter nuclei) to generate two unequal cells in wild type can be faithfully observed using these markers. However, in sli mutant embryos the GMC-1 undergoes a symmetric cytokinesis with two nuclei of equal sizes to generate two equal sized cells was observed (Fig. 2B and D). (The method of visualization of GMC-1 division in live embryos using green fluorescent protein was not possible since it takes >3 hours for this protein to become fluorescent after it is made and this window of time is too long as the GMC-1 completes its division by then.) Finally, transformation of some other neuroblast into NB4-2 was not observed in sli mutants as judged by the expression of Huckebein, a NB4-2 specific marker (Chu-LaGraff et al., 1995; Bhat, 1996). Thus, these results indicate that GMC-1 in sli or sim mutants undergoes a symmetrical division to generate two RP2s.

The generation of an RP2 at the expense of the sib was further confirmed using additional cell-type specific markers. First, mutant embryos were double stained for Eve and Zfh-1. In wild type, Zfh-1 is never expressed in GMC-1, GMC1-1a, sib, pCC, or newly formed RP2 and aCC. Zfh-1 begins to be expressed in RP2 and aCC at approx. 9 hours of development (at 22°C) and continues to be expressed thereafter (Fig. 3A and B). In approx. 10-hour old sli embryos, both the progeny of GMC-1 of the RP2/sib lineage co-express high levels of Eve and Zfh-1 (Fig. 3C) and they continue to co-express high levels of Eve and Zfh-1 in approx. 13-hour or older mutant embryos (Fig. 3D). In the aCC/pCC lineage, both the progeny of GMC1-1a co-express Eve and Zfh-1 (Fig. 3E and F) in 8% of the hemisegments (n=1100), indicating that the two daughter cells have adopted an aCC identity in these hemisegments. Consistent with the possibility that the duplicated Eve- and

Fig. 1. The GMC-1 in sli and sim mutants symmetrically divide to generate two RP2s. Embryos are stained for Eve. Anterior is up, vertical lines indicate the midline. RP2, larger arrow; sib, smaller arrow; GMC-1, arrowhead. Scale bars, 15 μm. (A-C) Wild-type embryos. (D-F) sli mutant embryos, the symmetric division of GMC-1 to generate two RP2s is shown. (G-I) Symmetric division of GMC-1 in sim mutant embryos. The midline in sim mutants become fused by approx. 8.5 hours of development. Note that RP2, aCC, pCC and other neurons are somewhat misplaced within a hemisegment in the CNS of 13-hour or older mutant embryos, however, in younger embryos (approx. 10 hours or less) these cells are not misplaced and do not cross the midline or segmental boundary.

Fig. 2. The nuclear division and cytokinesis of GMC-1 in sli mutants is symmetric. Embryos in A-D (half segments) are double stained for Eve (red) and spectrin (green). RP2 is indicated by large arrow, sib by small arrow. Scale bars, 5 μm. Anterior is up. Both nuclear division and cytokinesis of GMC-1 are asymmetric in wild type (A,C) while in sli mutants (B,D), they are symmetric and yield two equal sized RP2s at the expense of the sib.
Zfh-1-positive cells are RP2 and aCC neurons in sli embryos, they express 22C10 and have axon trajectory that of an RP2 (Fig. 3H) and aCC (Fig. 3I), respectively.

The relationship between Slit, Insc and Nb during the asymmetric division of GMC-1

The symmetric division of GMCs in sli mutants was similar to that observed in insc, Notch or rapsynoid (raps; also known as pins) mutants and opposite to that of nb (compare Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999; Yu et al., 2000). Previous results show that the cytoplasmic adaptor protein Insc is required for the asymmetric division of GMC-1 into RP2 and sib (Buescher et al., 1998). During GMC-1 division, Insc protein localizes to the apical side (see Fig. 4A) and Nb to the basal side. The Nb-negative daughter cell becomes specified as sib by Notch signaling whereas the cell that inherits Nb becomes an RP2 owing to the blocking of Notch signaling by Nb. Thus, in insc mutants, both cells inherit Nb and are specified as RP2 while in nb mutants both progeny becomes sib. Given the similarity of sli, sim and insc mutant phenotypes, we examined the relationship between Sli and Insc. First, in sli mutants the localization of Insc in GMC-1, when examined, was not asymmetric (Fig. 4B). About 7% of the hemisegments (n=440) showed this phenotype. A similar non-localization of Insc was also observed in GMC1-1a of the aCC/pCC lineage (Fig. 4D). In raps mutant embryos, Insc is also not localized and as in sli the GMC-1 divides symmetrically to generate two RP2s (Yu et al., 2000). Thus, failure to localize Insc in these GMCs in sli mutants is responsible for their symmetric mitosis.

In insc, nb double mutants both the daughters of GMC-1 are specified as sib by Notch signaling. In sli, nb (or sim, nb) double mutant embryos also, both the progeny of GMC-1 adopt a sib fate (Fig. 4F). Thus, Sli is required upstream of Nb during the asymmetric division of GMC-1. Since the GMC-1 symmetrically divides to yield two RP2s in Notch; nb double
Fig. 5. Symmetrical division of GMC-1 in sli mutants is caused by the up regulation of Nub. Anterior end is up, only half segments are shown in these panels. Embryos in panels A-H are double stained for Nub (red) and Eve (green; Eve only panels are not shown). GMC-1 is marked by arrow; U and aCC/pCC neurons are marked by arrowheads. Scale bars, 10 μm. A,C,E and G show Nub staining; B,D,F and H show Eve, Nub merged images. (A,B) Wild-type embryo; note the high levels of Nub in GMC-1 (arrow). (C,D) sli mutant embryo. (E,F) Wild-type embryo; note the down regulation of Nub in GMC-1. (G,H) sli mutant embryo; note that the level of Nub in GMC-1 is not down regulated. Similar results were also observed with Mit. (LJ) Eve-stained hs-miti embryos, the GMC-1 symmetrically divides to generate two RP2s. (K) Eve and Zfh-1 stained wild-type embryo, the asymmetric division of GMC-1 has generated an Eve-positive but Zfh-1-negative smaller sib and a larger Eve- and Zfh-1-positive RP2. (L) Eve and Zfh-1 stained hs-miti embryo. In the upper right hemisegment, the GMC-1 has generated two RP2s whereas in the lower right hemisegment, the GMC-1 has divided normally to generate RP2 and sib. (M) Eve (green) and Insc (red) stained hs-miti embryo where miti is ectopically expressed before GMC-1 division. Note that Insc expression is non-asymmetric. Similar results were also observed with over-expression of nub.

mutants (Wai et al., 1999) and two sibs in sli, nb double mutants, Sli is also upstream of Notch signaling during the asymmetric division of GMC-1. These results also indicate that when the GMC-1 in sli mutants symmetrically divides, both daughters inherit Nb.

Slit signaling regulates the asymmetric division of GMC-1 by down regulating Nubbin and Mit

Since previous results tie the two POU genes, miti and nub, to the normal elaboration of the GMC-1→RP2/sib lineage (Yang et al., 1993; Bhat and Schedl, 1994; Bhat et al., 1995; Yeo et al., 1995), we examined the expression of these genes in sli mutant embryos. In wild type, the levels of Nub (or Mit), which are normally high in a newly formed GMC-1 (Fig. 5A,B), are down regulated prior to the asymmetric division of GMC-1 (Fig. 5E,F). In sli mutants the expression of Nub (or Mit) in a newly formed GMC-1 was comparable to that of wild type, but, in a late GMC-1 the level remained high compared...
to wild type (Fig. 5G and H). Previous results showed that a brief ectopic expression of these POU genes from the hsp70 promoter prior to GMC-1 division induces GMC-1 to divide symmetrically to generate two GMC-1s; each then divides asymmetrically to generate an RP2 and a sib (Yang et al., 1993; Bhat et al., 1995). If the symmetric division of GMC-1 in these mutants has anything to do with the lack of down regulation of Nub and Miti in GMC-1, ectopic expression of miti or nub should also induce GMC-1 to divide symmetrically to generate two RP2 neurons. Indeed, a brief over-expression of miti (or nub) in a late GMC-1 causes this GMC to divide symmetrically into two RP2 neurons (Fig. 5I,J,L) in 27% of the hemisegments (n=770).

**Insc is non-localized in GMC-1 expressing high levels of Miti**

The loss-of-function effects of *sli* on the distribution of Insc in GMC-1 (and thus the symmetrical division of GMC-1) could be due to this lack of down regulation of Miti and Nub in GMC-1. To test this possibility, the miti transgene was ectopically expressed from the hsp70 promoter. A 25-minute induction of miti was sufficient to alter the localization of Insc and the distribution of Insc in these embryos resembled the distribution of Insc in *sli* embryos (Fig. 5M).

### The penetrance of the symmetrical division phenotype in *sim* mutant is sensitive to the dosage of *nub* and *miti* genes

The penetrance of the symmetric division of GMC-1 phenotype in *sli* and *sim* mutants was approx. 10%, indicating a partial genetic redundancy for this pathway. Since the loss of asymmetric division of GMC-1 in *sli* or *sim* appears to be due to a failure in the down regulation of Nub and Miti, we reasoned that the penetrance of the phenotype might be enhanced by increasing the copy numbers of these POU genes in *sli* or *sim* background. Since a duplication for *nub* and *miti* exists but the duplication is on the second chromosome (Dp (2; 2) GYL), we examined *sim* GYL embryos for the GMC-1 division phenotype. As shown in Fig. 6C and D, the penetrance of the phenotype in these embryos was enhanced to 42% (n=70); symmetrical division of GMC-1 in GYL is approx. 2% (n=840). Similarly, halving the copy numbers of the two POU genes in *sim* background using a small deficiency [sim/2; Df (2L) GR4/+] that eliminates these two genes (Yeo et al., 1995) suppressed the phenotype to 1.4% (n=74; see also Fig. 6E).

The above results indicate that the symmetrical division of GMC-1 in *sli* mutants is due to the up regulation of the two POU genes and that these two POU genes are the targets of Sli signaling in GMC-1; however, the partial penetrance of these phenotypes in *sli* mutants indicate that additional pathways also mediate this very same process and regulate the levels of the two POU proteins in GMC-1. Since the penetrance in *insc* mutants is also partial, additional pathways must exist to mediate the asymmetric division of GMC-1 to partially complement the loss of the Insc/Sli pathway.

**Robo, a key receptor for Sli, partially mediates GMC-1 asymmetric division**

How is the Sli signal transmitted from outside to inside? Previous results show that one of the receptors for Sli is the transmembrane protein encoded by the robo locus (Kidd et al., 1999; Wang et al., 1999; Brose et al., 1999; Li et al., 1999; Ba-Chavret et al., 1999). To determine if the effect of Sli signaling on GMC-1 is mediated via the expression of Robo, the GMC-1 asymmetric division phenotype was observed only in robo null mutants (or robo1null/robo2null/robo2deficiency), GMC-1 and GMC1-1a were found to divide symmetrically to generate two RP2s and two aCCs at the expense of sib and pCC (Fig. 7C,D). Although the penetrance of the RP2 lineage phenotype was low in robo mutants (~2.3%, n=1300), the fact that Robo is expressed in GMC-1 and that the phenotype was observed only in robo null mutants, argue that Robo at least partially transmits the Sli signal and promotes the asymmetric division of GMC-1 into RP2 and sib. Since three additional robo genes, robo2, robo3 (Rajagopalan et al., 2000; Simpson et al., 2000) and robo4 (our unpublished results) exist in *Drosophila*, the weak penetrance is likely to be due to genetic redundancy between these robo genes.

**DISCUSSION**

Several recent studies have shown that Sli-Robo signaling regulates growth cone repulsion, axon growth and axon branching (Kidd et al., 1999; Brose et al., 1999; Ba-Chavret et al., 1999; Li et al., 1999; Wang et al., 1999). In this study, we provide evidence that Sli signaling promotes asymmetric cell division of secondary neuronal precursor cells that are located several cell diameters away from the source of the signal.

The results presented here show that in *sli* mutants the GMC-1 divides symmetrically to produce two RP2s. Several lines of evidence support this possibility. For instance, initially only one large cell with the characteristics of a GMC-1 (in terms of its location and marker gene expression pattern: Eve, Miti and
possibility is the finding that localization of Insc in these genes function in the same pathway. Consistent with this mimicked those phenotypes in two aCC neurons at the expense of a pCC cell. Similarly, in the aCC/pCC lineage, the GMC1-1a generates direct evidence for the symmetric division of GMC-1. That problem of asymmetric mitosis of secondary neuronal cytokinesis to yield two identical cells as revealed by spectrin This GMC-1 undergoes a symmetrical nuclear division and Nub positive but Zfh-1 negative) appears in a hemisegment. The GMC-1 and GMC1-1a phenotypes in sli mutants mimicked those phenotypes in insc mutants indicating that these genes function in the same pathway. Consistent with this possibility is the finding that localization of Insc in sli mutants was affected in both GMC-1 and GMC1-1a (Fig. 4). A similar non-asymmetric localization of Insc and duplication of RP2 neurons was also observed in embryos mutant for raps, which encodes a protein required for the proper localization of Insc (Yu et al., 2000). Thus, these results tie Sli signaling to insc, a gene known to regulate GMC-1 and GMC1-1a asymmetric division. Our results show that the phenotypes in sli or sim null mutants are partially penetrant. Since the penetration in sim mutants is also partial, additional pathways must also regulate asymmetric division of GMCs. Moreover, it should be pointed out that in insc mutants the GMC-1 division is normal in approx. 30% of the hemisegments (n=280) despite having no insc. Similarly, the penetrance of the symmetrical division of GMC-1 in raps (where Insc localization is affected as in sli mutant embryos or embryos over expressing miti or nub genes) or nb is also partial, indicating the presence of additional (partially redundant) pathways mediating the asymmetric division of GMC-1 independent of Insc or Nb. These very same additional pathways must also contribute to the partial penetrance of the phenotypes in sli mutants. Nonetheless, we emphasize that while the penetrance of the phenotype is weak in sim, sli, and robo mutants, it is significantly enhanced by doubling the copy numbers of the two downstream target genes, miti and nub, nearly to the same extent as in insc mutants.

In sli mutants, the distribution of Insc in GMC-1 is not asymmetric and this is likely to be the reason for the symmetric division of GMC-1 or GMC1-1a. While the regulation of Insc localization appears to require down-regulation of the two POU genes, it is not known how elevated levels of these POU genes alter the localization of Insc. Since Mit and Nub have the structural motif of DNA-binding proteins, elevated levels of Mit or Nub might repress genes that are required for the localization of Insc.

Our results indicate that the loss of Sli signaling affects the expression and localization of Insc in GMC1-1a as it does in GMC-1 of the RP2/sib lineage. Interestingly, nub and miti appear not to be the targets of Sli signaling in GMC1-1a. This is based on the finding that ectopic expression of miti or nub which alters the division pattern of GMC-1, has no effect on the division pattern of GMC1-1a (Yang et al., 1993; Bhat and Schedl, 1994; Bhat et al., 1995). Similarly, while the loss of miti and nub genes leads to a mis-specification of GMC-1 identity, this has no effect on GMC1-1a (Bhat and Schedl, 1994; Bhat et al., 1995; Yeo et al., 1995). Moreover, altering the dosage of miti and nub in sim mutant background had no effect on the division of GMC1-1a. However, Sli signaling appears to regulate the terminal asymmetric division of GMC1-1a by regulating Insc localization. This is consistent with the fact that in insc mutants GMC1-1a often generates two aCC neurons at the expense of a pCC cell.

The results described here indicate that GMC-1 also divides symmetrically into 2 RP2s in embryos mutant for robo. Furthermore, we have shown that Robo is expressed on the surface of GMC-1. Thus, Sli secreted from the midline acts as a long-range signal and interacts with Robo on GMC-1 in row 4, column 2 of the ventral nerve cord to regulate its division. The effect of loss of robo on GMC-1 division is weakly penetrant. One possibility is that there is a genetic redundancy between robo, robo2, robo3 (see Simpson et al., 2000; Rajagopalan et al., 2000) and robo4 (our unpublished results).

In summary, the following picture emerges from this study. The Sli-Robo signaling down regulates the levels of Nub and Mit in late GMC-1, allowing the asymmetric localization of Insc and the asymmetric division of GMC-1. We entertain the possibility that loss of sibling cells in sli mutants would mean that some projections will be duplicated, while others are eliminated. Depending upon the extent, this might have an overall bearing on the pathfinding defects in sli mutants. Since Sli signaling is conserved in vertebrates, it is possible that this signaling may regulate generation of asymmetry during vertebrate neurogenesis as well.

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