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A requirement for *Notch* in the genesis of a subset of glial cells in the *Drosophila* embryonic central nervous system which arise through asymmetric divisions

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SUMMARY

In the *Drosophila* central nervous system (CNS) glial cells are known to be generated from glioblasts, which produce exclusively glia or neuroglioblasts that bifurcate to produce both neuronal and glial sublineages. We show that the genesis of a subset of glial cells, the subperineurial glia (SPGs), involves a new mechanism and requires Notch. We demonstrate that the SPGs share direct sibling relationships with neurones and are the products of asymmetric divisions. This mechanism of specifying glial cell fates within the CNS is novel and provides further insight into regulatory interactions leading to glial cell fate determination. Furthermore, we show that Notch signalling positively regulates *glial cells missing* (*gcm*) expression in the context of SPG development.

Key words: Notch signalling, Asymmetric cell division, *Drosophila*, CNS, Glia

INTRODUCTION

Drosophila is an excellent species to use to determine how the vast cellular diversity and complexity is created in the central nervous system (CNS). The generation of the embryonic CNS is a lineage-based process in which neural progenitors, called neuroblasts (NBs), give rise to largely invariant lineages of neural/glial cells. NBs divide in an asymmetric manner to bud off a set of ganglion mother cells (GMC), which in turn divide once to produce two postmitotic daughters. Cell lineage analysis techniques have been used to analyse most of the embryonic NB lineages at the histological level. These studies have elucidated the cellular composition and the specific nature of each NB lineage, as well as the morphologies of identified cells (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999).

Neural progenitor divisions are asymmetric. Both intrinsic cues, e.g. basally localised and asymmetrically segregated cell fate determinants like Numb and Prospero (Pros) (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana et al., 1995), as well as extrinsic cues (Guo et al., 1996; Spana and Doe, 1996) (mediated by Notch and Delta and cell-cell interaction), are involved in the specification of daughter cell fates. NBs and some GMCs divide with their mitotic spindles oriented along the apical-basal axis, enabling the preferential segregation of basally localised cell fate determinants to just one daughter (Kraut et al., 1996; Buescher et al., 1998; Schuldt et al., 1998). Recent studies have shown that this polarity of neural progenitors is mediated by several molecules, Inscuteable (Insc) (Kraut and Campos-Ortega, 1996; Kraut et al., 1996), Bazooka (Baz) (Schober et al., 1999; Wodarz et al., 1999), and Partner of Inscuteable (Pins) (Raps – FlyBase; Yu et al., 2000; Parmentier et al., 2000; Schaefer et al., 2000) which form a complex that localises to the apical cortex. Correct asymmetric Numb distribution has been shown to be crucial for the specification of distinct fates of sibling neurones in several contexts (e.g. Guo et al., 1996; Spana et al., 1995; Buescher et al., 1998). Numb acts to downregulate Notch signalling, thereby providing a direct link between intrinsic and extrinsic mechanisms for the specification of distinct fates of sibling neurones. However, this model is based upon only a handful of known CNS sibling neurones, which represent early born cells from their respective NB lineages. Therefore, it is still an enigma how later born cells, including glial cells, are specified in the context of NB/neuroglioblast (NGB) lineage development.

In terms of cell numbers, glia comprise ~10% of the cells in the embryonic nervous system (Ito et al., 1995). Glial cells can derive from the mesectoderm as well as from the neuroectoderm. A common characteristic of all neuroectodermally derived glia is the strict requirement for the gene, *glial cells missing* (*gcm*), which serves as a binary switch between neuronal and glial cell fates (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). However, mesectodermally derived glia do not require *gcm*, suggesting a different mechanism for their development. Moreover,

different types of glial progenitors derived from the neuroectoderm have been identified. Glioblasts (GBs) give rise to only glial cells, whereas NGBs produce mixed glial/neuronal lineages. For at least two NGB lineages, the thoracic NGB6-4T and NGB5-6, it has been demonstrated that an early bifurcation of the glial versus the neuronal sublineages takes place during the first division of the parental NB. This process involves the asymmetric expression of *gcm* in the glial sublineage (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). However, it is unclear whether this mechanism is generally applicable for other NGB lineages.

In order to better understand how a complete lineage of a specific NGB with all its progeny, including its glial cells, might be created we chose NB1-1 for a detailed analysis. NB1-1 has been extensively used for cell fate specification studies and a sound basis of information about this NB lineage is available (Udolph et al., 1993; Broadus et al., 1995; Skeath and Doe, 1998). NB1-1 is a NB that develops differential lineages in the thoracic versus the abdominal segments (Udolph et al., 1993; Prokop and Technau, 1994; Bossing et al., 1996). In our study, we focused on the abdominal NB1-1A because only these abdominal NB1-1 lineages contain glia. In addition to the aCC/pCC sibling neurones which are the progeny of the first GMC produced from this lineage, NB1-1A generates 2 to 3 glial cells and 4 to 5 clustered interneurones (cN), yielding a total of 9 to 10 cells. The three glial cells belong to the group of subperineurial glia (SPG) that lie at the periphery of the nerve cord and enwrap the entire ventral nervous system (Ito et al., 1995). Two of the glia, the A- and B-SPGs (Klämbt and Goodman, 1991), can be found in dorsal positions, with a third glia, the LV-SPG, located at ventral positions of the nerve cord. All SPGs, including the A- and B-SPG and LV-SPG of NB1-1A, are specifically labelled by two enhancer trap lines, *M84* and *P101* (Klämbt and Goodman, 1991).

In this study, we used available mutants, genetic markers and clonal analyses to investigate the development and sibling cell fate decisions of later components of the NB1-1A lineage. We present data to indicate that the glial cells within the NB1-1A lineage are direct siblings to neurones and each glia/neurone pair arise through asymmetric divisions. We provide evidence which indicate that Notch is required to resolve distinct neuronal/glia sibling fates. In this context, Notch acts upstream of *gcm* to specify SPG fate. Our findings reveal a novel mechanism by which glial cells can be generated in the CNS.

MATERIALS AND METHODS

Fly stocks

The following fly stocks were used: N^{55e11}, N^{ts1}, $pros^{17}$ (from Bloomington Stock Center); mam^{GA345}, nb^{796} (from G. Tear, London); *M84* (2nd chromosome), *P101* (3rd chromosome), *M84/P101* (from O. Vef); *pnt-lacZ* (from C. Klämbt); $gcm^{N7.4}$, $gcm\text{-}lacZ^{rA87}$ (from A. Giangrande); *UAS-*$N^{\Delta 242}$ (from Y-N. Jan); and *1407-Gal4* (from J. Urban). *1407-Gal4* is a neural specific driver and we know it is expressed later than *sca-Gal4*. Unlike *sca-Gal4*, in combination with *uas-N-intra*, *1407-Gal4* does not drastically affect neuroblast delamination.

Transplantation technique

The transplantation procedure was performed as described previously (Prokop and Technau, 1993). The heterogenetic transplantations were generally performed as follows: cells were removed from mutant donors at 0-20% ventrodorsal diameter. Single cells were subsequently implanted into an equivalent position in wild-type host embryos. Homozygous mutant donor embryos were identified by staining each donor separately for the presence of a Blue-balancer chromosome (see also Udolph et al., 1998).

N^{ts1} transplantations

Embryos, both N^{ts1} donors and wild-type hosts, were kept at the permissive temperature of 20°C until 1-2 hours after transplantation when the host embryos with the implanted N^{ts1} cells were subjected to the non-permissive temperature of 29°C until late stages of embryogenesis. This procedure assured that Notch was selectively removed only during its requirement for sibling cell fate specification and earlier functions during lateral inhibition were largely unaffected. Subsequently embryos were subjected to the standard protocol after transplantation (Prokop and Technau, 1993).

Overexpression transplantation studies

An activated form of Notch (*UAS-*$N^{\Delta 242}$, a homozygous viable insertion on the second chromosome) was crossed to the *sca-Gal4* driver, which is also homozygous viable. All F_1 progeny possessing one copy each of both the driver and the transgene were used as donors. Cells were taken from these donors and implanted into wild-type hosts according to standard procedures.

Generation of FLP-tau*lacZ* clones

Females carrying the Actin5c promoter/FRT cassette/tau*lacZ* construct (Buenzow and Holmgren, 1995) were crossed to males carrying a HS-FLP construct. From this cross eggs were collected on standard medium for 3 hours on 25°C, embryos were aged for another 4 hours at 25°C. The sequence separating the constitutive actin5c promoter from the tau*lacZ*-coding sequence is flanked by FRT sites (/) and contains a transcriptional termination signal. When this sequence is excised from a cell after activation of FLP, tau*lacZ* is expressed and marks the lineage derived from this cell. After ageing, embryos were dechorionated by bleach and incubated at 34°C in a water bath for 20 minutes. Heat-shocked embryos were raised overnight at 18°C, and were subsequently subjected to a standard antibody staining protocol using an anti-β-gal antibody. After staining, embryos were examined and two cell clones were analysed. Our conditions generated on average one clone every two hemisegments.

Immunohistochemistry

Antibody staining was as described previously (Schmidt-Ott and Technau, 1992). Following primary antibodies were used: anti-Even skipped (Frasch et al., 1986); anti-Repo (Halter et al., 1995); anti-β-gal (Promega). Secondary antibodies used throughout this study were coupled either to FITC, to Cy3 or to HRP (Jackson).

RESULTS

The temporal profile of A-, B- and LV-SPG development

The expression patterns of the enhancer-trap lines *M84* and *P101* (Klämbt and Goodman, 1991) are indistinguishable in abdominal segments: both are expressed in the SPGs including the A- and B-SPG and LV-SPG cells produced from NB1-1A. To investigate the relationship between the three glial cells derived from NB1-1A, we stained *M84/P101* (a stock double homozygous for both the *M84* and *P101* insertions, obtained from O. Vef, Mainz) embryos with anti-β-gal. In stage 12/13 embryos, a single *M84/P101*$^+$ cell appeared in dorsal positions

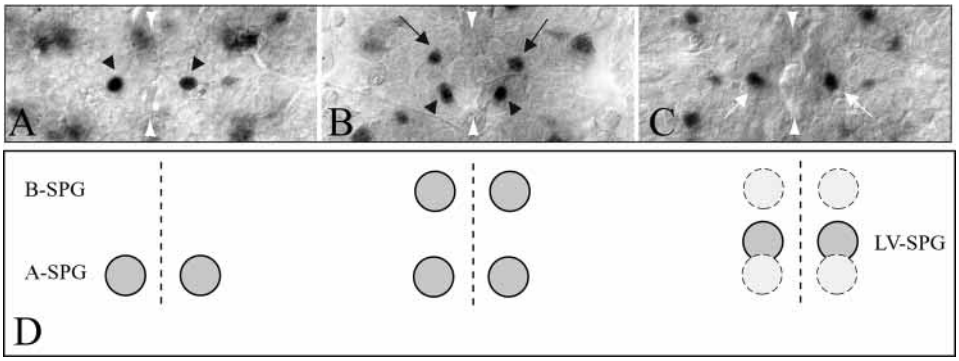

Fig. 1. Developmental profile of the M84/P101 expression pattern. (A-C) Dorsal views of one segment of *M84/P101* embryos stained with anti-β-gal. *M84/P101* embryos are viable and phenotypically wild type. Anterior is towards the top. White arrowheads indicate the ventral midline. (A) In a stage 12/13 embryo, each hemisegment contains one dorsally located *M84/P101*$^+$ cell (black arrowheads). Based on the position posterior to the Eve$^+$ cluster of NB1-1 and NB7-1 (data not shown) this cell is the A-SPG. (B) Slightly older embryo (Stage 13). A second dorsally located *M84/P101*$^+$ cell can be detected anterior to the A-SPG. This cell represents the B-SPG (black arrows). (C) A stage 14 embryo shown in a more ventral focal plane than that shown in A,B. After the A- and B-SPG can be detected, we find a third, more ventrally located *M84/P101*$^+$ cell, the LV-SPG (white arrow). A- and B-SPGs cannot be seen in this ventral focal plane. (D) The temporal profile of the *M84/P101* staining pattern is schematised; broken lines represent the ventral midline.

of each hemi-neuromere (Fig. 1A). Embryos double labelled with anti-Even-skipped (anti-Eve) indicated that this cell is located posteriorly to the Eve$^+$ cluster of NB1-1 (aCC/pCC) and NB7-1 progeny (CQ-neurones; data not shown). Slightly later a second *M84/P101*$^+$ cell appeared anterior to the first cell (Fig. 1B). According to the positioning within the developing nerve cord, the posterior cell represents the A-SPG, whereas the anterior cell is the B-SPG. A third glial cell, representing the LV-SPG (Ito et al., 1995), could be detected in ventral positions only after the A- and B-SPG were already present, suggesting that this cell is the last born glia within the lineage (Fig. 1C). The temporal order of the *M84/P101* expression pattern is summarised schematically in Fig. 1D.

Phenotypes of *Notch*, *mam* and *numb* mutants raise the possibility that SPGs are the products of asymmetric cell divisions

As a first step towards elucidating the origin of the glial cells of the NB1-1A lineage, we examined the effects of loss of function mutants in several genes, *Notch*, *mastermind* (*mam*) and *numb*, which are known to affect the resolution of distinct sibling cell fates, for their effect on the development of A-, B- and LV-SPGs. Embryos hemizygous/homozygous for a conditional *Notch* allele, *N^{ts1}*, and also carrying one copy each of *M84* and *P101* (*N^{ts1}/M84/P101*) were subjected to the non-permissive temperature of 29°C after 6 hours of development. This regime allows *Notch* to function during the singling out of NBs and removes *Notch* during the crucial period when it is required for sibling cell fate resolution. Double staining with anti-Eve and anti-β-gal was performed (Fig. 2). As expected, in most hemisegments, *N^{ts1}/M84/P101* embryos duplicated the RP2 neurone at the expense of its sibling cell. Moreover, in 96% of the hemisegments (*n*=144) *M84/P101*$^+$ cells could not be found in typical dorsal or ventral positions (Fig. 2B). We conclude that *Notch* function is required for the specification of the *M84/P101*$^+$ A-, B- and LV-SPGs. But we cannot rule out that these SPGs derived from NB1-1A are still physically present and the removal of *Notch* only downregulated *M84/P101* expression. However, transplantation experiments with *Notch* mutant cells revealed that cells with glial morphology could not be detected in the mutant NB1-1A lineages (see below). In wild-type embryos, *M84/P101* is expressed in about eight SPGs per hemisegment, including the A- and B-SPGs and the LV-SPG (Fig. 2A; Udolph et al., 1993). Removing *Notch* function results in the near complete abolishment of all *M84/P101* expression, indicating a more general function for *Notch* in SPG specification (Fig. 2B).

These findings prompted us to investigate whether *Notch* is required in the specification of CNS glia in general. We performed experiments in which glia-specific markers, either enhancer trap lines (*gcm-lacZ^{rA87}* and *pnt-lacZ*) or an antibody (anti-Repo; Halter et al., 1995) were used. *gcm-lacZ^{rA87}* is an insertion into the *gcm* gene and expresses β-gal in the pattern of *gcm*. *pointed* (*pnt*) is specifically expressed in glial cells (Klämbt, 1993; Klaes et al., 1994) and has been reported to act downstream of *gcm* (Giesen et al., 1997). *N^{ts1}* embryos were shifted to the non-permissive temperature after 6 hours of development, and subsequently stained with anti-Eve and anti-β-gal at late stage 16; taking into account the missing SPGs, both enhancer traps were expressed in a pattern reminiscent of wild-type embryos (Fig. 3A-D). However, an increase in the number of β-gal-positive cells was observed, which could be in part due to a mild neurogenic phenotype caused by *N^{ts1}*. Furthermore, we found that the glial-specific protein Repo (Campell et al., 1994; Xiong et al., 1994; Halter et al., 1995) was widely expressed in the CNS of *N^{55e11}* (an amorphic *N* allele) embryos (Fig. 3F). Thus, general glial specific markers like *gcm*, *pnt* and *repo* are expressed in embryos that lack or have strongly reduced *Notch* function, indicating that although *Notch* is required for the formation of SPGs, there is not a global requirement for *Notch* in the specification of all CNS glia.

We also tested another neurogenic gene, *mastermind* (*mam*), which has been linked to the Notch signalling pathway by its genetic interactions with *Notch* and its strikingly similar phenotype in early and late neurogenesis. It has been shown that *mam* acts downstream of *Notch* during sibling cell fate specification in the embryonic nervous system (Schuldt and Brand, 1999). The hypomorphic *mam^{345}* allele used in our study shows only a mild hypertrophy of the nervous system but clearly has an effect on sibling cell fate specification (Buescher et al., 1998). We observed a severe reduction (94%; *n*=56 hemisegments) of *P101*$^+$ cells in *mam^{345}; P101* embryos similar to that seen with *N^{ts1}/M84/P101* embryos (Fig. 2C).

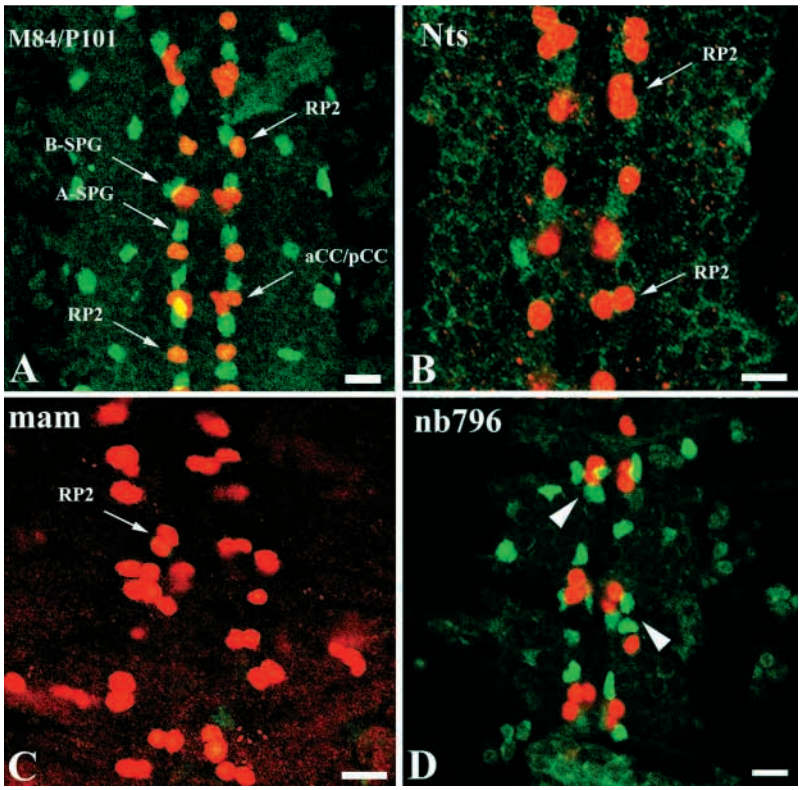

Fig. 2. *Notch*, *mastermind* and *numb* mutations affect SPG development. *M84*/*P101* staining pattern was assayed in different genetic backgrounds. Double staining was performed with anti-Eve and anti-β-gal. Anterior is upwards. (A) *M84*/*P101* embryo showing the typical wild-type pattern of SPGs (green). Anti-Eve staining (red) serves as a positional landmark. (B) *M84*/*P101* expression in N^{ts1} embryos which have been upshifted to the non-permissive temperature after 6 hours of development. Arrows point to duplicated RP2 neurones which are characteristic of N^{ts1} embryos. *M84*/*P101* expression is essentially abolished. (C) Embryo with the genotype *P101;* mam^{GA345}. Like N^{ts1} embryos, all *P101* expression is lost. Slight distortion of landmarks in the CNS is due to the hypomorphic neurogenic phenotype of the *mam* mutation. (D) In the $numb^{796}$ mutant background, additional $P101^{+}$ cells can be detected in the position of the A- and B-SPG. In many of the hemisegments, up to four cells can be found (white arrowheads), which indicates a duplication of the A- and B-SPG cells. Scale bars: 20 μm.

These data suggest that both genes are strictly required for the specification of SPGs, most likely in a linear pathway.

However, it is unclear how *Notch* acts in the specification of the SPGs. We consider the possibility that SPG glial cells could arise from a series of asymmetric cell divisions, with Notch being required to specify the glial daughters of these divisions. Based on its function as a negative regulator of Notch signalling, the expected *numb* phenotype is opposite to Notch in terms of sibling cell fate transformation. We tested the *P101* expression pattern in the background of a strong *numb* mutation (nb^{796}) (Buescher et al., 1998; Skeath and Doe, 1998). In contrast to *Notch* and *mam*, we found additional $P101^{+}$ cells in the vicinity of the aCC/pCC position. In most of the examined hemi-neuromeres, we detected up to four β-gal-positive cells in dorsal positions close to aCC/pCC (Fig. 2D; 67%; *n*=125 hemisegments). This is indicative of a duplication of the A- and B-SPGs. We also found additional $P101^{+}$ cells with glial morphology in lateral and ventral positions of the nerve cord, presumably duplications of other SPGs (data not shown). These findings are consistent with our asymmetric cell division model for the genesis of the SPGs.

Clonal analysis of mutant NB1-1A lineages

Our analyses of SPG formation using molecular markers in various mutant backgrounds do not provide any information pertaining to their relationship with other cells of the NB1-1A lineage. To overcome this limitation, we used the transplantation technique to create defined mutant NB clones in an otherwise wild-type background to enable the effects of a mutant gene to be assessed in the context of complete NB lineages. HRP-marked cells were taken from the neuroectoderm of hemi- or homozygous *Notch* mutants (N^{55e11} or N^{ts1}), or from transgenic donor embryos misexpressing a constitutively active form of the Notch receptor fused to the yeast upstream activating sequence (*UAS-*$N^{\Delta 242}$) driven by the *scabrous Gal4* driver (*sca-Gal4*). These mutant donor cells were implanted into wild-type hosts (see Materials and Methods). After implantation, the embryos were raised until late stages of embryogenesis (stage 16 according to Campos-Ortega and Hartenstein, 1985). Resulting clones of NB1-1A lineages were screened and analysed.

Thirteen NB1-1A clones derived from N^{55e11} mutant donors were obtained. In all 13 mutant clones, the pCC neurone was transformed into its sibling aCC, as revealed by the loss of the pCC projection and the duplication of the aCC motorprojection (Fig. 4A-D; Table1; data not shown). In addition, the correct muscle DO1 was innervated by the duplicated aCC (data not shown). These results confirm and extend on previous findings based on molecular markers for aCC/pCC (Buescher et al., 1998; Skeath and Doe, 1998) and indicate that the transformed pCC exhibits the characteristic axon morphology of its sibling aCC. Furthermore, the later components of the Notch mutant NB1-1A lineages show additional abnormalities that have not been previously described; with the exception of one clone, the glial components of these lineages were totally absent (compare Fig. 4A,B with Fig. 4C,D) based on two criteria: first, we could not detect cells in the expected glial positions and second, there were no cells present that exhibited typical glial morphologies, i.e. extensive glial protrusions. In addition, we found that either the number of cluster neurones was increased above the wild-type number, or additional cells with neuronal-like morphologies appeared in close vicinity of the cluster located ventrally to the duplicated aCC neurones (Table1).

Our clonal analysis demonstrates that the loss of *Notch*

function results in the loss of SPGs and a concomitant gain of neuronal cells within the NB1-1A lineage, consistent with the notion that a sibling cell fate relationship exists between cluster neurones and glial cells in this lineage and that *Notch* is required for the asymmetric divisions that generate these postulated neurone/glia sibling pairs. One prediction of this model would be that ectopic Notch function achieved by the overexpression of a constitutively active form of *Notch* should increase the number of glial cells and decrease the number of cluster neurones within the NB1-1A lineage. To test this prediction, we transplanted cells from donor embryos, which were genotypically *UAS-N$^{\Delta 242}$/sca-Gal4*. This combination of driver and transgene results in ectopic expression of a constitutively activated form of *Notch*, exclusively in the implanted cell and the resulting lineages. Seven such NB1-1A clones were obtained and as predicted, in all seven NB1-1A clones we found additional glial cells exceeding the number of SPGs that are normally found in wild-type clones and a concomitant decrease in the number of cluster neurones (Fig. 4E; Table 1). However, the locations of the additional glial cells were somewhat variable, occupying dorsal/lateral to ventral positions, possibly due to an effect on cell migration. Furthermore, in all these clones in which additional glial cells were found, we also detect a clear reduction in the number of cluster neurones (Table 1). Therefore, it seemed that additional glial cells were produced at the expense of cluster neurones. These data underline our hypothesis that glial cells and cluster neurones share a sibling relationship within the NB1-1A lineage.

However, these results do not rule out the possibility that (after its first division to produce GMC-1 and the aCC and pCC neurones) the second or a later division of NB1-1A results in a bifurcation, producing a glioblast and a neuroblast and that *Notch* might be require for this asymmetric division. In this scenario, increases or decreases in the number of glial cells in this lineage caused by the various mutants would also result in a reciprocal change in the number of neurones. If this were the case, however, one would expect that in any given (mutant) NB1-1A clone, glial cells would either be all present or all absent, depending on whether the glioblast is correctly specified.

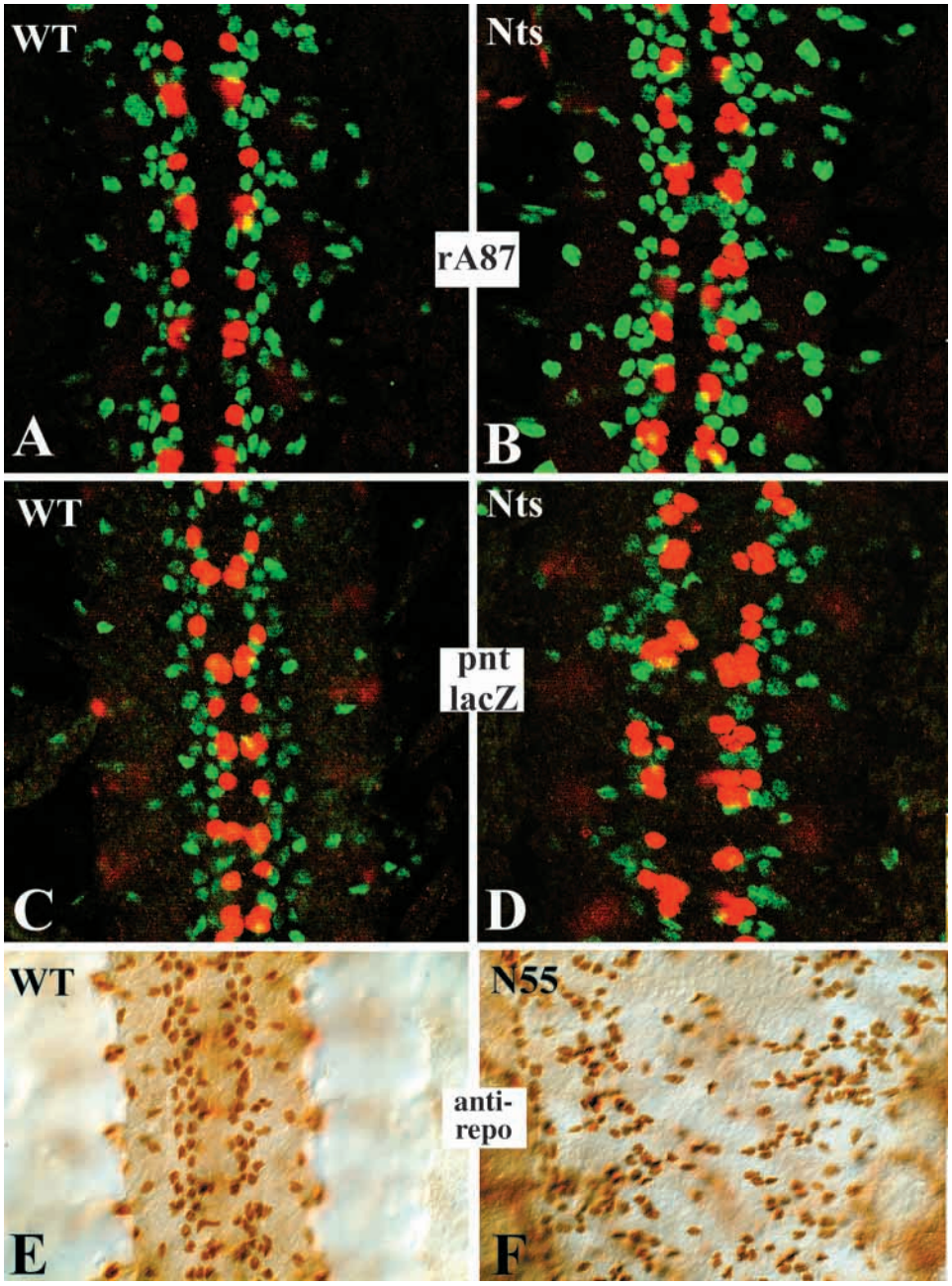

Fig. 3. *Notch* does not affect gliogenesis in general. Glia-specific markers were examined in wild-type and *Notch* embryos. Dorsal views are shown. (A-D) All embryos were double stained with anti-Eve (red) and anti-β-gal (green). (A,B) Expression of the *gcm-lacZ ^{rA87}* enhancer trap in wild-type (A) and *N^{ts1}* (B) stage 16 embryos. These patterns are very similar. (C,D) Expression of the glial specific *pnt-lacZ* enhancer trap in wild-type (C) and *N^{ts1}* (D) embryos. As with *gcm-lacZ^{rA87}* the *pnt-lacZ* expression patterns are highly similar in wild type and *N^{ts1}*. The extent of the neurogenic phenotype seen in the different embryos varies owing to slight differences in the developmental stage at which *N^{ts1}* is inactivated. (E,F) Staining with anti-Repo, which specifically labels all glial cells in the nervous system, except midline glia. (E) Expression of Repo in a wild-type embryo. (F) In the amorphic Notch allele *N^{55e11}*, Repo expression is still detectable. The obvious increase of labelled cells is caused by the strong neurogenic phenotype of *N^{55e11}*. WT, wild type; *N^{ts}*, *N^{ts1}*; *N^{55}*, *Notch^{55e11}*.

Table 1. Summary of the NB1-1A clones resulting from the transplantation assay

	Number	aCC	pCC	cN	SPG
Wild type	-	1	1	4 to 5	3
	1	2	0	8 to 10	0
	2	2	0	8 to 10	0
	3	2	0	8	0
	4	2	0	8	0
	5	2	0	4 to 5 (2)	0
	6	2	0	5 (3)	0
N^{55}	7	2	0	5 (2)	0
	8	2	0	5 to 6 (2)	0
	9	2	0	5 to 6 (2)	0
	10	2	0	5 to 6 (2)	0
	11	4	0	7	2
	12	~4	0	>12	0
	13	4	0	8 to 10 (?)	0
	14	2	0	~ 8	0
	15	2	0	6 to 7 (1)	0
	16	2	0	5 (2)	1
	17	2	0	4 to 6 (1)	2?
	18	2	0	6	0
	19	2	0	5	2
N^{ts}	20	2	0	4	3
	21	2	0	?	3
	22	2	0	?	2
	23	1	1	5 to 6	2
	24	1	1	5	2
	25	1	1?	4 (2)	?
	26	1	1	?	0
	27	0	2	0	5
	28	0	2	2	3
	29	0	2	2?	4
N^{intra}	30	0	2	0	4
	31	1	1	?	4
	32	0	2	1	5
	33	0	2	0	4

NB1-1A clones that result from different mutant cells are compared with wild-type NB1-1A clones. Cells originating from donors with different genotypes (indicated on the left) were implanted into wild-type hosts. Clones were analysed with respect to the histotypes and cell number at embryonic stage 16.

Note that in three of the N^{55e11} clones, four cells were found in the aCC/pCC position, all of them showing the aCC phenotype and neither pCC nor glial cells were present (Numbers 11-13). The nature of these clones is consistent with the interpretation that, for these cases, two *N* donor cells had been transplanted, resulting in multiple NB1-1A lineages.

cN, cluster neurones; SPG, A-, B- and LV-SPGs; the numbers in parenthesis (in column cN), the number of neuronal-like cells found outside the cN cluster; N^{55}, N^{55e11} allele; N^{ts}, N^{ts1}; N^{intra}, UAS construct of a constitutively active form of Notch ($N^{\Delta 242}$) driven by *sca-Gal4*.

? means number unclear.

To address this possibility, we transplanted donor cells taken from N^{ts1} embryos. In this experiment, the transplantation procedure was performed under the permissive temperature (20°C) and host embryos were subject to the non-permissive temperature 1 hour after transplantation. Analysis of 13 such clones yielded results that were qualitatively similar to those of transplanting N^{55e11} cells; we observed pCC to aCC transformation, loss of SPG and gain of neurones. However, the expressivity was incomplete for all of these cell fate transformations (Table 1). In the majority of the N^{ts1} NB1-1A lineages, where pCC was transformed into aCC, loss of SPG was incomplete; we could detect between zero and three glial cells in these clones. This is in contrast to the N^{55e11} NB1-1A clones, where essentially all SPGs cells of the NB1-1A lineage were absent. This might indicate that for the conditions we used, the N^{ts1} cells retain some residual *Notch* function, and that the specific threshold of *Notch* activity required to resolve aCC/pCC fates might be greater than that required to specify SPG fate. More importantly, the fact that partial loss of SPGs appears to be the predominant effect in N^{ts1} clones argues against the late bifurcation model. Taken together, our data favour a model in which a series of three GMCs produced from NB1-1A can each divide asymmetrically to produce a neurone and a glia, with Notch signalling required to specify the glia fate, and Numb and the absence of Notch signalling required for the neuronal fate.

Neurones and SPG-glia constitute sibling pairs

To directly demonstrate that SPG-glia and neurone form sibling pairs, we used the FRT/FLP tau-bGal 'flip-out' cassettes method (Buenzow and Holmgren, 1995) (see Materials and Methods) to generate a series of two cell clones in the wild-type CNS. Sixty hemisegments were obtained containing scoreable two cell clones. In all six cases where the clone contained one SPG on the basis of position and morphology, its sibling had a neuronal morphology (rounded cell body) with clearly visible axonal projections into the neuropil (Fig. 4F). These results further substantiate that SPGs, in general, derive from an asymmetric cell division, suggesting that not only are the SPGs derived from NB1-1A but probably all SPG in the CNS share sibling relationship with neurones.

Notch acts upstream of *gcm* during SPG development

As shown above, development of the SPGs requires Notch signalling. It is also known that *gcm* acts as a master regulator in gliogenesis because it has been described as a binary switch between glia and neurones (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). As expected, *gcm* appears to be required for the formation of SPGs. *P101* marker gene expression was abolished in embryos homozygous for $gcm^{N7.4}$, an amorphic *gcm* allele (Fig. 5B). The similarity of *gcm* and *Notch* phenotypes suggests the possibility that both genes share a common pathway required for SPG specification.

We reasoned that if *Notch* acts downstream of *gcm*, then overexpression of $N^{\Delta 242}$ in a *gcm* minus genetic background should result in additional SPGs; however, if *Notch* acts upstream of *gcm*, then in a *gcm* minus background the overexpression of $N^{\Delta 242}$ should not result in the production of additional $P101^{+}$ cells. We misexpressed *UAS-*$N^{\Delta 242}$ in a *P101* genetic background by using the *1407-Gal4* driver (Jo Urban, Mainz; see Materials and Methods). *1407-Gal4* driven misexpression of *UAS-*$N^{\Delta 242}$ leads to an increase above the number of normal wild-type $P101^{+}$ SPG glial cells (data not shown), which confirms the *UAS-*$N^{\Delta 242}$ transplantation results (see Table1). Using the same combination of markers and transgenes (*P101*/*1407-Gal4*/ *UAS-*$N^{\Delta 242}$), but in a *gcm* minus background, we observed a complete loss of the *P101* staining pattern within the nervous system (Fig. 5C; *n*=25 embryos). In contrast, the ectodermal component of the *P101* expression pattern was retained. Thus, in a genetic background lacking *gcm* function, $N^{\Delta 242}$ expression was not able to induce SPG development as indicated by the loss of marker gene expression.

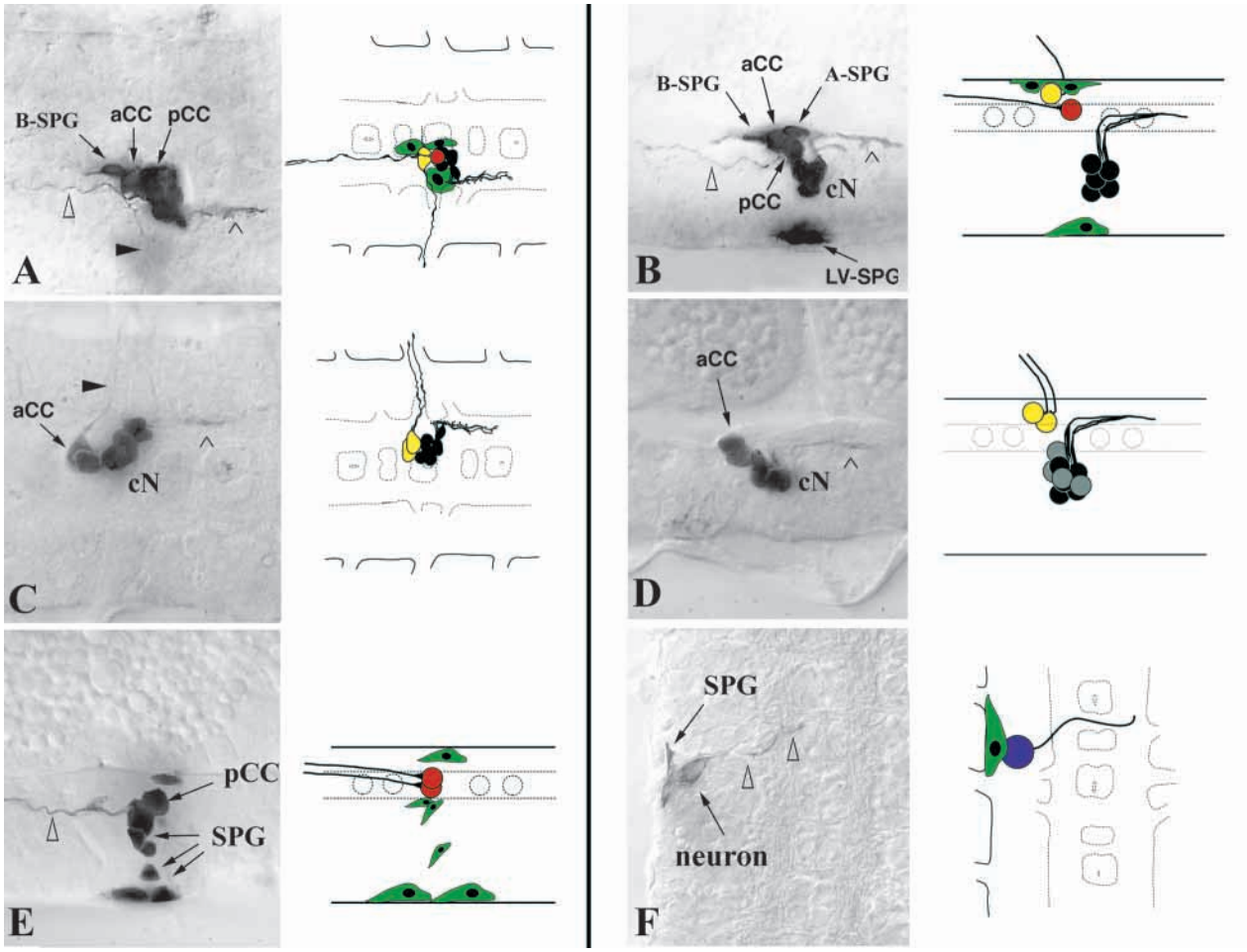

Fig. 4. SPG glial cell numbers in the NB1-1A lineage are affected by modulating *Notch* function. (A-E) Clones of NB1-1A which developed from wild-type, mutant or transgenic donor cells in wild-type hosts. Each panel consists of a HRP-labelled clone (left) and a schematised representations of the same clone (right). Anterior is towards the left. (A,B) Isogenic transplantations of wild-type donors into wild-type hosts. (A) Horizontal view of a wild-type NB1-1A lineage. The aCC neurone sends a motorneuronal projection into the periphery (black triangle/yellow) whereas its sibling pCC extends its axon along the ipsilateral connective (open triangle/red). The posterior fascicle of the cluster neurones (cN) is indicated (arrowhead). (B) Lateral view of the same clone as in A. Three glial cells are part of the NB1-1A lineage (green). Two SPGs lie at the dorsal border of the ventral nerve cord. The B-SPG is located anterior to aCC and the A-SPG is posterior to pCC. The LV-SPG is positioned ventral to the cluster neurones (cN). (C,D) Heterogenetic transplantations of N^{55e11} donor cells into wild-type hosts. Shown is a representative clone of NB1-1A. (C) Horizontal view. The pCC neurone is transformed into its sibling aCC (yellow), as no pCC projection can be detected. Instead, two aCC axons can be observed in the periphery (not shown). The projections of the cN are formed correctly (arrowhead). (D) Lateral view of the same clone as in C. The glial cells are completely absent from the lineage. In addition, the cluster of neurones contains an increased number of cells (see Table 1). (E) Heterogenetic transplantations of *scaGal4::UAS-*$N^{\Delta 242}$ donor cells implanted into wild-type hosts. Lateral view of a NB1-1A lineage that developed from such a donor cell overexpressing activated Notch. Additional glial cells can be detected in the clone in ventral as well as in ventrolateral positions (arrows/green; also see Table 1). The pCC neurone (red) is duplicated at the expense of its sibling aCC. The open triangle indicates the pCC projections. Cluster neurones are not detected in this clone, indicating a sibling cell fate transformation of cN into SPGs. (F) Dorsal view, anterior is to the top. A representative two cell clone created by activating an FRT-tau*lacZ* cassette (see Materials and Methods). This clone comprises of one SPG (green) at the lateral border of the CNS and one interneurone (purple) projecting contralaterally through the anterior commissure of the corresponding hemisegment (open triangles). cN, cluster neurones.

These findings are consistent with the notion that *Notch* functions upstream of *gcm* in the context of SPG development.

DISCUSSION

Subperineurial glial cells and neurones form sibling pairs in the NB1-1A lineage

Two types of neuroectodermally derived glial progenitors in the embryonic nervous system of *Drosophila* have been described. Glioblasts (GB) generate only glial progeny, and NGBs produce both neurones and glial cells within the same lineage (Udolph et al., 1993; Bossing et al., 1996; Schmidt et al., 1997). A mechanism by which both neurones and glial cells can arise within the lineage of a thoracic NGB, NGB6-4T, has recently been described. NGB6-4T represents a thoracic-specific NGB in which glial and neuronal sublineages bifurcate from each other during the first division of the parental NGB

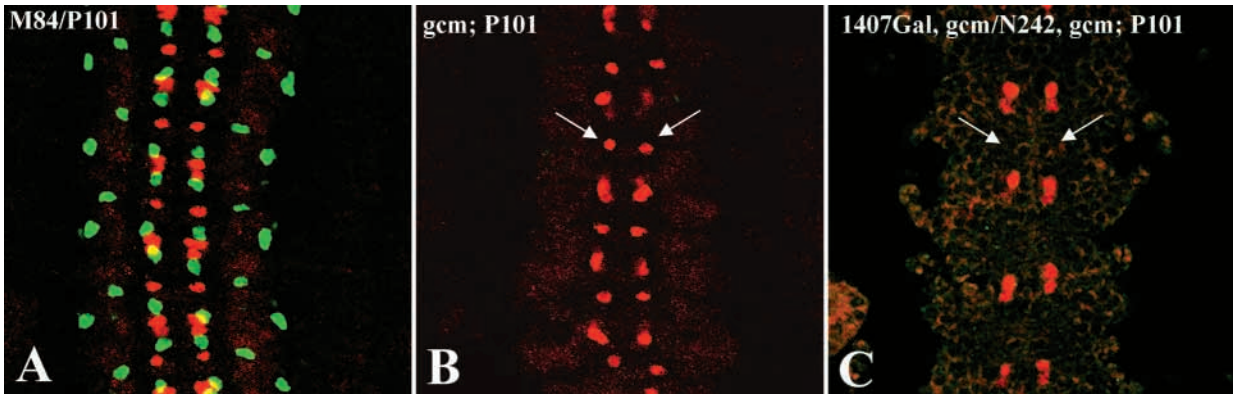

Fig. 5. With respect to SPG formation, *gcm* is epistatic to *Notch*. Anti-β-gal (green) and anti-Eve (red) antibody staining was performed. Anterior is towards the top, dorsal views of dissected nerve cords are shown. (A) wild-type pattern of the *P101* enhancer trap line (green) and Eve (red) expression. (B) A *P101*/*gcm* embryo. Note that owing to the loss of *gcm* no *P101*$^{+}$ SPGs can be detected. Ectodermal expression of *P101* is maintained in these embryos (data not shown). The RP2 neurones are marked by arrows. (C) An embryo with the genotype *1407-Gal4*, *gcm/UAS-N*$^{\Delta 242}$, *gcm*; *P101*. In these embryos, the RP2 neurone is transformed into RP2 sib owing to the overexpression of N$^{\Delta 242}$, and therefore no longer expresses Eve (arrows). Note that in the absence of *gcm* function no *P101*$^{+}$ SPGs can be detected despite the overexpression of constitutively activated Notch.

(Akiyama-Oda et al., 1999; Bernardoni et al., 1999). Only one of the two daughters expresses Gcm, a master regulator of glial cell fate that is involved in regulating the expression of other glia-specific target genes (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). In the NGB6-4T lineage, the asymmetric distribution of Gcm results in a cell that is specified as a GB within a neuroglioblast lineage. The GB will exclusively generate the glial components, whereas its sibling will exclusively give rise to the neuronal components of the lineage.

In this study, we report a novel mechanism by which glia can be generated during CNS development. Our data suggest that NB1-1A gives rise to a set of three glial cells through a series of three GMC asymmetric divisions. Several lines of evidence support the notion that within a NGB lineage GMCs can produce both glial and neuronal cells. First, immunohistochemical analyses indicate that the A-, B- and LV-SPG arise at different times in development, and their non-simultaneous birth, in conjunction with the fact that their formation can be differentially affected by inactivation of Notch, suggests that they do not derive from a common precursor. Second, we showed that mutations in genes involved in specifying alternative sibling cell fates affect SPG development in a fashion that suggest these cells are siblings with non-glial components of the lineage. Third, transplantation experiments indicate that the 3 SPGs share sibling relationships with the neuronal components of the NB1-1A lineage, the cluster neurones (cN). Finally, analysis of two cell FLP-clones demonstrate that SPG glia and neurones share a direct sibling relationships.

Our proposed mechanism for the genesis of SPGs (Fig. 6) is fundamentally different from the ones described for GBs, e.g. anterior glioblast (Jacobs et al., 1989), and for NGBs, e.g. NGB6-4T and NGB5-6A (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). The first division of NB1-1A produces a neurogenic GMC that gives rise to a pair of sibling neurones (aCC/pCC), and at the level of the first division of the parental NB no GB sublineage is bifurcated. In addition, the NB1-1A derived glial cells share a direct sibling relationship with neurones and does not involve the generation of a lineage internal GB at all. The asymmetric origin of glia described here provides a novel mechanistic framework of glial origin that might be used as a model system to gain further insights into the regulatory networks involved in glial cell fate specification in the CNS. We conclude that the mechanisms leading to glial cell fate specification are complex and that multiple developmental mechanisms lead to glial cell fate specification. These mechanisms will be likely to require

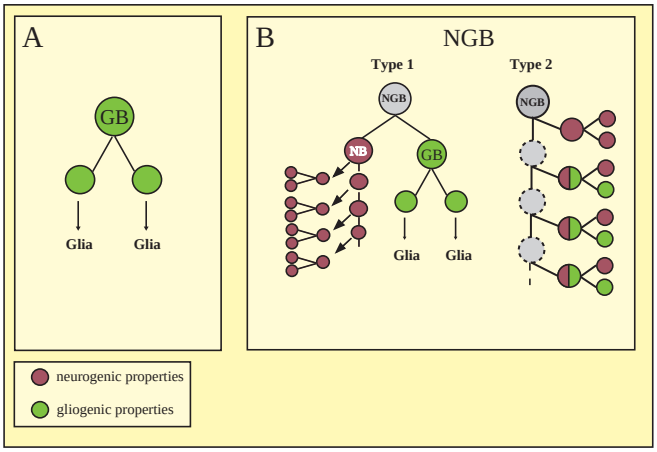

Fig. 6. Three modes of glial origin. Glia cells originate from different types of progenitors, glioblasts (GB) or neuroglioblast (NGB). (A) GBs possess only gliogenic properties and give rise exclusively to glial cells. (B) NGBs generate glial and neuronal components in a mixed lineage. Two different types of NGBs exist. Type 1 NGBs bifurcate into a GB and NB during their first division and create precursors with restricted developmental potential that give rise either to neurones or to glial cells (e.g. NB6-4T). Thus, within the lineage, specialised precursors are generated. In contrast, Type 2 NGBs create intermediate precursors that have the potential to generate neurones as well as glia via an asymmetric cell division (e.g. NB1-1A). *Notch* is used for specifying the glial part of the lineage.

different molecular machinery that involve distinct sets of genes or genetic hierarchies; for example, Notch being required specifically for the SPGs but apparently not for other types of glia. However, most of the glial cells (except midline glia) strictly require the *gcm* gene. Thus, there seems to be a common molecular basis that is context and cell specifically regulated during development.

It is unlikely that NB1-1A is the only lineage that generates glial cells in an asymmetric manner. Our studies using the SPG glial specific marker *M84/P101* showed that *Notch*, *mam* and *numb* mutants affect the formation of all SPGs, not just those derived from NB1-1A. All SPGs derive from NGB because GBs do not give rise to SPGs (Schmidt et al., 1997). Lineage analysis techniques have revealed eight NGBs (NB1-1A, NB1-3, NB2-2T, NB2-5, NB5-6T, NB5-6A, NB7-4) that produce both glial and neuronal histotypes, most of them also generate SPGs, either exclusively or with other glial cell types (NB1-1A, NB2-2T, NB5-6T, NB5-6A, NB7-4). The similarity of their genealogy, and their common response to the loss and gain of Notch function, as well as analyses of two cell clones, indicate that SPG glial cells in general might derive from asymmetric cell divisions that generate one glial and one neuronal progeny.

Notch is crucial for SPG development and acts upstream of gcm

Our study provides the first evidence that *Notch* is a crucial component in the specification of a subclass of CNS glial cells, the subperineurial glia (SPG). As revealed by the transplantation experiments, *Notch* is not only required for the expression of the *M84/P101* marker, but loss of Notch function leads to the loss of the SPG cell fate per se. In the case of NB1-1A, our data indicate that the loss of glia is accompanied by the conversion of these cells into neurones. In contrast, *Notch* gain of function results in the overproduction of SPG-like cells at the expense of the cluster neurones. Hence, Notch signalling is sufficient for the specification of these cells. A second gene, *gcm*, is also crucially required for the specification of the SPGs; in a *gcm* mutant background *M84/P101* expression is completely absent. If an activated form of the Notch protein is ectopically expressed in the embryonic CNS of animals lacking *gcm* function, SPGs do not form; in addition, *M84/P101* expression cannot be detected in the CNS of these embryos. This indicates that *gcm* function is strictly required for *Notch*-mediated SPG specification, suggesting that *Notch* functions upstream of *gcm*. It is interesting to note that Notch also appears to play an instructive role in gliogenesis for mammalian neural crest stem cells in culture (Morrison et al., 2000; reviewed by Wang and Barres, 2000).

In this context, *gcm* acts as an effector of Notch signalling during sibling cell fate specification. Our results provide a framework of a signal transduction paradigm in which a receptor (Notch) is involved in receiving external signals by cell-cell interactions, leading to the activation of *gcm*, which ultimate results in the specification of the glial fate for a SPG-glia/neurone sibling pair. However, the components of this proposed pathway other than *gcm* remain to be defined.

With respect to the requirement for *Notch*, CNS glial cells can be classified into two distinct groups. First, there are the glial cells (SPGs) that arise through asymmetric cell divisions that depend on Notch signalling; the specification of a second group of glial cells is independent of Notch function and these glia are probably born in a non-asymmetric fashion. We also suggest the existence of at least two different classes of NGBs, referred to as type I and type II NGBs. Type I NGBs bifurcate a glial sublineage during their early development; whereas the type II NGBs give rise to glial cells by producing GMC(s) that then undergo asymmetric division to produce a *Notch*-dependent glial daughter and a neurone (Fig. 6).

A model of NB1-1A development

Our results, in conjunction with what is already known about the origin of the aCC and pCC neurones, as well as the terminal lineage of NB1-1A, allow us to propose the following for the NB1-1A lineage: in total, NB1-1A gives rise to five pairs of sibling cells. The first pair comprises the aCC/pCC neurones resulting from GMC1-1a. In addition, three later born GMCs each give rise to one cluster neurone and one SPG-glia. A fifth GMC gives birth to two additional cluster neurones. The order of appearance and the position of the *MP84/P101*-labelled cells would suggest that, of the three glia-producing GMCs, the earliest born GMC gives rise to the A-SPG; the latest born GMC gives rise to the LV-SPG; and the GMC born in the middle gives rise to the B-SPG. We cannot place the time of birth of the fifth GMC in this lineage, which we postulate produces two cluster neurones. Clearly, refinement and proof for this proposal will require experiments that provide direct temporal information.

In summary, we provide evidence for a novel mechanism of glial cell fate specification where a subclass of glial cells, the SPG, derive from a series of GMCs, each of which divide asymmetrically to produce one neurone and one glia. *Notch* and *gcm* are both crucial for this event, and in this context, it is likely that *Notch* acts upstream of *gcm*.

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