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Transcriptional regulation of *Notch* and *Delta*: requirement for neuroblast segregation in *Drosophila*

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SUMMARY

Segregation of a single neural precursor from each proneural cluster in *Drosophila* relies on Notch-mediated lateral signalling. Studies concerning the spacing of precursors for the microchaetes of the peripheral nervous system suggested the existence of a regulatory loop between Notch and its ligand Delta within each cell that is under transcriptional control. Activation of Notch leads to repression of the *achaete-scute* genes which themselves regulate transcription of *Delta*, perhaps directly. Here we have tested a requirement for transcriptional regulation of *Notch* and/or *Delta* during neuroblast segregation in embryos, by providing Notch and Delta ubiquitously at uniform levels. Neuroblast segregation occurs normally under conditions of uniform *Notch* expression. Under conditions of uniform *Delta* expression, a single neuroblast segregates from each proneural group in 80% of the cases, more than one in the remaining 20%. Thus transcriptional regulation of *Delta* is largely dispensable. We discuss the possibility that segregation of single precursors in the central nervous system may rely on a heterogeneous distribution of neural potential between different cells of the proneural group. Notch signalling would enable all cells to mutually repress each other and only a cell with an elevated neural potential could overcome this repression.

Key words: *Drosophila*, Notch signalling, Delta, neuroblast segregation, cell fate

INTRODUCTION

During metazoan development cell-cell interactions play a fundamental role in the choice of differentiation pathways followed by specific populations of cells. Inductive interactions take place between signalling cells that produce the ligand and receiving cells that express the receptor, transduce the signal and respond appropriately. Lateral signalling, however, involves interactions between equivalent cells that initially each produce both ligand and receptor, but later resolves to a single signalling cell. One well studied example is the determination of neural precursors in *Drosophila*.

In the neuroectoderm, groups of cells, called proneural clusters, express proneural genes which confer competence to become neural precursors (for review see Campuzano and Modolell, 1992). All cells with high levels of proneural proteins have the potential to adopt the neural fate and indeed, in the absence of lateral signalling, they all undergo neural differentiation (Campos-Ortega, 1993). Neural competence, however, is progressively restricted to a single signalling cell that causes downregulation of proneural genes in the other, inhibited cells (Brand and Campos-Ortega, 1988; Skeath and Carroll, 1992; Ghysen et al., 1993). Signalling is mediated by the receptor Notch and its ligand Delta, activation of Notch leading to a repression of proneural genes (Artavanis et al., 1995).

All cells express Notch and Delta (Fehon et al., 1991; Kooh et al., 1993) and have the capacity to inhibit, or be inhibited by, their neighbours. Indeed, expression of an activated form of Notch before neuroblast segregation, uniformly represses the neural fate in all cells (Struhl et al., 1993; Rebay et al., 1993; Lieber et al., 1993). Furthermore, careful examination has shown that two cells occasionally emerge from a proneural cluster, although finally only one precursor cell differentiates, suggesting that they remain in competition for the precursor fate (Huang et al., 1991; Doe, 1992). In the grasshopper, neuroblast ablation results in the determination of another neuroblast from an immediate neighbour suggesting that the delaminating neuroblast actively inhibits the neighbouring cells, but that a release of this inhibition allows these cells to then adopt the neural fate (Taghert et al., 1984; Doe and Goodman, 1985). Cells can also switch to the alternative fate when transplanted into younger host embryos (Technau et al., 1988).

It is not known how the transition from a group of equivalent cells to only a single signalling cell takes place. Two extreme models can be envisaged. The first implicates the Notch signalling pathway, via a feedback loop linking production of ligand with that of receptor, in the choice of precursor, which could, in some cases, be stochastic. The second postulates that the precursor is pre-determined by some mechanism other than Notch signalling, and that Notch signalling then

only serves to mediate mutual, uniform repression of the other cells and ensure development of a single precursor.

A random choice of precursor has been postulated for the precursors of the small bristles or microchaetes that vary slightly in number and position between flies. Changes in relative levels of Notch and Delta between cells can bias the choice of fate of these precursors (Heitzler and Simpson, 1991). Furthermore, *Notch* mutant cells were shown to have a stronger capacity to send the inhibitory signal than their wild-type neighbours, an activity that is dependent on both Delta and the proneural genes (Heitzler and Simpson, 1991, 1993; Heitzler et al., 1996). This was interpreted as evidence in favour of the existence of a feedback loop between receptor and ligand where activation of Notch results in a downregulation of the proneural genes that themselves control expression of *Delta*. Thus a small difference in the relative levels of Notch or Delta could be amplified by means of this regulatory loop until a single cell, chosen stochastically, emerges as the signalling cell.

This first model predicts probable changes in the expression of receptor and ligand between the cells with time, and that ubiquitous expression of receptor and ligand might resolve to ligand expression in the presumptive precursor and receptor expression in the inhibited cells. While there is no evidence for regulation of *Notch* expression, the *Delta* promoter does appear to be regulated by the proneural proteins in imaginal discs (Heitzler et al., 1996; Hinz et al., 1994) and in embryos where this has been shown to be direct (Kunisch et al., 1994). No differential regulation of the expression of *Notch* and *Delta* proteins has been detected in the equivalence groups in embryos or imagos, however, during the selection process (Fehon et al., 1991, Kooh et al., 1993), but small differences in RNA levels seen by in situ hybridisation and in the expression of *lacZ* from a *Dl* enhancer trap line have been reported, suggesting that such regulation may occur (Haenlin et al., 1990; Ghysen et al., 1993). In *C. elegans*, where LIN-12-mediated lateral signalling has been extensively investigated, a stochastic choice of fate between the anchor cell and the ventral uterine cell involves progressive restriction of ligand and receptor to reciprocal cells (Wilkinson et al., 1994; Wilkinson and Greenwald, 1995).

In contrast to the extreme possibility discussed above, where lateral signalling allows a random selection of the signalling cell, in other cases another, extrinsic signal may bias this choice by introducing a change in the level of receptor or ligand in one cell such that it becomes more competitive. This mechanism would still require lateral signalling and the regulatory loop but the choice of fate would no longer be random. Such a process may allow preselection of the precursors for the large bristles or macrochaetes. These precursors arise at precise positions within the proneural clusters that may be influenced by the levels of Extramacrochaetae, a negative regulator of the proneural proteins, Achaete and Scute (Cubas and Modolell, 1992). Cells with less Achaete-Scute would produce less Delta and so be less competitive for the neural fate. In *C. elegans* the choice of primary versus secondary vulval precursor cells is predetermined by an EGF-like signal from an outside cell but still relies on lateral signalling between the precursor cells themselves (Horvitz and Sternberg, 1991). In yet other cases an intrinsic signal may bias lateral signalling, an example is the protein Numb which is assymetrically segregated to one daughter cell after division and downregulates Notch in that cell (Gho et al., 1996).

The second model postulates that the cells of a proneural cluster are not truly equivalent and that one cell is predestined to be the precursor. In this case Notch signalling would merely play a permissive role in that all cells would inhibit each other mutually and only the predetermined cell with the highest neural competence would be able to overcome the inhibitory signal and develop as a neural precursor. In this model, transcriptional regulation of *Notch* and *Delta* and the feedback loop would not be necessary. Muskavitch (1994) postulated an extreme form of this model whereby Notch signalling provided mutual inhibition but factors independent of Notch signalling were required to select the precursor and to render it insensitive to Notch signalling so that it escapes inhibition.

Here we ask whether neural potential differs between cells of the proneural clusters from which the embryonic neuroblasts segregate, and whether transcriptional regulation of *Notch* and *Delta* is required for segregation. We show, from the analysis of identified neuroblasts in *Notch* and *Delta* mutant embryos, that neural potential is not homogeneously distributed throughout the proneural cluster, that two or three cells have a higher neural potential which we suggest is due to differing levels of proneural proteins. Furthermore uniform, non-regulated expression of *Notch* and *Delta* allows the segregation of almost normal numbers of neuroblasts suggesting that a uniform level of Notch signalling is sufficient to repress the neural fate of most cells. However, our data do indicate that regulation of the *Delta* promoter by proneural genes may be required both to mediate competition between the two or three most competent cells, and also to maintain the choice of fate for some time in a dynamic process. We suggest a three step process for the segregation of neuroblasts that includes features of both of the models discussed above which are thus not mutually exclusive.

MATERIALS AND METHODS

Fly stocks

Delta^{revF10} (Haenlin et al., 1990) and *N*^{55e11} (Lindsley and Zimm, 1992) were used as *Delta* and *Notch* null alleles. The *Notch* germ-line mutant clones resulted from mitotic recombination in the gonad induced by the FRT/FLP method. 24-hour egg collections were made and embryos were heat-shocked for 1 hour at 37°C between 24 and 48 hours after egg laying. *N*^{55e11} *FRT101/ ovo*^{D1} *FRT101; FLP38 Bc/+* virgins were then collected and crossed to *w Pw*$^{+}$*[ftz, lacZ]* males. The male progeny of this cross lack both maternal and zygotic *Notch* expression. The *Pw*$^{+}$*[ftz, lacZ]* allowed us to recognise them after staining with antibodies against β-galactosidase.

The enhancer trap lines *Pw*$^{+}$*(735)* (homozygous lethal) and *Pw*$^{+}$*(439)* (homozygous viable) were generated by us and have the same expression pattern and the same cytological localisation of *seven-up* and *scratch* respectively (L. S. and M. H., unpublished observations). For this reason, we assume that they are inserted in these genes and refer to them as *svp-lacZ* and *scratch-lacZ* enhancer-trap lines.

TM3 Ser, Pw^{+}*[twist, lacZ]* and *TM6C Sb Tb, Pw*$^{+}$*[twist, lacZ]* balancers allowed us to recognise the genotype of homozygous *Delta* mutant embryos, after staining with antibodies against β-galactosidase.

Flies were raised at 25°C unless otherwise stated.

Constructs used to provide ubiquitous expression of *Notch* and *Delta*

The Gal-4 targeted expression system was used (Brand and Perrimon,

1993). The UAS *Delta* plasmid is an insertion in the pUAST vector of an *Nru*I-*Dra*I fragment from the c3.2 cDNA (Vässin et al., 1987). The UAS *Notch* plasmid is an insertion in pUAST vector of an *Xba*I fragment containing the last three introns of the *Notch* gene and the 5′ and 3′ untranslated region of the mRNA. The UAS *Serrate* was kindly provided by E. Knust (Speicher et al., 1994). The *arm-FRT-yellow-FRT-Gal4-VP16* (*arm-FRT-Gal4-VP16*) construct was made by consecutive insertion of a fragment comprising the *armadillo* promotor (Vincent et al., 1994), the FRT-*yellow*-FRT cassette fragment from the J35 construct (Struhl et al., 1993), a fragment encoding the GAL4-VP16 fusion protein (Tora et al., 1989), and the hsp70 3′ fragment from the pGaTN plasmid (Brand and Perrimon, 1993), into the pCaSpeR vector (Pirrota, 1988).

Verification of the ubiquitous expression of the GAL-4 constructs

Several *arm-FRT-Gal4-VP16* inserts were obtained and crossed to induce mitotic recombination in the male germ-line and embryonic *Gal4* expression, as described by Struhl et al. (1993). Two of them, which provided good rescue of the Notch zygotic phenotype with *UAS Notch (UASN)*, were selected. For these two, we estimated at 80% the rate of excision of the FRT-*yellow*-FRT cassette when crossed to β_2-tubulin-*flp#3* (Struhl et al., 1993) in absence of any UAS constructs. The remaining 20% non flipped embryos provided us with an internal control in our crosses. We obtained similar rescue of *Notch* and *Delta* mutant embryos using these two *arm-FRT-Gal4-VP16* inserts together with two different UAS *Delta* (*UASDl*) and *UASN* inserts. Expression of *Gal4* was examined with *UAS lacZ* or an *UAS CD2* construct. Two other ubiquitous Gal4 driver strains were also used. *daG32* is a fusion of the *daughterless* promoter to the Gal4 cDNA (Nakao and Campos-Ortega, 1996), and *e22c* an enhancer trap line (kindly provided by H. A. Brand).

The quality of rescue obtained with these constructs was estimated by counting the number of supernumerary neuroblasts formed after SI segregation in segments A-5 to A-8 in at least three different embryos. Midline neuroblasts were not included in these counts. Since each hemisegment contains 10 neuroblasts at this stage, the final number of supernumerary neuroblasts corresponds to the analysis of 240 equivalence groups. In five different wild-type embryos, no supernumerary neuroblasts were observed.

Antibody staining

Antibody staining were performed as described by Haenlin et al. (1994). Notch monoclonal antibodies were kindly provided by S. Artavanis-Tsakonas and staining was detected with the abc peroxidase kit. In each case at least five embryos from the same stage were examined.

RESULTS

Markers that identify individual neuroblasts

Genes such as *achaete (ac), scute (sc),* and *snail* are expressed in all or many neuroblasts during embryogenesis and thus provide general markers of neural cell fate. These markers have been used to describe the position and size of the clusters of cells that initially express *ac-sc* and are thought to represent equivalence groups (Skeath and Carroll, 1992). Here we have made use of an enhancer-trap line with an insertion in the pan-neural gene *scratch* that also marks all neuroblasts (Roark et al., 1995; Campos-Ortega and Haenlin, 1992). In mutant embryos in which more than one neuroblast segregates from each equivalence group, however, the proximity of the clusters makes it difficult to assign the neuroblasts to specific groups. In order to follow this process in more detail we have used an enhancer-trap line with an insertion in the *seven-up* gene (*svp-lacZ*, see Materials and Methods) which marks specific, identified neuroblasts. *seven-up* is eventually expressed in all the neuroblasts but expression is initiated at different times (Doe, 1992; Broadus and Doe, 1995). During early neurogenesis, *svp-lacZ* expression is restricted to a single neuroblast within each hemisegment just anterior to the band of *engrailed* expression (neuroblast 5-2; Fig. 1A). In the neurectoderm of wild-type embryos, it is initiated at a low level in all cells of the proneural cluster which will give rise to neuroblast 5-2, just before segregation of this neuroblast. After segregation, neuroblast 5-2 starts to express high levels of *svp-lacZ*, whereas expression fades in the overlying neuroectoderm. Shortly after, expression begins in a second neuroblast (neuroblast 7-4) that falls within the band of *engrailed* expression. In this case, expression is not detectable in the corresponding proneural cluster and begins soon after delamination of the neuroblast (Fig. 1D).

Segregation of neuroblasts from the 5-2 group occurs progressively in *Notch* and *Delta* mutants

With the use of general neural markers, the equivalence groups from which a single neuroblast will segregate have been estimated to contain 5-6 cells (Skeath and Carroll, 1992; Skeath et al., 1994). Expression of these markers alone, however, does not provide an absolute indication of neural competence. In *Notch (N)* and *Delta (Dl)* mutant embryos all cells of the group end up developing as neuroblasts since no cells are left to form the epidermis (Campos-Ortega, 1993). Therefore the number and time of appearance of neuroblasts formed from each group in these cases provides an independent measure of the neural competence of cells in these clusters. We used *svp-lacZ* to follow neuralisation in the 5-2 and 7-4 neuroblast groups in embryos lacking both maternal and zygotic *N* or zygotic *Dl*. Similar observations were made for both mutants: two or three (but occasionally one or four) 5-2 neuroblasts segregate instead of a single one per hemisegment (Fig. 2A,B). Later, at the time when neuroblast 7-4 appears, the number of 5-2 neuroblasts has increased up to four to five per hemisegment. Neuroblasts 7-4 behave similarly, two appear initially, but three to five are found later, at the time when neuroblasts 3-2 and 7-1 segregate (Fig. 2C,F). Neuralisation of 5-2 and 7-4 neuroblasts is therefore progressive. It is also very irregular from one hemisegment to another, especially in *Notch* mutant embryos. It is possible that neuralisation may be even more extensive at later stages, but the behaviour of neuroblast groups 5-2 and 7-4 (and other proneural groups) was not analysed further because of the complexity of the pattern.

Thus, in the absence of N signalling, the cells within an equivalence group do not enter the neural differentiation pathway at the same time. This suggests that neural potential is not evenly distributed between these cells.

Segregation of neuroblasts from the 5-2 group in *Notch* hemizygotes

In hemizygous *Notch* mutant embryos derived from heterozygous mothers, the *Notch* product derived from maternal expression allows early neurogenesis to occur normally, despite the absence of any transcriptional regulation (Martin-Bermudo et al., 1995). However, at later stages the embryos become

strongly neuralised. In order to measure the duration of maternal perdurance, we followed neuralisation in these embryos with the *scratch* enhancer-trap line. Stage 9 embryos display a neuroblast pattern that is indistinguishable from wild type, as shown previously (Struhl et al., 1993 and see Fig. 4D). The first abnormalities appear at stage 11 when the number of neuroblasts in the most ventral part of the CNS is already greater than in the wild type (not shown). This increases and is considerably enhanced at stage 12 when a large number of extra cells are present in the CNS (see Fig. 4I). The A and P precursors of the PNS also segregate normally in the beginning, but many additional cells are found later. At later stages, the embryos show a very strong neurogenic phenotype, similar to that of *Delta* mutants where neurogenesis is abnormal from the very beginning (not shown).

To look more closely at the segregation process we followed the fate of 5-2 and 7-4 neuroblasts with *svp-lacZ*. Here segregation of neuroblast 5-2 is initially normal in *N* hemizygotes and only one neuroblast forms per hemisegment (Fig. 1B). Occasionally two are found, a phenomenon that is also seen at lower frequency in wild-type embryos. However, at the time when neuroblast 7-4 arises, all cells of the 5-2 cluster become neural and 5 or 6 cells strongly express *svp-lacZ* (Fig. 1E). The same sequence of events was seen in the case of neuroblast 7-4 (not shown).

These results indicate that inhibition of the neural fate in surrounding cells, by the neuroblast that forms first, is not an irreversible process and that inhibition must be maintained after neuroblast selection. This may mean that the quantity of maternal products is insufficient to maintain inhibition, or, alternatively, perhaps transcriptional regulation of *Notch* is required for normal neuroblast segregation.

Replacement of endogenous *Notch* and *Delta* gene function by expression from *Notch* and *Delta* transgenes that cannot be transcriptionally regulated

In order to test for a requirement for transcriptional regulation of *N* and/or *Dl*, we expressed them under the control of the *armadillo* promoter (*arm-GalVP16/UASN* or *UASDl)*, by means of the *Gal4-UAS* targeted expression system (see Materials and Methods). In order to look at the distribution of *Gal4* expression from this promoter, we examined embryos carrying *UAS lacZ* or *UAS CD2*. Expression of *Gal4* begins at stage 8 and is initially somewhat heterogeneous. Levels vary from one cell to another and the pattern is different in each embryo (not shown). It is not impossible that such differences could affect the number of neuroblasts that segregate from a given group in our experiments, by, for example, providing some cells with greater levels of Dl. However this should result in a wide range in the numbers of neuroblasts per group and a great variability between embryos since no regulation is possible. In fact little variability is observed. Furthermore we have also examined the segregation of the A and P precursors in the PNS that develop later. Here the *GalVP16/UAS* system provides a strong expression in all cells, since the levels increase rapidly

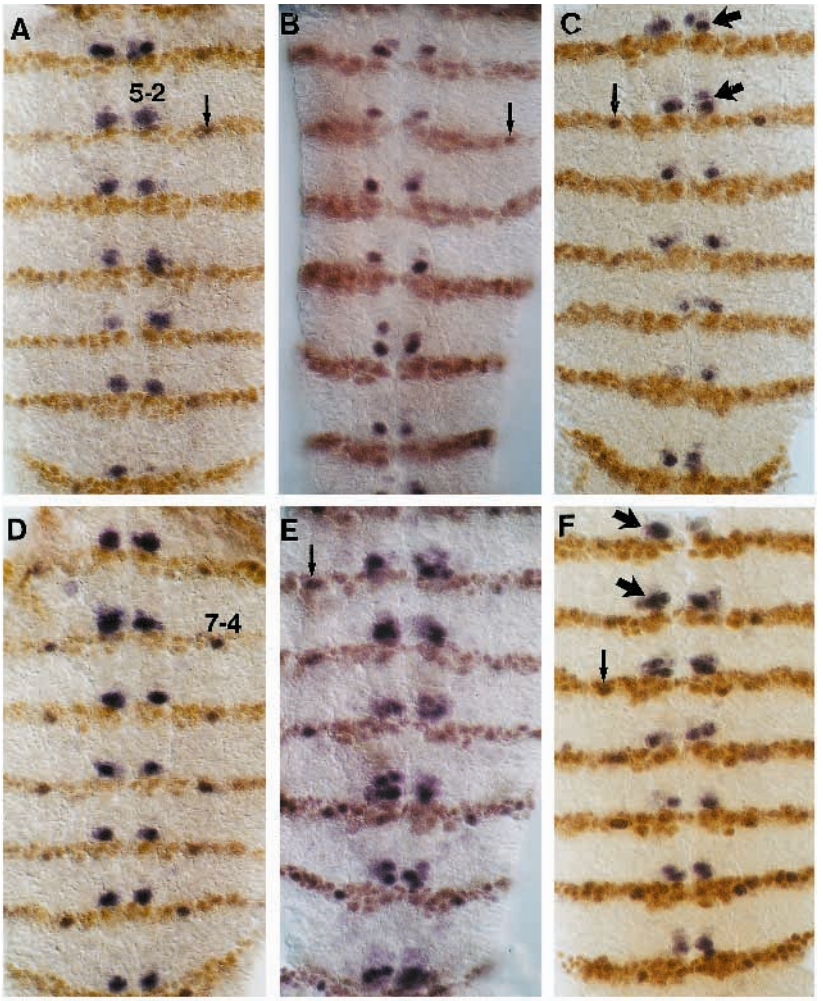

Fig. 1. Segregation of neuroblasts 5-2 and 7-4 in wild-type and mutant embryos. Ventral view, anterior up. (A,D) Wild type; (B,E) hemizygous *Notch* mutant; (C,F) mutant *Delta* embryos rescued by the *FPΔEbox-mgDl* minigene. Flat preparations of early (A-C) and late (D-F) stage 9 embryos, double stained for *svp-lacZ* (β-galactosidase expression revealed by alkaline phosphatase-coupled secondary antibodies, in blue) and *engrailed* protein (peroxidase-coupled secondary antibodies, in brown). The most anterior segment is C2. (A,D) Wild-type embryo, showing the segregation of 5-2 neuroblasts at early stages (A) and of 7-4 neuroblasts later (D). In the cephalic segments the segregation of neuroblast 7-4 occurs earlier than in thoracic segments (thin arrow in A). The diffuse staining around each 5-2 neuroblast is due to persistant expression in the proneural cluster. There is no expression in the proneural cluster in the case of neuroblast 7-4, which expresses *svp-lacZ* only after delamination. (B,E) Hemizygous *Notch* embryos containing maternal *Notch* product. The segregation of neuroblast 5-2 is close to normal at early stages (B) but the proneural cluster is neuralised later (E). Note that at this stage the segregation of neuroblast 7-4 is also close to normal (thin arrow). (C,F) Mutant *Delta* embryos rescued by the *FPΔEbox-mgDl* minigene. At early stages (C) a single (thin arrow), or occasionally two (thick arrows), 5-2 neuroblast(s) segregates in each hemisegment. Later (F), the number of 5-2 neuroblasts increases to three to four (thick arrows). Neuroblast 7-4 also begins to delaminate as a single (or occasionally two) cell(s) within the *engrailed* band (thin arrow in C and F).

at stage 9 and appear to be completely uniform by stage 11. Segregation is normal and there is no subsequent increase in numbers. Finally two other ubiquitous *Gal4* driver strains, *daG32* and *e22c*, were also used: *daG32* gave similar results to *arm-GalVP16* whereas *e22c* was only sufficient for a partial rescue of the cuticular phenotype of *Dl* embryos (not shown).

With *UASN*, the expression of *N* protein in *N* mutant embryos, detected with a N monoclonal antibody, begins at stage 8-9 and remains at a level close to wild type until the end of embryogenesis. *Delta* mRNA expression obtained with *UASDl* in *Dl* mutant embryos has been analysed in whole-mount embryos with a digoxigenin-coupled probe. Expression is first detected at stage 7, at a level comparable to that of the wild type. From stage 11 until the end of embryogenesis *Dl* mRNA expression remains significantly higher than in the wild type where the levels of Dl decrease.

Uniform expression of Notch

We looked at the CNS and PNS precursors with *scratch-lacZ* and the axonal projections with 22C10 antibodies and found that hemizygous *N* embryos carrying *arm-GalVP16*/*UASN* are indistinguishable from wild type (Fig. 3C,D). This result suggests that transcriptional regulation of *Notch* is not necessary for many aspects of development of the larval CNS and PNS. In particular, it is dispensable both before and after neuroblast segregation.

Uniform expression of Delta

In contrast to *N*, previous results suggest that transcriptional regulation of *Dl* may be necessary for normal neuroblast segregation. Embryos in which the endogenous *Dl* gene has been replaced by the uniformly expressed transgene (*arm-GalVP16*/*UASDl*), present a pattern of neuroblast segregation at stage 9 that is close, but not identical, to that of the wild type (Fig. 4B,G). *scratch-lacZ* staining shows that in general one neuroblast segregates from each equivalence group but in some groups two appear. The positions of the additional neuroblasts vary from one embryo to another. We observed 50 extra neuroblasts for 240 extant ones in segments A5 to A8 (results from three different embryos, see Materials and Methods and Fig. 6F). Rare cases of missing neuroblasts were also observed. By providing an additional *UASDl* insert, a higher level of *Dl* expression can be obtained. In this case fewer ectopic neuroblasts form (22 extra neuroblasts per 240 were counted in segments A5 to A8).

The number of neuroblasts in each equivalence group does not increase subsequently and at the end of embryogenesis, the size of the CNS is still close to normal, but its overall shape is somewhat irregular and this varies from one embryo to another (Fig. 3E). mAb22C10 staining at this stage shows that the axonal scaffold in the CNS as well as the axonal projections coming from the PNS also show extensive abnormalities (Fig. 3E,F). However, from the very beginning segregation of the P precursors of the larval PNS in mutant *Delta* embryos rescued with *GalVP16/UASDl* also appears normal (Fig. 5B). Thus, unlike similar experiments with *N*, ubiquitous expression of *Dl* at a uniform rate does not allow normal development of the CNS and PNS. Nevertheless, rescue is considerable, and in the early stages segregation of a single neuroblast does take place within many groups; only a small percentage of them bear more than one.

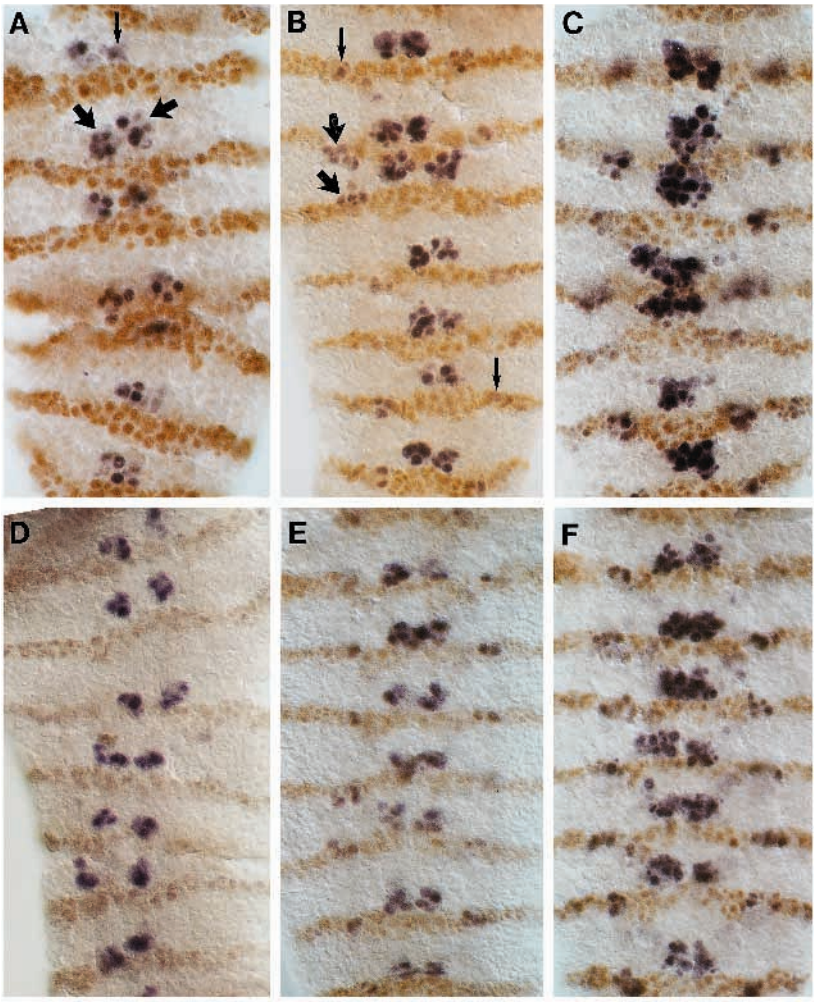

Fig. 2. Analysis of neuralisation in the equivalence groups corresponding to neuroblasts 5-2 and 7-4, in *Notch* (A-C) and *Delta* (D-F) mutants. Flat preparations (anterior is up, ventral view, C2 is the most anterior segment) of early stage 9 (A,D), late stage 9 (B,E) and early stage 10 embryos (C,F), stained as in Fig. 1. (A-C) *Notch* mutant embryos without maternal contribution (derived from germ-line clones mutant for *Notch*). The segmentation is disturbed in some segments, and some 5-2 equivalence groups are fused (probably due to abnormal determination of the mesectoderm, Menne and Klambt, 1994; Martin-Bermudo et al., 1995). Three to four 5-2 neuroblasts, but occasionally one (thin arrow) or five (thick arrows) initially segregate in each hemisegment (A). This number increases at later stages (B,C) in a very irregular manner. Two to three 7-4 neuroblasts first segregate in each hemisegment (B). Occasionally one (thin arrows in B) or four to five cells (thick arrows in B) are also observed at this stage. At later stages (C) five to six 7-4 neuroblasts are formed in each hemisegment. (D-F) *Delta* mutant embryos: neuralisation of the equivalence groups corresponding to 5-2 and 7-4 neuroblasts occurs similarly to that of *Notch* mutant embryos without any maternal contribution.

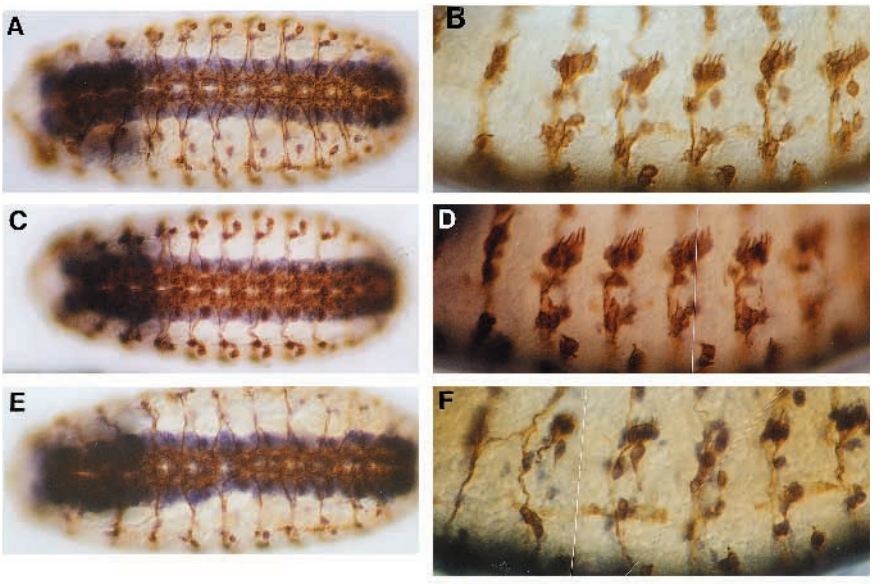

Fig. 3. Differentiation of late CNS and PNS in *Notch* and *Delta* mutant embryos rescued by uniform *Notch* and *Delta* expression. Stage 14 embryos (anterior is left, ventral view in A,C,E, lateral view in B,D,F) double stained with *scratch-lacZ* (β-galactosidase expression revealed by alkaline phosphatase-coupled secondary antibodies, in blue) and also with the 22C10 antibody (peroxidase-coupled secondary antibodies, in brown) which labels the processes of all sensory organs as well as some neurones in the CNS (Zipursky et al., 1984). (A,B) Wild-type embryos showing a normal CNS and PNS axonal scaffold. (C,D) Embryos in which *Notch* expression has been replaced by *GalVP16*/*UASN* are indistinguishable from the wild type. (E,F) Numerous abnormalities are induced in *GalVP16*/*UASDl* rescued embryos. The CNS shows an abnormal axonal scaffold (E), and the PNS contains less cells than the wild type (F).

We also replaced *Dl* expression by a ubiquitous expression of *Serrate*, that encodes another ligand for N but which is not required for neurogenesis. The rescue was similar to *UASDl* at early stages, but the size of the CNS and the number of cells in the PNS were very strongly reduced at later stages (Fig. 4C,H). This result shows that Serrate can functionally substitute for Dl and confirms a previous report (Gu et al., 1995).

We conclude that the transcriptional regulation of *Delta* is dispensable to obtain a pattern of neuroblast segregation close to normal. However, we observed that sometimes more than one neuroblast segregates per cluster in mutant *Delta* embryos rescued by *arm-GalVP16*/*UASDl*. These abnormalities could be either due to insufficient expression of *Delta* locally, or to the fact that this expression is not regulated by the proneural genes.

Addition of a *Delta* minigene complete with E boxes improves rescue obtained from uniform *Delta* expression

Previous studies of the regulatory sequences of *Dl* revealed the presence of binding sites (E boxes) for proteins of the *achaete-scute* complex proteins that are required for function during early neurogenesis (Kunisch et al., 1994). *Ebox-mgDl* is a *Delta* minigene containing a region of the promoter bearing the Ac and Sc binding sites, but lacking the upstream 5′ regulatory region that is required for early embryonic expression (mg30; from Haenlin et al., 1994). This construct does not rescue mutant *Dl* embryos: *scratch-lacZ* and *svp-lacZ* staining reveals a neurogenic phenotype (Fig. 6D). To see whether expression of *Ebox-mgDl* can improve the pattern of neuroblast segregation seen with uniform *Dl* expression, we complemented *arm-GalVP16*/*UASDl* expression with *Ebox-mgDl*. Rescue at stage 9 is significantly improved: 12 extra neuroblasts for 240 extant ones were observed in segments A5 to A8 (Fig. 6C). This rescue is stronger than that obtained with two *UASDl* inserts. Thus *Ebox-mgDl* may allow a small local increase in *Dl* expression through the activity of the proneural proteins, and this transcriptional regulation is sufficient to reduce the number of cases in which more than one neuroblast segregates.

Early neuroblast segregation and requirement of E-box-dependent transcriptional regulation of *Delta*

It was shown earlier that mutation of the Ac-Sc binding sites in a large fragment of the *Dl* promoter is sufficient to abolish the capacity of the corresponding minigene to rescue neurogenesis in *Dl* mutant embryos (Kunisch et al., 1994; mg48, referred to here as *FPΔEbox-mgDl)*. However, the pattern of neuroblast segregation at stage 9 is close to normal in such embryos, whereas at stage 11 a neurogenic phenotype comparable to that of *Dl* mutants is observed (Kunisch et al., 1994). We have re-examined this phenotype with *svp-lacZ*. A single 5-2 neuroblast segregates initially in most hemisegments, although two or three segregate in a few cases, generating abnormalities in the pattern (Fig. 1C). At the time of appearance of neuroblast 7-4, the number of 5-2 neuroblasts increases to reach in most cases two to three cells, although a few hemisegments still bear a single one at this stage (Fig. 1F). Virtually all 7-4 neuroblasts segregate as single cells at early stages, whereas later on most hemisegments bear two of them. As with 5-2 neuroblasts, a few hemisegments still bear a single 7-4 neuroblast at late stages (not shown). At even later stages many other cells of the 5-2 and 7-4 groups become neural.

Thus the pattern of neuroblast segregation with *FPΔEbox-mgDl* is initially close to normal despite some abnormalities and it is only later that extensive neuralisation occurs. Regulation of *Dl* by Ac and Sc therefore seems not to be absolutely required for the initial selection of a single neuroblast, but is necessary to maintain the fates of the inhibited cells.

DISCUSSION

Neural potential is not distributed homogeneously between cells of the neuroblast proneural groups

The equivalence groups from which the embryonic neuroblasts are selected comprise five to six cells. This is the number of cells expressing AS-C genes and also the number of neuroblasts that segregate from each group in *N* and *Dl* mutants. The process of neuralisation in the mutant embryos, however,

suggests that neural potential is not equally distributed between cells of the equivalence group. Two to three neuroblasts segregate initially, followed only later by the other cells of the group, suggesting that a subset of cells have a higher level of competence. Similar observations have been made in the larval PNS (Goriely et al., 1991). There may be a morphological correlate to this subset: a small number of cells in neuroblast equivalence group 4-2 have been seen to move their nucleus basally just before one of them delaminates as a neuroblast (Doe, 1992).

A greater neural potential of some cells may be correlated with higher levels of proneural proteins. Indeed in the case of the macrochaete proneural clusters in the imaginal wing discs, which are considerably larger than those of the embryo, confocal analysis has revealed that a subset of cells (the proneural field) do accumulate higher levels of Ac and Sc, and the sensory organ precursor is always chosen from one of these (Cubas et al., 1991; Cubas and Modolell, 1992). The gene *extramacrochaetae*, that encodes a negative regulator of Ac and Sc, could be partly responsible for such local differences in proneural proteins (Cubas and Modolell, 1992). Another factor that is required for Ac-Sc accumulation at some sites on the notum is the secreted signal Wingless (Phillips and Whittle, 1993). Wingless has also been shown to affect N signalling (Axelrod et al., 1996). Quantitative differences in the levels of Ac and Sc have not been reported so far in the embryonic proneural clusters, but small changes may be hard to detect. If they do exist, probable signals causing local increases in proneural gene expression would include Wingless, a requirement for which has also been demonstrated for the formation and identity of specific neuroblasts (Chu-LaGraff and Doe, 1993).

Overall our results are in favour of the idea that the presumptive neuroblast may be predetermined, or that, at least, it is selected from among a subset

Fig. 4. The first wave of neuroblast segregation is almost normal in the absence of transcriptional regulation of *Notch* and *Delta*. Flat preparations (anterior is left, ventral view) of stage 9 embryos (A-E) showing the SI neuroblast pattern, and late stage 11 embryos (F-J) showing the condensing CNS. *scratch-lacZ* staining (β-galactosidase expression revealed by alkaline phosphatase-coupled secondary antibodies, in blue) labels all the neural cells. Double staining with antibody against the *engrailed* protein (peroxidase-coupled secondary antibodies, in brown) allows the identification of each segment. (A,F) Wild-type embryos. (E,J) *Delta* mutant embryos in which each proneural cluster is neuralized from the very beginning of neurogenesis. (B,G) Replacement of *Delta* zygotic expression by *GalVP16*/*UASDl* expression rescues the early pattern of neuroblast segregation (B), despite some abnormalities. At later stages the development of the CNS is still close to normal, although the number of cells is slightly reduced (G). (C,H) Replacement of *Delta* zygotic expression by *GalVP16*/*UAS-Serrate*. The rescue at early stages is comparable to the one obtained with *UAS Dl* (C; experiment performed at 29°C) whereas a conspicuous reduction of the CNS is observed later (H; normal temperature). (D,I) Rescue of early neurogenesis by the maternal contribution of *Notch* in *Notch* hemizygous embryos. Early neuroblast segregation is nearly normal in these embryos, showing that *Notch* transcriptional regulation is not necessary at this stage (D). Late embryos are strongly neuralized in the absence of zygotic expression (I).

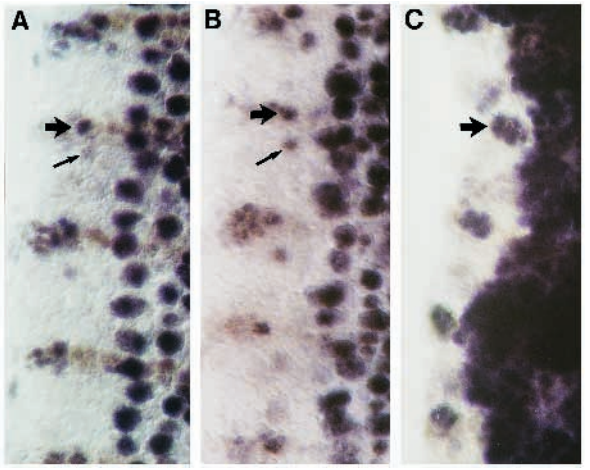

Fig. 5. Segregation of the P precursors of the larval PNS in wild-type (A), mutant *Delta* rescued with *GalVP16/UASDl* (B), and mutant *Delta* (C) embryos, at stage 10-11. Embryos were stained with *scratch-lacZ* and *engrailed* as in Fig. 4. The arrow points to the P precursor of segment A4. The P precursors delaminate normally in *GalVP16/UASDl* rescued embryos (B), whereas they segregate as a group of cells in mutant *Delta* embryos (C). Note that other PNS precursors also segregate individually in *GalVP16/UASDl* rescued embryos (thin arrow). The P precursor is recognizable by virtue of its localisation in the *engrailed* band of expression, and its position ventral to a cluster of ectodermal cells weakly expressing *scratch-lacZ*. The *engrailed* band is not in the focal plane of these pictures.

of two or three cells of the cluster. This would imply that the neuroblast itself, or the two or three early formed 5-2 neuroblasts seen in *N* or *Dl* mutants, arise from cells that always occupy the same position in the cluster. This is difficult to ascertain with present markers. For the notal macrochaetes, it is the excentric positioning of sensory organ precursors within the proneural clusters that has been used as an argument that they may indeed arise consistently from a cell in the same position (Cubas et al., 1991).

Transcriptional regulation of *Notch* and *Delta* is largely dispensable for neuroblast selection and maintenance of cell fates

Microchaete precursors on the notum do not arise at predictable positions (Usui and Kimura, 1993) and their number is somewhat variable from fly to fly suggesting that the precursor cells are not predetermined. It has been postulated that precursors of the microchaetes are chosen in a stochastic manner that relies on *N*-mediated lateral signalling between equivalent cells (Heitzler and Simpson, 1991). Indeed choice of the precursor cell can be influenced by the relative levels of *N* or *Dl* in mosaic animals (Heitzler and Simpson, 1991). Cells with little or no receptor send a very strong signal that is dependent on *Dl* and *ac-sc* activity (Hietzler and Simpson, 1993; Heitzler et al., 1996). Furthermore Ac-Sc proteins have been shown to bind to regulatory sequences in the *Dl* promoter and levels of Dl decrease in *ac-sc* mutants (Kunisch et al., 1994; Ghysen et al., 1993). Taken together these results favour the existence of a regulatory loop between *N* and *Dl* within each cell, that involves the *ac-sc* genes themselves and is under transcriptional control. An initial small, stochastic, quantitative, difference in any component of this regulatory loop between cells, could be amplified such that one cell would finally dominate. Detailed analyses of the expression patterns of *N* and *Dl* during formation of the microchaete precursors have not yet been reported. During formation of the macrochaete precursors, however, no differences in protein levels have been seen, but small differences in RNA levels and in the expression of *lacZ* from a *Dl* enhancer trap line have been detected suggesting that such regulation may occur (Ghysen et al., 1993). The mechanism of selection of microchaete precursors resembles that used for segregation of the AC and the VU cells in *C. elegans*. The choice between these two cells is naturally variable and in this case the existence of a feedback loop between the receptor, LIN-12, and the ligand, LAG-2, is well documented and progressive restriction of ligand and receptor to reciprocal cells has been demonstrated (Seydoux and Greenwald, 1989; Wilkinson et al., 1994).

Here we have tested whether segregation of embryonic neuroblasts occurs in a similar fashion to that of the microchaete precursors. We have replaced endogenous *N* expression by uniform expression and this experiment shows that transcriptional regulation is dispensable both for selection of the neural precursor and maintenance of the epidermal fate. Similar experiments with *Dl* revealed that here, too, selection occurs normally, in that a single neuroblast segregates in most cases and cell fate choice is maintained. Transcriptional regulation of *Dl*, either up- or down-regulation (Muskavitch, 1994), and the feedback loop are thus not absolute requirements for neuroblast segregation in embryos.

Nevertheless it remains possible that in our experiment destined to express *Dl* uniformly with the GAL4-UAS system, the levels of Dl still vary somewhat from cell to cell in a random fashion. This could, for example, result in a higher activation of N in some cells and thus introduce differences between cells. If autoregulation of *N* were to take place then such differences could perhaps be amplified and maintained. Although there is no evidence for autoregulation of *N* in flies, autoregulation of *lin-12* has been demonstrated in *C. elegans* (Wilkinson et al., 1994; Christensen and al., 1996). However, it is unlikely that such an effect would lead to precise segregation of a single cell per cluster. For one thing, such an effect of autoregulation would be cell autonomous. These cells would have no knowledge of the status of N activation in neighbouring cells and they would be unable to influence their neighbours through regulation of *Dl*, since lateral signalling has been interrupted. Such a phenomenon would probably lead to rather chaotic segregation and highly variable numbers of precursors. In contrast to these predictions we observe uniform effects with a single precursor per cluster in 80% of the cases. Furthermore this result is consistent with the observation that the presence of maternal *N* products only, which allows no regulation or autoregulation, is also sufficient to permit the early stages of neuroblast segregation to occur normally.

We thus suggest that choice of the neuroblast is not dependent on the transcriptional feedback loop mechanism which cannot therefore provide the main mechanism of selection. Indeed early neurogenesis takes place normally in *Dl* mutants carrying the *FPΔEbox-mgDl* minigene, in which the binding sites for the AS-C proneural proteins in the promoter

have been mutated, although a partial neuralisation occurs later. No differences in the levels of transcription of *Dl* during selection of the embryonic neuroblasts have been detected (Haenlin et al., 1990).

If the neuroblast precursors are predetermined by an independent mechanism, then one possibility is that *N* signalling merely mediates uniform repression, which would not require transcriptional regulation of *N* and *Dl*. Uniform repression of the neural fate may be sufficient for an almost normal pattern and together with the uneven distribution of neural potential, it would allow only cells with the highest competence to overcome the inhibition and become neural. Such a mechanism resembles events occurring during selection of the different vulval precursor fates in *C. elegans*. Here one cell is predetermined by a signal from the overlying anchor cell and Lin-12 signalling prevents neighbouring cells from adopting the same fate in the absence of transcriptional regulation of ligand and receptor (Wilkinson and Greenwald, 1995). Thus details of the gene regulation occurring during lateral signalling may differ in the neuroectoderm at different stages of development.

It remains possible, of course, that posttranscriptional regulation of Dl and/or N may occur that would allow regulation even after uniform expression. However, a uniform expression of *Serrate* not normally expressed at these stages in wild-type embryos (Thomas et al., 1991), can also rescue *Dl* embryos which makes posttranscriptional regulation less likely. So far no evidence for posttranscriptional regulation has been presented.

Transcriptional regulation of *Delta* by proneural proteins may refine the process of neuroblast segregation

In spite of the almost normal neuroblast segregation observed after uniform expression of *Dl*, two neuroblasts instead of one do segregate in 10-20% of the proneural groups. A minigene bearing sequences that allow regulation by the proneural proteins substantially improves selection and the number of ectopic neuroblasts decreases. Thus transcriptional regulation of *Dl* probably does take place in the wild type and may be important to ensure that a single neuroblast segregates in every case. We propose that neuroblast segregation in the embryo is progressive and involves several steps.

First, neural potential is unequally distributed between cells of the equivalence group. This is most likely to be due to differing levels of proneural gene products (as observed in the macrochaete proneural clusters; Cubas et al., 1991). Notch signalling would then mediate uniform repression and only the cell(s) with the highest levels of proneural proteins would initiate neural development. In most cases this is sufficient to allow a single cell to segregate. Increasing the amount of uniform Dl improves selection, so it is possible that transcriptional activation of Dl by the proneural proteins would also sustain high levels of *Dl* and provide a sufficient level of uniform repression. If a specific cell is predestined to become the precursor, and if this predisposition is determined by a higher level of proneural protein, then regulation of *Dl* by the proneural proteins would also enable this cell to more effectively inhibit the other cells.

Heterogeneous neural potential together with uniform repression is not, however, efficient 100% of the time since sometimes two or three cells take up the neural fate. The transcriptional feedback loop may therefore mediate competition

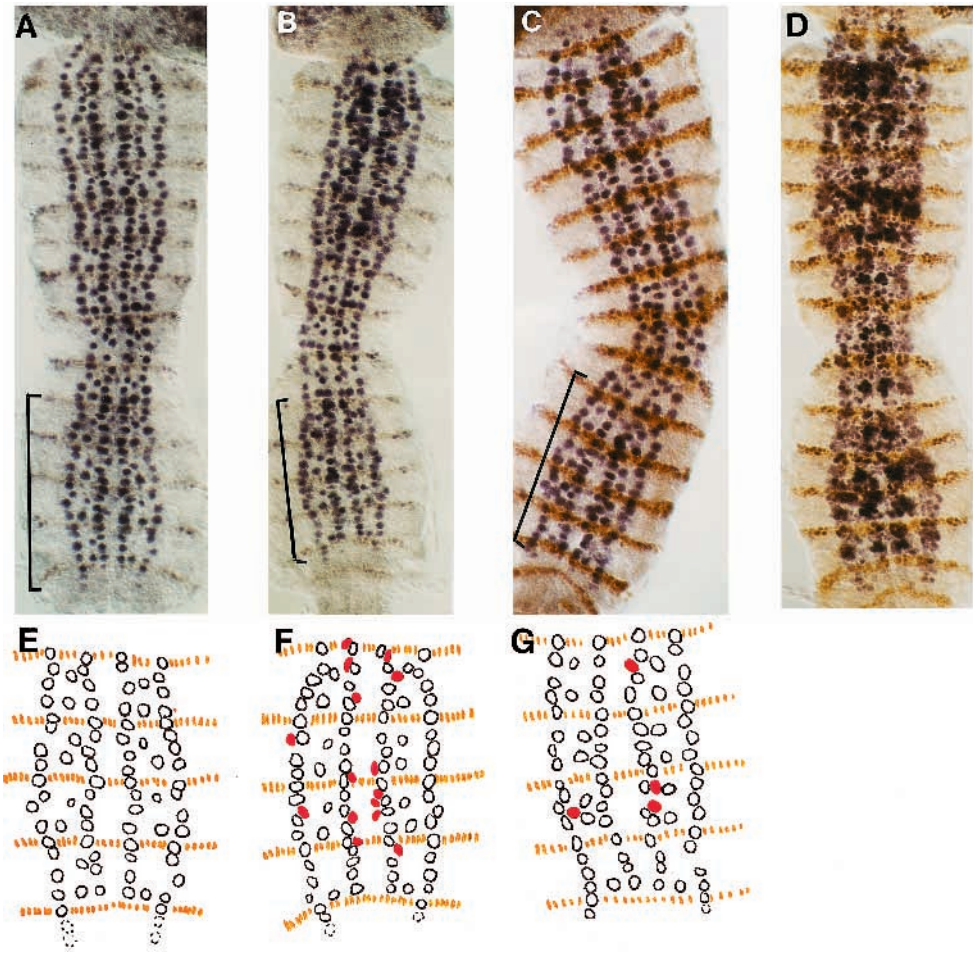

Fig. 6. Comparison between rescue obtained by replacing zygotic *Delta* expression by *GalVP16/UASDl* (B,F) and that obtained with a combination of the non regulated *GalVP16/UASDl* and expression of the *Ebox-mgDL* minigene (C,G). Embryos were stained with *scratch-lacZ* and *engrailed* as in Fig. 4. (A,E) Stage 9 wild type. (D) *Delta* mutant embryo expressing *Ebox-mgDL*: *Ebox-mgDL* expression is not able to rescue mutant *Delta* embryos. (E-G) Drawings of the pattern of neuroblasts in segments A5 to A8 of embryos corresponding to (E) wild type, (F) rescue obtained with *GalVP16/UASDl* alone, and (G) rescue obtained with *GalVP16/UASDl* combined with *Ebox-mgDL*. White circles symbolise the normal neuroblast array, and red ones indicate the supernumerary cells. Yellow lines symbolize *engrailed* expression.

between these cells, until a single one finally dominates. This hypothesis calls for a transcriptional modulation of *Dl* similar to that proposed for segregation of microchaete precursors, but only to mediate competition between the two to three most competent cells of the proneural cluster. Interestingly it has been observed that when two cells in the larval PNS are committed to the neural fate at the same time, only one finally differentiates as a precursor cell (Huang et al., 1991; Doe, 1992). Regulation by proneural genes would thus refine the selection process to ensure segregation of a single precursor in every case.

Regulation of *Dl* by the proneural proteins may also be necessary to maintain for a considerable time the choice of fates. Neuroblast segregation in *N* hemizygous embryos, where the presence of maternal *N* products allow normal segregation but is followed by later neuralisation, shows that choice of cell fate is reversible. Considerable flexibility has also been observed in the grasshopper, where ablation of an embryonic neuroblast is followed by the segregation of a single new one from one of the remaining cells of the proneural cluster (Taghert et al., 1984). This suggests that selection of the neural precursor is followed by a maintenance step and that continuous cell-cell interactions are necessary to strengthen the choice of cell fate. After its selection, the neuroblast may require high levels of Dl to inhibit the other cells over a longer time period. Such high levels may depend on a transcriptional regulation of *Dl* linked to the accumulation of large amounts of proneural proteins in the precursors.

The last possibility that we cannot exclude is that of redundant mechanisms that would allow neuroblast segregation to occur normally 80% of the time in the absence of transcriptional regulation of both *Delta* and *Notch.* Mutation of other genes, such as *bigbrain* (Rao et al., 1990), *kuzbanian* (Rooke et al., 1996), *scabrous* (Baker et al., 1990; Mlodzik et al., 1990), *pecanex* (LaBonne et al., 1989) and *nervig* (Vaessin, personal communication) encoding secreted or transmembrane proteins, also leads to a neurogenic phenotype. Indeed, null mutants of *bigbrain*, display two neural precursors, instead of one at many sites of the larval and adult PNS (Rao et al., 1992) and this gene has been shown to act independantly of the *Notch* pathway (de la Concha et al., 1988).

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