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Successive specification of *Drosophila* neuroblasts NB 6-4 and NB 7-3 depends on interaction of the segment polarity genes *wingless*, *gooseberry* and *naked cuticle*

Nirupama Deshpande, Rainer Dittrich, Gerhard M. Technau and Joachim Urban*

Institut für Genetik, Universität Mainz, Saarstraße 21, D-55122 Mainz, Germany
*Author for correspondence (e-mail: jurban@mail.uni-mainz.de)

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SUMMARY

The *Drosophila* central nervous system derives from neural precursor cells, the neuroblasts (NBs), which are born from the neuroectoderm by the process of delamination. Each NB has a unique identity, which is revealed by the production of a characteristic cell lineage and a specific set of molecular markers it expresses. These NBs delaminate at different but reproducible time points during neurogenesis (S1-S5) and it has been shown for early delaminating NBs (S1/S2) that their identities depend on positional information conferred by segment polarity genes and dorsoventral patterning genes. We have studied mechanisms leading to the fate specification of a set of late delaminating neuroblasts, NB 6-4 and NB 7-3, both of which arise from the *engrailed* (*en*) expression domain, with NB 6-4 delaminating first. In contrast to former reports, we did not find any evidence for a direct role of *hedgehog* in the process of NB 7-3 specification. Instead, we present evidence to show that the interplay of the segmentation genes *naked cuticle* (*nkd*) and *gooseberry* (*gsb*), both of which are targets of *wingless* (*wg*) activity, leads to differential commitment to NB 6-4 and NB 7-3 cell fate. In the absence of either *nkd* or *gsb*, one NB fate is replaced by the other. However, the temporal sequence of delamination is maintained, suggesting that formation and specification of these two NBs are under independent control.

Key words: Neuroblast, *Drosophila*, Naked cuticle, Gooseberry, Engrailed, Wingless, Segment polarity

INTRODUCTION

The specification of unique cell fates as a function of their position within a developing organism is a fundamental process during the development of multicellular organisms. The development of the *Drosophila* central nervous system (CNS) serves as an ideal model system to elucidate mechanisms that link pattern formation to cell-type specification (Skeath, 1999). The fly CNS is derived from a population of neural stem cells, the neuroblasts (NBs), which delaminate from the neurogenic region of the ectoderm into the interior of the embryo. These NBs are formed in a segmentally repeated pattern (Hartenstein and Campos-Ortega, 1985), each having a unique identity (Doe, 1992), which leads to the formation of a specific set of neurons and/or glial cells (Bossing et al., 1996; Schmidt et al., 1997). In the thoracic and abdominal region, delamination of NBs occurs in five waves, S1-S5, and after S5 each hemisegment contains a subepidermal layer of 30 NBs (Doe, 1992).

The early NBs, delaminating during S1 and S2, form an orthogonal array of four rows (2/3,4,5,6/7) and three columns (medial, intermediate and lateral). Work carried out so far suggests that specification of these NBs is based on a combination of positional information along the anteroposterior (A/P) and the dorsoventral (D/V) axes (reviewed by Bhat, 1999; Skeath, 1999). For example, positional cues provided by segment polarity genes like *gooseberry* (*gsb*), *wingless* (*wg*) and *engrailed* (*en*) establish identities of cell rows along the A/P axis of the neuroectoderm, which is a prerequisite for the formation of specific S1 and S2 neuroblasts within each hemisegment (Bhat, 1996; Bhat and Schedl, 1997; Chu-LaGraff and Doe, 1993; McDonald and Doe, 1997; Skeath et al., 1995; Zhang et al., 1994).

However, about half of the NBs delaminate in the later segregation waves (S3-S5) and acquire a different identity, despite the fact that many originate from positions similar to the early NBs. Additionally, the three-column and four-row arrangement pattern is only transitory during early stages of neurogenesis and is obscured by late emerging neuroblasts (Doe and Goodman, 1985; Goodman and Doe, 1993). As a first step to understand how late delaminating NBs are specified, we have concentrated on studying the function and interactions of segment polarity genes within a specific neuroectodermal region: the En expressing domain. This domain gives rise to row 6 and row 7 NBs and is under the influence of the segmentation genes *wg* and *hedgehog* (*hh*). Wingless, which is a secreted protein, is expressed in row 5 and influences the specification of the fate of NBs in row 5 and in the adjacent

rows 4 and 6 of the neuroectoderm (Chu-LaGraff and Doe, 1993). However, the maintenance of En expression in row 7 is also dependent on the Wg signal, so that the question arises as to how row 6 and row 7 NBs become differently specified.

To investigate this, we have chosen the S3 neuroblast NB 6-4 in row 6 and the S5 neuroblast NB 7-3 in row 7 as 'model' NBs. Both NBs are missing in embryos mutant for *en* (Lundell et al., 1996). We show, that in contrast to what was proposed earlier (Matsuzaki and Saigo, 1996) Hh, which is co-expressed in the En domain (Tabata et al., 1992), has no direct role in the formation or specification of any of these NBs. Instead, we provide evidence that Wg is the key player in this process. We show that the activity of the segment polarity gene *naked cuticle* (*nkd*), which is a target of the Wg pathway (Zeng et al., 2000), specifically inhibits the other Wg target gene *gsb* (Li and Noll, 1993; Hooper, 1994) in the posterior En domain, but does not affect *en* expression itself. The combined expression of *gsb* and *en* in the anterior En domain leads to the specification of NB 6-4, while the repression of *gsb* in the posterior En region is necessary for NB 7-3 identity. Furthermore, our analysis reveals that the mechanisms controlling the timing of delamination of these neuroblasts seem to be independent from those controlling their specification.

MATERIALS AND METHODS

Immunohistochemistry

Antibody staining and dissection of embryos were carried out as previously described (Nose et al., 1992; Patel, 1994). The following primary antibodies were used: mouse anti-Invected at 1:3 dilution; rabbit anti-Eagle at 1:1000 dilution; mouse anti-Eagle; rabbit anti-Repo at 1:1000 dilution; rabbit anti-Eyeless at 1:100 dilution; rat anti-Gooseberry-distal at 1:3 dilution; and rabbit anti-β-Galactosidase (Cappel) at 1:1000 dilution.

Fly strains

The following fly strains were used: Oregon R (wild type), hh^{AC}, wg^{CX4}, en^{E}, ptc^{H84}, gsb^{IIX6} (all described by Bhat and Schedl, 1997), sgg^{m1H} FRT101 (a gift from K. Basler), nkd^{2}, *Hs-en* (both strains from Bloomington stock center), *en-Gal4* (a gift from A. Brand), *UAS-nkd* (a gift from M. Scott), gsb^{IIX6};nkd^{2} and *UAS-wg*;hh^{AC} (a gift from B. Sanson).

Heat shock protocols

Embryos from 1 hour egg layings were collected on apple juice agar (2% agar) plates and aged accordingly at 25°C to the required stage. The heat pulse was then given at 37°C for 20 minutes followed by a recovery phase of 15 minutes at 25°C and again a heat pulse of 20 minutes at 37°C. After this the embryos were aged at 25°C to stage 14-15 and then fixed for immunostaining.

Staging and mounting of embryos

Embryos were staged according to standard morphological markers (Hartenstein and Campos-Ortega, 1985). After antibody staining, the embryos were dissected so that the CNS was exposed and mounted in 70% glycerol in phosphate-buffered saline (PBS).

Documentation

The analysis of embryos was carried out on a Zeiss Axioplan microscope mainly using Normaski optics. Embryos labelled with fluorescent dyes were analysed with a Leica TCS confocal microscope. Quantitative analysis such as cell counts were made using 63× or 100× oil objectives. Non-fluorescent images were digitally recorded with a CCD video camera. Combination of different focal planes in Figs 2D-F, 4A-F was carried out using Adobe Photoshop 5.1.

RESULTS

We were interested in clarifying how late delaminating neuroblasts in row 7 become specified differently from those in row 6, as both rows are lying within the En domain. For our investigations, we focused on two late neuroblasts in the En stripe: NB 6-4 in row 6 and NB 7-3 in row 7, which give rise to distinct types of lineages (Bossing et al., 1996; Schmidt et al., 1997). NB 6-4 characteristically generates glia that can be identified by the co-expression of *eagle* (*eg*) and *reversed polarity* (*repo*) (M-CBG and MM-CBG; Ito et al., 1995; Dittrich et al., 1997; Halter et al., 1995), whereas NB 7-3 typically generates serotonergic neurons (Lundell et al., 1996; Dittrich et al., 1997) and can be identified with anti-Eyeless (Ey) or anti-Eg, in combination with anti-En antibodies. As the segmentation genes *wg* and *hh* influence the En domain, we analysed the individual role of these genes in detail, in addition to the role of En.

En is a key factor for NB 7-3 formation and Hh has no independent role in this process apart from En maintenance

The only factor known so far, to distinguish row 6 from row 7 is Gsb, which is expressed in row 6 neuroectoderm. It is also known that Gsb is a target of the Wg signalling cascade and specifies the identities of neuroblasts in rows 5 and 6 (Skeath et al., 1995). However, as row 7 is also under the influence of the Wg signalling, how is Gsb prevented from being expressed here? One mechanism that could be involved in this process is Hh signalling, because previous work by Matsuzaki and Saigo (Matsuzaki and Saigo, 1996) has postulated that NB 6-4 and NB 7-3 show differences in their dependence on Wg and Hh signalling: NB 6-4, which originates from the anterior En stripe, was missing in *wg* as well as in *hh* mutant embryos, whereas NB 7-3, which delaminates around 30 minutes later from the posterior En stripe, always appeared to be present in the absence of Wg or Hh alone, but was no longer found in a *wg;hh* double mutant. Based on these results, it was proposed that Wg and Hh signalling pathways converge or compensate for each other to specify NB 7-3 fate, while both Wg and Hh are equally important for NB 6-4 formation (Matsuzaki and Saigo, 1996). One important consequence of this would be that Hh could have an autocrine function in specifying NB 7-3. However, as both NBs delaminate from the En-positive neuroectodermal domain and En activity represses *ptc* (*patched*; Hidalgo and Ingham, 1990), the only known receptor binding to Hh directly (Marigo et al., 1996), such an autocrine function of Hh would at least need a different receptor. We therefore investigated in more detail the role Hh plays in the formation and/or specification of NB 6-4 and NB 7-3. We chose null mutant alleles of *wg* and *hh* for our investigations, whereas previously (Matsuzaki et. al., 1996) a hypomorphic allele of the *hh* gene was used. We found that the formation of NB 7-3 is affected in both *wg* and *hh* single mutants. NB 7-3 is missing in 75% (n=88) of wg^{CX4} and in 40% (n=202) of hh^{AC}

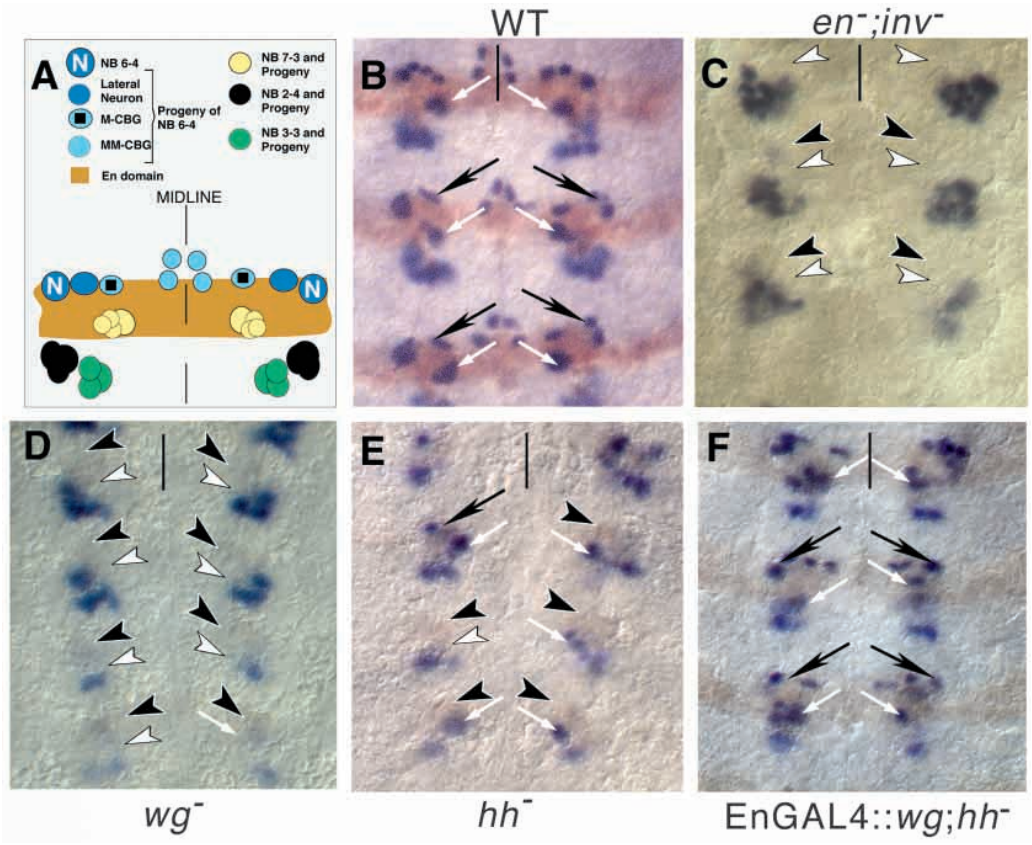

Fig. 1. Loss of NB 7-3 in *hh* mutant embryos can be rescued by ectopic *wg* expression. Flat preparation of embryos at stage 12, anterior is upwards. Eg expression is seen in blue and En expression in brown with the first three segments from the top being thoracic. The black bar represents the midline. In B-F, arrows denote the presence of an NB and arrowheads denote the absence of the NB in a given hemisegment. (A) Wild type Eg/En expression pattern: two thoracic hemisegments on either side of the midline are shown. Eg antibody stains four NBs and its progeny belonging to NBs 6-4 (shown in shades of blue) and 7-3 (yellow cells) in the En domain, and NBs 2-4 (black cells) and 3-3 (green cells) outside the En domain. NB 6-4 in the thoracic segments characteristically produces three glial cells (two MM-CBG glia that migrate towards the midline and one M-CBG glia) and a cluster of laterally located neurons. (B) Wild-type embryos show Eg expression in two cell clusters in the En domain: NB 6-4 and its progeny are anteriorly located (black arrows); NB 7-3 and its progeny are posteriorly located (white arrows). (C) en^{E} embryos, which are double mutant for *en* and *inv*, show no Eg expression at the position of NB 6-4 (black arrowheads) and NB 7-3 (white arrowheads). In all (n=52) of the hemisegments counted, En expression is completely abolished. (D) wg^{CX4} mutant embryos look similar to en^{E} mutant embryos: Eg expression is absent at the position of NB 6-4 in 100% (n=54) (black arrowheads) and, for NB 7-3, in 75% (n=88) (white arrowheads) of hemisegments counted. (E) hh^{AC} mutant embryos show Eg expression to be missing in 40% (n=60) at NB 6-4 position (black arrowheads) and in 40% (n=202) for NB 7-3 position (white arrowheads) of the hemisegments counted. Occasionally, residual En expression can be seen around the Eg-positive cells clusters. (F) Expression of *UAS-wg* by *en-Gal4* in hh^{AC} mutant embryos rescues the formation of NB 6-4 to 98% (n=109) (black arrows) and NB 7-3 to 95% (n=66) (white arrows) of the hemisegments counted. En expression is rescued.

mutant hemisegments counted (Fig. 1D,E). These results also show that the effect on NB 7-3 is more severe in wg^{CX4} than in hh^{AC} mutant embryos. As En expression is fading away earlier in wg^{CX4} (~stage 8) than in hh^{AC} mutant embryos (~stage10) (Bejsovec and Wieschaus, 1993), we assume that the number of remaining NB 7-3 correlates with the degree of the residual En expression. Indeed, embryos that are deficient for *en* and *inv* (*invected*, a homeobox gene that shows some functional redundancy to *en*) show that NB 7-3 is missing in 100% (n=50) of the hemisegments counted (Fig. 1C). These results suggest that NB 7-3 formation needs Hh indirectly for the maintenance of En expression via Wg. We confirmed this by analysing hh^{AC} mutant embryos in which Wg was ectopically expressed within the En domain using EnGal4 as a driver of *UAS-wg*. In these embryos, the dependency of Wg expression on Hh is uncoupled and therefore En expression was rescued (Sanson et al., 1999). In accordance with our hypothesis, these embryos show a very efficient rescue of NB 7-3 to 95% (n=66) of the hemisegments counted (Fig. 1F). Thus, under these conditions NB 7-3 does not need any additional input by the Hh signalling pathway to be formed and specified. We conclude that NB 7-3 normally requires Hh only for maintenance of Wg expression, which in turn leads to En maintenance.

Naked Cuticle activity is essential for NB 7-3 identity

Having shown that Hh is not involved in the differential regulation of Gsb we decided to look at inhibitors of the Wg signalling cascade: *naked cuticle* (*nkd*) and *shaggy* (*sgg*). Analysis with anti-Ey antibody showed that NB 7-3 specific Ey expression is missing in 83% of *sgg* mutant (n=92, data not shown) and in 81% of *nkd* mutant (n=80) hemisegments of embryos at stage 12-13 (Fig. 2B), although Eg-positive cells were always observed at the position of the NB 7-3 cluster using anti-Eg antibody (both NBs 6-4 and 7-3 are Eg positive). This suggests that the fate of NB 7-3 is mispecified (see below). By contrast, NB 6-4 was found to be always present in these mutants, as judged by anti-Eg and anti-Repo antibody staining.

We selected *nkd* mutants for further analysis, as Nkd (like Gsb) is a target of the Wg signalling cascade and is thought to establish a negative feedback loop by downregulating the Wg signal (Zeng et al., 2000). As a first step, we tested whether Gsb was derepressed in *nkd* mutations in regions from where NB 7-3 normally delaminates. Indeed, we found that while in

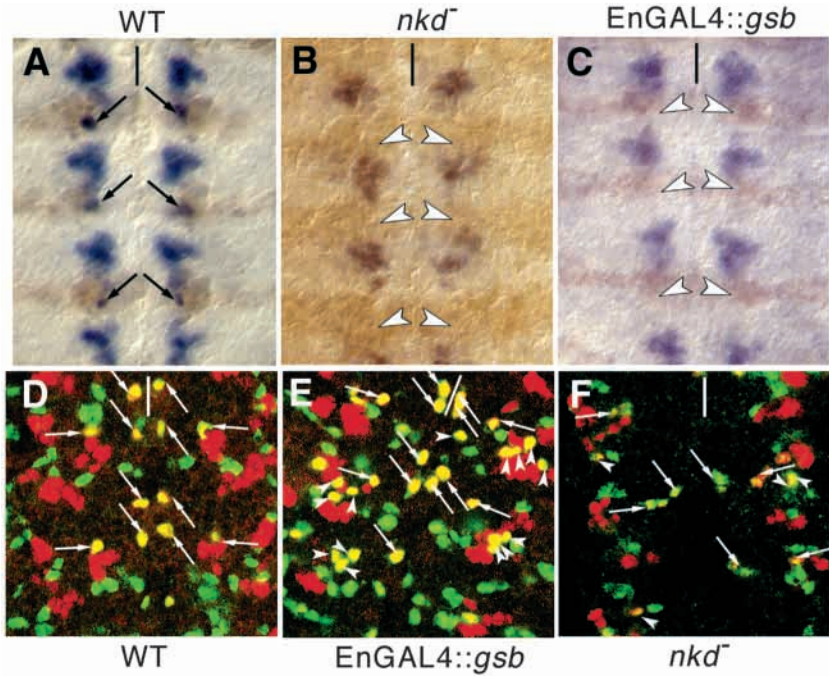

Fig. 2. NB 7-3 is transformed to NB 6-4 fate in embryos mutant for *nkd* and in embryos with ectopic Gsb expression in the whole En domain. Flat preparation of embryos with anterior upwards. (A-C) Ey expression in blue and En expression in brown at late stage 12; the first three segments from the top are thoracic. The black bar represent the midline. (D-F) Combined sections of confocal images of fluorescence antibody staining against Eg in red and Repo in green at stage 13. Double-labelled cells are seen in yellow with the first two segments from the top being thoracic. The white bar represents the midline. (A) In the En domain of wild-type embryos, Ey expression is seen only in the position of NB 7-3 (black arrows). Ey is expressed additionally in five NBs and their progeny outside the En domain. (B) In *nkd* mutant embryos, Ey expression is absent at the position of NB 7-3 in 81% (*n*=80) of the hemisegments counted (white arrowheads). (C) In embryos with ectopic Gsb expression in the En stripe, Ey expression is absent at the position of NB 7-3 in 84% (*n*=86) of the hemisegments counted (white arrowheads). (D) Wild-type embryos showing cells double labelled for Eg and Repo, which are unique to the glial cells produced by NB 6-4 (white arrows). In each thoracic hemisegment, two of these cells are seen along the midline (MM-CBG) and one more laterally (M-CBG). (E) In embryos with ectopic Gsb expression in the En domain, cells co-expressing Repo and Eg are seen in addition to the three genuine NB 6-4 progeny (white arrows), which are present in the ventral focal planes. At the position of NB 7-3, such cells are seen in the dorsal focal plane in 52% (*n*=40) (white arrowheads) of the hemisegments counted, suggesting a transformation of NB 7-3 to NB 6-4 fate. (F) In *nkd* mutant embryos phenotype similar to that in E is seen. At the position of NB 7-3, cells co-expressing Repo and Eg are seen in 54% (*n*=40) at stage 12 (white arrowheads) of the hemisegments counted, suggesting a transformation of NB 7-3 to NB 6-4 fate. For the purpose of clarity, not all sections of the confocal images are combined here.

the En domain of wild-type embryos, only row 6 NBs and NB 7-1 expressed Gsb (Skeath et al., 1995), in *nkd* mutants, the Gsb-expressing neuroectodermal region was broadened. As a result the more lateral row 7 NBs also expressed Gsb, which must include NB 7-3 (Fig. 3). Because, in this situation, row 7 is similar to row 6, it could have the ability to give rise to an additional ectopic NB 6-4. Staining of *nkd* mutant embryos with the glia specific anti-Repo antibody in combination with anti-Eg antibody indeed revealed an additional NB 6-4-like fate in 54% (*n*=40) of hemineuromeres counted (Fig. 2F). Co-expression of these markers is characteristic for NB 6-4 derived cells. To ensure that this is not due to secondary effects of the *nkd* mutation, we ectopically expressed Gsb in the En domain using the *UAS*/*Gal4* system, which yielded the same result as *nkd* mutations: a replacement of NB 7-3 by an ectopic NB 6-4 in 52% (*n*=40) of the hemisegments (Fig. 2E).

That Gsb expression acts a switch between row 6 and row 7 identity in the En-positive neuroectoderm is additionally supported by earlier work analysing the role of Gsb in the CNS (Matsuzaki and Saigo, 1996; Patel et al., 1989), where it was shown that in hemineuromeres of *gsb* mutant embryos an additional NB 7-3 fate was formed. Taking this result further, we confirmed that this additional NB 7-3 fate is at the cost of NB 6-4, which is converse to the situation in *nkd* mutants. Analysis of *gsb* mutant embryos with anti-Ey antibody showed that a duplicated NB 7-3 was formed in 70% (*n*=82) of the hemisegments counted (Fig. 4D) and NB 6-4 markers were missing in 100% of hemisegments counted (data not shown). Additionally, in the absence of both Nkd and Gsb, ectopic NB 7-3 was found in 76% of hemisegments (*n*=86) (Fig. 4E). Similarly, 40% of the hemisegments (*n*=80) showed duplicated NB 7-3 fate when Nkd was ectopically expressed in the En domain using the *UAS*/*Gal4* system (Fig. 4F). Thus, we

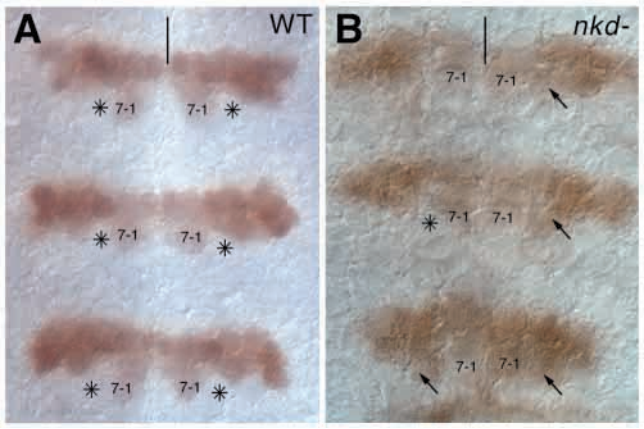

Fig. 3. Gsb expression is expanded posteriorly in embryos mutant for *nkd*. Flat preparation of embryos (early stage 12), Gsb expression as revealed by anti-Gsbd serum (brown); anterior is upwards. The first three segments from the top are thoracic. The black bar represents the midline. (A) Wild-type embryos show Gsb expression in all NBs belonging to rows 5 and 6. Only one NB, NB 7-1, belonging to row 7 is Gsb positive. The region of NB 7-3 is Gsb negative (black asterisks). (B) In *nkd* mutant embryos, Gsb expression is derepressed and now expressed in additional NBs belonging to row 7, which must include NB 7-3 (black arrows).

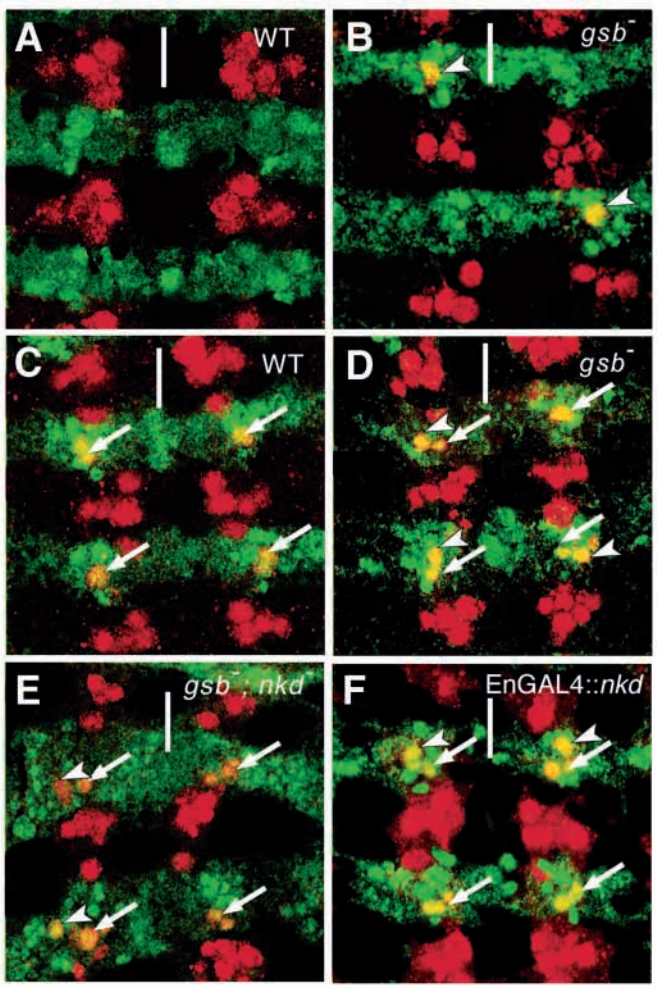

Fig. 4. Loss of Gsb function as well as ectopic Nkd expression in the En-domain results in an additional NB 7-3-like fate. Confocal images of embryos between stage 10-13 with anterior upwards. Ey expression seen in red, En in green and double staining in yellow. The first three segments from the top are thoracic. The white bar represents the midline. (A) Wild-type embryo at stage 10 shows no Ey expression in the En domain. (B) *gsb* mutant embryos at stage 10 show Ey expression in 65% (*n*=60) of the hemisegments counted (white arrowheads). (C) Wild-type embryo at late stage 11 shows Ey expression in the En domain at the position of NB 7-3 (white arrows) and its progeny in 100% (*n*=50) of the hemisegments counted. (D) *gsb* mutant embryos at late stage 11 show Ey expression at the position of NB 7-3 (white arrows) and its progeny. An additional Ey-positive cell cluster in a different focal plane is seen at the position of NB 6-4 (white arrowheads) in 70% (*n*=82) of hemisegments counted. (E) *gsb;nkd* double mutant embryos show a phenotype similar to *gsb* mutant embryos. Ey expression is found at the position of NB 6-4 (white arrowheads) in 76% (*n*=86) of the hemisegments counted. (F) *UAS-nkd* driven by *en-Gal4* results in Ey expression at the position of NB 6-4 (white arrowheads) in 40% (*n*=80) of the hemisegments counted.

conclude that NB 7-3 formation, as opposed to formation of NB 6-4, requires the absence of Gsb, which is inhibited by Nkd function. Taken together the above results suggest that row 6 and 7 neuroectoderm can potentially produce NBs with the same identities, and that the differential effects of Wg

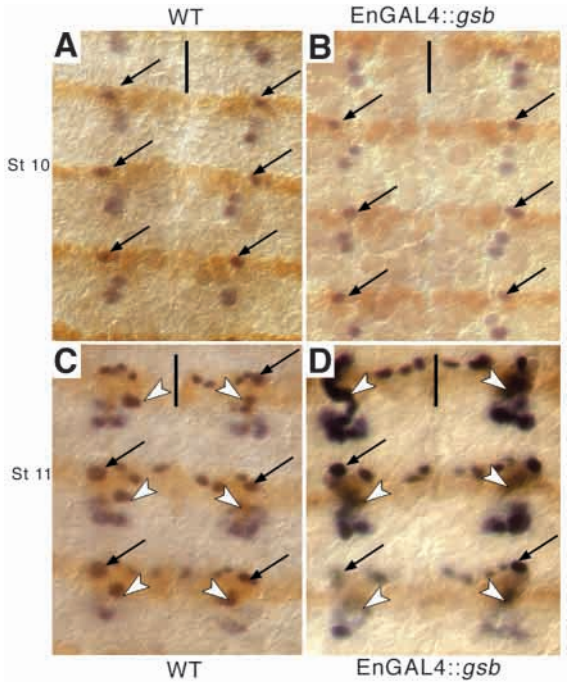

Fig. 5. Duplicated NB 6-4 in embryos with ectopic Gsb expression is born at the time of NB 7-3. Flat preparation of embryos at stages 10 (A,B) and 11 (C,D) stained against Eg in blue and En in brown, anterior is upwards. The first three segments from the top are thoracic. The black bar represents the midline. (A) Wild-type embryos at stage 10 show Eg expression in the En domain only at the position of NB 6-4 (black arrows) and not in the position of NB 7-3, as it is not yet delaminated. (B) Eg expression in embryos with ectopic Gsb expression in the En domain at stage 10 is indistinguishable from that in wild-type embryos. (C) Wild-type embryos at stage 11 show Eg expression in the En domain at the position of NB 6-4 (black arrows) and NB 7-3 (white arrowheads). (D) Eg expression of embryos with ectopic Gsb expression in En domain at stage 11 is again similar to that of wild-type embryos, although the cells at NB 7-3 position (white arrowheads) now express characteristic markers of NB 6-4 progeny (see Fig. 2E).

signalling are responsible for bringing about the different fates of the two late NBs from this region.

NB 6-4 and NB 7-3 specification is independent of time of NB formation

The above results show that in *nkd* mutants, an extra NB 6-4 is formed in the position of NB 7-3. As NB 6-4 normally delaminates earlier than NB 7-3, the question arises as to when the duplicated NB 6-4 delaminates. In wild-type embryos, NB 6-4 delaminates during S3 (stage 10), followed by NB 7-3 in S5 (stage 11) from the En domain (Broadus et al., 1995; Doe, 1992). Therefore, embryos either mutant for *nkd* or expressing ectopic *gsb* in the En domain (EnGal4::*gsb*) of stage 10 and stage 11 were examined with anti-Eg antibody to look for the timing of NB duplication. In wild-type embryos at stage 10, Eg is detected only at the position of NB 6-4 and never at the position of NB 7-3 (Fig. 5A). At stage 11, Eg-positive cells are visible in the En domain at the position of NB 7-3, as well as NB 6-4. (Fig. 5C). Surprisingly, in EnGAL4::*gsb* embryos this temporal sequence is maintained: Eg is first detected at the position of NB 6-4 (Fig. 5B) and later at the position of NB 7-

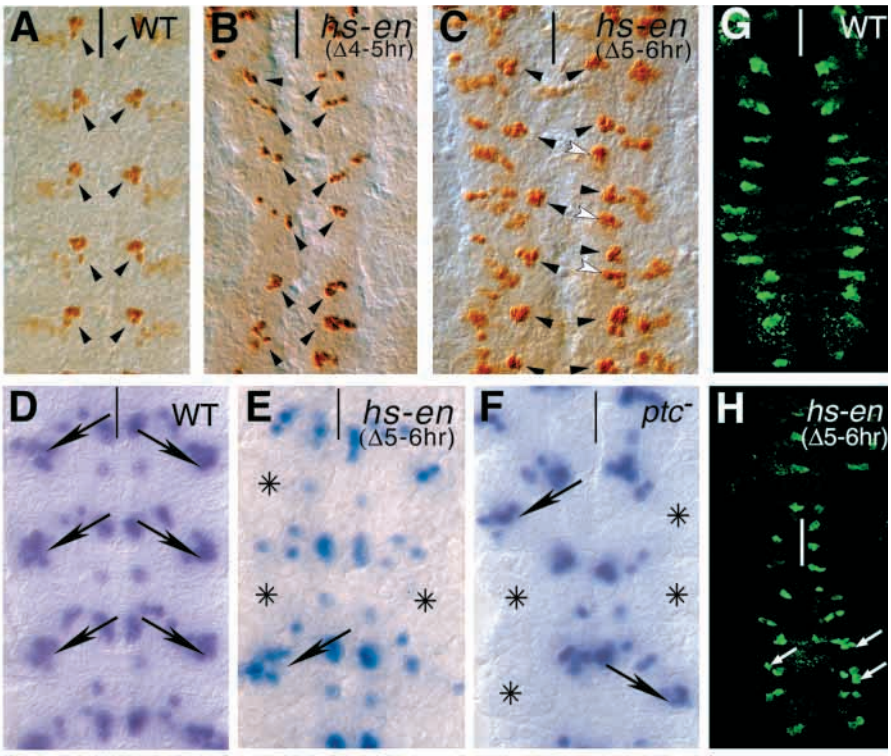

Fig. 6. Ectopic En expression results in duplication of NB 7-3 fate. Flat preparation of embryos at stage 15 with the midline represented by a black bar (A-F) and an isolated CNS of a first instar larva (G,H) with the midline represented by a white bar; anterior is upwards. (A-C) Eg expression in brown; (D-F) Eve expression in blue; (G-H) serotonin expression in green. (A) Wild-type embryos showing strong Eg expression in the position of NB 7-3 (black arrowheads). (B) *Hs-en* embryos subjected to a heat pulse between 4 and 5 hours after egg laying: the CNS is malformed but NB 7-3 and its progeny (black arrowheads) can still be identified. (C) *Hs-en* embryos subjected to heat pulse between 5 and 6 hours after egg laying: ectopic NB 7-3 like cluster (white arrowheads) can be found just below the wild-type NB 7-3 (black arrowheads) in 20% (*n*=100) of the hemisegments counted. (D) Wild-type embryos showing Eve expression in the position of EL cells (black arrows), which are the progeny of NB 3-3. (E) *Hs-en* embryos subjected to heat pulse between 5 and 6 hours after egg laying: Eve expression is often missing in the position of EL cells (black stars). (F) *ptc* mutant embryos shows Eve expression missing in the position of EL cells in many hemineuromeres (black stars). (G) The CNS of a first instar wild-type larva. Two serotonergic neurons are seen per hemisegment. (H) The CNS of a first instar *Hs-en* larva. Heat shock was applied as in C. Ectopic serotonin expression was found in several hemisegments (white arrows).

3 (Fig. 5D). Therefore, the ectopic NB 6-4 is delaminating at S5, at the time NB 7-3 would normally appear. Conversely, in *gsb* mutants, an extra NB 7-3 is formed at the cost of NB 6-4. This NB 7-3 is detected by anti-Ey antibody staining in embryos mutant for *gsb* at stage 10 (Fig. 4B), whereas in wild-type embryos, no Ey-positive cell is present in the En domain at this stage (Fig. 4A). Thus, this ectopic NB 7-3 delaminates at the time of NB 6-4. We conclude that, with respect to NB 6-4 and NB 7-3, the timing of NB formation appears largely independent of NB specification, and that the segmentation genes *nkd* and *gsb* are essential to bring about the specification of the two NB fates investigated.

Late ectopic En expression induces an ectopic NB 7-3 fate in row 3 neuroectoderm

As the above results suggest that the prerequisite for NB 7-3 fate specification is En in absence of Gsb expression, we examined embryos with ectopic En expression in order to see whether we could induce ectopic NB 7-3 cells outside of the normal En domain. A heat pulse given to *Hs-en* embryos just before the delamination of NB 7-3 (i.e. 5-6 hours after egg laying at 25°C) results in an ectopic NB 7-3 formation in 20% (*n*=100) of the hemisegments, based on anti-Eg antibody staining (Fig. 6C). This ectopic NB 7-3 is also able to give rise to characteristic progeny cells. We detected additional serotonergic neurons in 20% (*n*=68) of the hemineuromeres counted in first instar larvae (Fig. 6H). The ectopic NB 7-3 seems to be formed at the cost of the Eg-positive NB 3-3 of row 3, as we find a loss of the Eve-positive EL cells, which are progeny of NB 3-3 (Fig. 6E; Schmidt et al., 1997). No additional NB 7-3 cluster is seen when the heat pulse is given between 4 and 5 hours after egg laying at 25°C (Fig. 6B), although the CNS is very malformed. Additionally, we analysed *ptc* mutant embryos, as a derepression of En in an ectopic stripe is seen in such mutants (DiNardo et al., 1988). Ptc, which is a receptor for Hh, is expressed in rows 2-5 of the neuroectoderm (Bhat, 1996). Ptc activity represses En expression (Hidalgo and Ingham, 1990), which results in a mutually exclusive gene expression pattern with respect to these two genes. We found that a NB 7-3 like fate was formed ectopically in 50% of *ptc* mutant hemineuromeres (*n*=60, see also Patel et al., 1989), which is similar to the ectopic expression experiments using *Hs-en*. Again this seems to be at the cost of NB 3-3, as 60% of the EL cells are missing in these mutant embryos as well (Fig. 6F). This is in accordance with the observation that the ectopic En stripe in *ptc* mutant embryos is in the region where the row 3 NBs delaminate (data not shown). Taken together these results suggest that in wild type, Ptc represses En expression in row 3 neuroectoderm, thereby enabling the specification of late delaminating row 3 NBs. In the absence of Ptc function, at least some of these neuroectodermal cells acquire row 7 identity, owing to the presence of En and the absence of Gsb.

DISCUSSION

The impact of segmentation genes and D/V patterning genes has been well studied for the early segregating neuroblasts (S1 and S2). The expression of these genes defines three dorsoventral columns and four rows in anteroposterior direction, thus creating a Cartesian coordinate system that

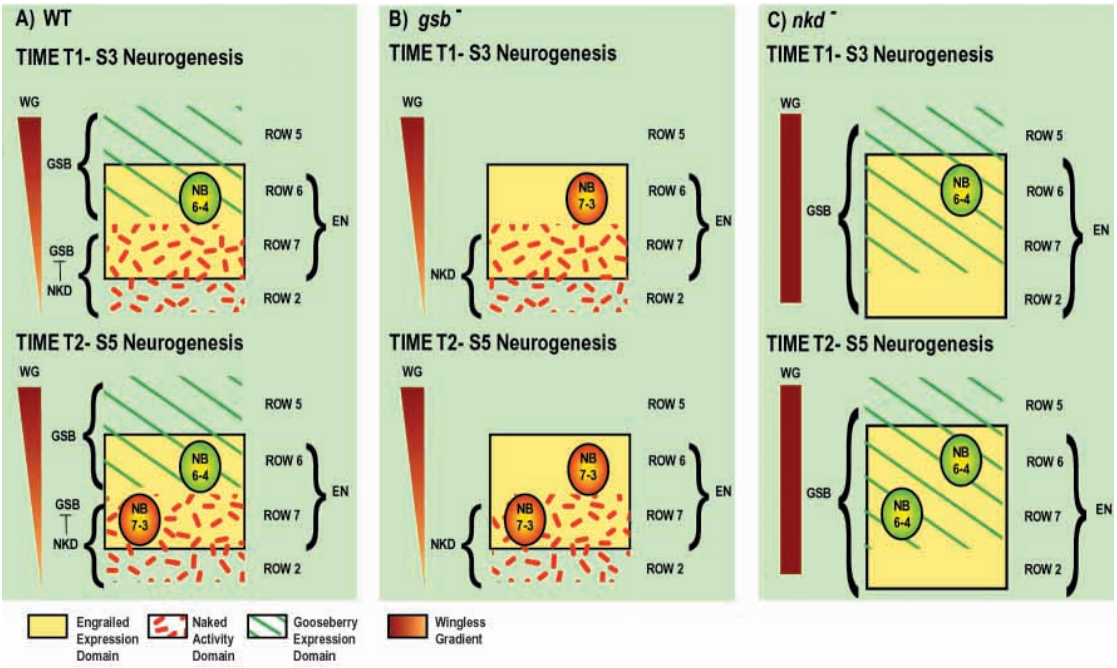

Fig. 7. A model for the mechanism leading to the formation of NB 6-4 and NB 7-3 identities. (A) The top figure shows the situation in wild type where at time T1 (during S3 NB delamination) in row 6, which is the overlapping domain of GSB (green hatched lines) and EN (yellow) expression, the delaminating NB takes a NB 6-4 identity. The bottom figure shows that at time T2 (during S5 NB delamination) NKD activity (red) inhibits GSB expression in row 7, and the delaminating NB in this region has a NB 7-3 identity. (B) In absence of GSB expression in *gsb* mutant embryos at time T1 (top figure) in row 6, an NB with an identity of NB 7-3 delaminates at the position of NB 6-4. At time T2 (bottom figure) in row 7, the normal NB 7-3 delaminates. (C) In absence of NKD activity in *nkd* mutants, the GSB and EN expression is broadened. At time T1 (top figure) in row 6, the normal NB 6-4 delaminates. At time T2 (bottom figure) in row 7, a NB with an identity of NB 6-4 delaminates at the position of NB 7-3.

assigns unique fates to individual NBs as a function of their position in the neuroectoderm (Bhat, 1999; Skeath, 1999). This pattern is partially obscured by the late segregating NBs and the progeny of early NBs (Doe and Goodman, 1985; Goodman and Doe, 1993). As a consequence, late delaminating NBs might face a very different situation, considering that substantial morphogenetic movements take place. Therefore we asked how segmentation genes interact to confer unique identities on late delaminating NBs and whether NB formation and specification is tightly linked in this case. We selected a pair of late segregating NBs in the En domain, namely the S3 neuroblast NB 6-4 in row 6 and the S5 neuroblast NB 7-3 in row 7 for our analysis.

Segment polarity genes separate NB 6-4 and NB 7-3 fates

In the En domain Wg plays a role both in NB formation and NB specification (Chu-LaGraff and Doe, 1993). The homeodomain transcription factor En is a prerequisite for the formation of the NBs 6-4 and 7-3, because in its absence both NBs fail to form (Lundell et al., 1996; Matsuzaki and Saigo, 1996). As Wg signalling is necessary for maintaining En expression (Hidalgo and Ingham, 1990), it is also essential for the formation of these two NBs. In addition Hh is co-expressed in the En domain, but we found no evidence for a direct function of Hh with respect to the formation and specification of these NBs, as opposed to a previous report (Matzuzaki and Saigo, 1996). En maintains Hh expression in rows 6 and 7, and Hh in turn is essential for Wg expression in row 5, thereby constituting a maintenance loop (Bejsovec and Wieschaus, 1993; Heemskerk et al., 1991; Hidalgo, 1991). Thus, for late NBs in row 6 and 7, the expression of En is crucial and Hh is required to maintain En expression via Wg. However, for the separate specification of NB 6-4 and NB 7-3, differential regulation of two Wg targets, *nkd* and *gsb*, is essential (Fig. 7).

Wg is a diffusible molecule expressed in row 5 and acts on neighbouring rows, which include rows 6 and 7 (Chu-LaGraff and Doe, 1993). However, row 6 differs from row 7 as it expresses *gsb*, which is, as stated above, a target of Wg signalling (Fig. 7A). The fact that row 7 does not express *gsb*, despite being under the influence of Wg raises the question of how this differential regulation is brought about. In this work we have shown that Nkd is essential for this regulation. Recently, Nkd has been identified as a negative regulator of the Wg signal transduction pathway, itself being a target of this pathway (Zeng et al., 2000). We have found that in the absence of Nkd, Gsb is derepressed, owing to Wg hyperactivity in row 7, leading to the generation of an ectopic NB 6-4 like fate (Fig. 7C). Thus, the distinct identities of NB 6-4 and NB 7-3 are brought about by the interplay of Gsb and Nkd. For NB 6-4 specification, Gsb is an essential factor. In the absence of Gsb NB 6-4 fails to be specified (Matsuzaki and Saigo, 1996; Skeath et al., 1995) and instead takes the identity of NB 7-3 fate (Fig. 7B). Conversely, for NB 7-3 specification, a Gsb-free environment, which is created by the activity of Nkd, is essential. In summary, NB 6-4 needs the expression of Gsb and En, whereas NB 7-3 needs En but the absence of Gsb.

However, the fact that *gsb* as well as *nkd* are targets of Wg

signalling makes it difficult to explain why *gsb* is repressed by *nkd* only in the posterior region of the En stripe. The posterior En domain is further away from the Wg source than the anterior En domain and therefore should receive a lower signalling input when compared with the anterior region. As a consequence, this should lead to higher Nkd activity in the anterior En cells, leading to a stronger Gsb repression in this region – the opposite of what we observe. A careful analysis of the expression pattern on the transcriptional level did not give any obvious clues to solve this apparent paradox (data not shown). We confirmed that during early germ band extension (stage 8-9) *nkd* transcription is nearly ubiquitous with higher RNA levels in the two to four cell rows posterior to the En stripe (Zeng et al., 2000). At late phase of germ band extension, *nkd* expression is most abundant anterior to the En stripe and lower just posterior to the En-stripe (stage 10-11; Zeng et al., 2000). No significant difference between the anterior and posterior En domain could be detected (data not shown). One explanation for the differential regulation of *gsb* could be that, owing to earlier pair rule gene activity of *paired* (Bouchard et al., 2000), the level of Gsb protein at the time of NB 6-4 delamination in the anterior En region is high enough to override repression by Nkd activity. Alternatively, a direct differential regulation of the two Wg targets that is due to the different levels of Wg signalling could be responsible for the observed regulatory differences. It could be that the regulation is such that the amount of Wg signalling within the En stripe causes a relatively homogenous level of *nkd* expression in this region. At the same time, the transcriptional activation of *gsb* could be more sensitive to Wg signalling levels, resulting in a very strong activation, especially near to the Wg-expressing cells. As a result, the relatively low Nkd activity in the whole En stripe might be able to inhibit *gsb* expression in the region of low *gsb* activation only: the posterior En domain. A hint that a differential regulation of Wg targets indeed exists comes from the Wg-dependent En regulation: it seems that a lower Nkd activity is sufficient to repress *gsb* but not to inhibit *en* expression. This conclusion was drawn from our finding that overexpression of *nkd* within the En stripe using an EnGal4 driver line led to a selective repression of *gsb* with no obvious effect on *en* expression itself. Clearly, additional work has to be carried out to clarify these points.

Row 3 has the potential to generate a late row 7 neuroblast

Besides row 6 neuroectoderm, row 3 neuroectoderm also has the potential to generate an ectopic NB 7-3. It has been shown previously that in embryos mutant for *ptc*, neuroectodermal cells in the area of row 3 begin to express En and additional serotonergic neurons can be found in these mutant embryos, which suggests the presence of an ectopic NB 7-3 like fate (Patel et al., 1989). We now show, additionally, that when En is ubiquitously expressed, only row 3 has the ability to give rise to an ectopic NB 7-3 fate. In all cases, this occurs at the cost of row 3 NBs such as NB 3-3. We think that this might reflect that row 3 neuroectoderm, which is right in the middle of the segment, represents something like a 'ground state' in the neuroectoderm: in this area neither Hh nor Wg signalling may take place. Therefore the decision to specify late row 3 or late row 7 NBs seems to be only dependent on the absence or presence of En, respectively.

Temporal aspects of NB specification

Previous work has indicated that genes expressed in proneural clusters are involved in specifying the individual fates of NBs that develop from these clusters (Chu-LaGraff and Doe, 1993; Matsuzaki and Saigo, 1996; Skeath et al., 1995). Our finding that NB 6-4 and NB 7-3 can be mutually transformed while the sequence of birth does not change suggests that the mechanism for the timing of late NB delamination is independent from mechanisms that regulate NB identity. This might be reminiscent of early NBs. Initiation of S1 NB formation requires the activity of proneural genes that have been shown to be dependent on pair-rule genes (Skeath et al., 1992). The identity of the NBs delaminating from these clusters, however, is dictated by the activity of segment polarity genes (Chu-LaGraff and Doe, 1993; Skeath et al., 1995). Thus, the control of proneural gene expression that enables NB formation and the control of segmentation genes conferring NB identity occurs in parallel. At later stages, pair-rule gene expression vanishes and can no longer be responsible for NB formation (Skeath et al., 1992). How is NB formation regulated in the following segregation waves? One possibility is that after the first segregation wave, NB formation and identity are more tightly linked; the finding that specific NBs like NB 4-2 are sometimes not transformed but missing in *wg* mutant embryos (Chu-LaGraff and Doe, 1993) seems to support this idea. However, our finding that the transformed NB 6-4 and NB 7-3 are delaminating according to the 'old identity' shows that, at least in these cases, NB formation and specification is independent. Our results favour the idea that the timing of the formation of proneural clusters within the neuroectoderm is generally independent of the segment polarity genes investigated here. This does not exclude permissive functions, such as those of En, which enable the proneural cluster formation as such. According to this hypothesis, intrinsic or extrinsic factors present in the position of the proneural cluster at the time of delamination govern the identities of the NBs. This might be not only true for the positional regulation of NB identity but also for the determination of NB identity along the temporal axis. Indeed, heterochronic transplantation experiments recently performed in our laboratory (Berger et al., 2001; in the same issue) strongly support the possibility that one or more extrinsic factors exist that lead to stage specific NB identities. It will be a challenge for the future to identify these factors, and to investigate whether similar mechanisms exist in higher organisms.

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