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hedgehog signaling independent of *engrailed* and *wingless* required for post-S1 neuroblast formation in *Drosophila* CNS

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SUMMARY

The *hedgehog* gene product, secreted from *engrailed*-expressing neuroectoderm, is required for the formation of post-S1 neuroblasts in rows 2, 5 and 6. The *hedgehog* protein functions not only as a paracrine but also as an autocrine factor and its transient action on the neuroectoderm 1-2 hours (at 18°C) prior to neuroblast delamination is necessary and sufficient to form normal neuroblasts. In contrast to epidermal development, *hedgehog* expression required for neuroblast formation is regulated by neither *engrailed* nor *wingless*. *hedgehog* and *wingless* bestow composite positional cues on the neuroectodermal regions for S2-S4 neuroblasts at virtually the same time and, consequently, post-S1 neuroblasts in different rows can acquire different positional values along the anterior-posterior axis. The average number of proneural cells for each of three *eagle*-positive S4-S5 neuroblasts was found to be 5-9, the same for S1 NBs. As with *wingless* (Chu-LaGraff et al., *Neuron* 15, 1041-1051, 1995), *huckebein* expression in putative proneural regions for certain post-S1 neuroblasts is under the control of *hedgehog*. *hedgehog* and *wingless* are involved in separate, parallel pathways and loss of either is compensated for by the other in NB 7-3 formation. NBs 6-4 and 7-3, arising from the *engrailed* domain, were also found to be specified by the differential expression of two homeobox genes, *gooseberry-distal* and *engrailed.*

Key words: *Drosophila*, neuroblast formation, CNS, *hedgehog*, *wingless*, *gooseberry*, *engrailed*, *eagle*

INTRODUCTION

In *Drosophila*, the central nervous system (CNS) develops through neural precursor or neuroblast (NB) formation, with delamination occurring at five waves, S1-S5 (Doe, 1992). At S5, the final stage of NB formation, each hemisegment contains a subepidermal layer consisting of 30 NBs arranged in a stereotyped spatial pattern. Each NB generates several ganglion mother cells (GMCs), which divide once to produce postmitotic neurons and/or glia.

Examination of cell ablation in *grasshopper* and in vitro culture in *Drosophila* (Doe and Goodman, 1985; Huff et al., 1989; Lüer and Technau, 1992) suggested that the unique properties of neurons and glia are due to intrinsic identity rather than circumstantial factors. The identity of neurons is determined by parental GMCs whose fates are controlled by parental NBs. NB identity appears dependent on the location of the neuroectodermal region from which a given NB is derived.

Initially, neuroectoderm is an equipotential two-dimensional sheet on which discrete proneural clusters are formed (reviewed in Doe and Goodman, 1993). Proneural genes including the *achaete-scute* complex (AS-C) are expressed in stereotyped positions and bestow general potential for following neural fate on the cells expressing them (Skeath et al., 1992, 1994). Within each cluster, only one cell is selected as an NB by lateral specification, while the remaining follow epidermal fate (for review, Campos-Ortega, 1993). Embryos lacking neurogenic gene expression show neural hyperplasia whereas proneural gene mutations cause neural hypoplasia (Lehmann et al., 1983; Jimenez and Campos-Ortega, 1990).

Pair-rule and segment polarity genes may quite likely provide the neuroectoderm with positional cues along the anterior/posterior axis. Pair-rule genes may regulate the expression of proneural genes for S1 NB development, since *achaete* (*ac*) and *scute* expression is controlled positively and negatively by *fushitarazu* (*ftz*) and *odd-skipped* (Skeath et al., 1992). Patel et al. (1989a) observed severe CNS defects associated with segment polarity gene mutations. For example, *patched* (*ptc*) mutants exhibit occasional lack or duplication of a class of NBs. *gooseberry-distal* (*gsb-d*) is required for S1, row-5 NB specification (Zhang et al., 1994; Skeath et al., 1995). Chu-LaGraff and Doe (1993) showed *wingless* (*wg*) to be essential for the formation and/or specification of NBs adjacent to *wg*-expressing domains. *wg* function required for NB development appears distinct from that for epidermal development.

Here, we show that *hedgehog* (*hh*), a segment polarity gene coding for a secretory protein (Hh; Mohler and Vani, 1992; Lee et al., 1992; Tabata et al., 1992; Tashiro et al., 1993), is essential for formation of most post-S1 NBs derived from *engrailed* (*en*)-expressing neuroectodermal regions and their

immediate neighbors. A transient action of paracrine and/or autocrine Hh on the neuroectoderm prior to NB delamination is necessary and sufficient to form normal NBs. In NB formation, Wg and Hh appear involved in separate, parallel pathways and, in particular, in NB 7-3 formation, endogenous Wg and Hh may mutually compensate for the loss of each other. The combined activity of *gsb-d* and *en* is also shown necessary for the specifications of NBs 6-4 and 7-3.

MATERIAL AND METHODS

Fly strains

Unless noted otherwise, hh^{IIO} (a strong allele; Mohler, 1988) was used as the *hh* mutant, while a temperature-sensitive *hh* allele, hh^{9K} (Mohler, 1988), was used for temperature-shift experiments. hh^{13C} (a presumed null allele; Mohler, 1988) was also used for some experiments. Other mutants examined were wg^{IL} (Chu-LaGraff and Doe, 1993), $Df(2R)gsb^{IIX}$ (a deletion of *gsb-d* and *gooseberry-proximal* (*gsb-p*)), en^{E} (a deletion of *en* and *invected*; Tabata et al., 1995) and $Df(1)N^{8}$ (a deletion of *Notch* (*N*)). As molecular markers, enhancer trap lines 5953 (*huckebein-lacZ* (*hkb-lacZ*); Doe, 1992), H162 (*seven-up-lacZ* (*svp-lacZ*); Mlodzik et al., 1990) and 17*en*40 (*wg-lacZ*; Kassis et at., 1992) were used, while K42 is a line having a *kinesin-lacZ* gene whose expression is regulated by the *eagle* (*eg*) enhancer (Higashijima et al., 1996). hh^{IIO} was introduced into the third chromosome of K42 or 5953 by recombination. Embryos homozygous for hh^{IIO} were identified using a balancer, TM3 *ftz-lacZ*. Embryos homozygous for wg^{IL} hh^{IIO} were identified based on the fact that *wg hh* embryos have no *en* expression in either the ectoderm or midline cells at S5 (Bejsovec and Wieschaus, 1993).

Immunohistochemistry

Immunohistochemisty was carried out as described by Higashijima et al. (1996). Unless otherwise noted, staged embryos raised at 27°C were collected. Developmental stages and stages of NB formation were according to Campos-Ortega and Hartenstein (1985), and Doe (1992). NB identity was based on the expression of molecular markers, cell position and morphology (Broadus et al., 1995). Primary antibodies used were: mouse anti-En (Patel et al., 1989b), mAb16F12 (anti-Gsb-d; Zhang et al., 1994), mouse anti-Ac (Skeath and Carroll, 1992), rat anti-RK2 (Repo; Campbell et al., 1994), mAbBP102 (Klämbt et al., 1991), rabbit polyclonal anti-β-gal (*Escherichia coli* β-galactosidase; Cappel), and mouse monoclonal anti-β-gal (Promega). To detect Eagle, rabbit polyclonal anti-Eagle antiserum, LU1, was used (Matsuzaki et al., unpublished data). Secondary antibodies used are: AP-conjugated anti-rabbit (Cappel) and anti-mouse (Promega), and biotin-conjugated anti-rabbit, anti-rat and anti-mouse (Vector) antibodies. ABC-HRP kit (Vector) and DAB/$NiCl_2$ were used for signal amplification. Double labeling was carried out by a combination of AP and ABC-HRP reactions, or ABC-HRP reactions with DAB/$NiCl_2$ and those without $NiCl_2$. The substrate for AP was NBT/BCIP and that for HRP was DAB.

Temperature-shift experiments

Embryos produced by crossing of hh^{IIO} K42/TM3 *ftz-lacZ* and hh^{9K}/TM3 *ftz-lacZ* flies were used for temperature shift-up and shift-down experiments along with transient *hh* activation and inactivation experiments. One hour after egg laying (AEL) at 18°C or 30 minutes AEL at 29°C, eggs were collected onto a wet mesh, which was then put in a Petri dish. Several Petri dishes were incubated simultaneously by floating in a water bath with an appropriate temperature. By transferring dishes to another water bath with different temperature, both incubation time and temperature could be easily regulated. At 14-16 hours AEL at 18°C or 7-8 hours AEL at 29°C, embryos were fixed and stained with anti-β-gal antibodies. Embryos lacking *lacZ* expression regulated by the *ftz*-promoter were collected and expression of *eg-kinesin-lacZ* was examined. Cuticlar patterns at 18°C and 29°C, respectively, were examined at 49 and 24.5 hours AEL.

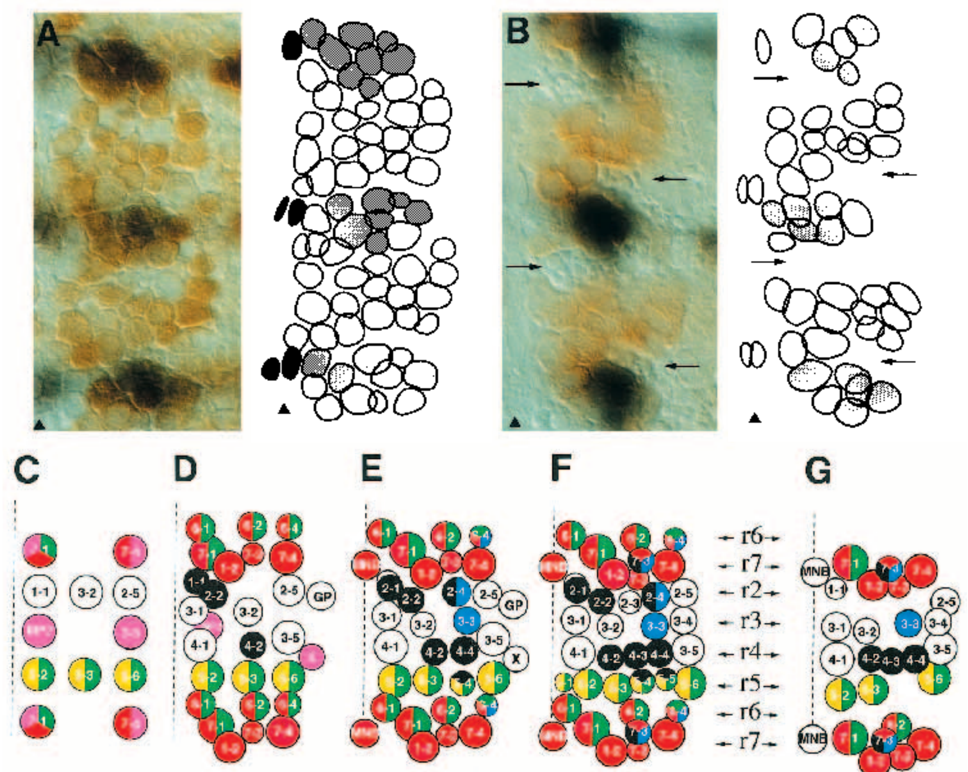

Fig. 1. NB patterns in wild type and the *hh* mutant. Top, anterior; triangles and dashed lines, midlines. (A,B) Brown and blue, respectively, show *svp-lacZ* and *en* expression. About 30 NBs are included in a wild hemisegment (A), while an *hh* mutant hemisegment (B) contains about 20 NBs and two large non-NB regions labeled with arrows. Right margin, Camera lucida. Stippled, NBs derived from *en/hh*-positive neuroectoderm; black, *en*-positive median neuroblasts (MNBs). (C-G) NB maps: (C-F) wild type; (C) S1; (D) S3; (E) S4; (F) S5; (G) *hh* mutant at S5. Wild-type maps are based on Broadus et al. (1995), while the *hh* mutant map, our data. Red, *en*; green, *gsb-d*; yellow, *wg-lacZ*; pink, *ac*; black, *hkb-lacZ*; blue, *eg*. Arrows, non-NB gaps.

RESULTS

Requirement of *hh* for post-S1 NB formation in rows 2, 5 and 6

The ventral ganglia consist of three types of segments, two of which, thoracic and abdominal, have been shown to have similar NB patterns (Doe, 1992; Higashijima et al., 1996). Attention in this study was directed to abdominal segments. At S5 (late stage 11), the final stage of NB development, the NB layer of each hemisegment consists of 6 rows (rows 2-7), each containing 3-6 NBs (Fig. 1F). For simplicity *en*-negative NB 1-1 and *en*-positive NB 1-2 were assumed to belong to rows 2 and 7, respectively. NBs in rows 6 and 7 are derivatives of neuroectodermal cells expressing *hh* and *en* (Doe, 1992).

Examination was first made of final NB patterns at S5, using *svp-lacZ* as a marker. Virtually all NBs (about 30) could be identified in the wild-type hemisegment by *svp-lacZ*-staining (Fig. 1A; Doe, 1992). Fig. 1B shows the *hh* mutant hemisegment to consist of about 20 NBs and two large gaps (non-NB regions) in rows 2, 5 and 6. Superimposition of wild-type and mutant patterns suggest that about 2/3 of NBs arising normally in rows 2, 5 and 6 are absent from the *hh* mutant hemisegment

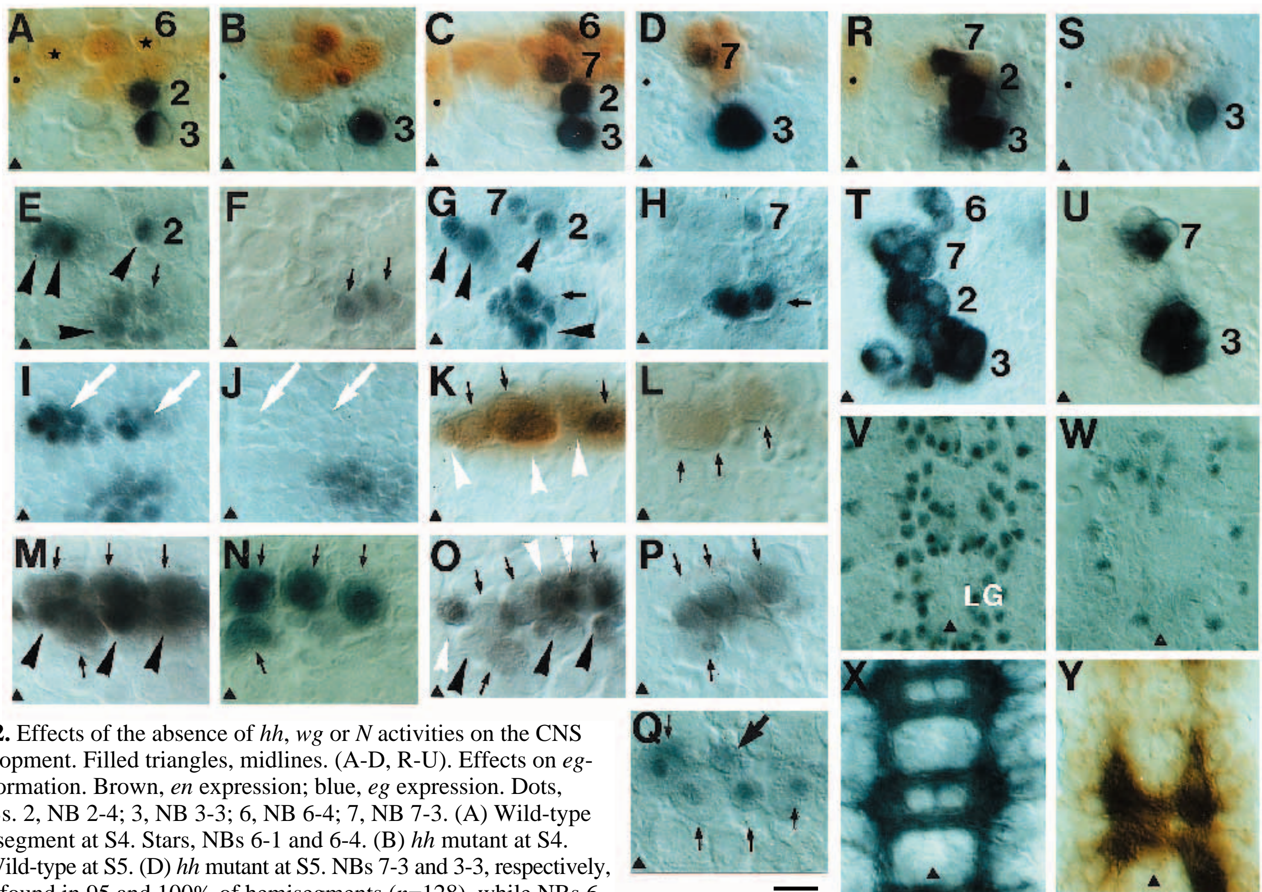

Fig. 2. Effects of the absence of *hh*, *wg* or *N* activities on the CNS development. Filled triangles, midlines. (A-D, R-U). Effects on *eg*-NB formation. Brown, *en* expression; blue, *eg* expression. Dots, MNBs. 2, NB 2-4; 3, NB 3-3; 6, NB 6-4; 7, NB 7-3. (A) Wild-type hemisegment at S4. Stars, NBs 6-1 and 6-4. (B) *hh* mutant at S4. (C) Wild-type at S5. (D) *hh* mutant at S5. NBs 7-3 and 3-3, respectively, were found in 95 and 100% of hemisegments (n=128), while NBs 6-4 and 2-4, 12 and 2%. (E-J) *hkb-lacZ* expression at S4 (E, F, I, J) and S5 (G, H). Small arrows, *hkb-lacZ*-positive NBs in row 4; arrowheads, *hkb-lacZ*-positive NBs in rows 2 and 5; 2, NB 2-4; 7, NB 7-3. In wild-type at S4 (E), *hkb-lacZ* is expressed in NBs 2-1, 2-2, 2-4, 4-2, 4-4 and 5-4. In the *hh* mutant (F), only two row-4 NBs are *hkb-lacZ*-positive. In S5 wild type (G), *hkb-lacZ* is expressed in nine NBs (2-1, 2-2, 2-4, 4-2, 4-3, 4-4, 5-4, 5-5 and 7-3 (see Fig. 1F)). *hh* mutant hemisegments (H) contain only NB 7-3 and three row-4 NBs as *hkb-lacZ*-positive NBs. (I, J) Expression of *hkb-lacZ* in the neuroectoderm at S4. In wild type (I), *hkb-lacZ* is expressed in putative proneural regions for row-2 NBs (2-1/2-2, and 2-4; labeled with white arrows), two row-4 NBs and NB 5-4. In the *hh* mutant (J), neuroectodermal regions for row-2 and row-5 NBs fail to express *hkb-lacZ*. (K, L) *wg-lacZ* expression at S5. Wild-type hemisegment (K) contained 6 *wg-lacZ*-positive NBs (5-2, 5-3, 5-6 (arrows), 5-1, 5-4 and 5-5 (white arrowheads). Only arrowed NBs were *wg-lacZ*-positive in the *hh* mutant (L). (M-P) *gsb-d* expression patterns. In wild type at S3 (M), *gsb-d* is expressed in 7 NBs (5-2, 5-3, 5-6, 7-1 (arrows), 6-1, 6-2 and 6-4 (arrowheads); see Fig. 1D). In wild type at S5 (O), three NBs (5-1, 5-4, and 5-5 (white arrowheads)) newly arise and, as a total, ten NBs express *gsb-d*. In S3 (N) and S5 (P) *hh* mutants, *gsb-d* expression occurs only in four arrowed NBs (5-2, 5-3, 5-6 and 7-1). (Q) *en* expression in the *hh* mutant at S4. As shown by thin arrows, *en* is expressed in four NBs (7-1, 7-2, 7-4 and 1-2), while, in this particular picture, NB 6-2, labeled with the thick arrow, is also *en*-positive. In wild-type embryos at S4, *en* is also expressed in two row-6 NBs (6-1 and 6-4; see stars in A). In hh^{IIO} embryos, 38% (n=56) and 51% (n=51) hemisegments, respectively, contained NBs 6-1 and 6-2. In hh^{13C} embryos, 40, 44 and 5% (n=62) hemisegments, respectively, contained NBs 6-1, 6-2 and 6-4. (R) *wg* hemisegment at S5. *eg* is expressed in NBs 7-3 (90%, n=90), 2-4 (100%) and 3-3 (100%). No NB 6-4 was detected by *eg* staining (0%). (S) S5 hemisegment homozygous for *hh* and *wg*. Almost all *wg hh* mutant hemisegments contained only one *eg*-NB (NB 3-3). *eg* expression at various NB positions (n=80): NB 6-4, 0%; NB 7-3, 1%; NB 2-4, 5%; NB 3-3, 100%. (T) *N* hemisegment at S5. Four *eg*-positive clusters were found. Except for the anterior-most, each cluster consisted of 5-9 cells. (U) S5 hemisegment homozygous for *N* and *hh*. Only two *eg*-positive clusters can be seen. (V,W) *repo* expression at stage 16 in wild type and *hh* mutant, respectively. (X,Y) mAbBP102 staining of wild type and the *hh* mutant embryos at stage 16, respectively. LG, longitudinal glia. Bar, 10 µm for A-U; 9.5 µm for V-Y.

without extensive dislocation of the remaining NBs. Since 1/3 of NBs in rows 2, 5 and 6 belong to S1 NBs (see Table 1) and all S1 NBs appear to arise normally in the *hh* mutant (see below), virtually all and only post-S1 NBs in rows 2, 5 and 6 would be probably abolished in the *hh* mutant.

Individual NBs abolished in the *hh* mutant may be identified using various molecular markers of NBs (Doe and Goodman, 1993). Four NBs (*eg*-NBs), 6-4, 7-3, 2-4 and 3-3, can be specifically marked by *eg-kinesin-lacZ* (Fig. 2C; Higashijima et al., 1996). *eg*-NBs are lined up along the anterior/posterior axis and this facilitates clarification of the *hh* effect on NB formation or specification in different rows. In contrast to the wild type (Fig. 2A,C), no *eg*-NB corresponding in position to NB 2-4 was recognized in the *hh* hemisegment at S4 and S5 (early and late stage 11; Fig. 2B,D). Similarly, no *eg* expression was found at 90% of NB 6-4 positions in the *hh* hemisegments (Fig. 2C,D). These findings appear consistent with the notion that the *hh* mutant cannot form NBs 2-4 and 6-4, post-S1 NBs in rows 2 and 6 (see Table 1).

hkb-lacZ is a marker specifically expressed at S4 in three row-2 NBs (2-1, 2-2, 2-4), two row-4 NBs (4-2 and 4-4), and a row-5 NB, 5-4, (Figs 1E, 2E; Doe, 1992; Chu-LaGraff et al., 1995). At S5, three additional *hkb-lacZ*-positive NBs, 4-3, 5-5 and 7-3, newly delaminate (Figs 1F, 2G). In the *hh* mutant, no *hkb-lacZ* expression was detected at positions corresponding to those of row-2 and row-5 post-S1 NBs positive to *hkb-lacZ* in the wild type (Fig. 2F,H), while no loss of *hkb-lacZ*-positive NBs in rows 4 and 7 was detected.

In an *hh* background, the expression of *gsb-d*, *wg-lacZ* and *en* in the NB layer was found to have changed. *gsb-d* expression at S3 suggested that post-S1 NBs 6-1, 6-2 and 6-4 are absent from about 60, 50, 90% of hh^{IIO} hemisegments, respectively (Fig. 2M,N). Similarly, a comparison of Fig. 2P with Fig. 2O suggests the failure of the formation of three row-5 NBs 5-1, 5-4 and 5-5 arising at S4 or S5. The absence of these row-5 NBs was also demonstrated by *wg-lacZ* expression at S5 (Fig. 2K,L). In contrast, *en* expression at S4 shows S1-S2 NBs in row 7 (7-1, 7-2, 7-4 and 1-2) to form normally in the *hh* mutant (Fig. 2Q).

hh is also required for the development of GP, an S3 glial precursor in row 2 (see Fig. 1D), which divides symmetrically to produce longitudinal glia (LG) and may not be included at S5 in the NB layer (Fig. 1F; Jacobs et al., 1989). *reversed polarity* (*repo*) is normally observed in all glial cells (Fig. 2V; Campbell et al., 1994). Thus, altered *repo* expression at stage 16 may be an indication of the absence of LG in the *hh* mutant (Fig. 2W). The frequent disruption of longitudinal connectives in the *hh* mutant may be due in part to the loss of LG (Fig. 2X,Y).

It should be noted here that, as summarized in Table 1, all post-S1 NBs in rows 2, 5 and 6 require *hh* for formation. In contrast, all S1 NBs formed independently of *hh*, and exhibited normal gene expression patterns at S1: putative NBs 5-2, 5-3, 5-6 and 7-1 expressed *gsb-d*, putative NBs 7-1 and 7-4 expressed *en*, and *ac* was expressed in putative NBs 7-1, 7-4 and 3-5, and MP2 (data not shown; see Fig. 1C).

Transient Hh action on the neuroectoderm necessary and sufficient to form *hh*-dependent NBs

To determine the critical period sensitive to *hh* activity (CPSH) in NB development, temperature shift-up and shift-down experiments were carried out using hh^{9K}, which produces a temperature-sensitive Hh (Mohler, 1988; Porter et al., 1995). Marking with *eg-kinesin-lacZ* makes it possible to follow the development of two *hh*-dependent NBs, 6-4 and 2-4, which are S3 and S4 NBs, respectively. At permissive temperature (18°C), hh^{9K}/hh^{IIO} embryos exhibited *eg*-NB patterns similar to those of the wild type (Fig. 3C). At a non-permissive temperature (29°C), NB 2-4 could not be detected in 96% of hh^{9K}/hh^{IIO} hemisegments (n=72), while mg glia, a putative progeny of NB 6-4 (Higashijima et al., 1996), was present in 37%, suggesting the leakiness of hh^{9K}/hh^{IIO} in NB 6-4 formation (Fig. 3F).

Incubation temperature was changed at various times and the numbers of mg glia or NB 2-4 were scored at stages 12 or 13 (Fig. 3A,B). Shift-up experiments (see filled circles) suggested that the Hh activity after 8.5 hours AEL at 18°C (equivalent to 4 hours AEL at 29°C) is dispensable for the formation of NBs 6-4 (Fig. 3A) and 2-4 (Fig. 3B). Shift-down experiments (see open circles) showed that Hh produced during 0-3 hours AEL at 29°C (0-6.5 hours at 18°C) is not essential for the formation of NBs 6-4 and 2-4. It may thus follow that CPSHs for the formation of NBs 6-4 and 2-4 are virtually identical to each other and range from 6.5 to 8.5 hours AEL at 18°C or 3 to 4 hours AEL at 29°C. This was further confirmed by transient Hh inactivation and activation experiments (lower margins of Figs 3A,B,D,E). NB 2-4 and mg glia were found in 80% hemisegments of embryos producing active Hh only during CPSH, while the absence of active Hh from CPSH brought about a considerable reduction of the fraction of hemisegments with NB 2-4 or mg. Thus, it is concluded that Hh produced during 6.5-8.5 hours AEL at 18°C (or 3-4 hours at 29°C) is necessary and virtually sufficient to form NBs 6-4 and 2-4 normally. Since S3 and S4 NBs, respectively, begin to delaminate at 9.5 and 10.5 hours AEL at 18°C (see upper margins of Fig. 3A,B), it would appear that Hh activity is required 1-2 hours (at 18°C) prior to the delamination of NBs 6-4 and 2-4, and hence target cells for Hh are not NBs but neuroectodermal precursors.

hh^{9K}/hh^{IIO} embryos in which Hh was inactivated only during CPSH for NB formation exhibited a cuticular pattern similar to that of the wild type (compare Fig. 3H with Fig. 3G). The

Table 1. *hh*-dependent NBs situated in rows 2, 5, 6 and 7 at S5

	Row 5	Row 6	Row 7	Row 2
S1	NB 5-2 NB 5-3 NB 5-6		NB 7-1 NB 7-4	NB 2-5 NB 1-1
S2		NB 6-2	NB 7-2 NB 1-2	NB 2-2
S3		NB 6-1 NB 6-4		(GP)
S4	NB 5-4			NB 2-1 NB 2-4
S5	NB 5-1 NB 5-5		NB 7-3	NB 2-3

NB delamination stages (S1-S5) and NB positions at S5 are shown. Solid underlines indicate NBs whose formation is *hh*-dependent, while a broken underline, NB 7-3 requiring *hh* for formation only in the absence of *wg*. GP, a glical precursor in row 2, is absent from the S5 NB layer.

cuticular pattern of embryos having active Hh only during the CPSH was indistinguishable from that of *hh* mutant embryos (Fig. 3I,J). Thus, Hh secreted during the CPSH for NBs 2-4 and 6-4 formation is not required for normal cuticular formation, previously shown to require the *hh* activity from 2.5 to 7 hours AEL at 25°C (equivalent to 5-14 hours AEL at 18°C; Mohler, 1988). Deduced CPSH for NBs 6-4 and 2-4 is presumed to overlap the earliest but least required part of the period in which *hh* activity must be available for epidermal development.

Size estimation of proneural regions of *eg*-NBs and alteration of *hkb-lacZ* expression in putative proneural regions in the *hh* mutant

To further clarify Hh functions in NB formation, the size and locations of proneural regions for post-S1 NBs should be determined. However, for most post-S1 NBs, no proneural genes have been identified to date. Thus, we first estimated the numbers of putative proneural cells for three *eg*-NBs.

All cells in a given proneural region are considered equipotential and hence should become NBs with identical gene expression in the absence of *N* (Struhl et al., 1993). Since, in three of four *eg*-NBs (NBs 2-4, 3-3 and 7-3), *eg* RNA expression is initiated during NB delamination (Higashijima et al., 1996), nearly all *N*-mutant NBs derived from proneural regions for these three *eg*-NBs should express *eg* RNA and, thus, it should be possible to identify them by *eg-kinesin-lacZ* expression. In NB 6-4, *eg* RNA is expressed only at the last stage of NB development (Higashijima et al., 1996). As shown in Fig. 2T, approximately 20 *eg*-positive NBs, making up four aggregates, were found at S5 in the hemisegment of *N* embryos produced by *N*/+ parents. In *hh N* double mutants, two *eg*-NB clusters, putative derivatives of *hh*-dependent NBs 6-4 and 2-4 proneural regions, disappeared (Fig. 2U). This made it possible to determine the average number of proneural cells for each of the three *eg*-NBs (2-4, 7-3 and 3-3) as 5-9, the same for S1 NBs (5-7; Skeath and Carroll, 1992).

In the wild-type background, *hkb-lacZ* is expressed not only in a particular set of NBs but also in their presumptive proneural regions in the neuroectoderm (Doe, 1992). Consistent with this notion, 6-8 ectodermal cells were *hkb-lacZ*-positive in the presumptive proneural region for NB 2-4 (Fig. 2I). Study was thus made to determine whether *hh* has any effect on *hkb-lacZ* expression in the neuroectoderm. At S4, three row-2 NBs, two row-4 NBs, one row-5 NB and their putative proneural regions were *hkb-lacZ*-positive (Fig. 2E, I). At least in the presumptive proneural region for NB 2-4, *hkb* RNA expression is initiated at stage 9 just prior to NB delamination (Doe, 1992; Chu-LaGraff et al., 1995), thus suggesting that, in the NB 2-4 proneural region, CPSH occurs considerably prior to the *hkb* RNA expression period. Fig. 2F, J shows that, in the *hh* mutant, *hkb-lacZ* expression is completely abolished in neuroectodermal cells and NBs in rows 2 and 5 throughout development, indicating Hh to be requisite for inducing *hkb* expression in all presumptive proneural cells for *hh*-dependent NBs.

Regulation of the formation and/or specifications of *hh*-dependent *eg*-NBs by *wg*, *en* and *gsb-d*

Chu-LaGraff and Doe (1993) showed that, in *wg* mutant embryos, post-S1 NBs in rows 4 and 6 cannot form normally. We confirmed this in the *eg*-NB system (Fig. 2R). In a *wg* background, no *eg*-positive NB was detected at any NB 6-4 position while no change in *eg* expression in NBs 7-3, 2-4 and 3-3 was observed. Since NBs 3-3 and 7-3 were present also in the *hh* mutant (Fig. 2B, D), *hh* and *wg* may not be essential at the same time for the formation of NBs 3-3 and 7-3. Both *hh* and *wg* are required for NB 6-4 formation, while only *hh* is essential for NB 2-4 formation.

Fig. 2S shows the *hh wg* mutant hemisegment to always contain NB 3-3 but to lack NB 7-3 virtually completely. This suggests that neither *wg* nor *hh* is required for NB 3-3 formation while both are involved in NB 7-3 formation in a redundant manner. In NB 7-3 formation, *wg* and *hh* appear to function in separate, parallel pathways so that at least one is always essential for NB 7-3 formation. The alternative requirement of *hh* and *wg* for NB 7-3 formation suggests that, in contrast to epidermal development (Ingham and Hidalgo, 1993), *hh* and *wg* are not regulated interdependently. The absence of *wg* results in the almost complete loss of all three row-6 NBs (Chu-LaGraff and Doe, 1993). About half of NBs 6-1 and 6-2 and 5-10% of NB 6-4 cells are still present in *hh* embryos (see Fig. 2 legend). The partial absence of row-6 NBs from *hh* mutants suggests that both Hh and Wg signals are required for row-6 NB formation; the loss of Hh activity is, however, partially compensated for by the activity of Wg or other unknown factors. The requirement of *hh* for NBs 7-3 and 6-4 formation in a *wg* background may indicate that Hh serves as an autocrine factor in the formation of NBs 7-3 and 6-4, since their neuroectodermal precursors are *hh*-positive (Fig. 5A).

A homeobox gene *gsb-d* has been shown essential in S1 NB specification. Loss of *gsb-d*, expressed in row 5 neuroectodermal cells at S1, causes row 5 NBs to be transformed into row 3 NBs in S1 development (Skeath et al., 1995). Since *gsb-d* is also expressed at stages other than S1 in all post-S1 NBs and neuroectodermal cells in rows 5 and 6 and another homeobox gene, *en*, is constitutively expressed in all NBs and neuroectodermal cells in rows 6 and 7 (see Fig. 1F), examination was made of the effects of *gsb-d* and *en* on the development of *hh*-dependent, post-S1 *eg*-NBs. The distribution of Eg was examined using anti-Eg antiserum. As shown in Fig. 4B, in the *gsb-d* mutant, Eg expression in row 6 was apparently altered. No Eg expression was detected at the NB 6-4 position but, instead, 72% of *gsb-d* mutant hemisegments (n=107) contained a new row-6 NB, similar in properties to NB 7-3 (Fig. 4B). This NB delaminated at S5 with the authentic NB 7-3, expressed both Eg and *hkb-lacZ* and divided quasi-symmetrically as also noted for NB 7-3 (Fig. 4C-E). Note that NB 6-4 delaminates at S3 and does not express *hkb-lacZ*. The putative progeny of NB 7-3 (EW and GW neurons; Higashijima et al., 1996) was also duplicated (data not shown). The absence of *gsb-d* would thus appear to cause the transformation of row 6 neuroectoderm into row 7 neuroectoderm so that NB 7-3 cell duplication can occur.

In the *en* mutant, Eg expression at NBs 6-4 and 7-3 positions disappeared in 100 and 81% of the hemisegments, respectively (n=89; Fig. 4F). No apparent effect on Eg expression in NBs 2-4 and 3-3 was detected, suggesting that the production of Hh required for NB 2-4 formation is unrelated to *en*. Eg-positive, row-7 NBs in 19% of *en* mutant hemisegments are somewhat larger than authentic NB 7-3, and, unlike NB 7-3, divide asym-

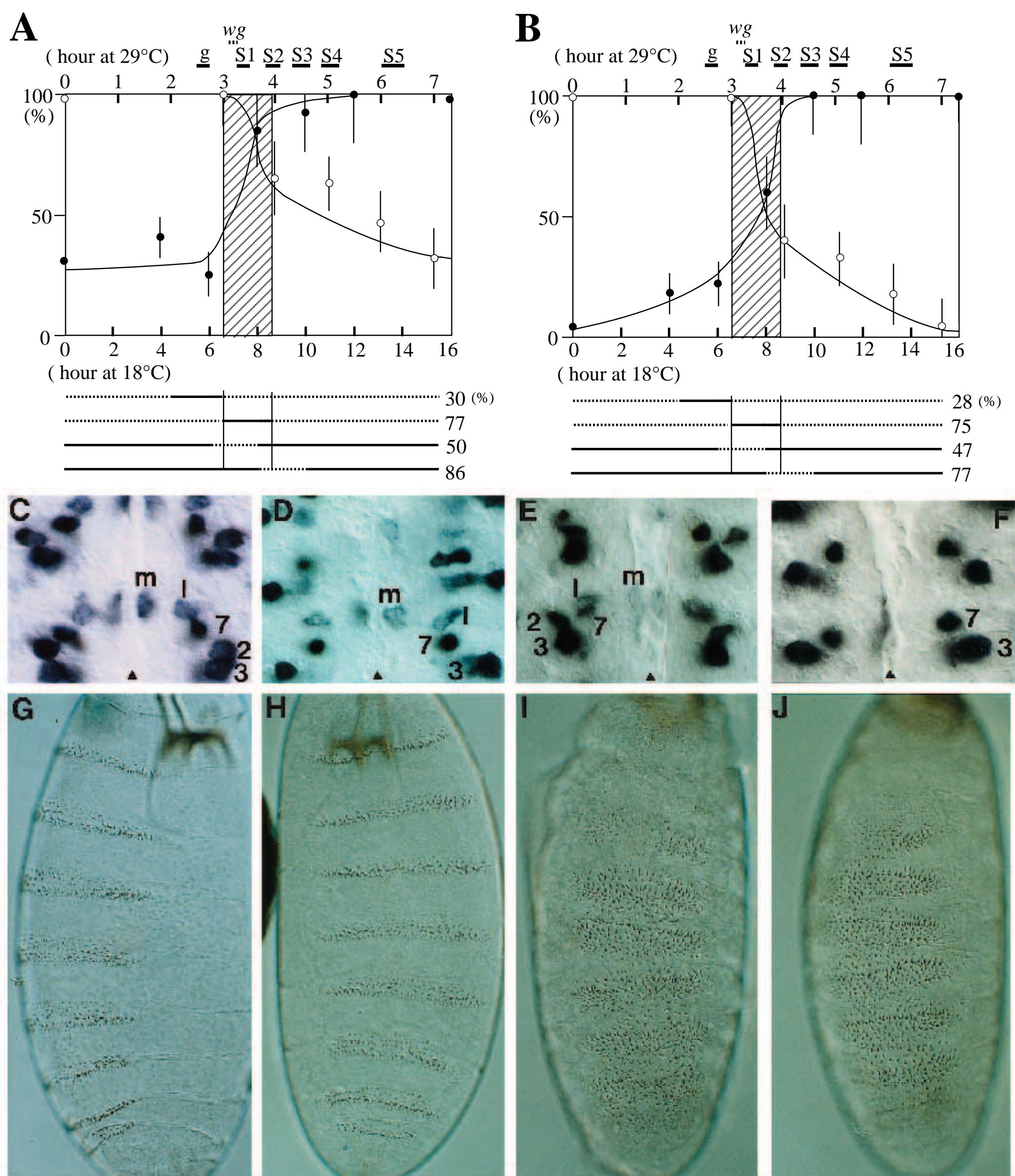

Fig. 3. Determination of CPSH (the critical period sensitive to the *hh* activity). hh^{9K} was used as a temperature-sensitive allele. Permissive temperature, 18°C; non-permissive temperature, 29°C. Results of shift-up and shift-down experiments are shown by filled and open circles, respectively. Results of transient activation or inactivation are shown in the lower margin: broken and solid lines, respectively, indicate non-permissive and permissive temperatures. Numbers of mg glia (A; a derivative of NB 6-4), and NB 2-4 (B) were scored during 14-16 hours AEL at 18°C or an equivalent period at 29°C , and shown as percentage (n=21-139). Vertical hatched belts, deduced CPSHs. g, gastrulation. Broken bars in the upper margin, *wg*-sensitive period for NB 4-2 development (Chu-LaGraff and Doe 1993). (C,G) *eg* expression and cuticular patterns of hh^{9K}/hh^{IIO} embryos raised at 18°C. (D, H) hh^{9K}/hh^{IIO} embryos raised at 18°C except for a brief period of non-permissive temperature corresponding to 6-8 hours AEL at 18°C. Cuticular patterns (H) are similar to those of wild type (see G), but NB patterns are similar to those of *hh* mutants (see F). (E, I) hh^{9K}/hh^{IIO} embryos raised at 29°C except for a brief period of permissive temperature, which corresponds to 3-4 hours AEL at 29°C. As with wild-type, NB 2-4 and mg glia were detected in most hemisegments at stage 13 (E), while cuticular pattern (I) was similar to that of the *hh* mutant (see J). (F, J) hh^{9K}/hh^{IIO} embryos grown at 29°C. NB 2-4 and mg glia were absent from most hemisegments at stage 13 (F). Cuticular patterns were severely disrupted (J). Triangles, midline. 7, NB 7-3 and its derivatives; 2, NB 2-4 and its progeny; 3, NB 3-3 and its progeny. m and l are glial cells derived from NB 6-4 (Higashijima et al., 1996).

metrically to generate progeny with no apparent morphological relationship to EW or GW neurons (data not shown). Abnormality in *en* mutant NB 7-3 lineage has been reported recently by Lundell et al. (1996).

Taken together, these results indicate four segment polarity genes, *hh*, *wg*, *gsb-d* and *en* to all function in concert to determine the formation and specifications of three *hh*-dependent *eg*-NBs (6-4, 7-3 and 2-4). The development of NB 3-3, *hh*-independent, however, is totally unrelated to any of these segment polarity genes (Fig. 5A).

DISCUSSION

This study shows *hh* to be essential for NB development in CNS. Secreted Hh acts on neuroectodermal cells adjacent to and within the *hh/en*-expressing domain to regulate the formation of eleven post-S1 NBs including a glial precursor. *hh* is not the sole element required for post-S1 NB formation. The present and other studies (Chu-LaGraff and Doe, 1993) indicate the functions of *hh, wg, gsb-d* and *en* to be essential in combination for the formation and specifications of post-S1 NBs. Unlike epidermal development for which interdependent expression of *hh*, *wg* and *en* is essential (Ingham and Hidalgo, 1993), *hh* expression required for NB formation is controlled by neither *wg* nor *en*, and thus the *hh* activity required for NB formation is likely only that regulated at the earliest stage by pair-rule genes (Lee et al., 1992).

Regulation of post-S1 NB formation by composite positional cues bestowed by Hh and Wg

Chu-LaGraff and Doe (1993) showed Wg to function in a

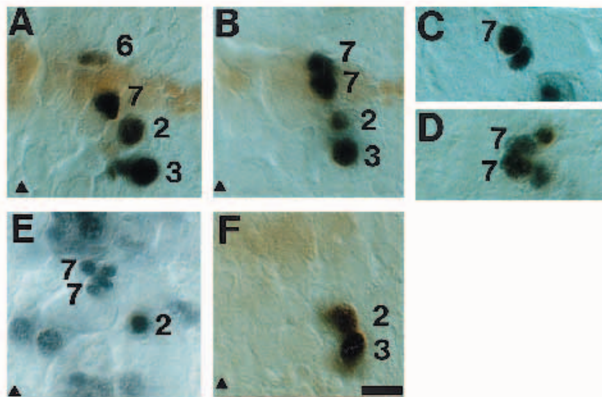

Fig. 4. Eg expression in *gsb-d* and *en* mutants. 2, NB2-4; 3, NB 3-3; 6, NB 6-4; 7, NB 7-3. Eg was detected by anti-Eg antiserum, LU1. (A, B) Brown, *en* expression; black, Eg expression. (A) Wild type at S5. (B) *gsb-d* hemisegment at S5. Note that NB 6-4 is lost (100%, *n*=107) and instead NB 7-3 is duplicated (72%). As with the authentic NB 7-3 in wild type (C), both authentic and ectopic NB 7-3 cells in *gsb-d* mutants (D and E, respectively) divide quasi-symmmetrically at late S5. (C,D) Eg expression; (E) *hkb-lacZ* expression. (F) *en* hemisegment at S5. Brown, *gsb-d* expression in rows 5 and 6; black, Eg expression. Percentage of Eg expression (*n*=89): 0% (NB 6-4), 19% (NB 7-3), 98% (NB 2-4) and 100% (NB 3-3). Bar, 10 µm for A, B, E and F; 14 µm for C and D.

manner similar to Hh in the formation of post-S1 NBs in rows 4 and 6. Interestingly, the critical period sensitive to *wg* activity for NB 4-2 (an S2 NB) is included in CPSH for *eg*-positive S3 and S4 NBs (see upper margins of Fig. 3A,B). Thus, Hh and Wg may endow neuroectodermal regions, from which S2-S4 NBs in rows 2 and 4-6 are singled out, with positional cues essential for NB formation at almost the same time (Fig. 5).

We showed all post-S1 NBs in rows 2, 5 and 6 to be *hh*-dependent. NB 7-3 in row 7 requires *hh* for formation only in the absence of *wg* activity. Chu-LaGraff and Doe (1993) showed *wg* to be essential for the formation of all post-S1 NBs in rows 4 and 6. Thus, as far as the formation of post-S1 NBs other than two row-7 NBs (1-2 and 7-2) is concerned, five different row-dependent combinations of positional cues are given by Hh and Wg (Fig. 5A). Row-4 and row-5 NB formation requires Wg and Hh, respectively. Both Wg and Hh are essential for row-6 NB formation. In NB 7-3 formation, Hh and Wg are functionally redundant to each other. Row-2 NB formation requires only Hh. Neither Hh nor Wg are required for row-3 NB formation. At present, we do not know the effects of *hh* on NBs 1-2 and 7-2 formation in the *wg* mutant. Thus, positional cues controlling NB formation along the anterior/posterior axis are quite likely to be provided mainly by *hh* and *wg*. S1 NB formation is totally independent of *hh* and *wg*, consistent with the notion that AS-C expression in S1 NBs is regulated by pair-rule genes but not by segment polarity genes (Doe and Goodman, 1993)

Possible function of Hh upstream of proneural genes and neurogenic genes

AS-C genes are essential for proneural fate determination in many nervous systems in *Drosophila*. However, in CNS, AS-C serves as proneural genes only for 25% of NBs, most being

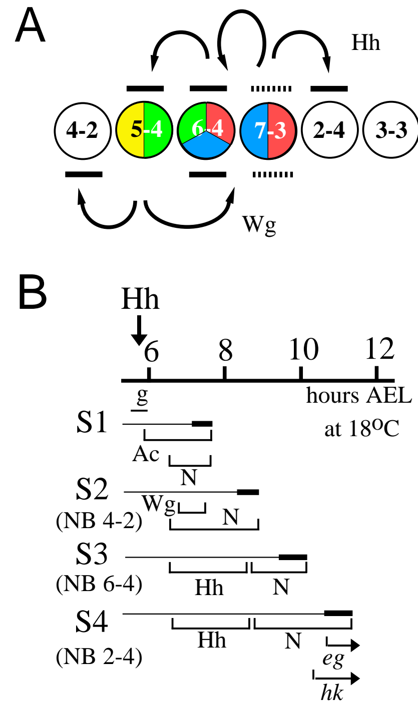

Fig. 5. (A) A model showing the action of Hh and Wg on NB development. Circles labeled with blue and yellow, respectively, show NBs derived from *hh*-expressing and *wg*-expressing neuroectodermal cells. Solid bars over and under circles, respectively, indicate that Hh and Wg are required for NB formation. Dashed bars indicate requirement for *hh* and *wg* to be redundant. Targets of Hh and Wg are not NBs but their neuroectodermal cells (see text). *en* (red) is expressed in both NBs and neuroectodermal cells in rows 6 and 7. Similarly, *gsb-d* (green) is expressed in both NBs and neuroectodermal cells in rows 5 and 6. NBs in these rows are specified by *en* and *gsb-d* expressed in neuroectodermal cells prior to NB delamination. NB 6-4 (or row-6 NB) fate may require both *en* and *gsb-d* activity while row-5 NB (NB 5-4) and row-7 NB (NB 7-3) fate may be solely determined by *gsb-d* and *en*, respectively. (B) Temporal sequences of gene expression and function in the formation of *hh*-dependent post-S1 NBs. Vertical arrow, the time of appearance of 14 stripes of strong Hh (Tashiro et al., 1993). Thick horizontal bars, periods for NB delamination. U-shaped bars labeled Wg and Hh indicate critical periods sensitive to Wg and Hh, respectively (Chu-LaGraff and Doe, 1993 and present work), while those labeled Ac and N, Ac-expressing (Skeath and Carroll, 1992) and N-sensitive periods (Hartenstein et al., 1994). N may not be necessary throughout the N-sensitive period. L-shaped arrows, *hkb-lacZ* expression in putative proneural regions and *eg* expression in delaminating NB 2-4.

S1 NBs (Skeath et al., 1992). Although the present results suggest that each post-S1 NB is derived from its own proneural region, similar in size to those for S1 NBs (see Fig. 2I, T), no proneural genes for most post-S1 NBs have been reported. Thus, at present, whether *hh* functions upstream, in parallel or downstream of putative proneural genes for post-S1 NBs remains unclear. However, in the case of S1 NB formation, AS-C expression occurs in a 1-1.5 hour period (at 18°C) just before delamination (see Fig. 5B; Skeath and Carroll, 1992) and this suggests that, in S3 and S4 NB formation, CPSH occurs prior to proneural gene expression and, hence, Hh functions upstream of proneural genes (see Fig. 5B).

Hartenstein et al. (1994) noted the expression of *hsp-Notch* (*intra*), a *hsp*-promoter-driven gain-of-function form of *N*, during 4-5 hours AEL at 25°C to prevent the segregation of most post-S2 NBs. Considering the lag time required for effective translation, the expression period of Notch (intra) may correspond to 9-11 hours AEL at 18°C, during which S3 and S4 NBs delaminate. As schematically shown in Fig. 5B, a period of N-dependency may follow CPSH.

At least in NB 2-4, the expression of *hkb* and *eg*, respectively, occurs shortly before and concomitant with NB delamination (see Fig. 5B). These genes are implicated in axon pathfindings in certain progeny neurons (Chu-LaGraff et al., 1995; Higashijima et al., 1996). Our results indicate *hkb* and *eg* to be situated downstream of the Hh pathway.

Control of post-S1-NB formation and specifications by concerted action of four segment polarity genes, *hh*, *wg*, *gsb-d* and *en*

gsb-d and *en* may not be involved in proneural fate acquisition, since, unlike *hh* and *wg* mutants, no appreciable gap regions in the NB layer were found in *gsb-d* or *en* mutants (unpublished data). Analysis of *eg*-NBs rather suggests that the absence of *gsb-d* in row 6 causes row-6 to be transformed into row-7 (see Fig. 4B), and this appears consistent with the finding that paired serotonergic neurons, putative derivatives of NB 7-3, are doubled in *gsb-d* mutant embryos (Patel et al., 1989; Lundell et al., 1996). At S1 stage, row-5 NBs in the *gsb-d* mutant are transformed into row-3 NBs, whereas ubiquitous *gsb-d* expression generates the opposite transformation (Skeath et al., 1995). *en* may be essential for the acquisition of row-6 and row-7 *eg*-NB identity, since the absence of *en* resulted in 100 and 81% loss of *eg*-NBs in rows 6 and 7, respectively (see Fig. 4F). 19% of NBs at NB 7-3 positions, still capable of expressing *eg* in the absence of *en*, were shown not to be of the NB 7-3 type (unpublished data). These NBs may possibly be relatives of *eg*-positive NB 2-4, in consideration of their locations and asymmetry in cell division. Thus, the identity of four *hh*-dependent neuroectodermal rows (rows 5-7 and 2) may be controlled through the concerted action of two homeobox genes *en* and *gsb-d* .

Due to the loss of NBs, no NB-fate alteration occurred in the *hh* mutant. But, *hh* may also be involved in NB specification, since (1) *hh* is essential for putative proneural cells to express *hkb* and acquire ability for *eg* expression on delamination and (2) *wg* has been shown to be involved in specification of NB 4-2 (Chu-LaGraff and Doe, 1993).

Unlinked transcriptional regulation of *hh*, *wg*, *en* and *gsb-d*

In contrast to epidermal development, *hh* activity required for NB formation is unrelated to either *en* or *wg*, since an *hh*-dependent NB 2-4 is normally produced in *en* and *wg* mutants (Figs 4F, 2R). However, Hh and Wg pathways required for NB formation may have in common with the late Hh/Wg system certain components required for epidermal development, since our preliminary experiments indicated that, as in epidermal development (Forbes et al., 1993; Siegfried et al., 1994), the *hh* mutant phenotype (loss of NB 2-4) is suppressed by an additional *patched* mutation and the phenotypes of *porcupine* and *dishevelled* are identical to that of the *wg* mutant (loss of NB 6-4).

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