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The differentiation of the serotonergic neurons in the *Drosophila* ventral nerve cord depends on the combined function of the zinc finger proteins Eagle and Huckebein

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SUMMARY

The *Drosophila* ventral nerve cord (vNC) derives from a stereotyped population of neural stem cells, neuroblasts (NBs), each of which gives rise to a characteristic cell lineage. The mechanisms leading to the specification and differentiation of these lineages are largely unknown. Here we analyse mechanisms leading to cell differentiation within the NB 7-3 lineage. Analogous to the grasshopper, NB 7-3 is the progenitor of the *Drosophila* vNC serotonergic neurons. The zinc finger protein Eagle (Eg) is expressed in NB 7-3 just after delamination and is present in all NB 7-3 progeny until late stage 17. DiI cell lineage tracing and immunocytochemistry reveal that *eg* is required for normal pathfinding of interneuronal projections and for restricting the cell number in the thoracic NB 7-3 lineage. Moreover, *eg* is required for serotonin expression. Ectopic expression of Eg protein forces specific additional CNS cells to enter the serotonergic differentiation pathway. Like NB 7-3, the progenitor(s) of these ectopic cells express Huckebein (Hkb), another zinc finger protein. However, their progenitors do not express *engrailed* (*en*) as opposed to the NB 7-3 lineage, where *en* acts upstream of *eg*. We conclude that *eg* and *hkb* act in concert to determine serotonergic cell fate, while *en* is more distantly involved in this process by activating *eg* expression. Thus, we provide the first functional evidence for a combinatorial code of transcription factors acting early but downstream of segment polarity genes to specify a unique neuronal cell fate.

Key words: serotonergic neuron, Eagle, Huckebein, *Drosophila*, zinc finger, ventral nerve cord, neuroblast

INTRODUCTION

The insect central nervous system (CNS) develops from neuroblasts (NBs) which delaminate from the neuroectoderm following gastrulation. NBs divide asymmetrically to produce a chain of smaller ganglion mother cells (GMCs); each GMC divides once to give rise to a pair of neurons and/or glia. The mechanisms regulating NB formation are quite well understood (for review, see Campos-Ortega, 1993), but the mechanisms leading to the specification of the individual NBs and the differentiation of their lineages are less well known. Each NB can be uniquely identified by its time of formation, position of delamination and pattern of gene expression (Doe, 1992; Broadus et al., 1995). In addition each NB produces a characteristic set of neuronal and/or glial progeny (Udolph et al., 1993; Bossing et al., 1996b). Laser ablation experiments (Doe and Goodman, 1985), in vitro NB cell culture (Huff et al., 1989; Lüer and Technau, 1992) and cell transplantation studies (Udolph et al., 1995) suggest that the lineages are largely controlled by intrinsic properties of the respective NBs. To clarify processes governing cell fates in the CNS, it is necessary to identify the genes employed and to understand their functional relationships.

This paper focusses on the NB 7-3 lineage in the *Drosophila* ventral nerve cord. Single cell labelings with DiI show that it is a very small lineage consisting of only 4 to 5 neurons at the end of embryogenesis (Bossing et al., 1996b), and 2 of these cells are likely to represent the ventral nerve cord serotonin cells (Lundell et al., 1996), comparable to the situation in the grasshopper (Taghert and Goodman, 1984).

To identify genes that might be involved in the specification of these cells, we screened for enhancer trap lines and found two, Mz360 and P289, with specific expression of the reporter gene in the NB 7-3 lineage. Both lines were found to be insertions at the *eagle (eg)* locus. *eg* encodes a putative zinc finger protein and the *eg* RNA is expressed in NB 7-3 as well as in three other NB lineages (Higashijima et al., 1996). Analysis of *eg* mutant embryos revealed impaired axogenesis in the putative progeny of NB 7-3 and NB 2-4, suggesting that *eg* plays a critical role in the development of these cells (Higashijima et al., 1996).

We find that Eagle (Eg) protein is expressed in NB 7-3 just after delamination and is present in all NB 7-3 progeny, including the serotonin neurons, until late stage 17. Mutant phenotypes of *eg* demonstrate that this gene is required for

correct axonal pathfinding, for the expression of serotonin and, in the thoracic NB 7-3 lineage, for the generation of the correct cell number. Furthermore, *eg* is not only necessary for correct serotonin expression but is also able to promote ectopic serotonergic differentiation of specific neurons at defined positions in the *Drosophila* CNS. Loss-of-function alleles of *huckebein (hkb)* and *engrailed (en*) lead to a nearly complete loss of serotonin immunoreactivity and suggest an important role for these two genes in NB 7-3 development (Lundell et al., 1996). We show that *en* function in NB 7-3 development is upstream of *eg* and is necessary for *eg* expression in this lineage. On the contrary, the ectopic serotonergic neurons do not derive from *en*-expressing NB(s) but do depend on *hkb*. This led us to conclude that Eg and Hkb are part of a 'combinatorial code' of transcription factors acting in the NB 7-3 lineage to control serotonergic differentiation while *en* function is required earlier for the activation of *eg* expression.

MATERIALS AND METHODS

Fly strains and genetics

The Gal4 P-element construct of Brand and Perrimon (1993) was used to generate more than 2000 independent enhancer-trap lines, including Mz360 (Ito et al., 1995), a homozygous viable fly strain, which shows an *eg* phenotype and failed to complement eg^{1}, eg^{2} and eg^{spy} obtained from Indiana stock center. Therefore, we refer to this line as eg^{Mz360}. eg^{P289}, an enhancer-trap line bearing a P-larB P-element construct (Wilson et al., 1989) in the *eg* locus, was kindly provided by B. Genisch and G. Korge, Berlin.

In order to obtain an *eg* deficiency, we remobilised the P-element of eg^{Mz360} by using the P[ry, Δ 2-3] chromosome (Robertson et al., 1980). We tested over 1000 excision events for lethality and non-complementation with eg^{Mz360} and obtained one line, eg^{18B}, that fullfilled both criteria.

Molecular mapping of the *eagle* alleles

The genomic DNA fragments flanking the Mz360 and P289 insertions were cloned by the plasmid rescue technique (Bier et al., 1989). Southern hybridisation (Sambrook et al., 1989) was used for mapping the flanking DNA of the P-element insertions and to characterise the deletion of eg^{18B}.

DiI labeling

DiI labeling of individual progenitors, in vivo identification of NBs and photoconversion of labelled clones was done as described elsewhere (Bossing and Technau, 1994; Bossing et al., 1996b).

Antibody generation

For protein expression and preparation, we made use of the QIAexpress system (Quiagen). A *Sac*I fragment from Exon C of the *eg* coding sequence (Higashijima et. al., 1996) was cloned into the *Sac*I site of pQE 31. After induction, the expressed protein was prepared under non-denaturating conditions as described in the QIAexpress manual (Quiagen). Immunisation was done in rabbits by the Eurogentec company, using a standard protocol. The obtained serum was tested in embryos for specific *eg* staining and used without further purification.

UAS-*eagle* flies

An *eg* RACE-type 1 clone (Higashijima et al., 1996), containing the A1 and B exon of the *eg* gene was cut with *Hin*dIII and *Nae*I and the fragment consisting of exon A1 and B was cloned into *Hin*dIII/*Nae*I-restricted H 3.7. This genomic clone contains exon B and exon C of the *eg* gene (Higashijima et al., 1996). The resulting construct was cut with *Kpn*I and *Hin*dIII and the fragment containing the entire *eg* coding sequence was inserted into a *Kpn*I/*Hin*dIII-restricted pBluescript II KS-vector. In a final step, this construct was cut with *Xba*I and the *eg* sequences were cloned into the *Xba*I site of p-UAST (Brand and Perrimon, 1993). Transgenic flies were generated, using standard techniques (Spradling and Rubin, 1982). Several insertions were isolated and tested for *eg* expression when crossed to *sca-Gal4*. The lines UAS-*eg*18.1 and UAS-*eg*43.1 were chosen for further experiments.

For *sca-GAL4*/UAS-*eg* expression in the hkb^{2}-mutant background, we crossed the lines UAS-*eg*43.1; hkb^{2}/*TM3, Sb* and *sca-GAL4*; hkb^{2}/*TM6b, Tb*. The *hkb* mutants were identified by their head involution defect.

In situ hybridization and immunocytochemistry

Whole-mount in situ hybridization was done according to Tautz and Pfeifle (1989). Antibody stainings in *Drosophila* embryos against β-galactosidase and En were performed as previously described (Schmidt-Ott and Technau, 1992). For anti-Eg antibody staining, embryos were incubated overnight at room temperature in a 1:500 dilution of preabsorbed anti-Eg antiserum in PBT (0.3% Triton X-100, 10% calf serum in PBS), washed with PBT and then incubated for 2 hours at room temperature with the HRP- or rhodamine-conjugated goat anti-rabbit antibody (Dianova) diluted 1:400 in PBT. The HRP was detected using 0.5 mg/ml DAB and 0.03% hydrogen peroxide. Embryos were mounted as flat preparations on coverslips in 90% glycerol in PBS. HRP-stained embryos were viewed under a Zeiss Axiophot microscope.

DDC, TH, β-galactosidase and serotonin staining of first instar larval nervous systems was done according to Beall and Hirsh (1987). The antibodies were used in the following dilutions: polyclonal rabbit anti-DDC (Scholnick et al., 1991) 1:100, polyclonal rabbit anti-TH (Pel Freeze) 1:50, monoclonal mouse anti-β-galactosidase (Promega) 1:1000 and monoclonal rat anti-serotonin (Accurate) 1:50. As secondary antibodies, we used FITC-conjugated goat anti-rat, FITC-conjugated goat anti-mouse and RITC-conjugated goat-anti rabbit (Boehringer) in a 1:400 dilution. Immunofluorescence was imaged on a Leica DM confocal microscope.

Note on figures

In Figs 1C-G, 3-7 we combined different focal planes by using Photoshop 3.0 (Adobe).

RESULTS

eg is expressed in NB 7-3 and all of its progeny

We identified the P-GawB (Brand and Perrimon, 1993) enhancer-trap line Mz 360 (Ito et al., 1995) and the *lacZ* enhancer-trap line P289 as alleles of *eg* and therefore refer to them as eg^{Mz360} and eg^{P289}. The embryonic reporter gene expression patterns (*Gal4, lacZ*) of the two lines are identical. In abdominal segments, double labeling with anti-Invected antibodies revealed a reporter gene expression in four NBs and their putative progeny: NB 2-4, NB 3-3, NB 6-4 and NB 7-3 (Fig. 1A,B). This expression pattern is also described for the enhancer trap line P179 by Higashijima et al. (1996) which also represents an insertion in the *eg* locus.

In order to determine the distribution of the Eg protein, we generated a polyclonal antibody against Eg. Here we describe the Eg expression pattern in the abdominal neuromeres as revealed by this antibody. At early stage 11 (embryo staging according to Campos-Ortega and Hartenstein, 1985), when the S4 NBs have formed, Eg expression first appears in NB 2-4

and NB 3-3 (Fig. 1C). At late stage 11, NB 2-4, NB 3-3, NB 6-4 and NB 7-3 show Eg expression; NB 6-4 has already divided and there are several small Eg-positive cells located in the dorsal vicinity of NBs 2-4 and 3-3, representing their putative progeny (Fig. 1D). By the end of stage 12, Eg expression in the glial descendants of NB 6-4, the MM-CBG and M-CBG (terminology of glial cells according to Ito et al., 1995), is no longer detectable. At this stage, 3 to 4 Eg-expressing cells are visible at a more medial position in addition to 10 to 15 laterally located Eg-positive cells (Fig. 1E). By the end of stage 15, the lateral Eg-positive cells are hardly detectable, while the medial cells (called EW and GW neurons by Higashijima et al., 1996) still show strong Eg expression. This cell cluster shows a typical morphology with the GW neuron lying just posterior to the EW cells (Fig. 1F). Eg expression fades from the entire CNS at late stage 17 (Fig. 1G).

The lineage of NB 7-3 is described in Bossing et al. (1996b; see also Fig. 4A). At stage 17, most NB 7-3 clones consist of 4 mediodorsal neurons: three interneurons, called 7-3I neurons, which project contralaterally across the posterior commissure and one ipsilaterally projecting motoneuron, called 7-3M. Differentiation of 7-3M starts rather late (stage 15/16). At stage 17 the projection of 7-3M reaches the posterior root of the intersegmental nerve. The same projection pattern is described for the EW and GW neurons (Higashijima et al., 1996). Based on cell position, cell number and projection pattern, we conclude that EW neurons and 7-3I neurons, on the one hand, and the GW neuron and the 7-3M cell, on the other hand, are identical. Therefore, combining lineage data and the Eg expression pattern, we show that Eg is expressed in NB 7-3 just after delamination and is present in all NB 7-3 progeny (7-3I; 7-3M) until late stage 17.

In the grasshopper, the serotonergic neurons represent contralaterally projecting interneurons which are progeny of NB 7-3, resulting from the division of the first ganglion mother cell (GMC) (Taghert and Goodman, 1984). In *Drosophila*, the serotonergic neurons of the ventral nerve cord also project contralaterally across the posterior commissure (White and Vallés, 1985; Vallés and White, 1986; Lundell and Hirsh, 1994), and it has recently been shown that the *hkb* and *en* genes are coexpressed uniquely in the serotonin cells and in NB 7-3. This suggests that, as in the grasshopper, NB 7-3 is the progenitor of the serotonergic neurons (Lundell et al., 1996). Double labeling for β-gal and serotonin in the CNS of eg^{Mz360}-*GAL4*/UAS-*lacZ* first instar larvae reveals colocalisation in two of the NB 7-3 progeny (data not shown). This confirms that the serotonin neurons are indeed among the progeny of the *eg*-positive NB 7-3.

eg is required for serotonin differentiation, axonal pathfinding and restriction of cell number in the NB 7-3 lineage

In order to investigate the function of *eg* within the NB 7-3 lineage, we analysed fly strains carrying mutations at the *eg* locus. The P-element insertions of eg^{Mz360} and eg^{P289} are homozygous viable and cause, as shown by P-element re-

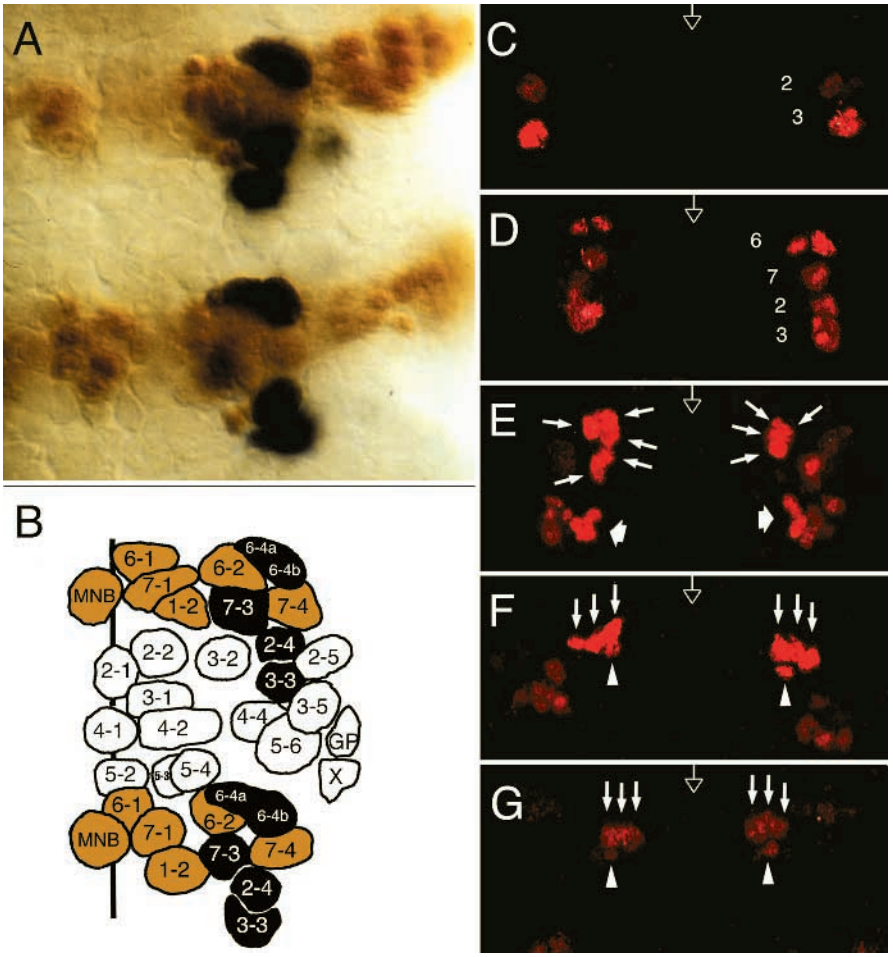

Fig. 1. *eagle* expression in abdominal segments as revealed by the enhancer trap line P289 and by the anti-Eg antiserum. (A,B) Abdominal neuromeres of an eg^{P289} embryo at stage 11 stained with anti-β-galactosidase (black) and anti-Inv (brown) (A) and the corresponding camera lucida drawing (B). NBs 2-4, 3-3, 6-4 and 7-3 express β-galactosidase.
(C-G) Neuromeres of a wild-type embryo stained with anti-Eg. (C) Early stage 11. NB 2-4 (2) and 3-3 (3) start Eagle expression. (D) Late stage 11, NB 2-4 (2), 3-3 (3), 6-4 (6) and 7-3 (7) are Eagle positive; 6-4 is already dividing, and there are several small Eagle-expressing cells located in the dorsal vicinity of 2-4 and 3-3. (E) Stage 12. The putative NB 6-4 descendants are no longer detectable; 3 to 4 Eagle-expressing cells are visible at the position of NB 7-3-derived neurons (small white arrows); in addition 10-15 lateral neurons are stained (thick white arrow). (F) Stage 15. Eagle expression in the lateral neurons is hardly detectable, while the three 7-3I neurons (white arrows) and the 7-3M neuron (white arrowhead) still show high level of Eagle protein. (G) Stage 17. Only 7-3I/7-3M neurons still show faint Eagle expression (7-3I: white arrows; 7-3M: white arrowhead). Anterior is up;dorsal views; open arrow, midline.

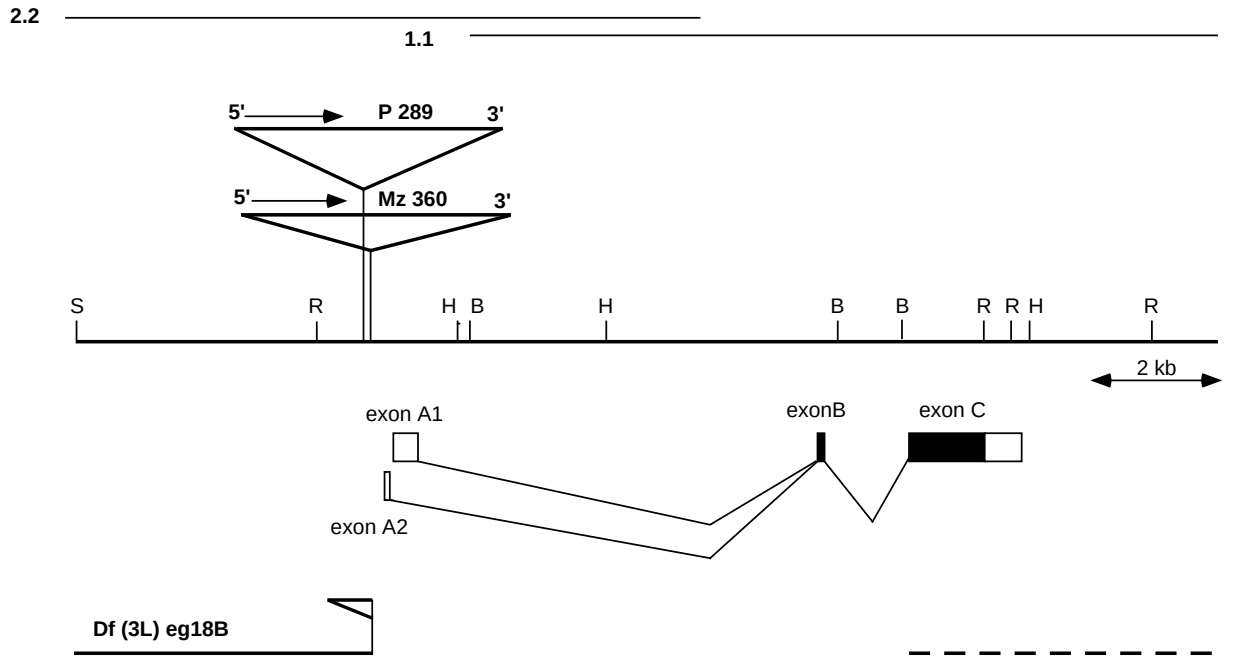

Fig. 2. Genomic organisation of the *eagle*-locus. The thick horizontal line represents the genomic region. Thin lines on top show sizes and locations of genomic clones. The organisation of the transcripts according to Higashijima et al. (1996) is shown below. Coding sequences are black. The positions of two enhancer-trap insertions, P[eg^{Mz360}-GAL4,w+] and P[eg^{P289}-lacZ; ry+] are indicated by triangles. Df(3L)eg^{18B} removes at least exon A1, exon A2 and exon B; broken lines indicate that the endpoint is not mapped.

mobilisation, a 'wings held out' phenotype, which is allelic to classical *eg* mutations (Lindsley and Zimm, 1992). We cloned the flanking regions of the P-elements by plasmid rescue and mapped the insertions at the molecular level. The P-elements are inserted only about 100 bp apart from each other and approximately 200 bp upstream of 5′-Exon A2 (Higashijima et al., 1996) of the *eg* gene (Fig. 2). After remobilisation of the P-element in eg^{Mz360}, we isolated eg^{18B}, a homozygous lethal line that is allelic to eg^{Mz360} and other *eg* mutations. Southern hybridisation indicated that in this line at least 7 kb of genomic DNA of the *eg* locus is deleted, including sequences of exons A1, A2 and B bearing the translational start site (Higashijima et al., 1996; Fig. 2). This suggests that eg^{18B} is a null mutation for *eg*. In support of this, the analysis of the homozygous mutant embryos of eg^{18B} revealed no detectable expression of Eg protein. This was also true for eg^{P289}, suggesting that the P-element insertion in eg^{P289} alone causes a severe embryonic loss-of-function mutation.

In order to analyse in detail the NB 7-3 development in an *eg* mutant background, we used the lipophilic fluorescent tracer DiI (Bossing and Technau, 1994) to label NB 7-3 and its progeny in eg^{P289}. As already mentioned, the wild-type NB 7-3 produces three interneurons (7-3I), projecting contralaterally across the posterior commissure and one motoneuron (7-3M) that develops an ipsilateral posterior axon (Bossing et al., 1996b; Fig. 3A,B). We generated three abdominal and two thoracic NB 7-3 clones and examined their cell numbers, positions and axonal projections (Fig. 3C-E). The abdominal clones correspond to wild type in cell number and positions and all show an ipsilateral posterior axon, suggesting normal 7-3M neurons. However, all mutant clones show severe pathfinding defects of the interneuronal projections. These projections no longer cross the midline, but stay ipsilaterally where the orientation of these projections is quite variable. In one case, the mutant cells project anteriorly and posteriorly (Fig. 3E) and, in the remaining cases, they project out of the CNS (Fig. 3C,D; thick arrows). Thus, NB 7-3 in eg^{P289} embryos produces interneurons (7-3I) with significant axon pathfinding defects, whereas 7-3M seems to have a normal axonal projection. In contrast, Higashijima et al. (1996) described a lack of axonal outgrowth of NB7-3-derived neurons in the *eg* mutant using kinesin-lacZ driven by the *eg* enhancer as a marker. This discrepancy might be explained by the higher resolution of the DiI method.

In addition to the interneuronal pathfinding defects mentioned above, eg^{P289} embryos have a thorax-specific abnormality in the NB 7-3 clones (Fig. 3C). The mutant clones consist of 6 to 8 cells, as compared to 4 to 5 cells in wild type. This suggests that *eg* is necessary to restrict the cell number in the thoracic NB 7-3 lineage.

Since the serotonergic neurons of the ventral nerve cord are among the progeny of NB 7-3, we further investigated serotonin expression within the *eg* mutant. In abdominal segments of a wild-type first instar larval CNS, there are two serotonergic neurons per hemineuromere, except for A8, where there is only one (White and Vallés, 1985; Vallés and White, 1986; Lundell and Hirsh, 1994; Fig. 4A). In eg^{P289} and eg^{18B} mutants of the same stage, truncal serotonergic cells are almost entirely lacking (Fig. 4B,C). Only 2 to 4 escaper serotonergic cells are generally present and occur mostly as single cells in a random distribution. *eg* is therefore an important factor for serotonin expression in the NB 7-3 lineage, yet other genes must exist that are able to promote serotonin expression at a low frequency in the absence of *eg* function.

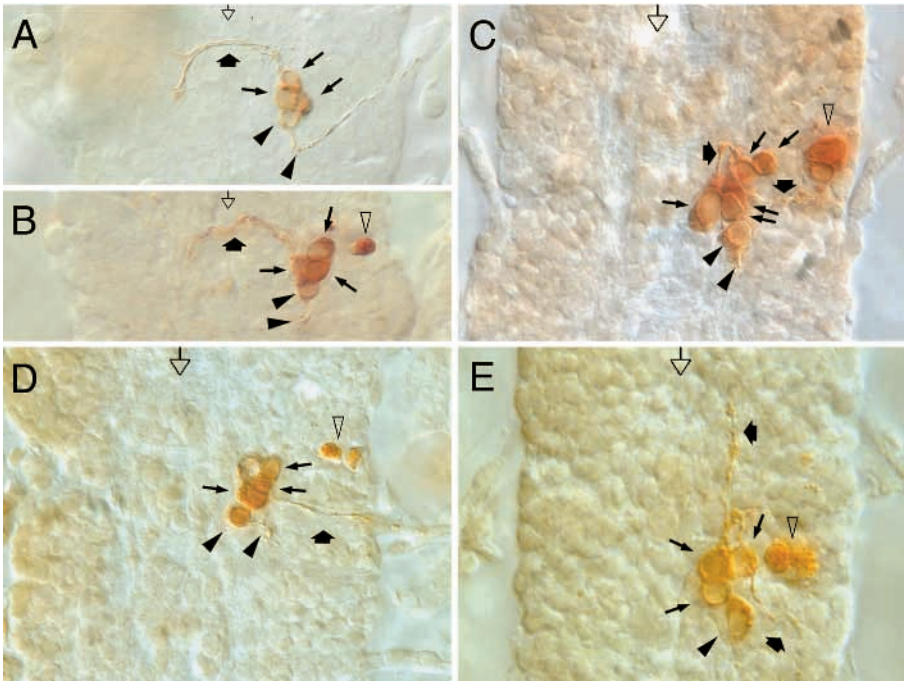

Fig. 3. NB 7-3 lineage in *eg* mutant embryos. DiI-labelled NB 7-3 clones in wild-type (A,B) and *eg* mutant (C-E) embryos. (A,B,D,E) abdominal clones; (C) thoracic clone. (A,B) Components of the wild-type NB 7-3 clone include three interneurons (7-3I; thin arrows) projecting contralaterally across the posterior commissure (thick arrow) and one motoneuron (7-3M) forming an ipsilateral posterior axon (arrowheads). Differentiation of the clone in A is more advanced compared to B. In *eg* embryos, the interneuronal projections stay ipsilateral, projecting in various directions (C-E; thick arrows). In contrast, the motoprojection appears normal (arrowheads). Cell number is wild type in abdominal clones (D,E), but exaggerated in thoracic ones (C). Cells marked by an open arrowhead in C-E occupy the position at which apoptotic cells are often observed in the wild-type lineage (open arrowhead in B; Bossing et al.. 1996b). Anterior is up; dorsal view; midline: open arrow.

eg is competent to promote ectopic serotonergic differentiation

eg is an important factor for the serotonergic differentiation in the NB 7-3 lineage. However, is it able to promote an ectopic serotonergic cell fate? To answer this question, we induced ectopic *eg* expression using the GAL4 system (Brand and Perrimon, 1993). *sca-GAL4* was used to drive GAL4 expression throughout the developing embryonic nervous system, reflecting the *scaborous (sca)* expression pattern (Mlodzik et al., 1990; Klaes et al., 1994). Consistent with this, *eg* expression, as revealed with the anti-Eg antiserum, was first detected as early as stage 10 in neuroectodermal cells and delaminating NBs. At later stages, it was present in most, if not all, NBs and their progeny until late stage 16.

First, the expression of various markers in the vNC of *sca-GAL4*/UAS-*eg* embryos was analysed. As revealed with the axon markers anti-Fasciclin II (Grenningloh et al., 1991), mAb22C10 (Fujita et al., 1982) and mAbBP102 (Patel, 1994; Seeger et al., 1993), the neuropile of these embryos is disorganised, indicating various pathfinding defects. The expression of *en* appears completely wild type (data not shown).

Despite these defects, most of the *sca-GAL4*/UAS-*eg* embryos hatch and therefore the CNS of the first larval instar could be analysed with an anti-serotonin antibody. In about 70% (n=100) of the hemineuromeres, we observe 1 to 2 ectopic serotonin-expressing cells (Fig. 5B). These es-cells (ectopic serotonergic cells) show neuronal cell morphology and occur at a reproducible, dorsolateral position. This suggests that ectopic serotonin expression does not occur randomly but in specific neurons of the CNS.

The position and number of the es-cells suggested that they could possibly be identical to the dorsolateral dopamine cells (DL-cells). In a wild-type larval CNS, there are 1 to 2 DL-cells expressing the enzymes DOPA decarboxylase (DDC) and tyrosine hydroxylase (TH) (Lundell and Hirsh, 1994). TH catalyses the first step of dopamine biosynthesis and is, within the *Drosophila* larval vNC, expressed only in the midline dopamine cell and the DL-cells (Levitt et al., 1965; Lundell and Hirsh, 1994). We examined serotonin, DDC and TH expression in the first larval instar CNS of *sca-GAL4*/UAS-*eg* animals either by double labeling with anti-serotonin and anti-DDC or with anti-serotonin and anti-TH antibodies (Fig. 5C-F). In both cases, we observed 2 serotonin cells and 1 to 2 es-cells per hemisegment expressing serotonin as well as DDC or

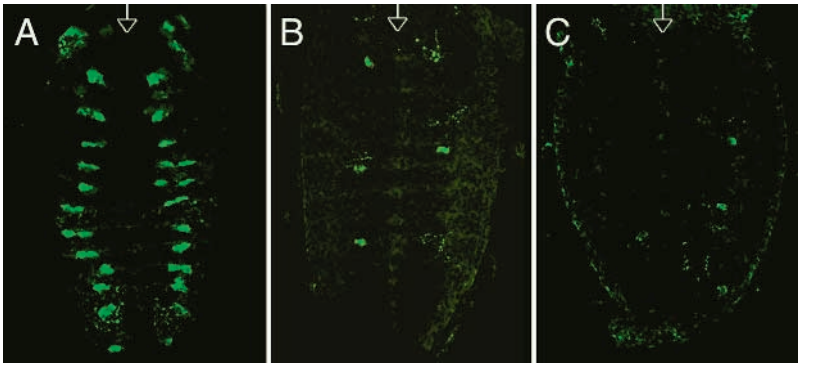

Fig. 4. Serotonin expression in *eg* mutant embryos. Serotonin expression in wild type (A) as compared to *eg* (B,C) 1st instar larvae. VNC of a wild-type (A), an eg^{P289} (B) and an eg^{18B} (C) first instar larva stained with anti-serotonin. Serotonin immunoreactivity is almost completely absent in the VNC of *eg* mutants (B,C). Anterior is up; dorsal view; midline: open arrow.

TH. TH expression in serotonergic neurons is never observed in wild type. In both experiments, we found 1 to 2 dorsally located cells expressing only DDC or TH in addition to the serotonin and es-cells. Due to their position and number, these cells are most likely the DL-cells. Thus, although the ability of *eg* to induce the expression of both DDC and TH obscured the results of the double labeling experiments, these observations show that the es-cells are not identical to the DL-cells. The fact that we get ectopic TH expression in the serotonergic neurons must be due to higher levels of Eg protein in the NB 7-3 lineage, as the same result is observed by overexpression of *eg* using eg^{Mz360}-*GAL4*/UAS-*eg* (data not shown).

eg is required in the progenitor cells to promote ectopic serotonergic differentiation

We asked further whether specification of es-neurons occurs at the level of the progenitor cells or at the level of the mature, differentiating cells. To induce ectopic *eg* expression only in postmitotic neurons, we used the *elav-GAL4* line to reflect the *elav* expression pattern (Robinow and White, 1988). Though *eg* expression could be evoked efficiently in *elav-GAL4*/UAS-*eg* embryos, we never observed ectopic serotonergic cells in the ventral nerve cord of *elav-GAL4*/UAS-*eg* first instar larvae (data not shown). This suggests that ectopic serotonergic expression in the *sca-GAL4*/UAS-*eg* line is due to the presence of Eg in NBs and/or GMCs.

en acts upstream of *eg* and is most likely not directly involved in serotonergic differentiation

At late embryonic stages, the serotonergic neurons are the only cells expressing *en* and *hkb* simultaneously, leading to the hypothesis that these genes act in combination to determine the serotonergic cell fate. In support of this, loss-of-function mutations in these genes lead to loss of serotonin immunoreactivity (Lundell et al., 1996). Thus, it is of interest to investigate the functional relationship between *en*, *hkb* and *eg* in order to clarify the serotonergic differentiation pathway within the NB 7-3 lineage.

To examine the functional relationship between *eg* and *en* a loss-of-function mutation, en^{IM99} (Heemskerk et al., 1991), was stained with the anti-Eg antiserum (Fig. 6B). In about 90% of mutant hemineuromeres (n=100), we observed a complete loss of *eg* expression at the position of the NB 7-3 neurons. This is in agreement with results of Matsuzaki and Saigo (1996) who found a loss of Eg expression in 81% of hemineuromeres. In the remaining hemineuromeres, we find *eg* expression in all NB 7-3 progeny (7-3I; 7-3M). Due to their position and their strong Eg expression, these cells are clearly distinguishable from the lateral Eg-expressing neurons. Thus, *en* function in NB 7-3 development must be upstream of *eg* and is therefore necessary for correct *eg* expression within this lineage.

To address whether *en* could have a second function in NB 7-3 development, which is more directly related to the serotonin expression, we examined whether *en* expression is necessary for promoting the es-cell fate. Using the UAS/GAL4 system, *eg* was ectopically induced in the *en* expression domain. Although Eg expression in *en-GAL4*/UAS-*eg* embryos was strong, it failed to promote an es-cell fate (data not shown). Since the onset, intensity and perdurance of *en-GAL4*-driven UAS-*eg* expression is comparable to that in *sca-GAL4*, we conclude that *en* is not necessary to establish *eg*-dependent ectopic serotonergic differentiation. Consistent with this, *invected (inv)*, which in the embryo is coexpressed with *en*, is not expressed in the es-neurons of *sca-GAL4*/UAS-*eg* first instar larvae (Fig. 7A). This is in contrast to the wild-type serotonergic cells, as they are *inv* positive at this developmental stage (Fig. 9A).

Taken together, these data indicate that *en* is not directly

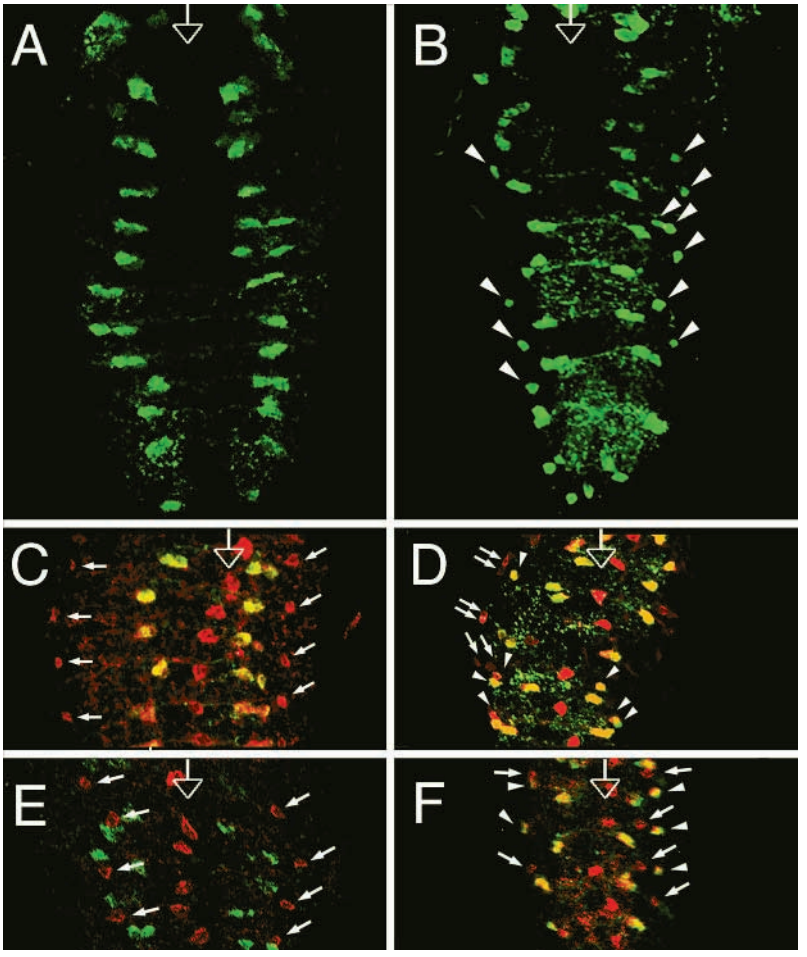

Fig. 5. *eagle* is competent to specify ectopic serotonergic neurons. VNCs of wild-type (A,C,E) and *sca-GAL4*/UAS-*eg* (B,D,F) first instar larvae. Wild-type (A) and *sca-GAL4*/UAS-*eg* (B) stained with anti-serotonin (green). In the *sca-GAL4*/UAS-*eg* vNC 1 to 2 ectopic serotonergic cells are apparent in most hemisegments (arrowheads). Wild-type (C,E) and *sca-GAL4*/UAS-*eg* (D,F) vNCs double stained with anti-serotonin (green) and anti-DDC (red; C,D) and with anti-serotonin (green) and anti-TH (red; E,F). In wild-type TH and DDC-expressing DL-cells are indicated by white arrows. In *sca-GAL4*/UAS-*eg*, serotonin and es-neurons (arrowheads) are double stained (yellow) whereas putative DL-cells express only DDC respectively TH (D,F) (arrows). Anterior is up; dorsal view; midline: open arrow.

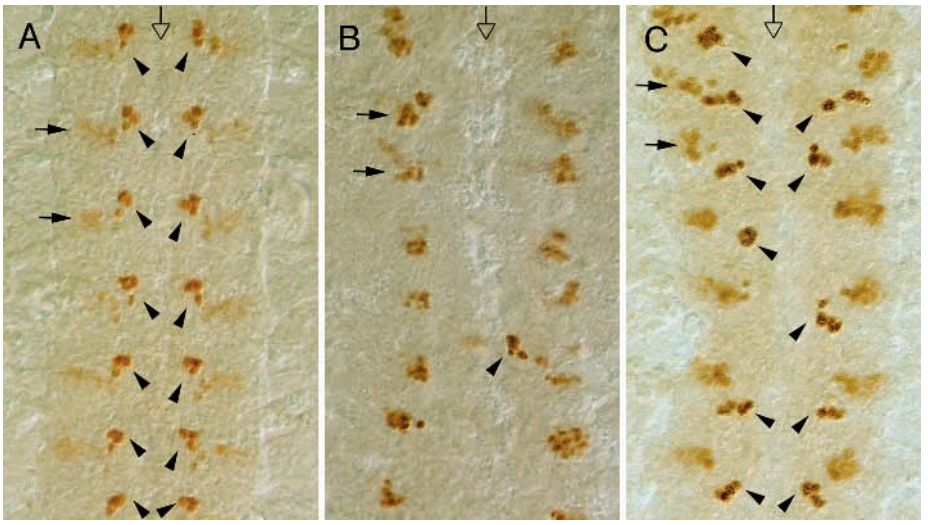

Fig. 6. Eagle expression in *en* and *hkb* embryos. Abdominal segments of wild-type (A), *en* (B) and *hkb* (C) stage 16 embryos stained with anti-Eg. (A) In wild type all four NB 7-3 progeny show strong Eagle expression (arrowheads). (B) In *en* embryos most hemisegments lack Eagle expression at the position of NB 7-3 neurons. Only in a few hemisegments, we observe Eagle expression at this position (arrowhead). (C) Most *hkb* hemineuromeres show wild-type Eagle expression in NB 7-3 neurons (arrowheads). However, in a few segments, there is no staining at the position of these cells. In A-C arrows point to lateral Eagle-expressing cells (out of focus in A and C). Anterior is up; dorsal view; midline: open arrow.

involved in the process of serotonergic differentiation, but acts further upstream by activating *eg* expression.

hkb acts in combination with *eg* to promote serotonergic differentiation

Another transcription factor important for NB 7-3 development is *hkb*. In *hkb* mutants, serotonin differentiation is severely disturbed (Lundell et al., 1996). Analogous to *en*, we wanted to elucidate the functional relationship between *hkb* and *eg*. We therefore stained hkb^{2}, a strong hypomorphic allele (Weigel et al., 1990), with anti-Eg antiserum. *eg* expression is observed in all NB 7-3 progeny (7-3I; 7-3M) in approximately 80% (n=100) of hkb^{2} hemineuromeres. A complete loss of *eg* expression at the position of NB 7-3 neurons occurs in only 20% (n=100) of mutant hemisegments (Fig. 6C). This loss of *eg* expression cannot exclusively account for the observed serotonin phenotype in *hkb* mutants, since earlier studies revealed a loss of 80% of the ventral ganglion serotonin cells in hkb^{62}, another hypomorphic *hkb* allele (Lundell et al., 1996). Thus, the majority of 7-3I cells in *hkb* embryos express *eg* but fail to exhibit serotonin expression.

To rule out the possibility that *eg* regulates *hkb* expression in the NB 7-3 lineage, we analysed *hkb* RNA expression in eg^{P289}. In early stage 11 wild-type embryos, *hkb* is expressed, among other cells, in a neuroectodermal cluster at the position where NB 7-3 will delaminate (Chu-LaGraff et al., 1995). At late stage 11, when the S5 NBs have formed, NB 7-3 is the posteriormost NB-expressing *hkb* (Chu-La Graff et al., 1995). This is also the case in eg^{P289} embryos (data not shown), indicating that *eg* is not necessary for correct *hkb* expression in NB 7-3, but rather acts in parallel with *hkb*.

To investigate whether a combinatorial function of *eg* and *hkb* is generally necessary to promote serotonergic differentiation, ectopic *eg* expression was induced by *sca-Gal4* in a hkb^{2}-mutant background. This was designed to test whether the differentiation of es-neurons is also dependent on the function of *hkb*. For the analysis of the first instar larval CNS, we took advantage of the fact that the wild-type and ectopic serotonin neurons can be distinguished by their position, and that the es-neurons show no *inv* expression (Fig. 7A; see also previous section). This experiment revealed that approximately 80% of the wild-type and ectopic serotonin cells are absent (Fig. 7B). Previous work on *hkb* indicates a cell autonomous function of this gene (Chu-La Graff et al., 1995; Bossing et al., 1996a). Therefore this finding strongly suggests that the ectopic serotonergic neurons are generated by a *hkb*-positive NB and, furthermore, together with the data regarding the *eg* and *hkb* mutant phenotypes of the NB 7-3 lineage (see above), implies that the Eg and Hkb transcription factors are part of a combinatorial code of regulatory proteins necessary for promoting the serotonergic cell fate.

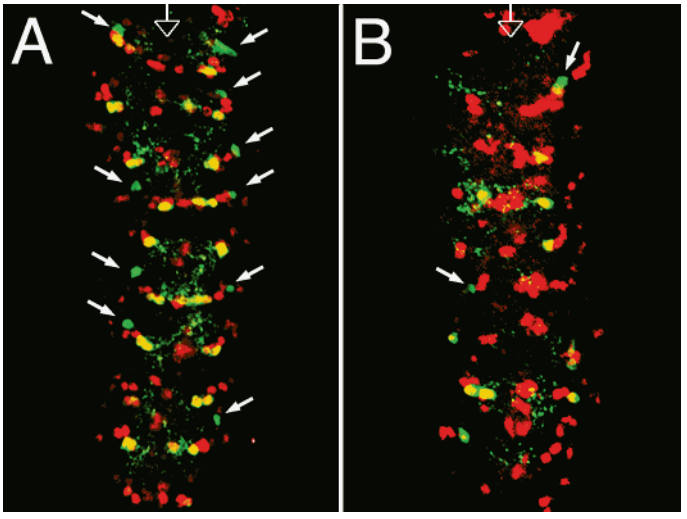

Fig. 7. Differentiation of ectopic serotonergic neurons depends on *hkb* function. VNCs of *sca-GAL4*/UAS-*eg* (A) and hkb^{2}-mutant *sca-GAL4*/UAS-*eg* (B) first instar larvae double stained with anti-serotonin (green) and anti-Inv (red). In contrast to the wild-type serotonin cells, the ectopic serotonergic neurons do not express *en/inv* (arrow). In the hkb^{2}-mutant vNCs 80% of wild-type and ectopic serotonergic neurons are absent (B). Anterior is up; dorsal view; midline: open arrow.

DISCUSSION

To investigate mechanisms controlling the development of a

specific NB lineage, we have chosen the lineage of NB 7-3 as a model system. NB 7-3 has the advantage of generating a small number of progeny (4 to 5), two of which representing serotonergic neurons. Here we analysed the role of *eg*, a gene coding for a zinc finger protein (Higashijima et al., 1996), which is expressed in NB 7-3 and all its progeny.

eg exerts multiple functions within the NB 7-3 lineage

Although the cell number of the NB 7-3 lineage is not tagma specific in wild-type embryos (Bossing et al., 1996b), we find a thorax-specific abnormality in NB 7-3 clones of *eg* embryos. The mutant clones in the thorax consist of 6 to 8 cells, as compared to only 4 to 5 cells in wild type. This suggests that there are differences in the proliferative capabilities within the thoracic and abdominal NB 7-3 lineages and the function of *eg* might be necessary to restrict these in thoracic neuromeres.

Besides this segment-specific phenotype, we observe striking defects in the differentiation of the 7-3I neurons in all truncal segments. These are pathfinding defects and lack of serotonin expression.

How does the lack of the *eg* gene lead to these differentiation defects? Since *eg* codes for a DNA-binding protein, one possible mechanism is that it directly regulates the genes whose products are involved in serotonin synthesis, pathfinding of the 7-3I fibres and/or restriction of cell proliferation in the thorax. This direct regulation is possible with respect to axonal guidance and NB proliferation, since Eg is expressed at the appropriate time, ie. during axogenesis and in the neuroblast after proliferation has ceased. However, it is not compatible with a direct function of *eg* in serotonin expression, since Eg protein fades in 7-3I neurons at the onset of DDC expression (this paper; Lundell and Hirsh, 1994) which catalyses the second step of serotonin biosynthesis. Thus, a more likely explanation for the differentiation defects observed in the mutant NB 7-3 lineage is that loss of *eg* leads to cell fate changes at an early stage. This is also supported by the observation that *eg* is able to promote ectopic serotonergic differentiation only after misexpression in progenitor cells.

Despite this abnormal development of the NB 7-3 lineage in *eg* embryos, certain features of the abdominal clones, like cell number, the average cell positions and the 7-3M projection appear normal. Together with the information about time and position of the NB delamination, these wild-type characteristics allow us to unambigously identify the mutant clones and indicate that loss of *eg* does not transform NB 7-3 into a different NB identity; instead, *eg* seems to be autonomously required for the execution of distinct differentiation features within the lineage. Similar observations have been made for the function of *hkb* in the NB 4-2 (Chu-LaGraff et al., 1995), NB 1-1 and NB 2-2 lineages (Bossing et al., 1996a).

Regulatory and functional interactions between *en*, *eg* and *hkb* in elaborating serotonergic differentiation

en, *eg* and *hkb* are all expressed in the NB 7-3 lineage, and loss-of-function mutations in any of these genes lead to a nearly complete absence of serotonin differentiation (this paper; Lundell et al., 1996). What is the regulatory and/or functional relationship between these three genes in the differentiation of the serotonergic neurons?

In 90% of *en* mutant hemisegments, we observed a complete loss of *eg* expression at the position of the NB 7-3 neurons and this is the case throughout neurogenesis. Taking into account that three additional NB 7-3 lineage markers (Inv, hkb-lacZ and serotonin) are lacking in *en* embryos (Lundell et al., 1996), we conclude that, in most neuromeres of these embryos, NB 7-3 is unable to develop properly or is not formed. Recent results indicate that genes belonging to the segment polarity group have a function in neuroblast formation (Chu-LaGraff and Doe, 1993; Hartenstein et al., 1994; Skeath et al., 1995; Bhat, 1996; Matsuzaki and Saigo, 1996). Thus, it would not be surprising if *en* has similar functions in the generation of NB 7-3. However, it is also possible that NB 7-3 still delaminates in *en* embryos but fails to express any marker tested so far. In any case, loss of serotonin differentiation in *en* embryos is preceded by the loss of *eg* and probably also *hkb* expression within the NB 7-3 lineage.

Although *en* mutant embryos show a loss of *eg*-positive NB 7-3 in most hemisegments, it is still present in approximately 10% of the hemisegments, even in *en/inv* double mutants (see also Matsuzaki and Saigo, 1996). Therefore, there must be additional genes turning on *eg* expression in the absence of *en/inv* function. One of these genes might be *hkb*. We observe a complete loss of Eg expression at the position of NB 7-3 and its progeny in 20% of *hkb* mutant hemisegments. This loss of *eg* expression indicates an early function of *hkb* in NB 7-3 development consistent with the fact that *hkb* is already expressed in the neuroectodermal cluster at the position where NB 7-3 will delaminate (Chu-LaGraff et al., 1995). Additionally, a combined function of *wingless (wg)* and *hedgehog (hh)* has been shown to be necessary for the formation of NB 7-3 (Matsuzaki and Saigo, 1996) and this may also include turning on *eg* expression in NB 7-3.

The nearly complete loss of serotonin immunoreactivity in *hkb* embryos (Lundell et al., 1996), while having *eg* expression in NB 7-3 progeny in most hemisegments, shows that in the absence of *hkb* the majority of 7-3I cells are present but fail to express serotonin. This indicates that *hkb* also has a late function in NB 7-3 development and is, like *eg*, necessary for establishing serotonergic cell fate within its lineage (Fig. 8).

Upon early expression of Eg in the entire developing nervous system, 1 to 2 es-neurons were found in most hemisegments. This shows that *eg* is not only necessary but in some cases also sufficient for the specification of these cells. The fact that only a few cells are able to switch towards a serotonergic pathway suggests that Eg needs the function of additional factors to promote serotonergic differentiation. One of these factors is Hkb since severe reduction of es-neurons caused by *eg* overexpression is seen in the absence of *hkb* function. However, since there are nine NBs per hemineuromere expressing *hkb*, the coexpression of *eg* and *hkb* cannot be sufficient for triggering serotonergic cell fate. This is consistent with the fact that two of the *eg*-expressing NBs, NB 2-4 and NB 7-3, coexpress *hkb* (Doe, 1992) but only NB 7-3 generates the serotonergic neurons. Since the NB 7-3 lineage is the only lineage expressing *en* in addition to *eg* and *hkb*, En might be another factor necessary for es-neuron development. However, we have circumstantial evidence that this is not the case: unlike the endogenous serotonergic cells, the es-cells do not show anti-Inv immunoreactivity, an antibody that labels all *en*-

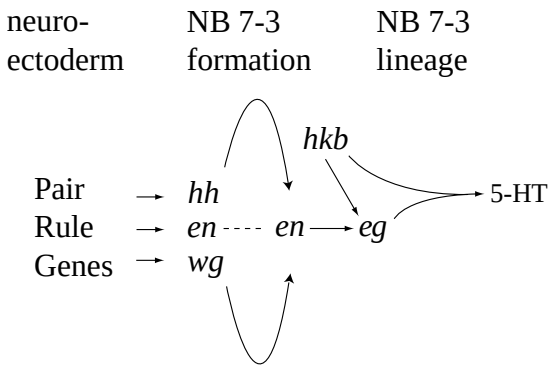

Fig. 8. Regulatory and functional gene interactions during NB 7-3 development. First, the pair rule genes establish *hh, wg* and *en* expression domains in the neuroectoderm. *hh* and *wg* are redundantly required for NB 7-3 formation. *en* and *hkb* are necessary to turn on *eg* expression in NB 7-3. The combined action of *eg* and *hkb* is subsequently required, most likely in the NB or GMCs, to establish serotonin (5-HT) differentiation in two of the NB 7-3 progeny.

expressing cells. In addition, ectopic expression of *eg* using *en-GAL4* did not reveal any es-neurons.

Thus, although necessary for turning on *eg* expression and possibly also for NB formation, *en* function seems to be dispensable with respect to serotonergic differentiation, once the expression of the essential combination of transcription factors is established. Among these essential factors are the Eg and Hkb proteins. Currently we do not know how and at which level these two zinc finger proteins interact, but judging by our ectopic expression studies, it is very likely that both proteins are needed early in lineage development.

Therefore, we propose that *eg* and *hkb* represent a subset of NB and/or GMC identity genes necessary for the generation of serotonergic neurons. Upon overexpression of *eg* the ectopic combination of *eg* and *hkb* together with other unknown factors confers a 'partial NB 7-3 lineage identity' to another unidentified NB lineage. The function of these genes is downstream of segment polarity genes that are involved in NB determination in the neuroectoderm (Chu-La Graff et al., 1993; Skeath et al., 1995; Bhat, 1996; Matsuzaki and Saigo, 1996; see Fig. 8). As already mentioned, in case of NB 7-3, it was recently shown that *wg* and *hh* are redundantly required for the formation of this NB (Matsuzaki and Saigo, 1996). Another player in NB 7-3 formation is *gooseberry (gsb). gsb* is expressed in the row 6 neuroectoderm and seems to repress NB 7-3 cell fate in this region (Patel et al., 1989; Matsuzaki and Saigo, 1996).

The combined function of *hh, wg* and *en* leads to the expression of *eg* and probably other NB 7-3 identity genes like *hkb*, necessary for the development of serotonergic neurons. At present, we do not know which genes act further downstream to finally execute the expression of serotonin. However, good candidates exist that may be directly involved in the regulation of DDC, including two POU domain proteins (for review see: Herr et al., 1988; Ruvkun and Finney, 1991) and a homeodomain protein with a zinc finger motif. One of the POU domain proteins, Cf1a (Johnson and Hirsh, 1990), also called Drifter (Drf) or Ventral veinlet (Vvl) (Anderson et al., 1995; deCelis et al., 1995) was originally identified by its ability to bind to a *DDC* upstream regulating element. Consistent with its possible role in *DDC* regulation, *Cf1a* is expressed in a subset of specific neurons including the serotonergic neurons (Treacy et al., 1991). The second gene, coding for the alternatively spliced transcripts *I-POU* and *twin of I-POU* (Treacy et al., 1992), is expressed in a highly overlapping pattern within the nervous system again including the serotonin cells. This is also true for the third gene, *zfh-2*, which codes for a DNA-binding protein possessing three homeodomains and sixteen zinc fingers, and which was also shown to bind to the *DDC* promoter region (Lundell and Hirsh, 1992). Further work needs to be carried out to elucidate if these three genes are really involved in DDC regulation and if they are downstream of *eg* and/or *hkb*.

Ectopic *eg* also promotes aspects of dopaminergic differentiation

Besides DDC the biosynthesis of serotonin is carried out by a second enzyme: Tryptophan Hydroxylase (TPH) (Wright, 1987). Both enzymes are expressed in serotonergic as well as dopaminergic cells, although TPH is not needed for dopamine synthesis (Vallés and White, 1986; Beall and Hirsh, 1987; Konrad and Marsh, 1987; Neckameyer and White, 1992). In contrast to this, only dopaminergic cells express TH. Upon ectopic *eg* expression, we found colocalisation of serotonin and TH in some of the es-cells and in the cells identified as the wild-type serotonergic cells. This ectopic TH expression is unexpected, since coexpression of serotonin and TH is not obtained in wild type. Here the serotonergic neurons are not TH positive although they express *eg* and, in *eg* mutants, the TH expression is not affected. The fact that the expression of high levels of Eg protein within the *eagle* lineages using eg^{Mz360}*-GAL4*/UAS-*eg* also leads to ectopic TH expression in the serotonergic cells shows that this relies on a cell autonomous mechanism. It suggests that TH, DDC and probably also TPH share common regulatory mechanisms that will be activated as a consequence of high *eg* expression. Interestingly, the expression patterns of TH, TPH and DDC in the vNC are identical, with the exception of TH not being expressed in the serotonin cells (Beall and Hirsh, 1987; Neckamayer and White, 1992; Lundell and Hirsh, 1994). We therefore postulate a mechanism within the developing NB 7-3 lineage that leads to a selective block of TH expression in the serotonergic neurons and which can be overridden by a high level of Eg protein. According to this hypothesis *eg* would be able to promote the decision towards a serotonergic and aspects of dopaminergic cell fate, which would be further specified through the action of additional genes. However, the fact that the dopaminergic cells are unaffected in the *eg* mutant shows that there must be at least one other pathway leading to dopaminergic differentiation.

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