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Hunchback is required for the specification of the early sublineage of neuroblast 7-3 in the *Drosophila* central nervous system

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SUMMARY

The *Drosophila* ventral nerve cord (VNC) derives from neuroblasts (NBs), which mostly divide in a stem cell mode and give rise to defined NB lineages characterized by specific sets of sequentially generated neurons and/or glia cells. To understand how different cell types are generated within a NB lineage, we have focused on the NB7-3 lineage as a model system. This NB gives rise to four individually identifiable neurons and we show that these cells are generated from three different ganglion mother cells (GMCs). The finding that the transcription factor Hunchback (Hb) is expressed in the early sublineage of NB7-3, which consists of the early NB and the first GMC (GMC7-3a) and its progeny (EW1 and GW), prompted us to investigate its possible role in NB7-3 lineage development. Our analysis revealed that loss of *hb* results in a lack of the normally Hb-positive neurons, while the later-born neurons (designated as EW2 and EW3) are still present. However, overexpression of *hb* in the whole lineage leads to additional cells with the characteristics of GMC7-3a-derived neurons, at the cost of EW2 and EW3. Thus, *hb* is an important determinant in specifying early sublineage identity in the NB7-3 lineage. Using Even-skipped (Eve) as a marker, we have additionally shown that *hb* is also needed for the determination and/or differentiation of several other early-born neurons, indicating that this gene is an important player in sequential cell fate specification within the *Drosophila* CNS.

Key words: *Drosophila*, NB sublineage genes, *hunchback*, Central nervous system, Serotonin, Corazonin

INTRODUCTION

The central nervous system (CNS) of all higher organisms consists of a large variety of different neuronal and glial cell types that build up a highly complex but reproducible cellular network. Thus, one challenging question in developmental neurobiology is how these many different neural cell types are specified in a correct spatiotemporal pattern during development. While several reports exist on neural fate specification with respect to position (reviewed by Bhat, 1999), virtually nothing is known about how neuronal diversity is organized with respect to the temporal axis. Therefore, it is of high interest to unravel genetic interactions that lead to the differential specification of neuronal cell fates in specific time windows. Owing to its accessibility and relative simplicity, the *Drosophila* ventral nerve cord (VNC) provides a very good model system with which to answer such questions.

The VNC of *Drosophila* develops from neural precursor cells, the so called neuroblasts (NBs), which delaminate in five successive waves. Each NB is characterized by its time of delamination, its position and gene expression (Broadus et al., 1995; Doe, 1992). Most NBs divide asymmetrically several times and are believed to generate one ganglion mother cell (GMC) at each division, which subsequently produces two neurons and/or glia cells (Hartenstein et al., 1987). At the end of embryogenesis, the VNC consists of ~320 neurons and ~30 glia cells per hemineuromere. Previous work indicates that NBs give rise to characteristic reproducible cell lineages (Bossing et al., 1996; Schmidt et al., 1997). As specific neurons have always been shown to derive from the first born GMC of the parental NB, it has been suggested that NB lineages are generated in a fixed temporal sequence (Brody and Odenwald, 2000). This has highlighted *Drosophila* NB lineages as being a very good model system for studying sequential neural cell fate specification.

Recently, the involvement of a set of genes encoding transcription factors that are expressed sequentially within most, if not all, NB lineages, has been detected (Kambadur et al., 1998; Brody and Odenwald, 2000). This ‘gene cassette’ consists of the genes *hunchback* (*hb*), the redundantly acting POU domain protein 1 (*pdm1*; *nub* – FlyBase) and POU domain protein 2 (*pdm2*), *castor* (*cas*), and *grainyhead* (*grh*) (Brody and Odenwald, 2000), which are expressed in early-, middle- and late-born neurons of NB lineages. Furthermore, *klumpfuss* (*klu*) has to be added to this list, as it is also expressed in NB sublineages (Yang et al., 1997). The successive time periods of *hb*, *pdm1* and *pdm2* and *cas* expression is partially established through the inhibition of *pdm1* and *pdm2* expression via *hb* and *cas* in early and late sublineages, respectively. The expression patterns of these

genes clearly suggest a function in sequential determination of neural cell types. Indeed, *pdm1* and *pdm2* (Johnson and Hirsh, 1990; Billin et al., 1991; Dick et al., 1991; Lloyd and Sakonju, 1991; Prakash et al., 1992; Yang et al., 1993; Bhat and Schedl, 1994; Ng et al., 1995; Yeo et al., 1995), *cas* (Mellerick et al., 1992; Cui and Doe, 1992; Cui and Doe, 1995) and *klu* (Yang et al., 1997) have a role in neural cell fate specification. However, a function of *hb* and *grh* in CNS development has not yet been shown.

In this study, we have analyzed the function of *hb* within NB lineages by focusing on the NB7-3. We chose this lineage for two reasons: first, it is small, consisting of only four neurons (Bossing et al., 1996; Schmid et al., 1999); and second, all NB7-3 neurons can be distinguished by their characteristic position, expression of marker genes and/or their transmitter phenotype (Bossing et al., 1996; Higashijima et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998) (this paper). Using BrdU incorporation, we show that the NB7-3 neurons derive from three GMCs. We found that *hb* is expressed in the early NB7-3, its first GMC (GMC7-3a) and its progeny, and that this gene is indeed necessary for the specification and/or maintenance of the early-born neurons within this lineage. Ectopic expression of *hb* in the later born sublineage is sufficient to transform these cells into neurons with the same fate as the GMC7-3a progeny. Moreover, additional neurons of early GMC7-3a fate are observed. We also found that *hb* might play a similar role in at least one other NB lineage – NB 7-1. Thus, our data support the hypothesis that *hb* is an important early cell fate determinant in the process of sequential cell fate specification within the CNS development.

MATERIALS AND METHODS

Fly strains and genetics

The following fly strains were used in this work: Oregon R (wild type); *eagle*Gal4 (*Mz360*) (Dittrich et al., 1997); *scabrous*Gal4 (Budnik et al., 1996); *engrailed*Gal4 (Tabata et al., 1995); *UAS-hb* (second and third chromosomal insertion) (Wimmer et al., 2000); *CycA*^{3}*/TM3, sb, Ubx-lacZ* (Lehner and O'Farrell, 1989); *Df(3L)H99/TM6b, abdA-lacZ*; *hb*^{12}*/TM3,Sb*; and *hb*^{9}*/TM3,Sb* (Bloomington stock center).

BrdU experiments

BrdU injection was as described previously (Prokop and Technau, 1991) with the following changes: the embryos were chemically dechorionized with 7.5% bleach shortly before injection. After injection they were allowed to develop until stage 15 and subsequently fixed with heptane saturated with 37% formaldehyde for 20 minutes.

Immunohistochemistry

Embryos and L1 larval brains were fixed, stained and mounted as described previously (Dittrich et al., 1997). Primary antibodies used were rat anti-Hunchback (1:100), rat anti-Sal (1:250), mouse anti-Zfh-1 (1:300), mouse anti-Zfh-2 (1:200), rabbit anti-Eagle (1:1000), mouse anti-Eagle (1:200), rabbit anti-Pdm-1 (1:500), rabbit anti-Serotonin (1:5000, Sigma), rabbit anti-Corazonin (1:2000), mouse anti-Eve (1:2, Developmental Studies Hybridoma Bank), rabbit anti-Eve (1:1000), rabbit anti-Castor (1:500) and anti-DIG-AP (1:1000, Roche). The secondary antibodies anti-rabbit-TRITC, anti-mouse-FITC and anti-rat-FITC from goat (Jackson Laboratories) were used at a 1:250 dilution. The mounted embryos were analyzed with a confocal laser scanning microscope (Leica TCS SPII). Scanning images were processed with Adobe Photoshop Macintosh Version 5.5.

Whole mount in situ hybridization

DIG-labeled RNA probe was synthesized with SP6 RNA Polymerase and pGEM-dSERT as a template according to the manufacturer's protocol (Roche). The hybridization on embryos was performed as described previously (Tautz and Pfeifle, 1989; Plickert et al., 1997).

RESULTS

All cells of the NB7-3 lineage can be identified individually

To investigate mechanisms involved in the specification of cell fates within the *Drosophila* CNS, we have chosen the lineage of the abdominal NB7-3 as a model system. The NB7-3 and all of its progeny express the transcription factor *eagle* (*eg*) (Higashijima et al., 1996; Dittrich et al., 1997), allowing a reliable identification of the NB7-3 derived neurons. The abdominal NB7-3 generates three contralaterally projecting interneurons (EW1, EW2, EW3) and one motoneuron [GW; nomenclature according to Higashijima et al. (Higashijima et al., 1996)] each of which can be identified by a combination of specific markers (see Fig. 1). As the GW motorneuron is always the most posterior cell within the NB7-3-derived cell cluster, it can easily be distinguished from the interneurons by its position (Higashijima et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998). Using this as a criterion we found that GW is the only NB7-3 derived neuron which expresses the transcription factor Zfh-1 (see Fig. 1E, Fig. 4D). Previous work has shown that from the second thoracic (T2) to the seventh abdominal segment (A7), the two most medially lying interneurons, EW1 and EW2 (see Fig. 1B), express the neurotransmitter Serotonin (5-HT) (Fig. 1D,E) (Lundell et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998). Furthermore, we found that the most laterally lying EW3 synthesizes the neuropeptide Corazonin (Crz; see Fig. 1C,D) (Veenstra, 1989; Cantera et al., 1994). As Serotonin as well as Corazonin antibody staining worked earliest in brains of freshly hatched L1 larvae, we used dSerT as a marker. The transcript for the *Drosophila Serotonin transporter* (*dSerT*) is specifically expressed in the serotonergic cells from stage 15 onwards (see Fig. 4J). Thus, we performed *dSerT* mRNA in situ hybridization to differentiate between the serotonergic and the corazonergic cells in the embryo. Additionally, anti-Zfh-2 antibody was used to distinguish between the two serotonergic neurons, as Zfh-2 is expressed only in the serotonergic EW2 and the corazonergic EW3 neuron.

The NB7-3 neurons are generated from three GMCs

To determine the sibling relationships of the individual neurons of the NB7-3 lineage, BrdU incorporation experiments were performed. BrdU is a thymidine analog that is incorporated into DNA during replication (S-phase). Thus, all cells going through an S-phase after BrdU application are labeled (Bodmer et al., 1989; Prokop and Technau, 1991). According to existing models of NB division, NBs produce a chain of GMCs, which subsequently divide once to generate a pair of neurons (Hartenstein et al., 1987). Based on this assumption, we expected the four progeny neurons of NB7-3 to be generated by two GMCs, which would mean that the two early- and the two late-born neurons should always co-label with BrdU. Conversely, if two neurons do not co-label, they must derive from different GMCs.

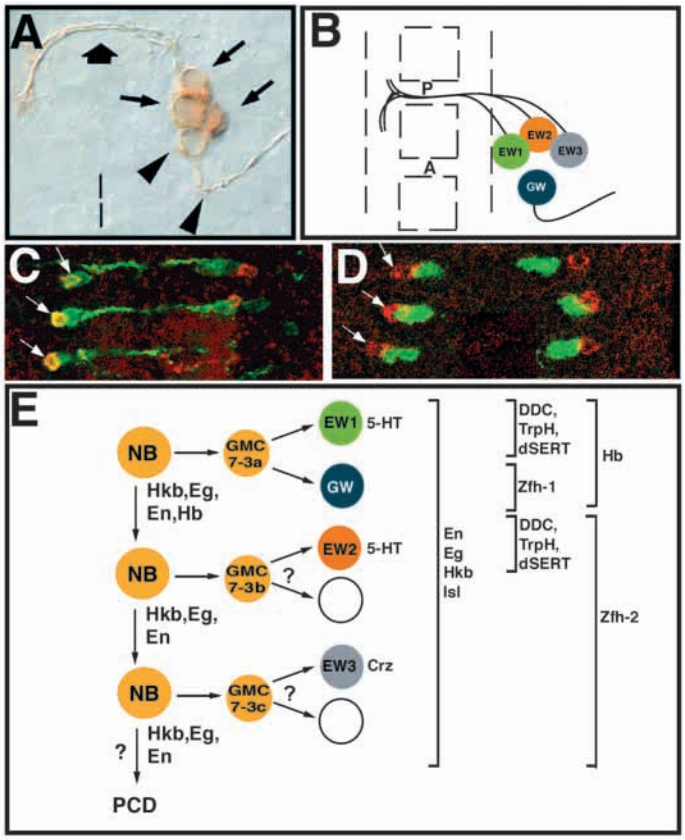


Fig. 1. All neurons of the NB7-3 lineage can be identified individually. (A) The NB7-3 lineage as revealed by DiI labeling (Bossing et al., 1996): three interneurons (arrows) with contralateral projections across the posterior commissure (thick arrow) and one motorneuron (arrowheads). Anterior is upwards; broken line indicates the midline. (B) Schematic representation of the NB7-3-derived neurons with their designations. Anterior is upwards; P, posterior commissure; A, anterior commissure. (C,D) Dorsal view of the VNC of an *eg*Gal4::GFP (C) and a wild-type (D) L1 larva. Anterior is upwards. Crz is stained in red, while GFP (C) and Serotonin (D) are stained in green. One Eg-positive NB7-3 derived neuron co-expresses Crz in the segments T2 to A6 (yellow in C; arrows). This neuron lies in most cases lateral to the serotonergic neurons (D, arrows) in the EW3 position. (E) NB7-3 lineage development showing markers and genealogy of the lineage. The markers listed are expressed in the postmitotic neurons as indicated by each bracket. 5-HT, Serotonin; Crz, Corazonin; DDC, Dopa-decarboxylase; dSERT, *Drosophila* Serotonin transporter; Eg, Eagle; En, Engrailed; Hb, Hunchback; Hkb, Huckebein; Isl, Islet; Pdm-1, POU-domain protein 1; TrpH, Tryptophan hydroxylase; Zfh-1 and Zfh-2: Zinc-finger homeodomain 1 and zinc-finger homeodomain 2; PCD, programmed cell death; NB, neuroblast; GMC, ganglion mother cell.

NB7-3 delamination takes place at about 6.5 hours after egg laying (AEL). Therefore BrdU was injected into embryos at two time points: between 6.5 and 7 hours AEL and between 7 and 7.5 hours AEL. After injection these embryos were allowed to develop until stage 15 and stained with anti-BrdU and anti-Eg antibody. At stage 15 all NB7-3 derived cells are postmitotic as they express the neuronal marker Elav (Robinow and White, 1991) (data not shown). Out of 111 abdominal NB7-3 lineages derived from injection between 6.5 and 7 hours AEL, 38% were BrdU positive in all four neurons, 55% in three neurons, 5% in two and 2% in one neuron. Injection at a later time point (7 to 7.5 hours AEL) resulted in an increase of NB7-3 lineages with less than three BrdU-positive neurons: 20% of hemisegments showed BrdU labeling in all four neurons, 28% in three neurons, 14% in two neurons and 39% in one neuron (n=153). Injection at around 8.5 hours AEL did not yield any BrdU staining in the NB7-3 cluster, indicating that after that time point there is no more S-phase in this lineage (Fig. 2E). By evaluating these results with respect to the position of the BrdU labeled neurons (see Fig. 2A-E,A′-E′), we found that GW and EW1 must be siblings as they are always labeled together (Fig. 2A,A′,B,B′). This is in contrast to EW2 and EW3, which are very often separately labeled (63%, n=264; Fig. 2B,B′,D,D′). Thus, according to our results, EW1 and GW are siblings, while EW2 and EW3 are generated separately.

We postulate that EW1 and GW are produced by the first GMC of NB7-3 (GMC7-3a) because both cells express *hb*, which has been shown to be expressed by the first cells generated from a neuroblast (Kambadur et al., 1998; Brody and Odenwald, 2000). This assumption is supported by the fact that NB7-3 already expresses *hb* before its first cell division and *hb* expression is subsequently maintained in its first progeny (Fig. 3A,B). This is also supported by the analysis of stage 15 embryos that are mutant for *Cyclin A* (*CycA*). In these mutants, cell division is blocked in the nervous system (Wai et al., 1999) and as a consequence only one, two or three cells were found in the position of the NB7-3 cluster (8%, 75% and 17%, respectively; n=63). When two cells were detected, one was Hb positive in 98% of these cases (n=47; Fig. 2H). This cell was often smaller and lay dorsal to the other cell, showing that it is indeed the first born GMC that stays Hb positive. In wild-type embryos, EW1 and GW are mostly found in the dorsal-most position that is typical for the first-born neurons of an NB lineage (Kambadur et al., 1998). Additionally, we found several NB7-3-derived clusters where EW2 and EW3 were BrdU labeled, while GW and EW1 were not (10%, n=264; Fig. 2C,C′). This indicates that the GMC giving rise to GW and EW1 has finished its S-phase before the precursors of the other two neurons in these cases (Fig. 2C″). However, the fact that a high number of clusters show EW2 to be the only unlabeled cell suggests that the S-phase of the first GMC is often delayed with respect to the S-phase of the EW2 precursor (39%, n=264; Fig. 2B″). This suggests that EW2 is generated by the second GMC (GMC7-3b), while EW3 derives from GMC7-3c supported by the fact that EW3 is always the last cell labeled by BrdU (Fig. 2D,D′). This means that two of the three GMCs produce only one neuron each, which would be unusual as it is believed that GMCs in the insect nervous system normally divide once to produce two sibling neurons (Hartenstein et al., 1987). Possible explanations for this are that either one of the resulting siblings is dying by programmed cell death (PCD) or the GMCs are able to differentiate directly without further cell division or there is a mixture of both. To test whether PCD occurs at all in this lineage, we stained cell death deficient mutant embryos at stage 14 (*Df(3L)H99*) (White et al., 1994) with anti-Eg antibody and found in 68% of hemisegments between six and ten cells at the position of the NB7-3 cluster when compared with four cells in wild type (n=38; Fig. 2F,G). Thus, PCD clearly occurs in this lineage raising the possibility that the siblings of EW2 and/or EW3 are indeed eliminated during wild-type development.

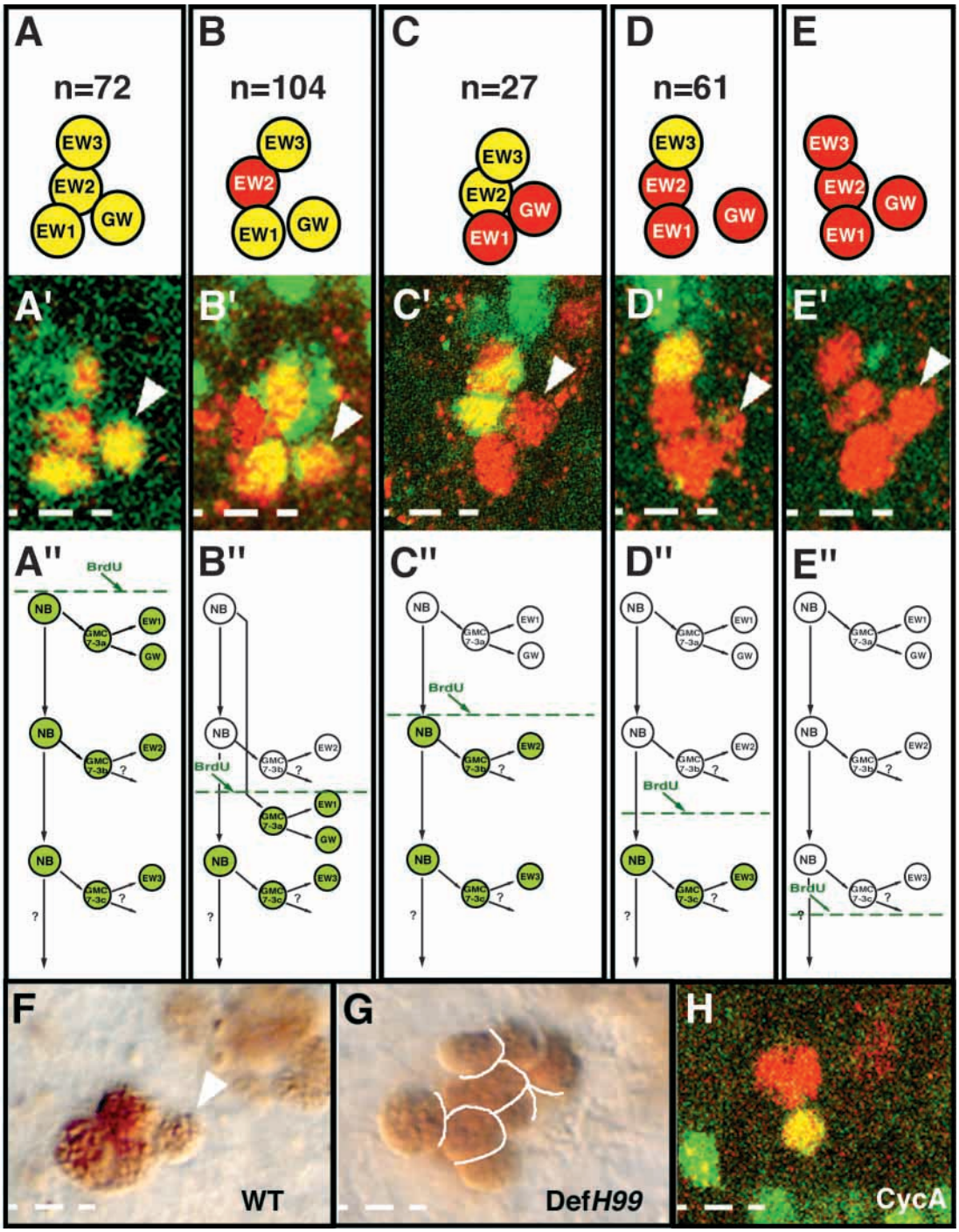


Fig. 2. BrdU incorporation reveals that the NB7-3 lineage generates three GMCs. Dorsal views; anterior is towards the left; the broken line indicates the midline; arrowheads, GW neuron. (A-E) Labeling obtained in NB7-3-derived neurons at stage 15 after BrdU injections between 6.5 and 7.5 hours AEL. The corresponding images are shown underneath (A′-E′). Green, BrdU; red, Eg; yellow, doublestaining. We found all (A,A′), three (B,B′), two (C,C′), one (D,D′) and none (E,E′) of the neurons double labeled (yellow). The EW3 neuron is always labeled last (D,D′). GW and EW1 are always either both labeled together (A,B) or not labeled (C,D) indicating that they are siblings. (A″-E″) Diagram to explain how BrdU incorporation at different times during NB7-3 lineage development affects labelling. Vertical axis is the time axis; green, BrdU incorporation; green horizontal line, timepoint of BrdU application. (F,G) An Eg-positive NB7-3-derived cell cluster in a stage 14 *Df(L3)H99* mutant embryo consisting of eight cells (G), compared with four in wild type (F). (H) In many hemisegments of *CycA* mutant embryos at stage 14, we find two cells in NB7-3 position where the most dorsal cell is smaller and Hb positive (yellow), indicating that this is the first born GMC. Eg is shown in red.

Hunchback is expressed in GMC7-3a and its progeny

After the detailed characterization of the NB7-3 lineage, we next analyzed candidate genes that could be involved in the specification of cell fate during lineage development. A recent publication (Kambadur et al., 1998) suggested that the sequential expression of the transcription factors Hb, Pdm-1, Pdm-2 and Cas might be involved in the specification of different cell fates within sublineages. Antibodies against Hb, Pdm-1 and Cas were used to test whether these proteins are also present in the NB7-3 lineage. As a result, we found *hb* and *pdm-1* but not *cas* to be expressed within the lineage (Fig. 3). At stage 15, Pdm-1 could be detected in all NB7-3-derived neurons (Fig. 3C), although often not all cells of a particular cluster were labeled (not shown). Hb was found only in the early born NB7-3 sublineage, i.e. GW and EW1 (Fig. 3B). Analyses of *hb* expression at earlier stages of development showed that Hb was already present in the NB7-3 shortly after delamination (Fig. 3A, left hemisegment). After the first division of NB7-3, the NB itself and GMC7-3a were Hb positive (Fig. 3A, right hemisegment). In NB7-3 clusters consisting of three cells, all cells expressed *hb* (Fig. 3A, middle hemisegment). Because at

later stages only two neurons were Hb positive, *hb* must be downregulated in the neuroblast after its first division, so that EW2 and EW3 stay Hb negative (Fig. 3B).

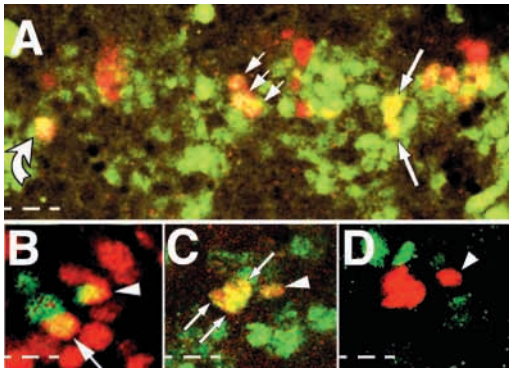


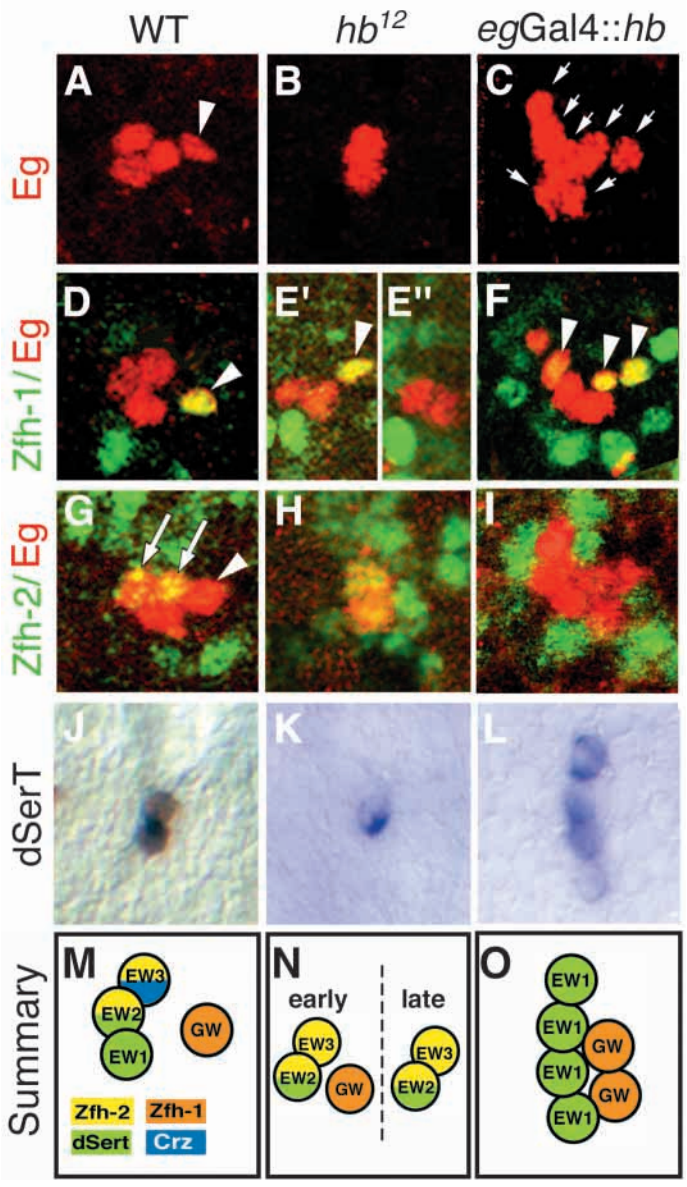
Fig. 3. *hb* and *pdm1*, but not *cas*, are expressed in the NB7-3 lineage. Flat preparations of wild-type embryos. Dorsal views, anterior is leftwards; broken line represents the midline; arrowheads indicate GW. (A) Three hemineuromeres of a stage 12 embryo stained for Hb (green) and Eg (red) showing different phases of NB7-3 lineage development in each hemisegment. Left position: Hb is expressed in NB7-3 (curved arrow) before the generation of progeny. Right position: two Hb-positive cells are detected, i.e. NB7-3 and GMC7-3a (long arrows). Medial position: three Hb-positive cells are seen corresponding to NB7-3 plus the early progeny (short arrows). At stage 15 (B-D), two of the four Eg positive neurons (green) in the NB7-3 lineage are Hb positive (yellow, B). These are always GW (arrowhead) and EW1 (arrow). Hb is shown in red. (C) Pdm1 (green) in the NB7-3 lineage is very variable in stage 15 embryos and is detectable in up to all four Eg-positive neurons (yellow) of the lineage. (D) Cas (green) is not detected in the NB7-3 progeny neurons (red).

Fig. 4. Hb specifies the identity of the first two GMCs. (A-L) Flat preparations of embryos at stage 15 (except E′, which is stage 13); one hemineuromere; dorsal view; anterior is towards the left; arrowheads indicate GW neuron. (A,D,G,J,M) Wild type, (B,E,H,K,N) hb^{12} and (C,F,I,L,O) *eg*Gal4::*hb*. Eg is red; double stained neurons are yellow. (A-C) NB7-3 lineage as revealed by anti-Eg staining. In *hb* mutant embryos at stage 15 (B), only two Eg-positive neurons are detected when compared with wild type (A). (C) Ectopic *hb* expression in the late sublineage leads to additional Eg-positive neurons. Shown is an NB 7-3 lineage consisting of seven Eg-positive cells (arrows). (D-F) Zfh1 (green) within the NB7-3 lineage. (D) In wild type, only the motorneuron (GW) of the NB7-3 lineage is Zfh1 positive (arrowhead). (E) In *hb* mutant embryos at stage 13, this Zfh1-positive neuron can still be detected in those hemisegments that show three Eg positive neurons (arrowhead, E′). At a later stage (stage 15, E″), this Eg-positive neuron has disappeared. (F) In *eg*Gal4::*hb* embryos, often two or more Zfh1-positive neurons can be found in the NB 7-3 lineage (arrowheads). (G-I) Zfh2 (green) within the NB7-3 lineage. (G) In wild type, Zfh2 labels exclusively the late born neurons EW2 and EW3 (arrows). (H) In *hb* mutant embryos, the remaining two neurons express Zfh2. (I) Ectopic expression of *hb* in the late part of the lineage results in absence of *zfh2* expression in all neurons of the NB7-3 lineage. (J-L) *dSerT* mRNA expression. (J) In wild type, each abdominal hemisegment except the last shows two *dSerT*-positive neurons (EW1 and EW2). (K) In *hb* mutant embryos, only one *dSerT*-positive neuron (EW2) is left, whereas after ectopic *hb* expression, up to four *dSerT* positive neurons (EW1) are obtained (L). (M-O) Summaries of the result obtained in A,D,G,J, B,E′,E″,H,K and C,F,I,L, respectively.

hb mutants exhibit a loss of the early NB7-3 sublineage

To test whether *hb* plays a role in the specification of GMC7-3a and its progeny we analyzed the NB7-3 lineage of embryos mutant for *hb*. To make sure that the obtained phenotypes are indeed related to Hb function we analyzed two independent mutations [the hypomorpic allele hb^{9} (Lehmann and Nüsslein-Volhard, 1987) and the null allele hb^{12} (Tautz et al., 1987; Hulskamp et al., 1994)] in parallel in most experiments. As similar results were obtained with both alleles, we will present the results found with hb^{12}, unless stated otherwise. Because in *hb* mutant embryos the thoracic segments and the last abdominal segment are missing, and segments 7 and 8 are fused (Lehmann and Nüsslein-Volhard, 1987), analyses were carried out only in the abdominal segments A1 to A6.

hb^{12} mutant NB7-3 lineages consisted of two (20%) or three (80%) Eg-positive neurons at stage 13 (Fig. 4E) (n=66) but only two remaining Eg-positive cells at stage 15 (98%, n=50; Fig. 4B,E″). To test which of the cells are missing, we



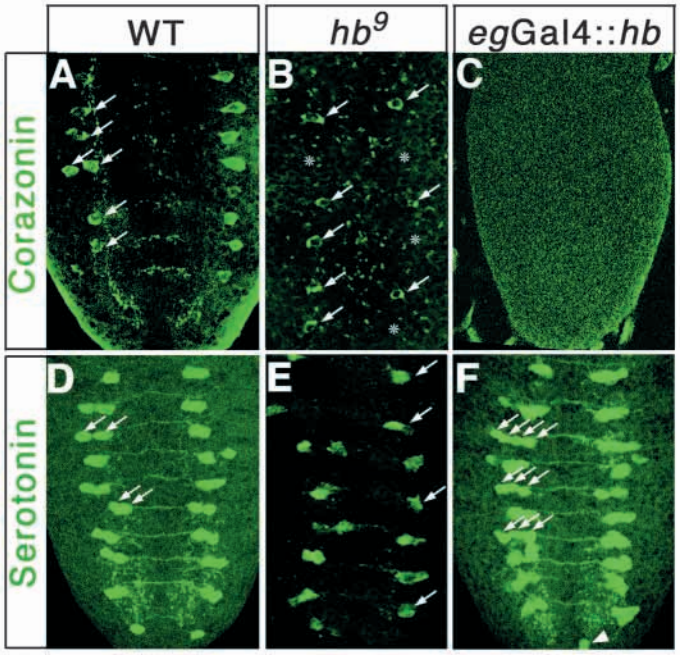


Fig. 5. *hb* affects Serotonin and Corazonin synthesis in the NB7-3 lineage. Ventral nerve cords of L1 larval stage; dorsal view; anterior is upwards. (A-C) anti-Crz; (D-F) anti-Serotonin. (A) In wild-type hemisegments, T2 to A6 show one corazonergic EW3 neuron each (arrows). (B) In the hb^9 allele, Crz (arrows) is missing in a significant number of hemisegments (asterisks). (C) Overexpression of *hb* leads to a complete loss of Crz. (D) In wild-type segments, T2 to A7 show two serotonergic neurons in each hemisegment (arrows), whereas the last segment shows only one serotonergic neuron. (E) In the hb^9 mutant a significant number of hemisegments shows only one serotonergic neuron (arrows). (F) Overexpression of *hb* yields often three to four serotonergic neurons per hemisegment (arrows).

performed double staining with antibodies against cell specific markers. Double staining against Zfh-1 and Eg at stage 13 showed that one posterior lying cell is mostly Zfh-1 positive (98%, *n*=53; Fig. 4E′) suggesting that the GW neuron is initially made, while at the later stage 15 only two Zfh-1-negative cells can be detected (*n*=49; Fig. 4E″). Thus, the GW neuron either does not survive or does not maintain *zfh1* expression at this later time point. The two remaining cells always express *zfh-2* (*n*=72; Fig. 4H), the marker for the late sublineage neurons (Fig. 1E, Fig. 4G). This strongly suggests that in hb^{12} mutant embryos, the neurons of the late NB7-3 sublineage (EW2 and EW3) are still present. These data were supported by RNA in situ hybridization against *dSerT* mRNA in *hb* mutant embryos, showing that only one neuron per hemisegment is labeled (*n*=56; Fig. 4K), which is probably the Zfh2-positive EW2 neuron. In addition, we tested for the presence of the neurotransmitters Serotonin and Corazonin in hb^9 mutant early larval VNCs (Fig. 5). In accordance with the decrease in *dSerT*-positive neurons, a significant reduction of Serotonin levels in the VNC was detected, with about 20% of hemisegments showing only one serotonergic cell (*n*=66; Fig. 5E). However, although the EW3 neuron seemed to be correctly specified as judged by *zfh-2* expression, the Corazonin staining is missing in 39% of the hemineuromeres (*n*=112; Fig. 5B). This suggests that, although present, the EW3 neuron is affected with respect to its transmitter phenotype.

Ectopic Hb leads to additional cells of the early sublineage fate

We next tested whether *hb* is able to transform the fates of late-born cells into those of early-born cells, by expressing *hb* ectopically in the late sublineage using the UAS/Gal4 system (Brand and Perrimon, 1993). By staining *eg*Gal4::*hb* embryos with anti-Eg, we found mostly five to eight NB7-3 derived cells in abdominal segments (82%, *n*=132; Fig. 4C). A subsequent analysis of the expression of marker genes revealed that 67% of the clusters have more than one Zfh1 positive cell (*n*=70; Fig. 4F), whereas 9% of these hemisegments show at least 3 Zfh1 labeled NB7-3 neurons. In addition, all cells of the NB7-3 cluster showed no *zfh2* expression (*n*=68; Fig. 4I). Upon ectopic *hb* expression, the number of *dSerT*-positive neurons was increased by up to three or four cells in 74% of hemisegments (*n*=111; Fig. 4L). Correspondingly, the number of serotonergic neurons was increased in 90% of hemisegments (*n*=70; Fig. 5F) and Corazonin could not be detected (Fig. 5C). Taken together these results indicate that ectopic *hb* expression has two effects: first, the total number of NB7-3-derived neurons is increased; and second, all neurons express markers of the early sublineage, while those of the late sublineage are missing.

Hb has a similar function in other NB lineages

Having found that Hb is an important determinant of early cell fate within the NB 7-3 lineage, we next tested whether this might also be true for other lineages of the *Drosophila* VNC. To do this we chose the *eve* (*even skipped*)-expressing cells, because a known subset of these are early-born progeny of identified NBs (see Fig. 6). Indeed, using antibodies against Eve and Hb in stage 15 embryos, we found co-expression in a dorsal subset of *eve*-expressing cells that consists of aCC, pCC (from GMC1-1a), RP2 (from GMC4-2a) (Fig. 6A′), fpCC and the dorsalmost CQ neuron (most likely the first born neurons from NB7-1; Fig. 6A″) (Bossing et al., 1996). Hb was not found in the NB 3-3-derived EL cells. We subsequently analyzed the function of Hb within these lineages using *spalt* (*sal*) as an additional marker for aCC, pCC and RP2 (Fig. 6B′). One of the EL neurons is also Sal positive (Fig. 6B″) but this cell can be reliably identified by its much more ventral position. We could show that Hb affected the NB7-1-derived fpCC and CQ neurons in a similar way as the GW and EW1 neurons in the NB7-3 lineage. In hb^{12} mutant embryos, the dorsal subset of the NB 7-1 derived neurons was missing in 97% of hemineuromeres (*n*=72): 80% showing a loss of two, 10% of one and 7% of three cells (Fig. 6C″). The missing cells are probably fpCC and/or the dorsalmost CQ neuron, which normally express *hb*. We assume that the missing third cell might be shifted into the EL cluster where it can no longer be identified (Fig. 6C″). Conversely, we observed an increase of the number of Eve-positive neurons in CQ position when we ectopically expressed *hb* via *sca*GAL4 or *engrailed*GAL4 (Fig. 6D″,E″) (Tabata et al., 1995). *en*GAL4 drives *hb* expression in all row 6 and 7 NBs. Among these neuroblasts, only NB7-1 gives rise to Eve-positive neurons (Broadus et al., 1995; Bossing et al., 1996). Therefore, we conclude that these additional neurons are most likely additional fpCCs and/or

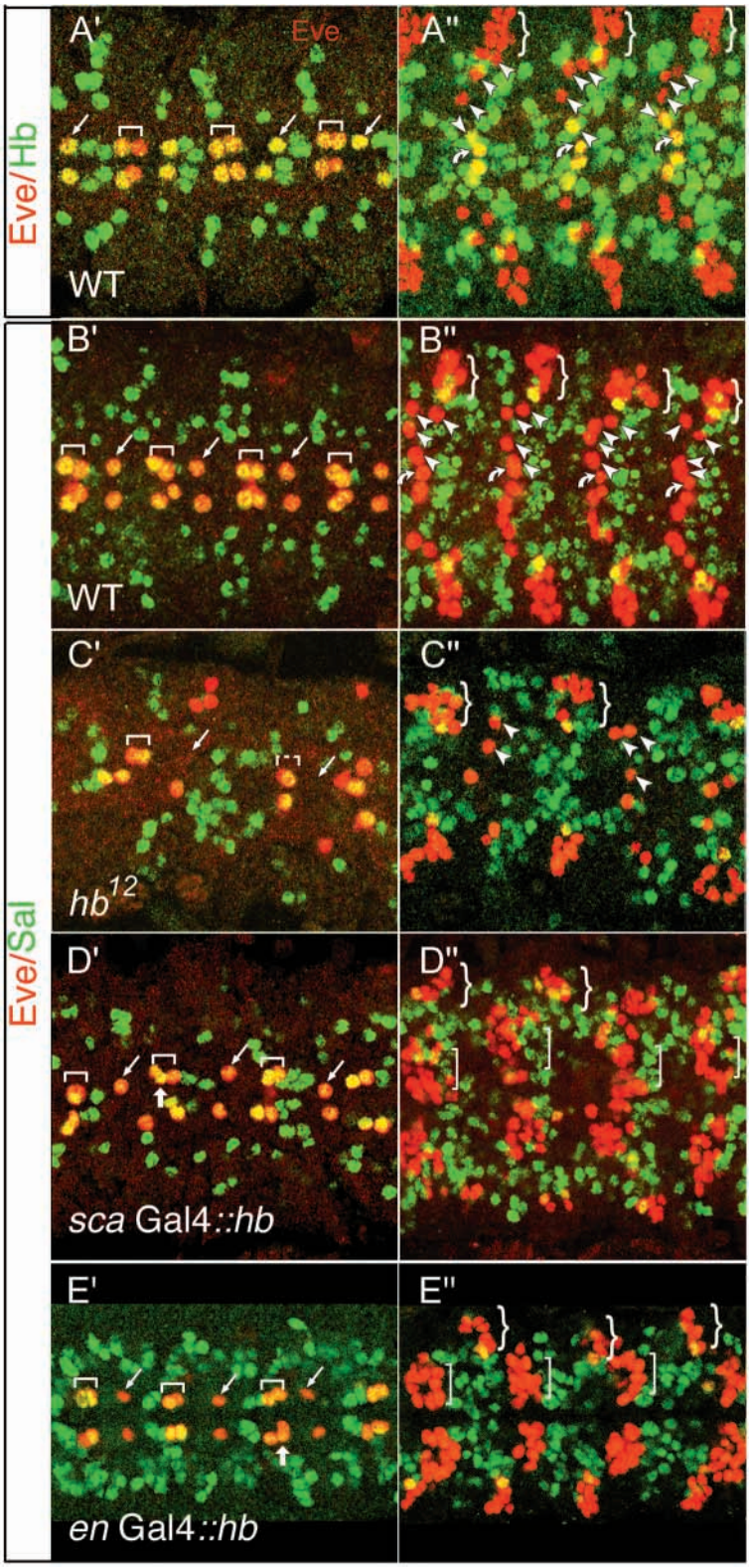


Fig. 6. *hb* affects the number of *eve*-expressing neurons. Flat preparations of embryos at stage 15, dorsal view, anterior is leftwards. Horizontal square brackets, aCC and pCC; vertical square brackets, NB 7-1 derived neurons; thin arrows, RP2; curled brackets, EL neurons; arrowheads, CQ neurons; curved arrows, fpCC. Sections of the confocal images were combined, but for the purpose of clarity presented in two layers: the left images (′) are dorsal planes, the right images are ventral planes (″). Eve is red; Hb (A) and Sal (B-E) are green; double stained neurons are yellow. (A) In wild type, *hb/eve* co-expression is detected in aCC, pCC and RP2 in the most dorsal layer (A′). In the ventral layer (A″) fpCC and the first innermost CQ neuron show *hb* co-expression. (B) In wild type, co-expression with *sal* is shown only in aCC, pCC, RP2 (B′), occasionally in fpCC (not shown) and in one of the EL neurons (B″). (C′,C″) In the *hb* mutant embryo, RP2 (arrow) and either aCC and/or pCC are missing in several hemisegments (C′, broken square bracket). In addition, the number of NB 7-1-derived neurons is decreased (C″, arrowheads). (D′,D″) Ectopic expression of *hb* using *sca*Gal4 as a driver leads to ectopic neurons in medial position (D″, square brackets) and occasionally to additional Sal-positive neurons in aCC/pCC/fpCC positions (D′, thick arrow). (E′,E″) Ectopic expression of *hb* using *en*Gal 4 as a driver gives rise to ectopic neurons in the position of the NB 7-1-derived neurons (large square brackets, E″). One hemisegment shows additional Sal staining in fpCC (thick arrow).

'dorsal' CQ neurons. However, owing to the lack of markers for the Eve-negative cells of the NB7-1 lineage, we could not check whether these additional neurons appear at the cost of other NB7-1-derived neurons.

In addition, other Eve-positive neurons are affected in *hb* loss of function mutant embryos, because either aCC or pCC were missing in ~39%, aCC and pCC in ~11%, and RP2 in ~67% of hemisegments (Fig. 6C′; n=60). In *sca*Gal4::*hb* embryos, rarely one or two additional Sal-positive neurons were observed in the vicinity of aCC and pCC (7%, n=104; Fig. 6D′). However, we could not assign identities to these cells, because the additional neurons could be due to a duplication of aCC and/or pCC or to additional fpCCs, as on rare occasions (less than 1%) fpCC is Sal positive too (Fig. 6E′).

DISCUSSION

In this work, we have analyzed the role of *hb* in CNS development. Using the NB7-3 lineage as a model system, we have found that Hb is an important factor in the determination and/or specification of the *hb*-expressing neurons in the NB7-3 lineage. Additionally, we have shown that other early born neurons are dependent on Hb as well, a result that is independently supported by Isshiki et al. (Isshiki et al., 2001).

NB7-3 generates three GMCs with the first GMC giving rise to the EW1 and GW neurons

By injecting BrdU at different time windows during development, we determined the sibling relationships of the progeny of NB7-3, which consists of two serotonergic interneurons (EW1 and EW2), one interneuron that expresses the neuropeptide Corazonin (EW3) and one motorneuron (GW) (Fig. 1) (Higashijima et al., 1996; Lundell et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998) (this work). According to our results, EW1 and GW are siblings, while EW2 and EW3 are generated sequentially from the NB7-3.

We have strong evidence that EW1 and GW are produced by the first GMC of NB7-3 (GMC7-3a). As EW3 is nearly always the last neuron labeled by BrdU, we hypothesize that this neuron is most likely to be generated by the third GMC, while GMC7-3b gives rise to EW2. The fact that we do not find any siblings for the later-born neurons can be explained in two ways: either the missing sibling is dying by PCD or the GMCs are able to differentiate directly without further cell division. We currently cannot conclude which of these possibilities is true but investigations in apoptosis-deficient mutant embryos clearly show that PCD occurs within the NB7-3 lineage. The frequent occurrence of more than six NB7-3 derived cells in these mutant embryos might be due to additional cell divisions of the surviving cells.

Hb is an important determinant for the specification of GMC7-3a

The restricted expression of *hb* in the early NB7-3 (before and shortly after its first division) and the first-born GMC, including its progeny suggest that *hb* is involved in the specification of these cells. Our analysis strongly supports this assumption: in stage 13 *hb* loss-of-function mutant embryos, we found either a complete loss of the GMC7-3a-derived EW1 and GW neurons or of the EW1 neuron alone. At stage 15 the GW neuron could also no longer be detected. Therefore, we conclude that the loss of Hb cannot transform GMC7-3a into GMC7-3b or GMC7-3c (Fig. 7B). This is different from the findings of Isshiki et al. (Isshiki et al., 2001); they found a duplication of EW2 neurons in a low number of cases. We do not know why this difference occurs, but it might be due to the use of different *hb* alleles. Our results suggest that either additional factors might be necessary for determining the fates of GMC7-3b and/or c, which are not present in the Hb positive GMC7-3a, or that GMC7-3a harbors additional factor(s) that suppress other GMC fates. However, ectopic expression of *hb* in all cells of the lineage leads to additional neurons of the EW1 and GW type at the cost of neurons normally generated by the later-born GMCs (Fig. 7C). This shows clearly that *hb* is sufficient to induce fates of the first GMC and to suppress those of the later-born GMCs. This can be interpreted in two ways. One possibility is that overexpression of *hb* in the progeny of GMC7-3a is sufficient to reiteratively cause both of these cells to adopt the fate of the parental GMC (GMC7-3a). But as *hb* is strongly expressed in the differentiating progeny of the first GMC anyway, it is very unlikely that a higher dose of Hb has such an effect. Therefore we favor the idea that ectopic Hb in the other GMCs and/or the late NB7-3 (after the first division) is able to transform the fate of these GMCs into the fate of the normally Hb-positive GMC (GMC7-3a). However, the fact that we found a high number of two to three additional EW1-like neurons (as judged by the presence of *dSerT* and absence of *zfh2* expression), but mostly only one additional Zfh1-positive GW neuron suggests that only one additional GMC is fully transformed in most cases. This hints at an additional factor being important for the generation of GW, which is present mainly in one additional GMC beside GMC-1, most probably in the second GMC (Fig. 7C). A good candidate for such a factor appears to be Krüppel (Kr) (Isshiki et al., 2001).

Interestingly, these overexpression experiments give rise to a maximum number of eight Eg-positive cells in the NB7-3 cluster, instead of six, as it would be expected when all three GMCs generate two cells. This could mean that *hb* is able to protect the neuroblast from apoptosis. The supernumerary cell(s) could then be additional progeny generated by the surviving NB or the NB itself.

Loss of Hb function affects the Hb negative corazonergic neuron

Our analysis of the NB7-3 lineage revealed that, in *hb* loss-of-function mutants, the neurons that are normally Hb positive are affected. However, using the hypomorphic allele hb^{9}, which develops into L1 larval stage we could additionally show that the Hb negative EW3 is also affected and fails to express Corazonin in a significant number of cases. Because not only this cell but also the immediate precursor cell(s) must have had a reduced Hb function, we can imagine two scenarios. It is possible that Corazonin expression can be switched on only in

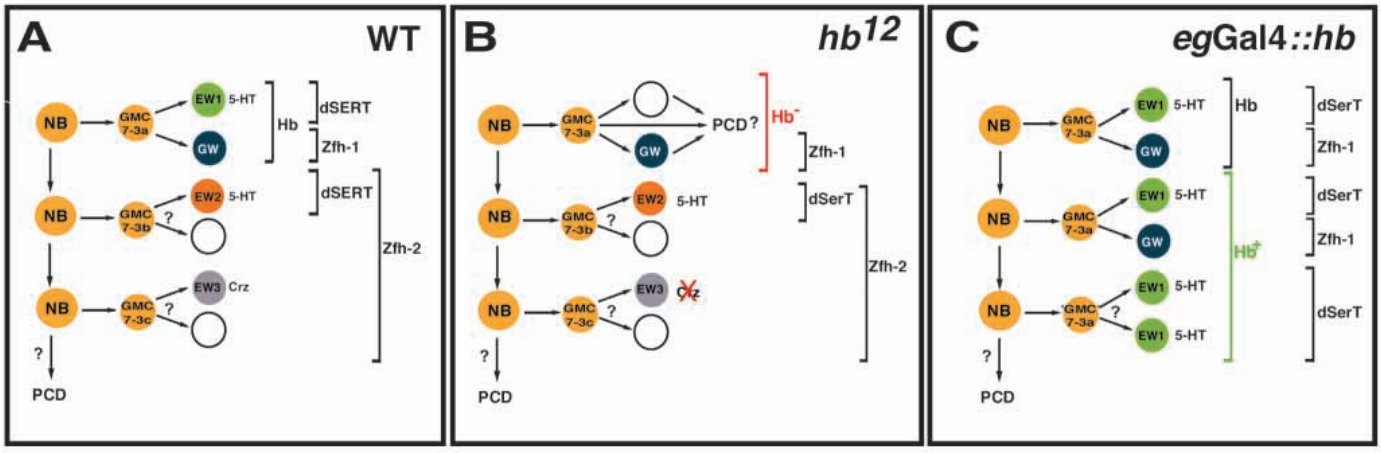


Fig. 7. A model explaining the loss-of-function and gain-of-function phenotypes of *hb* with respect to the NB7-3 lineage. (A) The development of the wild-type NB7-3 showing the relevant markers in the postmitotic neurons. (B) Lack of Hb leads to a complete loss of EW1 and only a transient presence of GW. By stage 15, the missing cells might have died by apoptosis (PCD). Although EW3 is detectable by markers, it does not express the neuropeptide Crz. (C) Overexpression of *hb* might transform the second GMC completely into a GMC7-3a fate, while the third GMC might often generate only EW1 type cells.

the last generated neuron when the NB receives Hb activity during the early phase of lineage development. This would favor the idea that at least some aspects of neuronal fate are dependent on transient gene activity within the stem cell long before the neuron is generated. Alternatively, initiation and/or maintenance of Corazonin expression might depend on cellular interactions such as synaptic inputs or the presence of extrinsic factors that could be altered in the *hb* mutant CNS. This could also explain the difference in our results and those of Isshiki et al. (Isshiki et al., 2001), who report no change in Crz expression in *hb* mutant embryos at a much earlier stage than we have analyzed. That extrinsic factors indeed can play a prominent role in specifying and maintaining neurons, including the transmitter phenotype, has been extensively shown in vertebrates (for a review, see Edlund and Jessell, 1999).

Hb is also needed for early sublineage fates in other lineages

hb-positive cells of other lineages are also affected in the *hb* mutant background: the Eve-expressing neurons RP2 (derived from GMC4-2a) (Doe, 1992) and aCC and/or pCC (derived from GMC1-1a) (Doe, 1992) are frequently undetectable. Additionally, fpCC and/or the dorsalmost of the four CQ neurons (derived from NB7-1) (Bossing et al., 1996) are not found in 97% of the hemineuromeres. These cells are most probably identical to the U1 and U2 neurons described by Isshiki et al. (Isshiki et al., 2001). The lower penetrance of the phenotype in RP2 and aCC/pCC (67% and 50%, respectively) could be due to other cell or lineage specific factors that are co-expressed with *hb* and are able to act redundantly in these cases. In particular, RP2 might be rescued by the presence of Pdm1 and Pdm2 activity in its parental GMC, because it has been shown that these two proteins are necessary for acquiring a GMC4-2a fate (Yang et al., 1993; Yeo et al., 1995). Maternally contributed *hb* (Lehmann and Nüsslein-Volhard, 1987) might also be able to partially rescue the phenotype in the lineages of the early delaminating NB4-2 and NB1-1.

In any case, the phenotype of the loss-of-function mutant embryos suggests that *hb* is, as in NB7-3, involved in the specification or at least maintenance of certain NB progeny. In particular, the role of *hb* with respect to the NB7-1 derived fpCC and dorsal CQ neuron seems to be comparable with what we found in the NB7-3 lineage, as pan-neural *hb* overexpression leads to additional Eve-positive fpCCs and/or CQ neurons. Also, additional Sal-positive neurons were observed in the position of aCC/pCC. This is in agreement with the results of Isshiki et al. (Isshiki et al., 2001) who found a duplication of aCC/pCC.

hb in comparison to other sublineage genes

Our results indicate that *hb* must be added as a functional member of the group of CNS sublineage genes that are involved in the specification of GMCs. The other three genes of this group, which have been investigated on the NB lineage level are the redundantly acting *pdm1* and *pdm2* genes and *klumpfuss* (*klu*). As mentioned above, *pdm1* and *pdm2* have been shown to be involved in the specification of the first GMC (GMC-1) in the NB4-2 lineage (Yang et al., 1993; Yeo et al., 1995). However, ectopic *pdm2* expression did not lead to a transformation of the later-born GMCs as we postulate for *hb* in the NB7-3 lineage, but transformed the progeny of GMC4-2a into the fate of the parental cell (Yang et al., 1993). Thus, although *pdm1* and *pdm2* are able to induce a NB4-2 specific first GMC fate, they can do this only in cells that derive from the first born GMC. This suggests that the 'quality of being GMC-1' (of NB4-2) is independent of Pdm1 and Pdm2 function.

However, in *klu* loss-of-function mutant embryos, the normally Klu-positive GMC-4-2b transforms into the fate of GMC-4-2a (Yang et al., 1997). Thus, in the absence of Klu, GMC4-2b has also a 'GMC-1 quality' and Klu is necessary for the switch to a 'GMC-2' fate. What determines this 'GMC-1 quality' in the NB4-2 lineage is not known, but our results imply that Hb might have such a function during NB7-3 lineage development, as it is able to induce ectopic GMC-1 fate(s) at the cost of the other GMC fates. Preliminary experiments show that, unlike in the NB4-2 lineage, Klu is not necessary for the fate of the second GMC of NB7-3 (data not shown). In accordance with this, Isshiki et al. (Isshiki et al., 2001) have shown that Kr is the factor that specifies the GMC 7-3b fate.

Conclusions

Taken together, our data suggest that there are principle mechanisms leading to the sequential cell specification in NB lineages, but different lineages seem to use either different genes to determine specific sublineages (e.g. *klu*) or use the same genes with different contributions (e.g. *hb*).

In any case, it is clear that an exact temporal regulation of their expression is crucial for the correct specification of neurons along the time axis. Therefore, an important aspect in understanding the sequential sublineage specification within NB lineages, is to know how sublineage genes like *hb* are switched on and off at the right time during lineage development. There are strong hints that the cell cycle plays an important role in this respect (Cui and Doe, 1995; Weigmann and Lehner, 1995). Owing to its accessibility, the NB7-3 lineage provides a powerful tool to investigate this and other related questions in the future.

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