Development 128, 3243-3251 (2001)
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DEV8800

Stage-specific inductive signals in the *Drosophila* neuroectoderm control the temporal sequence of neuroblast specification

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SUMMARY

One of the initial steps of neurogenesis in the *Drosophila* embryo is the delamination of a stereotype set of neural progenitor cells (neuroblasts) from the neuroectoderm. The time window of neuroblast segregation has been divided into five successive waves (S1-S5) in which subsets of neuroblasts with specific identities are formed. To test when identity specification of the various neuroblasts takes place and whether extrinsic signals are involved, we have performed heterochronic transplantation experiments. Single neuroectodermal cells from stage 10 donor embryos (after S2) were transplanted into the neuroectoderm of host embryos at stage 7 (before S1) and vice versa. The fate of these cells was uncovered by their lineages at stage 16/17. Transplanted cells adjusted their fate to the new temporal situation. Late neuroectodermal cells were able to take over the fate of early (S1/S2) neuroblasts. The early neuroectodermal cells preferentially generated late (S4/S5) neuroblasts, despite their reduced time of exposure to the neuroectoderm. Furthermore, neuroblast fates are independent from divisions of neuroectodermal progenitor cells. We conclude from these experiments that neuroblast specification occurs sequentially under the control of non-cell-autonomous and stage-specific inductive signals that act in the neuroectoderm.

Key words: *Drosophila*, CNS, Neuroectoderm, Neuroblast specification

INTRODUCTION

The development of the central nervous system (CNS) involves the transformation of a uniform epithelial sheet, the neuroectoderm, into a complex three-dimensional organ that consists of an enormous diversity of neuronal and glial cell types. To study the mechanistic principles that lead to this diversity and reproducible spatial arrangement of neural cell types, *Drosophila* is a well-suited organism. The segmented CNS (ventral nerve cord) of the *Drosophila* embryo is relatively simple, consisting of approximately 400 cells per hemineuromere. These originate after gastrulation from the ventral neurogenic region of the ectoderm. About 25% of the neuroectodermal cells delaminate into the embryo as CNS progenitor cells, called neuroblasts (NBs). The singling out of the NBs from among neuroectodermal cells is achieved by the activity of proneural and neurogenic genes (reviewed by Skeath and Carroll, 1994; Campos-Ortega, 1995). In each hemisegment approximately 30 NBs delaminate from the neuroectoderm according to a stereotyped spatiotemporal pattern. Each NB delaminates from a specific region of the neuroectoderm to occupy a particular place within the subectodermal NB layer. The process of delamination has been divided into five successive waves (S1-S5) with particular subpopulations of identified NBs delaminating during each wave (Doe, 1992; Fig. 1). Thus, each NB is characterized by a typical position and time of delamination. Furthermore, it expresses a specific set of molecular markers (Doe, 1992; Broadus et al., 1995). Finally, the unique identity of each NB is revealed by the production of a characteristic cell lineage (Bossing et al., 1996; Schmidt et al., 1997).

Crucial steps in the specification of the various NB identities appear to take place before delamination by the interpretation of positional information in the neuroectoderm encoded by segmentation genes and dorsoventral patterning genes (reviewed by Skeath, 1999). Heterotopic transplantation experiments have shown that neuroectodermal cells become committed by these spatial cues to different degrees (Prokop and Technau, 1994; Udolph et al., 1995). For example, whereas dorsal neuroectodermal cells are able to adjust their fate when transplanted to more ventral positions, ventral neuroectodermal cells exhibit firm commitment and produce lineages consistent with their origin. These experiments refer to a given developmental stage (early gastrula, stage 7). However, the time of delamination differs between NBs, and the identity of a given NB correlates with a certain time of delamination. This implies that NB specification requires temporal cues in addition to positional information.

The mechanisms behind the temporal sequence of NB specification are unknown. Different modes of regulation could be envisaged. For example, all NB identities, including the respective times of delamination, might become firmly determined at an early stage and are cell-autonomously expressed during further development. Alternatively, progenitor cells might acquire NB-identities sequentially under the influence of extrinsic signals. To test whether the developmental

potencies of neuroectodermal progenitor cells change over time and whether inductive signals are involved, we have manipulated the temporal axis independently from spatial cues by performing heterochronic transplantations of neuroectodermal cells. Neuroectodermal cells were transplanted from stage 7 donors (early gastrula, before S1) into stage 10 hosts (after S2), and vice versa. The identities assumed by these cells were determined by analyzing their lineages in the host embryos at stage 16/17. We show that in both experimental situations, neuroectodermal cells are able to adjust their fate to the new environment. Late neuroectodermal cells can generate early (S1, S2) NBs. Early neuroectodermal cells preferentially produced late (S3-S5) NB lineages, despite having been exposed to the neuroectoderm for a significantly reduced period of time. We also show that late NB fates are independent of previous divisions of neuroectodermal progenitor cells. These data suggest that extrinsic inductive signals exist in the neuroectoderm that change over time to control the specification of temporal subsets of neuroblasts.

MATERIALS AND METHODS

Flies

Oregon R was used as wild-type strain. Standard methods were used to rear flies and collect embryos.

Cell transplantations

Cell transplantations were performed as described (Prokop and Technau, 1993). Donor embryos were labeled by injecting a mixture of 4% fluorescein-isothiocyanate-dextran (FITC-dextran, Sigma) and 6% horseradish peroxidase (HRP, Boehringer Mannheim) diluted in 0.2 M KCl. Transplantation of cells was monitored using an inverted fluorescence microscope (Leica Fluovert). Ectodermal cells were transplanted either isotopical heterochronically (stage 10 to stage 7 or stage 7 to stage 10), heterotopical isochronically (stage 7 to stage 7), heterotopical heterochronically (stage 10 to stage 7), or isotopical isochronically (stage 10 to stage 10; see Fig. 2). The position for removal of cells at stage 7 was defined with respect to the first morphological landmarks and according to the early gastrula fate map (see Technau and Campos-Ortega, 1985; Technau, 1987). Because of germ band extension, the ventral neurogenic region narrows down from about 15 cell diameters to seven cell diameters; neuroectodermal cells at stage 10 were therefore removed from donors mounted on the ventral side using the midline as a landmark. Neuroectodermal cells were transplanted from all dorsoventral levels of the ventral neurogenic region, which spans between 0% (ventral midline) and 50% VD (% ventrodorsal perimeter) at stage 7, and between 0% and 25% VD at stage 10. Dorsal ectodermal cells at stage 10 were removed about three cell diameters apart from the amnioserosa from donor embryos mounted on the lateral side. Donor cells were individually transplanted into the ventral neurogenic region of host embryos. Host embryos were allowed to develop until stage 16/17. Flat preparations or whole mounts were fixed and then stained for HRP-labeled cell clones (Prokop and Technau, 1993). Clones were analyzed and documented with a Zeiss Axioplan equipped with a Kontron camera (Progress 3012). Different focal planes were combined using Adobe Photoshop 5.0 and 5.5. All embryonic stages given are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

RESULTS

To test the impact of cell-autonomous properties versus inductive signals on the temporal sequence of NB specification, we traced the fate of neuroectodermal cells upon transplantation into a heterochronic background (see Materials and Methods; Fig. 2). HRP-labeled cells were taken from and transplanted into the neuroectoderm of embryos at two different stages: stage 7 (early gastrula), before delamination of NBs from the neuroectoderm, and stage 10 (elongated germ band), after the first two waves of NB delamination. The identity assumed by the transplanted cells under these experimental conditions was determined by the identification of their lineages (Bossing et al., 1996; Schmidt et al., 1997) upon staining for HRP of host embryos at stage 16/17.

We will refer to NBs that normally delaminate during stage 8 and 9 (S1, S2) as ‘early NBs’, and those that normally delaminate during stages 10 and 11 (S3-S5) as ‘late NBs’.

Late neuroectodermal cells retain competence for extrinsic signals leading to early NB fates

In one set of experiments, neuroectodermal cells from stage 10 embryos were heterochronically transplanted into the neuroectoderm of 2 hours younger, early gastrula (stage 7) hosts

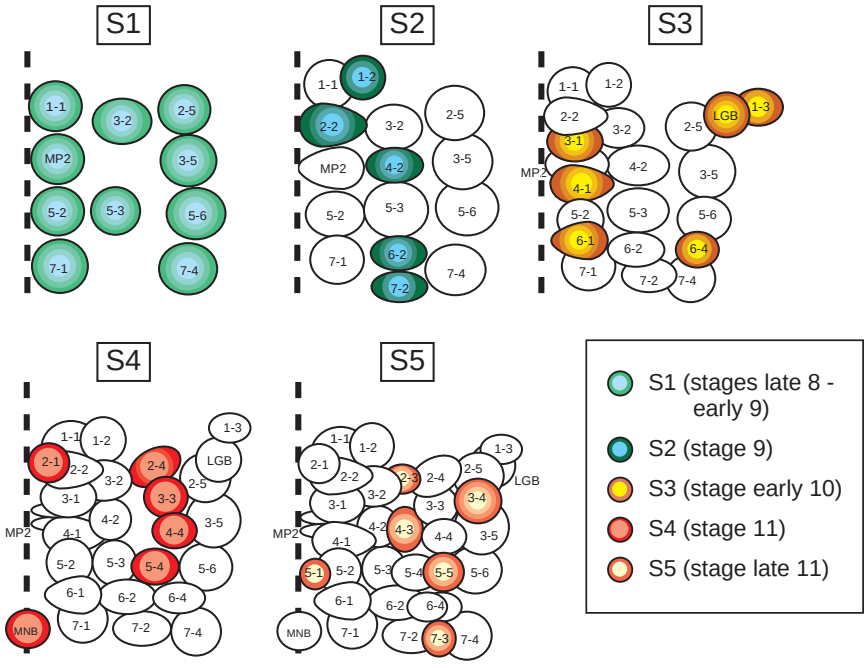

Fig. 1. Spatial arrangement and temporal sequence (S1-S5) of segregating neuroblasts (Doe, 1992; Bossing et al., 1996). Each map represents the pattern of one hemisegment (thorax, abdomen) with those NBs highlighted that are added during the respective wave of segregation. Anterior is upwards; ventral midline is marked by broken line.

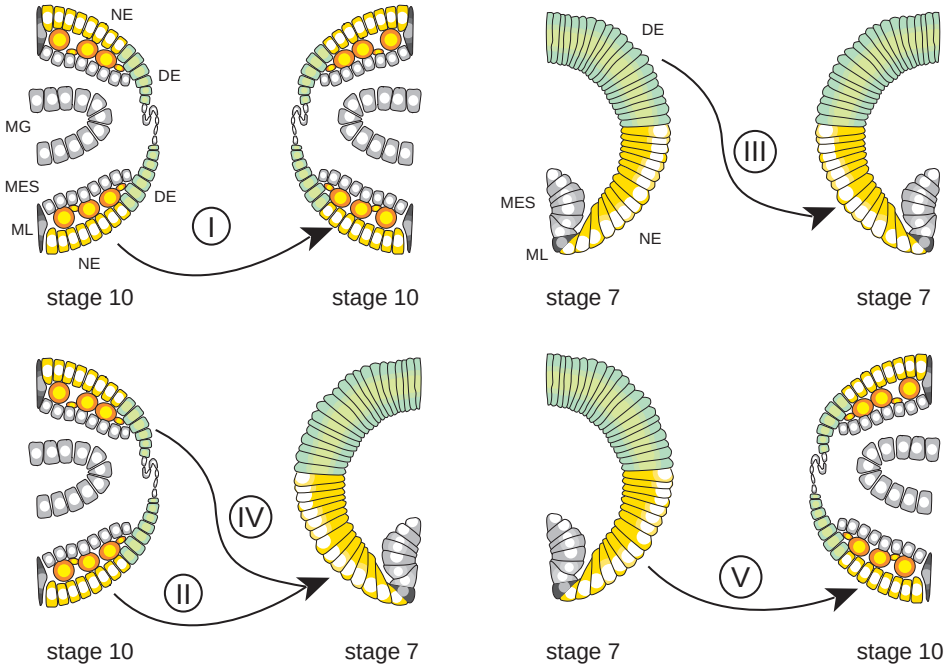

Fig. 2. Transplantation experiments. Half cross sections through embryos at stage 7 (early gastrula) and stage 10 (elongated germ band). Dorsoventral extent of the neuroectoderm (NE) is marked in yellow. Owing to germ band extension, the respective anlagen appear twice in cross sections through abdominal segments at stage 10. Cells were transplanted isochronical isotopically (experiment I, stage 10 to stage 10), isochronical heterotopically (experiment III, stage 7 to stage 7), heterochronical heterotopically (experiment IV, stage 10 to stage 7) or heterochronical isotopically (experiment II, stage 10 to stage 7 or experiment V, stage 7 to stage 10). DE, dorsal ectoderm (green); MES, mesoderm, MG, midgut, ML, midline (gray for all three tissues); NE, neuroectoderm (yellow); S1 and S2 NBs, which are already delaminated at stage 10, are marked in orange.

(see experiment II in Fig. 2). The transplanted cells gave rise to CNS clones, or to epidermal clones, or to mixed CNS/epidermal clones (see Table 1). This is consistent with previous data (Technau et al., 1988), and shows that despite their more advanced age, the implanted cells participate in the cell interaction process that leads to the decision of neurectodermal cells between an epidermogenic and a neurogenic fate. Remarkably, however, among the cells that follow the neural pathway, about 50% produced lineages typical for early NBs (S1, S2), as for example, NB1-1, MP2, NB2-2 or NB4-2 (Table 2 and Fig. 3). This indicates that neuroectodermal cells at stage 10, which normally only give rise to late NB lineages, have not lost the potency to assume identities of early NBs.

Why do the late neuroectodermal cells maintain this potency under the given experimental conditions? One possibility is that cells select an arbitrary NB fate, owing to technical artifacts. Alternatively, the cells express early NB fates autonomously after being released from signals that normally inhibit these fates in the late neuroectoderm. Finally, inductive signals leading to early NB fates might exist in the early neuroectoderm and the implanted late cells are competent to interpret these signals. We tested these possibilities by two further experiments.

First, neuroectodermal cells were isochronically transplanted from stage 10 donors into stage 10 hosts (experiment I, Fig. 2). With one exception (S2), all CNS clones obtained from these cells (*n*=17) corresponded to the lineages of late NBs (Table 2). For comparison, when

Table 1. Distribution of neural and epidermal clones obtained from transplanted ectodermal precursor cells

Transplantation experiment	Neural clones (Clones from midline precursors)	Neural clones (Clones from neuroblasts)	Mixed neural/epidermal clones	Epidermal clones
(I) Isotopic (ventral), isochronic (stage 10)	–	*n*=16 (*n*=16 (43%))	*n*=1 (3%)	*n*=20 (54%)
(II) Isotopic (ventral), heterochronic (stage 10 to stage 7)	(*n*=13 (20%))	*n*=44 (*n*=31 (48%))	*n*=4 (6%)	*n*=17 (26%)
(III) Heterotopic (dorsal to ventral), isochronic (stage 7)	(*n*=5 (20%))	*n*=16 (*n*=11 (44%))	–	*n*=9 (36%)
(IV) Heterotopic (dorsal to ventral), heterochronic (stage 10 to stage 7)	(*n*=2 (20%))	*n*=6 (*n*=4 (40%))	*n*=4 (40%)	–
(V) Isotopic (ventral), heterochronic (stage 7 to stage 10)	–	*n*=18 (*n*=18 (14%))	*n*=6 (5%)	*n*=108 (81%)
Isotopic (ventral), isochronic (stage 7)*		(61%)	(16%)	(23%)

*Distribution of clones obtained from isotopic (ventral), isochronic (stage 7) transplantations (data from Technau and Campos-Ortega, 1987 are shown for comparison).

For transplantation experiments I-V, see Fig. 2.

n, number of identified clones for each category (% refer to total number).

Table 2. Types of NB clones obtained from transplanted ectodermal cells

	NB clones				
Transplantation experiment	S1	S2	S3	S4	S5
(I) Isotopic (ventral), isochronic (stage 10)		*n*=1 (6%) 7-2 (1)	*n*=8 (47%) 1-3 (3) 3-1 (1) 6-4 (4)	*n*=8 (47%) 2-1 (1) 2-4 (3) 3-3 (2) 4-4 (1) 5-4 (1)	
(II) Isotopic (ventral), heterochronic (stage 10 to stage 7)	*n*=8 (23%) 1-1 (1) 3-2 (1) 3-5 (1) MP2 (2) 5-6 (2) 7-1 (1)	*n*=11 (31%) 1-2 (1) 2-2 (6) 4-2 (2) 6-2 (1) 7-2 (1)	*n*=8 (23%) 3-1 (1) 6-1 (1) 6-4 (6)	*n*=6 (17%) 2-1 (3) 3-3 (1) 4-4 (1) 5-4 (1)	*n*=2 (6%) 5-1 (1) 7-3 (1)
(III) Heterotopic (dorsal to ventral), isochronic (stage 7)	*n*=2 (18%) 2-5 (1) 7-4 (1)	*n*=1 (9%) 2-2 (1)	*n*=8 (73%) 1-3 (5) 6-4 (3)		
(IV) Heterotopic (dorsal to ventral), heterochronic (stage 10 to stage 7)	*n*=2 (25%) MP2 (1) 5-6 (1)	*n*=6 (75%) 1-2 (1) 2-2 (2)			
(V) Isotopic (ventral), heterochronic (stage 7 to stage 10)	*n*=3 (12.5%) 1-1 (2), MP2 (1)	*n*=2 (8%) 6-2 (2)	*n*=6 (25%) 1-3 (1) 3-1 (1) 6-4 (4)	*n*=10 (42%) 2-1 (4) 3-3 (1) 4-4 (2) 5-4 (3)	*n*=3 (12.5%) 4-3 (1) 7-3 (2)
Isotopic (ventral), isochronic (stage 7)*	(49%)	(39%)	(7%)	(2%)	(3%)

n, number of identified clones for each category (% refer to total number).
Individual identities of NB lineages are indicated (number of cases shown in parentheses).
For transplantation experiments I-V, see Fig. 2.
*Distribution of NB clones obtained from isotopic (ventral), isochronic (stage 7) transplantations (data from Udolph et al., 1998 are shown for comparison).

neuroectodermal cells are isochronically transplanted from stage 7 donors into stage 7 hosts, they develop the entire spectrum of NB fates including all temporal subsets (Udolph et al., 1995; Udolph et al., 1998). This excludes any arbitrary behavior of the cells that is due to the technical procedure itself. Instead it shows that the implanted cells react to the surrounding host tissue in a stage-specific manner.

Second, cells were heterotopically transplanted from the dorsal ectoderm, which normally does not contribute to CNS formation, into the ventral neurogenic ectoderm (experiments III, IV; Fig. 2; Table 2). Donors were either at stage 7 (experiment III) or stage 10 (experiment IV); hosts were at stage 7. In both cases, the dorsal ectodermal cells were able to adopt a CNS fate (see also Technau and Campos-Ortega, 1986; Technau et al., 1988; Stüttem and Campos-Ortega, 1991). This transdetermination process occurs under the influence of the surrounding neuroectodermal cells, as dorsal ectodermal cells are unable to autonomously develop as neuroblasts in single cell cultures (Lüer and Technau, 1992). Furthermore, our analysis of the CNS lineages produced by the heterotopically and heterochronically transplanted dorsal ectodermal cells revealed their ability to assume identities of early NBs (Table 2).

Taken together these data indicate, that late ectodermal cells (stage 10) are not irreversibly specified, and that signals exist in the early neuroectoderm (stage7) that are sufficient to induce early NB fates. Thus, instead of being merely based on cell-autonomous properties, the temporal regulation of early NB determination appears to be mediated by extrinsic inductive signals that are active in the early neuroectoderm.

Reduced time of exposure to the neuroectoderm does not prevent formation of late NBs

Having shown that the determination of early NB fates depends on stage specific inductive signals, we next tested whether inductive signals are also involved in the generation of late NB fates. We transplanted cells from the early neuroectoderm (stage 7) heterochronically into the neuroectoderm of stage 10 host embryos (experiment V; Fig. 2). Among 132 identifiable clones obtained from these cells, 24 (19%) were CNS clones and 108 (81%) epidermal clones (Table 1). Closer analysis of the 24 CNS clones revealed that about 80% (*n*=19) of them corresponded to lineages typical for late NBs, like 2-1, 5-4, 6-4 or 7-3, and only 20% (*n*=5) to early NB lineages (Table 2; Fig. 4). Therefore, the transplanted cells tend to adopt to the new temporal environment regarding the identities of NBs to be formed. Although having skipped two hours of exposure to the neuroectoderm, a significant proportion of them can compensate for this lack of time. Thus, the cells are not bound to an intrinsic timer to become specified as late NBs, but are able to react to inductive signals in the late neuroectoderm. The

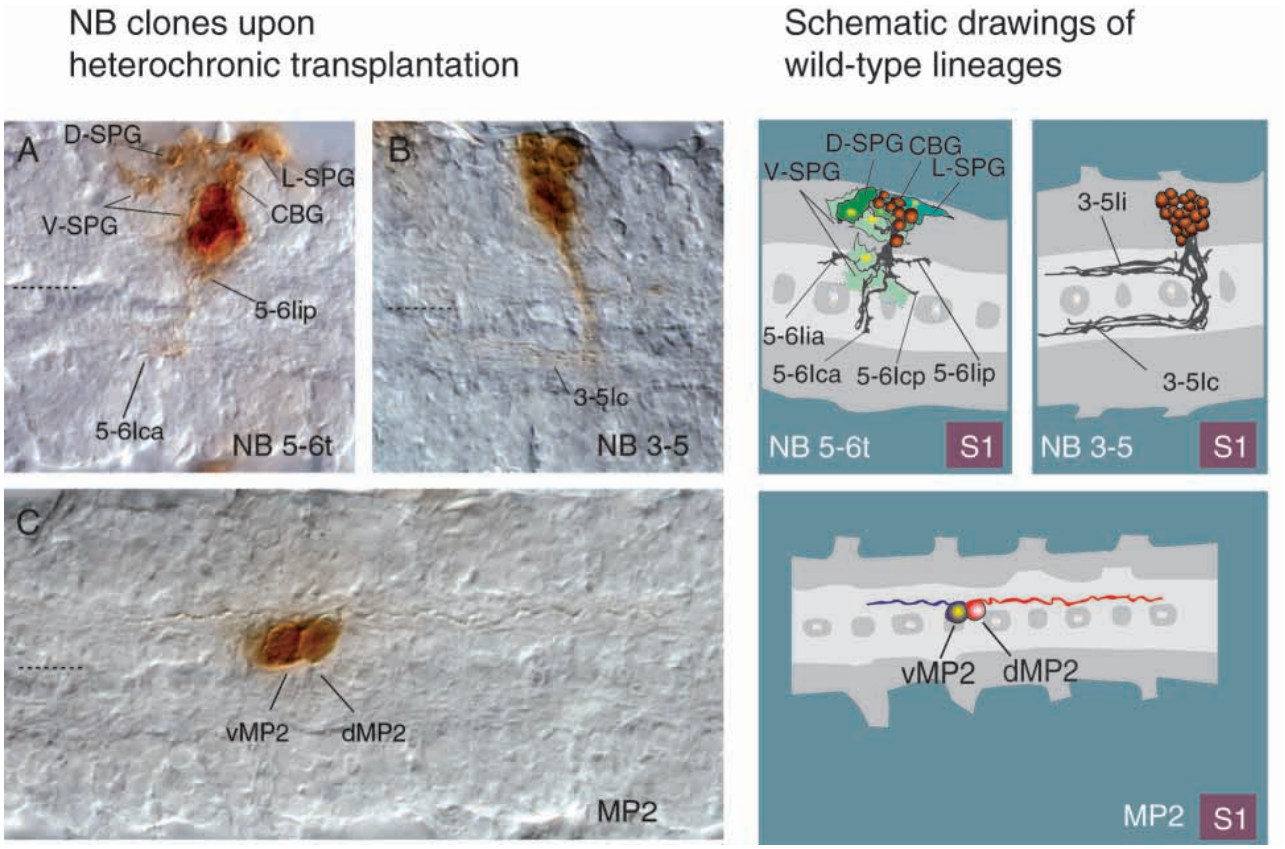

Fig. 3. Upon heterochronic transplantation from donors at stage 10 into the neuroectoderm of host embryos at stage 7 (see Fig. 2, experiment II) neuroectodermal cells can acquire an early NB fate. Horizontal views of HRP-labeled cell clones in the ventral nerve cord at stage 16/17 (A-C); anterior is towards the left; orientation of the CNS midline is marked by broken lines. Schematic drawings of wild-type lineages are shown for comparison (Bossing et al., 1996; Schmidt et al., 1997). (A) NB 5-6 thoracic clone consisting of a cluster of about six neurons with fibers projecting contralaterally through the anterior commissure (5-6Ica), and short ipsilateral projections (5-6 Iip; out of focus), three subperineural glia cells (V-, D-, L-SPG), and one cell body glia cell (CBG). (B) NB 3-5 clone consisting of about 22 interneurons with contralateral projection (3-5Ic) extending through the anterior commissure. Ipsilateral projections normally appear at later stages. (C) Cell clone derived from MP2 consisting of the two interneurons dMP2 and vMP2 with their typical ipsilateral projections posteriorly and anteriorly, respectively.

20% of cells that developed an early NB fate might point to differences in the degrees of commitment of neuroectodermal cells at a given stage or to an insufficient exposure to signaling in the late neuroectoderm under the experimental conditions.

Determination of late NBs does not depend on previous division in the neuroectoderm

As opposed to early NBs the lineages of S4 and S5 NBs, and some of the S3 NBs have an epidermal sister clone (Bossing et al., 1996; Schmidt et al., 1997). This is due to the postblastodermal division pattern of neuroectodermal progenitors (Foe, 1989; Hartenstein et al., 1994). Progenitors developing as S1 and S2 NBs do not divide before delamination from the neuroectoderm, some of those giving rise to S3 NBs divide, and those giving rise to S4 and S5 NBs (NBs 1-3, 2-1, 2-4, 3-3, 4-3, 4-4, 5-1, 5-4, 5-5 and 7-3) always divide in the neuroectoderm. Only one of the daughter cells that results from this division subsequently delaminates as a late NB, the other remains in the periphery to develop as an epidermoblast. We wondered whether this neuroectodermal division is required for late NBs to form and become properly specified.

When neuroectodermal cells are heterochronously transplanted from stage 7 donors into stage 10 hosts (experiment V), they are deprived from the phase in which the first wave of divisions normally runs through the neuroectoderm. As outlined above, most of the CNS clones obtained from these cells corresponded to lineages of late NBs. However, whereas S4 and S5 NBs normally have an obligatory epidermal sister clone, the situation is variable under the experimental conditions (Table 3). For example, the NB2-1 (S4) clone normally has an obligatory epidermal sister clone consisting of two to four cells (Bossing et al., 1996). Fig. 5 shows two NB 2-1 clones obtained upon heterochronic transplantation of a progenitor cell from early to late neuroectoderm. One of these clones has a sister clone consisting of four epidermal cells (Fig. 5A), the other clone is lacking an epidermal sister clone (Fig. 5B).

These data show that: (1) proliferation of individual neuroectodermal progenitors can be influenced by surrounding tissue; (2) late NBs can segregate from the neuroectoderm without having previously divided; and (3) late NBs do not depend on a previous division to acquire an individual identity and to produce their specific and complete CNS lineage. These observations lend further support to the idea that the temporal pattern of NB determination depends on inductive signals in

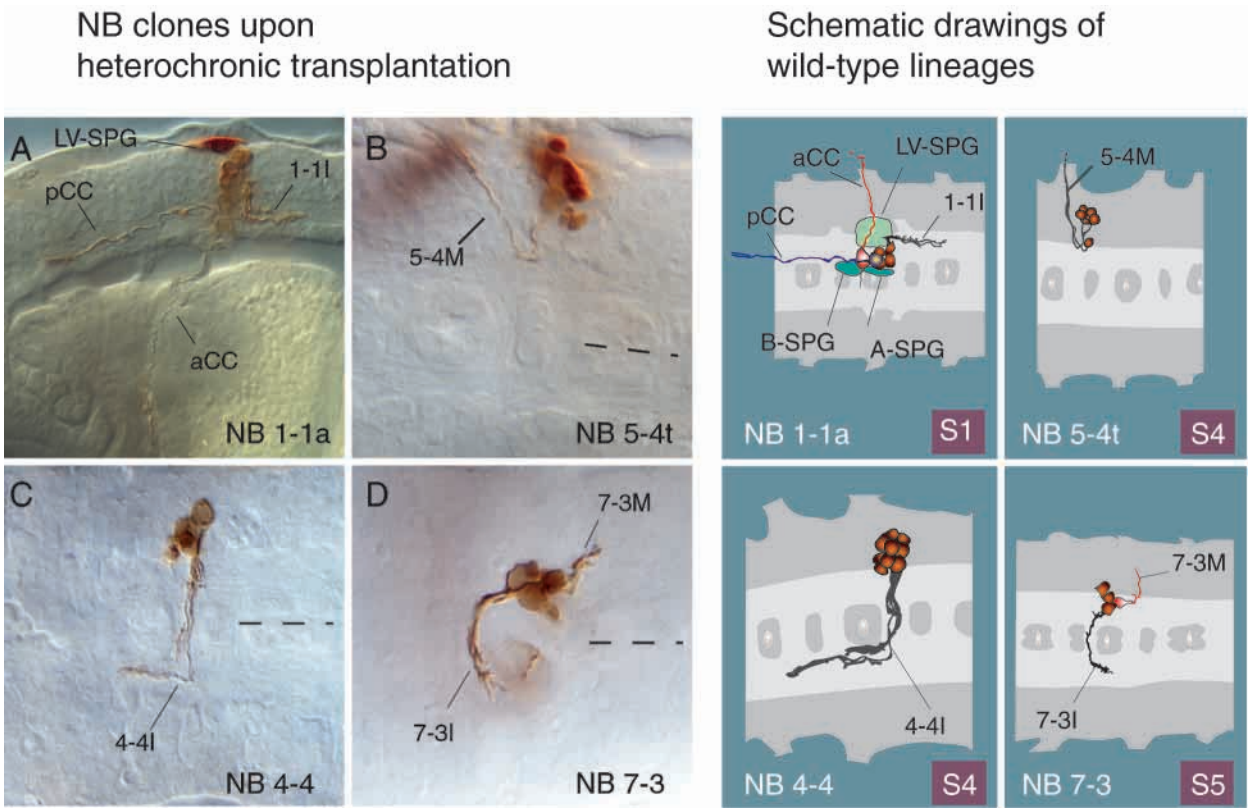

Fig. 4. Neuroectodermal cells at stage 7 transplanted heterochronically into the neuroectoderm of host embryos at stage 10 (see Fig. 2, experiment V) can produce late NB lineages (B-D) or early NB lineages (A). Lateral (A, ventral to the top) and horizontal (B-D) views of HRP-labeled cell clones in the ventral nerve cord at stage 16/17; anterior is towards the left; orientation of the CNS midline is marked by broken lines. Schematic drawings of wild-type lineages are shown for comparison (Bossing et al., 1996; Schmidt et al., 1997). (A) NB 1-1 abdominal clone consisting of a cluster of about eight neurons with ipsilateral interneuronal projection (1-1I), the aCC with its ipsilateral motoneuronal projection and the pCC with its anteriorly extending interneuronal projection. Of the three subperneurial glia cells (LV-SPG, A-SPG and B-SPG) only LV-SPG is present. (B) NB 5-4 thoracic clone consisting of a cluster of about 6 neurons in the lateral cortex and a more medially located neuron. The typical ipsilateral motoneuronal projection (5-4M) exits through the segmental nerve. (C) NB 4-4 clone consisting of about 10 neurons in the ventrolateral cortex with interneuronal contralateral projections (4-4I) extending through the anterior commissure. (D) NB 7-3 clone consisting of four neurons. The motoneuron is lying posteriorly in the cluster of cells with its typical projection extending ipsilaterally (7-3M). The three interneurons form a fascicle (7-3I) that extends contralaterally across the posterior commissure.

the neuroectoderm instead of following a stereotype cell autonomous clock.

DISCUSSION

Neuroblast (NB) formation in the *Drosophila* embryo involves a reproducible spatial pattern as well as a specific temporal sequence. NBs giving rise to the ventral nerve cord delaminate from the neuroectoderm upon gastrulation within a period of approximately 3.5 hours. This period has been subdivided into five phases (S1-S5), and it has been shown that the identity of a given NB also correlates with a specific phase of NB delamination (Doe, 1992; Broadus et al., 1995; Bossing et al., 1996; Schmidt et al., 1997). Although the spatial cues that control identity specification of NBs have been subject of intensive investigations (reviewed by Skeath, 1999; Bhat, 1999), the impact of the temporal sequence of NB delamination is largely unknown. We have used heterochronic transplantations of neuroectodermal cells to test whether the timing of NB formation is crucial for their specification and what is the influence of cell autonomous properties versus extrinsic cues. We show that extrinsic inductive signals in the neuroectoderm play an essential role in the specification of temporal subsets of neuroblasts. Neuroectodermal cells placed into a heterochronous environment are competent to adjust their fate according to these signals. We also provide evidence that cell divisions that normally occur in the neuroectoderm before the delamination of late NBs are not involved in the determination of the identity of late NBs.

Irreversible and reversible effects on determination of neuroectodermal progenitors

The transplantation assay allows to test when and to which degree a certain progenitor cell becomes determined to a specific aspect of its fate, and it allows to distinguish between cell autonomous and non-autonomous influences on the expression of this fate. Heterotopic single cell transplantations have previously revealed differential effects of the parameters of positional information on the degree of commitment of *Drosophila* neuroectodermal progenitors. For example, along the anteroposterior axis, neuroectodermal progenitors are

Table 3. Late NB clones with or without an epidermal sister clone

Transplantation experiment	Late NB clones with epidermal sister clone	Late NB clones without epidermal sister clone
(II) Isotopic (ventral), heterochronic (stage 10 to stage 7)	*n*=4 2-1 7-3 5-4t 5-1	*n*=4 2-1 (2) 3-3 (1) 4-4 (1)
(V) Isotopic (ventral), heterochronic (stage 7 to stage 10)	*n*=6 2-1 4-4 3-3 5-4t 7-3 (2)	*n*=11 2-1 (3) 4-4 (1) 6-4 (4) 5-4 (2) 4-3 (1)

Under normal conditions, an epidermal sister clone is obligatory for NBs 1-3, 2-1, 2-4, 3-3, 4-3, 4-4, 5-1, 5-4, 5-5 and 7-3, and optional for NB6-4.

Epidermal sister clones generally vary between two and four cells (Bossing et al., 1996; Schmidt et al., 1997).

n, number of identified clones for each category.

Individual identities of NB lineages are indicated (number of cases shown in brackets).

For transplantation experiment see Fig. 2.

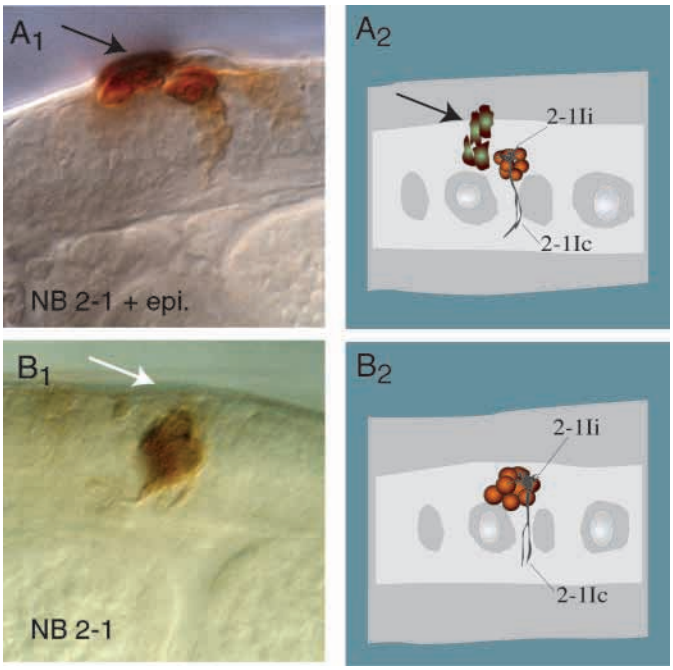

Fig. 5. Late NBs do not require a previous division in the neuroectoderm to be specified. (A1, B1) Lateral views of HRP-labeled cell clones in the ventral nerve cord at stage 16; anterior is towards the left, ventral on top. (A2,B2) Drawings show horizontal views of the same preparations (upon rotation). The clones derived from stage 7 neuroectodermal cells after heterochronical transplantation into the neuroectoderm of host embryos at stage 10. Both cells were determined to delaminate as NB 2-1 (S4), and produced about eight neurons with typical short ipsilateral fibers (2-1Ii) and a contralateral projection through the anterior commissure (2-1Ic). Normally, NB 2-1 is associated with an obligatory epidermal sister clone consisting of four cells, owing to a division of the neuroectodermal precursor (Bossing et al., 1996). The NB2-1 clone in A is associated with a sister clone of four epidermal cells (black arrow). The NB 2-1 clone in B has no epidermal sister clone.

irreversibly committed with regard to segmental identities of NB lineages already at the beginning of gastrulation (stage 7). Cells transplanted from the thoracic into the abdominal neuroectoderm retain their thoracic identities and vice versa (Prokop and Technau, 1994; Prokop et al., 1998). Along the dorsoventral axis, cells at ventral sites of the neuroectoderm are also firmly committed at stage 7 as they retain their fate upon transplantation to more dorsal sites (Udolph et al., 1995). Thus, in these cases the respective positional cues render irreversible effects on cellular identities very early and are cell-autonomously expressed during further development. However, neuroectodermal cells at more dorsal sites at stage 7 are still able to change their fate when exposed to ectopic ventral positions (Udolph et al., 1995; Udolph et al., 1998). Our results from heterochronic transplantations reveal such a plasticity of cell fate also along the temporal axis. Neuroectodermal cells are able to adjust their fate in both directions, i.e. from late to early as well as from early to late NB identities. In these cases, the cells remain competent to sense and properly interpret extrinsic signals.

Taken together, specification of NBs depends on a combination of factors that equip the neuroectoderm with specific spatial and temporal cues. Some of these cues become very early permanently adopted by the developmental program of neuroectodermal progenitors while others influence the fate of these progenitors without restricting their potencies.

Neuroblast fate is independent from divisions of neuroectodermal progenitor cells

Once a neuroblast has formed, the generation of different cell fates within its lineage depends on the sequential expression of particular genes (Goodman and Doe, 1993; Kambadur et al., 1998; Brody and Odenwald, 2000) and the asymmetric distribution of cell fate determinants (reviewed by Fuerstenberg et al., 1998). These processes are tightly associated with cell divisions. Late NBs (S4, S5) have already performed the first postblastodermal division before they delaminate from the neuroectoderm (Foe, 1989; Hartenstein et al., 1994). This division always results in two different cell fates: an epidermoblast and a neuroblast (Schmidt et al., 1997). This is clearly different from early NBs (S1, S2), which delaminate without a previous division in the neuroectoderm. Therefore, one could assume that the first postblastodermal division in the neuroectoderm might be required by precursors to become assigned to the fates of late NBs. However, our data show that this is not the case. Neuroectodermal cells that are deprived of this division are still able to acquire a late NB fate. Furthermore, late cells (having already divided), when transplanted back into the early neuroectoderm, sometimes performed a further division. This extra division had no obvious effect on cell fate as it produced an epidermoblast and a late NB (Table 3). We conclude that cell divisions taking place in the neuroectoderm are not involved in the specification of NBs. Independency of cell fate determination from the pattern of precursor cell divisions has also been reported for the developing eye imaginal disc. In the *Drosophila* eye disc, a second mitotic division increases the pool of cells from which photoreceptor cell types are recruited. If this second mitotic wave is blocked, each cell type is still specified (de Nooij and Hariharan, 1995). In the vertebrate CNS, differentiation of oligodendrocytes starts after

a specific period of time during which cells are proliferating. This period is precisely controlled by intrinsic and extrinsic factors. An intrinsic timing mechanism measures the elapsed time, but is independent from the number of cell divisions taking place during this period. Extrinsic signals are required to stop cell divisions and initiate differentiation at the appropriate time (Durand and Raff, 2000). According to our experiments, the separation of early and late NB fates is independent from neuroectodermal cell divisions, but they do not rule out an intrinsic timing component. If such an intrinsic timer exists in neuroectodermal cells it would have to work like a resetable clock that requires extrinsic signals as a trigger.

Extrinsic signals are involved in specifying temporal subsets of neuroblasts

There is ample evidence that the specification of NBs crucially depends on positional information in the neuroectoderm provided by the products of segmentation genes and dorsoventral patterning genes (reviewed by Skeath, 1999; Bhat, 1999). Part of this information becomes integrated into the cell-autonomous program of the cells before neurogenesis. Another part, however, is subsequently provided by extrinsic signals. For example, the segment polarity gene *wingless* (*wg*) is segmentally expressed in a single row of neuroectodermal cells and the secreted Wg protein is non-autonomously required in adjacent anterior and posterior neuroectodermal cells for the formation and specification of NBs (Chu-LaGraff and Doe, 1993). Along the dorsoventral axis, the secreted Spitz and Vein proteins are involved in conferring NB identities. (Skeath, 1998; Udolph et al., 1998). Our heterochronic transplantation experiments now show that extrinsic signals are also involved in NB specification along the temporal axis. Although neuroectodermal cells of stage 10 embryos normally never produce NBs belonging to the group of S1 and S2 NBs, they do so after being transplanted into stage 7 neuroectoderm. The possibility that the cells follow this fate autonomously after being released from signals that normally inhibit these fates in the late neuroectoderm is incompatible with the following evidence. Cells from the non-neurogenic dorsal ectoderm of stage 10 donors are able to adopt a CNS fate upon heterotopic transplantation (Technau et al., 1988; Stüttem and Campos-Ortega, 1991), and to become specified as early NBs. However, they are unable to autonomously develop as a NB in cell culture (Lüer and Technau, 1992). Thus, the transplanted late cells do react to signals in the early neuroectoderm and adjust their development accordingly. This also seems to be possible in the other direction. Upon transplantation of stage 7 neuroectodermal cells into the neuroectoderm of hosts at stage 10, most of the CNS lineages obtained were typical for NBs that normally delaminate late. Similar to the situation in *Drosophila*, heterochronic transplantations using the developing ferret brain have revealed an interaction scenario of extrinsic cues and intrinsically changing properties for the sequential birth of neuronal cell types from ventricular zone progenitor cells (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). Progenitor cells from very young embryos can adjust their fate to older host tissues. By contrast, cells from older tissue transplanted into younger host brains adopt only fates typical of their origin. The latter experiment reveals an irreversible intrinsic change of the developmental properties of older cells. Intrinsic changes over time are likely to occur also in the *Drosophila* neuroectodermal cells; however, they are reversible under the influence of external signals. It remains to be tested as to how far this is also the case for NBs once they have delaminated from the neuroectoderm.

Our experiments suggest that the entire temporal sequence of delamination of specific subsets of NBs is not readily determined in the early neuroectoderm but is controlled by the dynamic expression of stage specific signals. Segment-polarity genes play an important role in the formation and identity specification of NBs (for a review, see Bhat, 1999). They are segmentally expressed in particular rows of neuroectodermal cells. As the expression domains of some of these genes evolve dynamically and, hence, differ at different stages, they are also good candidates for being involved in the temporal control of NB formation/specification. Work from our laboratory (Deshpande et al., 2001; in this issue) shows that the differential commitment of the late neuroblasts NB 6-4 (S3) and NB 7-3 (S5) is mainly controlled by the interplay of the segment polarity genes *naked* (*nkd*) and *gooseberry* (*gsb*). Mutation of either *nkd* or *gsb* leads to the transformation of one NB fate to the other. Interestingly, however, the temporal sequence of their delamination is maintained, i.e. independent from these genes. This suggests that formation and specification of these two NBs is under independent control. Further work will have to test whether this is the case also for other NBs and to uncover the signals that regulate the temporal pattern of NB fate determination.

We thank Andreas Prokop and Nirupama Deshpande for critical comments on the manuscript, Christof Rickert for his advice in the analysis of neuroblast lineages, and Karin Lüer for introducing C. B. to the cell transplantation technique. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Te 130/7-4) and the Fonds der Chemischen Industrie to G. M. T.

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