The Embryonic Central Nervous System Lineages of Drosophila melanogaster

I. Neuroblast Lineages Derived from the Ventral Half of the Neuroectoderm

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Central nervous system development in Drosophila starts with the delamination from the neuroectoderm of about 30 neuroblasts (NBs) per hemisegment. Understanding the mechanisms leading to the specification of the individual NBs and their progeny requires the identification of their lineages. Here we describe 17 embryonic NB lineages derived from the ventral half of the neuroectoderm and we assign these lineages to identified medial and intermediate NBs. The lineages are composed of interneurons (NB 1-2, NB 2-1, MP2, NB 4-1, NB 5-1, NB 5-3, NB 6-1, NB 6-2, and NB 7-2), interneurons and motoneurons (NB 3-1, NB 3-2, NB 4-2, NB 5-2, NB 7-1, and NB 7-3), or interneurons, motoneurons, and glial cells (NB 1-1 and NB 2-2). NB 1-1, NB 2-2, and NB 3-1 form segment-specific lineages. Neuroectodermal progenitors forming NB 2-1, NB 5-1, and NB 7-3 divide while still in the ectoderm to give rise to an additional epidermoblast. Expression of segmentation genes is not linear in the clones of NB 1-2 and NB 7-3 (engrailed), NB 1-1, NB 4-2, and NB 7-1 (even-skipped), and NB 7-1 (gooseberry-proximal). The timing of delamination for individual NBs as well as the number of their progeny is not strictly invariant. The 17 NBs produce about 200 neurons and only three glial cells, corresponding to about 70% of the estimated total number of neurons and 10% of the glial cells per thoracic and abdominal hemisegment. Previously identified neural cell types were linked to particular lineages and we introduce a systematic terminology for the ventral nerve cord neurons. The wild-type clones provide a foundation for the analysis of mutants, expression patterns, and experimental manipulations.


INTRODUCTION

The central nervous system (CNS) consists of an impressive number of interconnected neuronal and glial cell types. This highly organized three-dimensional tissue derives from a two-dimensional layer of uniform neuroectodermal cells. In insects, the formation of the CNS starts with the delamination of neural precursors, called neuroblasts (NBs), from the neuroectoderm (Wheeler, 1891, 1893). The pattern of NBs in each segment is invariant (Bate and Grunewald, 1981; Goodman et al., 1984; Taghert and Goodman, 1984; Bastiani and Goodman, 1986), allowing individual NBs to be uniquely identified (Bate, 1976; Doe and Goodman, 1985). The powerful genetic and molecular tools of Drosophila favor its use as a model system to study the mechanisms controlling cell fate specification in the CNS. In Drosophila about 30 NBs delaminate from the neuroectoderm per thoracic and abdominal hemisegment (Doe, 1992; Broadus et al., 1995). Although the genetic network controlling NB formation is well understood (e.g., reviewed in Campos-Ortega, 1993; Jan and Jan, 1994), little is known about the mechanisms leading to the specification of the individual NBs and their progeny. Approaching these mechanisms by means of molecular genetic and experimental techniques...
critically depends on detailed information on wild-type CNS development. For the NBs, detailed descriptions are available showing that they delaminate according to a stereotypy segmentally reiterated spatiotemporal pattern (Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987), and the construction of a NB map has allowed the identification of each NB by molecular markers as well as by position and time of segregation (Doe, 1992; Broadus et al., 1995). These data have already aided the examination of aspects of NB determination (Duffy et al., 1991; Chu-LaGraff and Doe, 1993; Zhang et al., 1994; Skeath et al., 1994, 1995; Prokop and Technau, 1994).

The NBs give rise to about 350 progeny cells per hemisegment (H. Schmidt, T. Bossing, and G. Technau, unpublished data). The axon and dendrite projection pattern is known for only a relatively small number of these progeny (Thomas et al., 1984; Sink and Whittington, 1991; Goodman and Doe, 1993), whereas a considerable number of molecular markers, such as antibodies and enhancer trap lines, have been found to be specifically expressed in subsets of CNS cells (e.g., reviewed in Patel et al., 1989a; Doe et al., 1991; Goodman and Doe, 1993; Ito et al., 1995). Most of these markers do not disclose the structural details of the differentiated cells. Furthermore, their expression patterns provide limited insight into the origin and the lineage relationships of the various types of cells. However, the clarification of NB cell lineages is a prerequisite to address fundamental problems of CNS formation: How much overlap is in the origin of neurons and glia, of motoneurons and interneurons, and among further cellular subtypes? How are cell types related to positional cues in the neuroectoderm? Is the muscle innervation pattern correlated with the dorsoventral position of the NB delamination? What is the fasciculation behavior among the fibers of clonal progeny? What is the proliferation pattern of the various NBs? Do segment-specific features arise from particular lineages? How are gene expression patterns related to lineage? Is the development of the individual lineages invariant?

Methods are available that allow individual progenitor cells to be labeled with a lineage tracer (e.g., Dil and HRP), to follow their development throughout embryogenesis, and to disclose the morphology and distribution of all their progeny (Technau, 1986; Bossing and Technau, 1994). Using these techniques the entire embryonic lineage of the CNS midline progenitors (Bossing and Technau, 1994) and of three neuroblasts, NB 1-1 (Udolph et al., 1993; Prokop and Technau, 1994), NB 2-2 (Bossing et al., 1996), and NB 4-2 (Udolph et al., 1995; Chu-LaGraff et al., 1995), have been clarified.

Here we describe 17 embryonic NB lineages derived from the ventral half of the neuroectoderm and we assign these lineages to identified medial and intermediate NBs. Using molecular markers we also link a number of well-known cells to particular lineages and we introduce a systematic terminology for neurons of the ventral nerve cord. Principle developmental features of the lineages are discussed in light of the previously mentioned questions. The lineages derived from the dorsal half of the neuroectoderm are currently being analyzed (H. Schmidt, T. Bossing, and G. Technau, unpublished data; A. Schmid and C. Doe, unpublished data).

**MATERIALS AND METHODS**

**Fly Stocks**

The labelings were performed in wild-type Oregon R embryos and in embryos heterozygous for hkb<sup>2</sup> (balanced over TM3; Weigel et al., 1990) that are morphologically indistinguishable from wild-type clones (e.g., projection pattern, cell numbers, position, and apoptotic cells; see Bossing et al., 1996). Expression patterns of clones were examined only in Oregon R embryos (see below).

**Labeling Procedure and in Vivo Identification of the Neuroblasts**

Individual progenitors were labeled with Dil as described in detail elsewhere (Bossing and Technau, 1994), except that the dye was dissolved at 65°C without sonication. Cells of the ventral half of the neuroectoderm (between 0 and 25% of the ventrodorsal perimeters; see Fig. 1) were labeled during embryonic stage 7 (10–20 min after the onset of gastrulation). The majority of the labelings were located within segment anlagen T1-A4. Up to four individual cells were labeled in each embryo at a distance of at least one segment anlage to avoid spatial overlap of the resulting clones. In most of the cases the development of the cells was followed in vivo at defined intervals up to end stage 11 using a fluorescein filter set and (100 W) halogen light. Under these conditions cells can be irradiated for several minutes without developmental disturbances.

The stages of NB delamination were defined by using the synchronous first division of the midline cells as time reference. Cells that were fully delaminated from the ectoderm 30 min (stage 8 or stage 9), 30–60 min (stage 9 to beginning of stage 10), and 60–100 min (stage 10) after this division were recorded as S1, S2, and S3 NBs, respectively. NBs segregating between mid stage 10 and the onset of stage 11 were scored as S4 NBs and NBs appearing later as S5 neuroblasts (Doe, 1992).

NBs were uniquely identified in vivo at mid to late stage 11 according to the following criteria: anterior–posterior and medio–lateral position relative to the tracheal pits, the segmental furrows, and the ventral midline (Fig. 2); dorso–ventral position in the NB layer; shape; time of segregation (see above); and number, position, shape, and movements of daughter cells. The typical positions and shapes of the NBs (at stage 11) are illustrated in Fig. 2.

The embryos were allowed to develop to stage 17. The differentiated clones were inspected in vivo with a rhodamine filter set and a mercury lamp. Afterwards most of the specimens were photoconverted to obtain permanent preparations.

All types of lineages presented in this paper were also labeled by using the transplantation method (Technau, 1986); single neuroectodermal cells were isotopically transplanted from HRP-labeled donors into unlabeled hosts and the lineages derived from the transplants were analyzed at stage 17 (data not shown). Because we did not find significant differences in the composition of the lineages labeled by two different methods, it is unlikely that labeled progenitors developed abnormally (see Udolph et al., 1993, 1995).
Embryonic CNS Lineages of D. melanogaster

**FIG. 1.** Fate map of the early gastrula stage [left, lateral view, according to Technau and Campos-Ortega (1985); right, half cross section]. The neurogenic region of the ectoderm is marked (gray). The procephalic neurogenic region (pNR) gives rise to the brain, the ventral neurogenic region (vNR) gives rise to the ventral nerve cord (G, gnathal; T, thoracic; A, abdominal segment anlagen). Dil labelings were performed in the ventral half of the vNR within segment T1 to A4 (dark gray). dEpi, dorsal epidermal anlage; MES, mesoderm; ML, midline precursor.

**Photoconversion of Dil**

Whole mounts of photoconverted preparations were obtained as described previously (Bossing and Technau, 1994). Alternatively, flat preparations were subjected to photoconversion. Most of the oil, covering the embryos that were mounted on a glue-coated coverslip, was removed. The fragment of the coverslip carrying the embryos was cut and transferred into a small petri dish and covered with phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM Na$_2$HPO$_4$, 3 mM NaH$_2$PO$_4$, pH 7.2). The residual oil was removed by a jet of 100% alcohol squirted toward the embryos in the PBS with a pulled Pasteur pipette. The solution was replaced by PBS and the procedure was repeated. Afterwards the fragment was conveyed onto an alcohol-cleaned coverslip (22 × 60 mm) surrounded with a plasticine dam and immersed with PBS. For the preparation of cuticularized embryos (late stage 17) a glued coverslip was used. Under PBS the embryos were pierced with a fine glass capillary from posterior to anterior along the dorsal side and pulled out of the vitelline membrane. They were stuck onto the surface of the coverslip (22 × 60 mm) and dissected longitudinally along the dorsal midline. The gut was removed and the body wall was carefully flattened onto the coverslip. The preparations were immediately fixed with a solution of 7% formaldehyde in PBS for 10–15 min or with 3.7% formaldehyde in PBS for only 7 min if antibody staining was intended (see below). After fixation the samples were washed three times with PBS and incubated for 1 hr in calf serum on a gently moving shaker. The calf serum was replaced by diaminobenzidine solution (2 or 3 mg/ml dissolved in warm 100 mM Tris–HCl, pH 7.4) and the samples were irradiated for 10–20 min with mercury lamp through a rhodamine filter set and a 63× oil-immersion objective. Because prolonged incubation in DAB results in an increased background staining, embryos were processed individually. The photoconversion was stopped when brown staining of the cells was clearly detectable under the microscope. The embryos were removed from the coverslip and mounted in 90% glycerol in PBS or subjected to immunocytochemistry.

**Immunocytochemistry of Photoconverted Flat Preparations**

After photoconversion the flat preparations were washed with PBT (0.22% Triton X-100 in PBS), removed from the coverslip, and transferred to small plastic dishes. The antibody stainings were executed at 4°C and were enhanced by the use of secondary antibodies coupled to alkaline phosphatase or by the Vectastain ABC Kit (Vector Labs). Preparations were incubated overnight at 4°C with the primary antibody [diluted 1:5000 (anti-even skipped, α-eve) or 1:5 (anti-invected, α-inv; anti-gooseberry-distal or -proximal, α-gsb-d or α-gsb-p) in PBT with 10% calf serum and 0.02% Na$_2$HPO$_4$]. The antibodies were kindly provided by M. Frasch (α-eve), R. Holmgren (α-gsb-d and α-gsb-p), and C. Klämbt (α-inv). Specimens were washed 10 times with PBT and then incubated for at least 6 hr at 4°C with a biotin-conjugated secondary antibody (polyclonal goat anti-rabbit, goat anti-mouse (Fab′2 fragment) or goat anti-rat (Fab′2 fragment); a plasticine dam and immersed with PBS. For the preparation of DAB precipitate was obtained by addition of 0.06% NiCl$_2$ to the staining solution. For the phosphatase staining reaction the preparations were washed once with alkaline reaction buffer (0.1 M NaCl, 0.02 M MgCl$_2$, 0.1 M Tris–HCl, pH 9.5, 0.1% Tween 20) and incubated in the buffer for 10–20 min with mercury lamp through a rhodamine filter set and a 63× oil-immersion objective. Because prolonged incubation in DAB results in an increased background staining, embryos were processed individually. The photoconversion was stopped when brown staining of the cells was clearly detectable under the microscope. The embryos were removed from the coverslip and mounted in 90% glycerol in PBS or subjected to immunocytochemistry.

**RESULTS**

The delamination of the NBs from the neuroectoderm occurs between embryonic stages 8 and 11 (staging according to Campos-Ortega and Hartenstein, 1985) and is divided into five phases (S1–S5). S1–S3 N Bs form three longitudinal columns in the NB-layer: medial, intermediate,
**FIG. 2.** Spatial arrangement and shapes of the NBs as observed in vivo around mid stage 11. Medial and intermediate NBs derived from the ventral half of the neuroectoderm are shown in color. The lineages of the NBs derived from the dorsal half of the neuroectoderm (gray) will be described elsewhere. The scheme shows the location of the various NBs relative to each other (as viewed from the ventral side; anterior to the top, lateral to the right) and with respect to morphological markers (tracheal pits, remnants of the parasegmental furrows, and midline). Two hemisegments are shown to underline variabilities in shape and location for specific NBs (e.g., NB 6-1 and NB 7-1). NBs marked red give rise to neuronal clones with interneuronal projections only; NBs marked purple give rise to neuronal clones with interneuronal and fully extended motoneuronal projections (with endplates); lineages of NBs marked light blue include interneuronal projections and putative motoneuronal projections that appeared to be still at an early stage of differentiation (ending in the nerve, no endplates); NBs marked pink give rise to clones comprising interneurons, motoneurons, and glial cells; green labels indicate segment-specific NBs; yellow labels indicate NBs of which the neuroectodermal precursor divides prior to delamination (i.e., they always have an epidermal sister clone).
FIG. 3. Dil-labeled NBs upon delamination at stage 10 (A) and stage 11 (B, C). Dorsal views, anterior up. Flattened embryos were photoconverted and double labeled with antibodies against engrailed (black, A, B), gooseberry-distal (brown, B, C), and even-skipped (gray, C). (A) Two NB 7-1 (arrows) located within the engrailed expression domain of consecutive segments. Neighboring NBs are indicated. (B) In vivo diagnosis of the two Dil-labeled precursors as NB 2-3 (upper arrow) and NB 5-3 (lower arrow) is confirmed by their location relative to molecular markers (en and gsb-d; Doe, 1992) and to neighboring NBs (as indicated). The epidermal sister clone of NB 2-3 (see text) is out of focus. Note the size difference between the S5 NB 2-3 and the S1 NB 5-3. (C) In vivo identification of the two Dil-labeled precursors as NB 5-2 (upper arrow; only the dorsal daughter cells are in focus) and NB 1-1 (lower arrow) is consistent with their position in relation to the molecular markers. Only one even-skipped expressing cell (gray, open arrow) is visible close to but outside the NB 1-1 clone. This cell presumably belongs to the NB 7-1 lineage (for eve expression see Broadus et al., 1995). Arrowheads mark the midline; asterisks (in A and B) denote engrailed expressing midline cells.

Identification of NBs in Living Embryos

We used multiple criteria to uniquely identify each labeled NB (see Materials and Methods). Despite these diverse criteria we were not able to identify every labeled NB with absolute certainty. First, recognition of unlabeled NBs is often difficult. The neural layer is characterized by intense proliferation, and NBs that are in the process of dividing are hardly detectable. Second, movements of NBs (e.g., during cell divisions; Bossing and Technau, 1994) can lead to positional changes of NBs. Finally, the time of delamination of the individual NBs is variable. Although in general NBs segregate at a particular stage (S1 - S5; Doe, 1992), we found that some occasionally deviate from this pattern (Table 1).

Reliability of NB diagnosis in vivo is reflected by the frequency of lineage types obtained. This reliability is NB specific and varies between 44 and 94% (Table 2). Furthermore, the rate of correct diagnosis was confirmed in an independent series of experiments in which specimens were fixed shortly after in vivo diagnosis and photoconverted NBs were identified by double labeling with antibodies against molecular markers (Fig. 3). Thus, although the criteria listed previously do not allow an unambiguous identification of NBs in vivo, the amount of data obtained allowed us to link each type of lineage to a particular NB in the map.

In the course of this analysis we found that NB 1-1, which is defined as the progenitor of the aCC/pCC neurons by analogy to NB 1-1 in the grasshopper (du Lac et al., 1986), maps to a position different than previously thought. This has been confirmed by cell-specific markers and has led to NB 2-2 (Doe, 1992) being renamed NB 1-1; in addition, NBs

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TABLE 1
Time of Delamination Observed in Vivo for Individual DiI-Labeled NBs (Number of Cases)

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Note. The preferred times of delamination are denoted in gray. A, 0–30 min after the division of the midline progenitors (admp; early stage 9); B, 30–60 min admp (stage 9/10); C, 60–100 min admp (early stage 10); D, mid stage 10 (parasegmental furrows appear) to beginning of stage 11 (tracheal placodes appear); E, late stage 11; S1–S5: waves of segregation (according to Doe, 1992).

1-1, 1-2, and 2-3 have been renamed NBs 1-2, 2-3, and 2-2, respectively (Broadus et al., 1995). We use the revised NB names of Broadus et al. (1995) throughout this paper.

Systematic Nomenclature for Neuronal Cell Types

The lineages described in the following comprise a large number of different neuronal cell types that together with those to be identified in the future require a systematic terminology. The nomenclature we propose is applicable to all neurons of the ventral nerve cord and may also be used for the brain as soon as the brain NBs and their lineages are identified. Our classification system is mainly based on the origin and projection patterns and addresses each type of cell by the following criteria: parent NB (X–X); motoneuron (M) vs interneuron (I); ipsilateral (i) vs contralateral (c) axonal projection; contralateral projections running through the anterior (a) vs the posterior (p) commissure. For example, “6-2Icp” is an interneuron derived from NB 6-2 that sends a projection contralaterally through the posterior commissure. Cells with bilateral axons are considered as contralaterally projecting neurons. Contralateral and ipsilateral fascicles of a clone are addressed separately although we cannot exclude at present that in some cases the same cells contribute to both fascicles. If a clone comprises motoneurons running through different nerve routes the cells are indicated by “ar” (intersegmental nerve, anterior root), “pr” (intersegmental nerve, posterior root), or “s” (segmental nerve, nomenclature of nerve roots according to Thomas et al., 1984). For example, “3-2Mar” is a motoneuron derived from NB 3-2 projecting through the anterior root of the intersegmental nerve (as opposed to “3-2Ms,” which runs through the segmental nerve). In cases in which two or more cells fulfill the same basic criteria but differ by further criteria, such as order of birth, fasciculation, dendritic branching, or gene expression pattern, they may receive additional numbers as soon as such differences have been uncovered (e.g., “6-2icp1” and “6-2icp2” both project through the posterior commissure but use different fascicles). Symbol usage is kept at a minimum. For example, the two to four interneurons derived from NB 5-1 form only one fascicle running contralaterally through the posterior commissure. Because there are no other interneuronal projections in the late embryo these cells are named “5-1I”; if further characteristics are discovered that allow to distinguish between these cells, this can be reflected by further symbols (e.g., “5-1I1”).
TABLE 2
Assignment of the Different Clonal Types to Individual NBs

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Note. Shown is the distribution of clonal types (vertical columns) obtained from the labeled NBs as diagnosed on their delamination in vivo (horizontal columns, number of cases); the type of lineage obtained with highest frequency (framed) was assigned to the respective NB (for the NB 7-1 lineage see text). Three of the clones were obtained from precursors diagnosed as NB 2-3 and NB 5-4 that derive from the dorsal neuroectoderm; the dorsal NB lineages will be dealt with elsewhere. R, reliability of NB identification (percentage of lineage type obtained most frequently from diagnosed NB identity); F, frequency of correlation of lineage type with the assigned NB identity.

Because within the lineages most cells are arranged as a tight cluster and their axons fasciculate, we are currently not able to link each axon to a particular cell body (see above). However, some cells are individually identifiable due to the fact that their position and/or axonal projection pattern differs from all other cells in their lineages. These include most of the previously described cells with well-known names, such as aCC, pCC, RP2, etc., as well as the newly identified TB neuron (see below). Because of the peculiar characteristics of these cells and their small number we accommodate these cells without changing their names.

Embryonic Lineages of the Medial NBs

Medial NBs with Segment-Specific Lineages

NB 1-1. NB 1-1 delaminates preferentially as SI NB (early stage 9; Table 1). Upon delamination it is located in the most dorsal region of the NB layer (Figure 3C). Around mid stage 11 the anterior–posterior position of NB 1-1 is in line with, or slightly anterior to, the tracheal pits (Fig. 2). At this stage it has a cluster of six to eight daughter cells on its dorsal side. At the beginning of germ band shortening (end stage 11) two of the progeny, the aCC and pCC (Thomas et al., 1984), leave the cluster and move anteriorly. They are the first progeny of NB 1-1 (Broadus et al., 1995). The remaining daughter cells stretch laterally.

The lineage of NB 1-1 has been described in detail elsewhere (Udolph et al., 1993). Although the aCC/pCC neurons are identical in thoracic and abdominal neuromeres, significant segment-specific differences arise among later components of the lineage. Each thoracic NB 1-1 gives rise to a cluster of 8–14 cells including ipsilaterally projecting interneurons (1-1i) and 1 or 2 motoneurons (1-1Ms; Figs. 4A and 5). In the abdomen NB 1-1 gives rise to a smaller cluster comprising four to six ipsilaterally projecting interneurons and no motoneurons; in addition the abdominal clone comprises 3 subperineural glial cells (the two dorsal A- and B-SPG and the ventral LV-SPG) (Klämbt and Goodman, 1991; Ito et al., 1995). Thus, NB 1-1 represents a segment-specific neuroglioblast. With the exception of the aCC/pCC neurons and glial cells, the clone is located in the ventrolateral cortex (Figs. 4A and 4B). The aCC motoneuron innervates a dorsal muscle (Sink and Whitington, 1991; for description of muscle pattern see Crossley, 1978; Bate, 1993). The thoracic 1-1Ms axons terminate among ventrolateral muscles without a detectable endplate. One clone was located in the labial (third gnathal) neuromere and corresponded to the thoracic type.
FIG. 4. Photos of the thoracic and abdominal lineages of NB 1-1, NB 2-2, and NB 3-1. Dorsal views (C, E) and lateral views (A, B, D, F; ventral to the bottom) of stage 17 nerve cords; anterior to the left. (A, B) NB 1-1 lineage in the thorax (A) and abdomen (B); open arrow, pCC projection; carat, posterior interneuronal fascicle; arrows, dorsal A-SPG and B-SPG and ventral LV-SPG. (C, D) Two NB 2-2 clones (left clone, thoracic; right clone, abdominal) and one epidermal clone (arrowheads) labeled in same preparation; carats, abdominal interneuronal projections; open arrows, ipsilateral motoprojections; solid arrow in D, A-SPG. (E, F) NB 3-1 lineage in the thorax (left clone in E) and abdomen (right clone in E and F); open arrows, contralateral projections of the RP1,3,4,5 motoneurons; white arrows, cell fragments and pycnotic cells only visible in the abdominal NB 3-1 clone; RP4 is out of focus in left clone of E and RP5 in F. Bar = 6 μm (B) and 8 μm (A, C–F). For further details see text and Fig. 5.
NB 2-2. NB 2-2 preferentially delaminates during S2 (late stage 9; Table 1) just ventral to NB 1-1 in the medial column of NBs. During delamination of NB 2-1 (mid stage 10 to early stage 11) it is shifted laterally between the rows of medial and intermediate NBs and just posterior to the level of the tracheal pits (Fig. 2). At stage 11 NB 2-2 is often shaped like a drop with two or three progeny extending laterally. Its distinct position and shape allow us to use unlabeled NB 2-2 as an additional reference point for the identification of neighboring NBs.

Like NB 1-1, NB 2-2 is a segment-specific neuroblast that produces both neurons and glia. In the thoracic neuromeres the lineage includes one dorsal subperineural glial cell, which is at the same position as the NB 1-1-derived A-SPG in the abdominal segments. Thus, thoracic and abdominal A-SPG cells derive from different progenitors. The neuronal elements of the thoracic NB 2-2 lineage are two or three motoneurons (2-2M) and about 10 interneurons (2-2I) in the ventral cortex. The abdominal NB 2-2 lineage differs from the thoracic lineage by the absence of the glial cell and by...
the interneuronal fascicle (2-2I), which is more prominent and turns anterior after reaching the contralateral connective; also, small ipsilateral processes are detectable. Often two cells are separated dorsomedially from the main cluster (Figs. 4C, 4D, and 5).

The 2-2M neurons are located close to the thoracic 1-1Ms neurons and their axons also project through the segmental nerve. Endplates can be seen at the ventrolateral muscle group. One NB 2-2 clone was labeled in the labial neuromere. It contained no glial cell, although the neuronal components corresponded to the thoracic type.

**NB 3-1.** NB 3-1 delaminates as medial S3 NB at early stage 10 (Table 1). At stage 11 NB 3-1 is positioned medial, posterior, and dorsal to NB 2-2. Together with NB 4-1 it forms a pair of ovoid-shaped cells in the medial NB row (Fig. 2). Daughter cells are arranged as a column dorsal to the NB.

The NB 3-1 lineage consists exclusively of neuronal cells in both thoracic and abdominal neuromeres. It comprises the four contralaterally projecting motoneurons RP1,3,4,5 (Goodman et al., 1984; Sink and Whitington, 1991). In addition to these motoneurons the clone includes a cluster of interneurons that are positioned in the ventral cortex region (3-1I; Fig. 4F). The lineage differs in the thoracic and abdominal neuromeres by the number of the interneurons and the arrangement of their cell bodies in the cortex (Figs. 4E and 5; Table 3). Because at stage 17 we generally find labeled cell fragments associated with the abdominal but not the thoracic NB 3-1 clones, the lower average number of cells in the abdominal clones may be due to cell death (Fig. 4E). We cannot exclude that cell death occurs at later stages in the thoracic lineage. Furthermore, the contralateral fascicle of the abdominal 3-1I bifurcation bifurcates in an anterior-posterior direction, after traversing through the anterior commissure, whereas at the same stage this bifurcation is not obvious in the thorax. This difference might also be due to a temporal delay in fiber growth in the thorax. The dorsal group of RP motoneurons (RP1,3,4) forms a distinct fascicle along the posterior border of the anterior commissure. The axon of the RP5 that is positioned ventrolaterally to the dorsal group (Sink and Whitington, 1991) extends first along the interneuronal fascicle and joins the RP fascicle inside the anterior commissure. The common RP fascicle leaves the nerve cord through the anterior root of the intersegmental nerve. The observed innervation pattern of ventral and ventrolateral muscles is consistent with previous descriptions (Sink and Whitington, 1991).

One NB 3-1 clone labeled in the labial neuromere of the subesophageal ganglion comprised about 17 cells and corresponded to the thoracic type.

**Medial NBs with Non-Segment-Specific Lineages**

**NB 1-2.** The time of delamination of NB 1-2 is highly variable (Table 1). This variability appears to have no effect on other features of NB 1-2 development. Like the neighboring NB 2-2, upon delamination it is shifted laterally toward the intermediate NBs. Around mid stage 11, NB 1-2 is located anterolateral from NB 2-2 (Fig. 2) and carries four to six daughter cells on its dorsal side.

The NB 1-2 clone consists of 16–24 interneurons, most of which project contralaterally (Figs. 6A and 7). A single neuron (which we name "TB neuron") is located apart from the clone and has a unique time of differentiation and projection pattern. Axonogenesis of this neuron starts very early, during stage 13, and it has a distinctive "bent" morphology (Fig. 7). The cell cluster forms three main fascicles established during stage 15 or 16. The first fascicle develops rather late (end stage 16) and contacts the TB axon but continues to grow ipsilaterally (1-2I).

The second fascicle projects through the posterior commissure of the neuromere (1-2Icp), and the third fascicle projects through the anterior commissure of the next neuromere (1-2Ia). One or two anterior neurons of the cluster are recognized by an anti-injected (engrailed) antibody (Fig. 6A). These cells together with the interneurons of NB 7-3 (see below) form the posterior intermediate (PIs) group of engrailed-expressing cells (Cui and Doe, 1992). At stage 17 this cluster consists of about 6 cells located lateral to the ventromedial cluster of engrailed-expressing neurons, which are derived from midline progenitors (Patel et al., 1989; Bossing and Tech-nau, 1994).

**NB 2-1.** At the beginning of stage 10 the neuroectodermal progenitor of NB 2-1 divides once while still in the ectoderm (in 32 of 33 cases). During the S4 wave of NB segregation (mid stage 10 to early stage 11) one daughter cell delaminates medially to become NB 2-1 (Fig. 2; Table 1) and the second daughter cell remains in the ectoderm to give rise to an epidermal subclone. The epidermoblast divides during or shortly after the delamination of its sibling. The NB performs its first division around mid stage 11.

In the late embryo the NB 2-1 clone consists of about eight interneurons located in the ventromedial cortex (Fig. 6B). Most project ipsilaterally (2-1Ii), although one projection traverses through the anterior commissure (2-1Ic; Fig. 7). The epidermal subclone generally consists of four cells (Table 3).

**MP2.** MP2 delaminates preferentially as a medial S1 NB (early stage 9; Table 1), and is one of the most dorsally located NBs (similar to NB 1-1). During the delamination of NB 3-1 and NB 4-1 (stage 10) MP2 is shifted slightly laterally into a position between the medial and intermediate row of NBs. It forms only one division (Doe et al., 1988a; Spana et al., 1995; this paper) at the end of stage 10. At stage 11, MP2 progeny show a unique decrease in the intensity of the membranous DII fluorescent staining that is correlated with an uptake of the label by intracellular granular structures. This dye transfer argues for a rapid membrane turnover that seems to be characteristic of the MP2 cells. Initiation of axon outgrowth is observed during stage 13.

According to previous descriptions (Goodman et al., 1984; Thomas et al., 1984; Goodman and Doe, 1993; Spana et al., 1995) the two closely associated MP2 progeny differ by their size and dorsoventral position, the ventral one (vMP2) being
smaller than the dorsal one (dMP2). Their initial dorsoventral position is subsequently lost as they migrate to maintain an anteroposterior position, with the anterior cell being the smaller MP2 progeny (except in one case). The anterior (vMP2) and posterior MP2 (dMP2) also differ in their axonal pattern (Figs. 6C and 7). vMP2 has an anterior axon, whereas dMP2 has a prominent posterior projection and a smaller anterior axon branch.

**NB 4-1.** NB 4-1 preferentially delaminates during the S3 segregation wave (early stage 10; Table 1). A round mid stage 11 it is located posterior to NB 3-1 (Fig. 2) with about three daughter cells attached to its dorsolateral surface. The lineage consists of 12–18 interneurons forming a cluster in the ventrolateral cortex. Their ipsilateral projections (4-1Ili) extend anteriorly and their contralateral projections cross the anterior (4-1Ica) and posterior commissures (4-1Icp; Figs. 6D and 7). The 4-1Icp projections develop rather late (mid to end stage 16).

In 1 of 43 cases the neuroectodermal precursor divided once before one daughter cell delaminated during the S3 wave to become NB 4-1. Its sibling gave rise to an epidermal subclone of two cells.

**NB 5-1.** NB 5-1 delaminates at late stage 11 (S5; Table 1). The neuroectodermal progenitor divides once or often twice before one daughter cell delaminates to become NB 5-1. At end stage 11 the clone typically consists of one unusually small NB located most ventrally in the NB layer (Fig. 2) and three cells in the overlyingectoderm. In the late embryo the NB5-1 clone includes only two to four interneurons positioned in the ventralmost cortex (Fig. 6E). They form a fascicle projecting through the posterior commissure (“5-1I”); Fig. 7). The epidermal subclone contains two to nine cells (mostly six).

The time of segregation, its small size, and the overlying labeled epidermal cells hinder the in vivo recognition of NB 5-1 (only four cases; Table 2). However, its position, the low number of neuronal cells, and its mixed neural/epidermal composition are consistent with the lineage being derived from a medial S5 NB.

**NB 5-2.** The delamination of NB 5-2 takes place during S1 (early stage 9; Table 1). Around mid stage 11 (Fig. 2) a column of five to seven daughter cells is attached to the dorsal side of the NB (Fig. 3C).

The late embryonic NB 5-2 lineage contains 17–26 cells in the ventral to ventrolateral cortex (Figs. 6A and 6H). Most of the cells are contraterally projecting interneurons. One fiber projects along the anterior border (5-2Ica) and two or three axons traverse along the posterior border of the anterior commissure (5-2Ica2). A prominent fascicle runs through the posterior commissure (5-2Icp; Figs. 6A, 6H, and 7). In all clones one fiber of this fascicle ends near or inside the anterior root of the intersegmental nerve. In some of the oldest embryos observed the fiber leaves the CNS to reach muscle VL2 (Fig. 6A). Thus, this fiber presumably represents a motoneuronal projection (5-2M) that develops rather late (late stage 16/early stage 17). At stage 16 all progeny of NB 5-2 express the gooseberry-proximal protein at different intensities (Fig. 6G; see Buenzow and Holmgren, 1995).

Two (of 65) clones were located in the labial neuromere and, except for the lack of 5-2M, corresponded to the clones in the thoracic and abdominal neuromeres.

**NB 6-1.** NB 6-1 preferentially delaminates during S3 (early stage 10; Table 1) to become located medially and ventrally posterior to NB 5-2 (Fig. 2). Typically, NB 6-1 is elongated in mediolateral direction and represents the largest NB at the border of the neuromere. At mid stage 11 NB 6-1 has two or three daughter cells on its dorsolateral side.

The mature NB 6-1 clone consists of 10–16 interneurons forming a tight cluster in the ventromedial cortex (Fig. 6I). They form ipsilateral posterior projections (6-1Ili) and a fascicle through the posterior commissure that bifurcates in the contralateral connective (6-1Ic; Figs. 6I, 6J, and 7).

Two (of 56) NB 6-1 clones were labeled in the labial neuromere and corresponded to the pattern described for the thoracic/abdominal neuromeres. In one case we obtained a mixed NB 6-1/epidermal lineage (with two epidermal cells).

**NB 7-1.** The progenitor of the lineage assigned to NB 7-1 delaminates during S1 (early stage 9; Table 1). Due to considerable variability in the position of the precursor at mid stage 11, we were not able to reliably identify NB 7-1 in

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**FIG. 6.** Photos of clones originating from medial NBs. Dorsal views (A, D, G, H, J, K, L) and lateral views (B, C, E, F, I; ventral to the bottom) of stage 17 nerve cords (except G, K, and L, which are stage 16); anterior to the left. (A) NB 5-2 clone (left) and NB 1-2 clone (right) in a preparation stained with an antibody against engrailed. Two cells in the NB 1-2 clone express engrailed (arrow); open arrows, contralateral motoprojection of NB 5-2 clone; arrowheads, TB neuron and its projection. (B) NB 2-1 clone; the epidermal subclone is out of focus. (C) MP2 clone; arrowheads, vMP2; arrows, dMP2. (D) NB 4-1 clone; only the projections are in focus. (E) NB 5-1 clone; the epidermal subclone is out of focus. (F) Three NB 5-2 clones labeled in neighboring neuromeres. (G) The NB 5-2 clone is located anterior and ventral inside the neural gooseberry expressing domain (μ-gooseberry-proximal antibody staining). (H) NB 7-1 clone (left) and NB 5-2 clone (right). Note the different dorsoventral positions of the two clones, which both originate from medial S1 NBs, and the absence of the contralateral motoprojection of the NB 5-2 clone at early stage 17. Open arrow, ipsilateral motoprojections of the NB 7-1 clone; arrowhead, fpCC. (I) NB 6-1 clone. (J) Projections of NB 6-1 clone (and cortical μ-en staining). (K, L) NB 7-1 clones. (K) NB 7-1 clone is partially located in the posterior part of the gooseberry expression domain (μ-gooseberry-proximal staining). (L) The NB 7-1 clone includes the eve expressing fpCC (arrowhead) and the CQ neurons (arrows); open arrow, ipsilateral motoprojections; the interneuronal projections are out of focus. Bar = 10 μm except in B, E, bar = 7 μm.
FIG. 7. Drawings of medial NB lineages at stage 17. Horizontal views; anterior to the left. Previously described neurons (vMP2, dMP2, fpCC, and CQ) and the TB neuron are denoted. For other abbreviations, see the legend to Fig. 5 and text.

* vivo* (Table 2). Often the precursor of the candidate 7-1 clone was diagnosed as NB 5-2. However, the designated NB 5-2 lineage was significantly more frequently obtained from precursors diagnosed as NB 5-2 compared to any other NB, and as reported previously for the NB 5-2 progeny (Zhang et al., 1994; Buenzow and Holmgren, 1995) all cells of the NB 5-2 lineage express gooseberry-proximal (see NB 5-2; Fig. 6G). Two observations lead us to the definition of the NB 7-1 progeny. Double labeling of photoconverted medial S1 NBs with an α-inv antibody in stage 10 embryos proves
that NB 7-1 was labeled (Fig. 3A) in a frequency comparable to other medial S1 NBs. Additionally, we were able to show that all described eve-expressing CQ neurons (Patel et al., 1989a; Broadus et al., 1995) of which two are known to originate from NB 7-1 (Broadus et al., 1995) are part of the candidate NB 7-1 lineage (Fig. 6L).

The late embryonic NB 7-1 clone comprises 16–22 neuronal cells in the lateral cortex (Figs. 6H, 6L, and 7). About three motoneurons form a fascicle that exits through the anterior root of the intersegmental nerve (7-1M). Immediately outside the nerve cord the fascicle splits into two branches. One branch arborizes over up to three muscles of the ventral external oblique group (nomenclature according to Bate, 1993). The second branch extends dorsally along the intersegmental nerve and ends near muscle DO2, but only in 3 of 10 cases could a prominent endplate be detected. Considering the projection pattern and some cells in the clone occupying a position ventromedial to the aCC motoneuron, these motoneurons might correspond to the previously described U1–U3 neurons (Goodman and Doe, 1993). Most cells of the NB 7-1 lineage represent interneurons that form two major contralateral fascicles (7-1ca and 7-1cp; Figs. 6H and 7). Beside the CQ neurons an interneuron positioned dorsomedially can be detected with an antibody against the eve protein (Fig. 6L). Expression and position just below and between aCC and pCC suggests this cell to represent the “friend of pCC” (fpCC; Goodman and Doe, 1993; “FP” cell of Jacobs and Goodman, 1989) or “candidate fpCC” (Broadus et al., 1995). The position of the CQ neurons inside the cluster does not allow to identify their individual projections. A few cells of the NB 7-1 clone are located in the posterior region of the gooseberry expression domain (Fig. 6K).

**Embryonic Lineages of the Intermediate NBs**

**NB 3-2.** NB 3-2 preferentially delaminates during S1 (early stage 9; Table 1) into the intermediate column of NBs. At the beginning of stage 11 during the lateral shift of NB 2-2 the ovoid-shaped NB 3-2 is pushed dorsally. At mid to late stage 11 NB 3-2 is located dorsal and lateral to NB 2-2 (Fig. 2) and carries four to six daughter cells on its dorsal side.

The late embryonic NB 3-2 clone comprises 10–18 cells extending from the lateral to dorsolateral cortex (Fig. 8B) of which 3 or 4 (most likely motoneurons) are separated dorsal and posterior from the main cluster (in 2 of 26 cases the group comprised 6 cells). One or two contralaterally projecting interneuronal fibers were observed that develop rather late (late stage 16) (3-2; Figs. 8A and 10). The majority of fibers in the lineage belong to motoneurons that form two fascicles. One fascicle with two or three fibers exits the nerve cord through the segmental nerve (3-2Ms); endplates can be detected in the same segment on one muscle of the ventral oblique group and two muscles of the LT group. The second fascicle also consists of about three fibers and departs through the anterior root of the intersegmental nerve (3-2Mar); two or three muscles of the dorsal lateral to dorsal group in the next posterior segment are innervated by these projections.

**NB 4-2.** NB 4-2 delaminates during S2 (stage 9; Table 1). NB 4-2 occupies a dorsoventral position in the NB layer comparable to NB B2-2. During the delamination of the lateral NBs in row 3 (stage 11) NB 4-2 is shifted more medially (Fig. 2).

In the late embryo the clone consists of 10–16 cells in the lateral cortex (Fig. 8D). In addition to the RP2 motoneuron (Goodman et al., 1984; Doe et al., 1988a; Patel et al., 1989a) it comprises about two additional motoneurons (4-2M; CoR in Chu-LaGraff et al., 1995) and several interneurons projecting contralaterally across the anterior commissure (4-2; Figs. 8C and 10; see Udolph et al., 1995; Chu-LaGraff et al., 1995). The RP2 has an ipsilateral anterior projection that exits through the posterior root of the intersegmental nerve and arborizes on one dorsal muscle (muscle DA3 or DA2; see Sink and Whitington, 1991). The 4-2M ar project ipsilateral and posteriorly through the anterior root of the intersegmental nerve and innervate muscles of the ventral external oblique group. Thus, the lineages of the adjacent NB 3-2 and NB 4-2 exhibit similar features (comparable to the situation described for NB 2-2 and NB 1-1).

**NB 5-3.** NB 5-3 delaminates during S1 (early stage 9; Table 1) into a dorsal position within the intermediate column of NBs. At stage 11 the neural progenitor is positioned lateral and dorsal to NB B5-2 with about three daughter cells attached dorsally (Figs. 2 and 3D).

NB 5-3 gives rise to a lineage of 9–15 cells with interneuronal projections only. The cells are arranged as two separate clusters (Figs. 8E and 10). One to three neurons are located medially close to the outer border of the connective and develop axons across the anterior commissure (5-3ca). The remaining neurons are located in the lateral cortex and send fibers through the posterior commissure (5-3cp). At stage 16 to early stage 17 the lineage of NB 5-3 is very similar to the NB 7-1 lineage with regard to both fascicles and the cortical position of the cells. During stage 17, however, (compare Figs. 8E and 8F) the 5-3ca fibers continue to grow anterior and lateral and the axons of the 5-3cp extend anterior and medial inside the connectives. In the fully differentiated NB 5-3 clone the two fascicles cross each other twice. At early stage 16 most, but not all, the NB 5-3 progeny can be labeled with an antibody against the gooseberry-proximal protein (n = 1; Fig. 8F).

Interestingly, labeled NB 5-3 clones are accompanied by an increased amount of stained fragments close to the cell clusters or incorporated into macrophages outside the CNS (Figs. 8E and 8F). In photoconverted clones we observed up to four pycnotic cells distributed in the lateral as well as the medial cluster. Even at end stage 11 Dil-labeled cell fragments at the dorsal border of the neural layer were detected, which is in contrast to all other lineages described in this study.
TABLE 3

Sizes (Cells/Clone) of the Particular Types of Clones, Their Neuronal and Glial Components, and Progeny Expressing Engrailed (en), Even-Skipped (eve), or Gooseberry-Proximal (gsb-p)

<table>
<thead>
<tr>
<th>Type of clone</th>
<th>n</th>
<th>Cells/clone</th>
<th>Interneurons</th>
<th>Motoneurons</th>
<th>Glia</th>
<th>en</th>
<th>eve</th>
<th>gsb-p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1 thor.</td>
<td>31</td>
<td>10-16</td>
<td>pCC, 1-1l</td>
<td>aCC, 1-1Ms</td>
<td>aCC, pCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-1 abd.</td>
<td>16</td>
<td>9-11</td>
<td>pCC, 1-1l</td>
<td>aCC</td>
<td>A-, B-, LV-SPG</td>
<td>aCC, pCC</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>3-2</td>
<td>26</td>
<td>10-18</td>
<td>3-2l</td>
<td>3-2Ms, 3-2Mar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP2</td>
<td>57</td>
<td>2</td>
<td>vMP2, dMP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-2</td>
<td>65</td>
<td>17-26</td>
<td>5-2l, 5-2lca2, 5-2lcp</td>
<td>5-2M</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-3</td>
<td>22</td>
<td>9-15</td>
<td>5-3lca, 5-3lcp</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-1</td>
<td>44</td>
<td>16-22</td>
<td>7-1lca, 7-1lcp</td>
<td>7-1M</td>
<td>fpCC, 4CQ</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>65</td>
<td>16-24</td>
<td>TB, 1-2l, 1-2lcp, 1-2lca</td>
<td></td>
<td>1 or 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-2 thor.</td>
<td>25</td>
<td>12-14</td>
<td>2-2l</td>
<td>2-2M, A-SPG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-2 abd.</td>
<td>30</td>
<td>12-16</td>
<td>2-2l</td>
<td>2-2M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-2</td>
<td>19</td>
<td>10-16</td>
<td>4-2l</td>
<td>RP2, 4-2Mar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-2</td>
<td>12</td>
<td>8-16</td>
<td>6-2l, 6-2lcp</td>
<td>6-2lcp2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-2</td>
<td>13</td>
<td>8-14</td>
<td>7-2l, 7-2lcp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-1 thor.</td>
<td>13</td>
<td>12-15</td>
<td>3-1l</td>
<td>RP1,3,4,5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-1 abd.</td>
<td>22</td>
<td>10-12</td>
<td>3-1l</td>
<td>RP1,3,4,5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>43</td>
<td>12-18</td>
<td>4-1l, 4-1lca, 4-1lcp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-1</td>
<td>56</td>
<td>10-16</td>
<td>6-1l, 6-1lcp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-1</td>
<td>33</td>
<td>5-10 (epid.: 1-4)</td>
<td>2-1l, 2-1lc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-1</td>
<td>27</td>
<td>2-4 (epid.: 2-9)</td>
<td>5-1l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-3</td>
<td>17</td>
<td>3-5 (epid.: 4-9)</td>
<td>7-3l Serotonergic neurons</td>
<td>7-3M</td>
<td>At least 3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. From top to bottom clones are listed according to the temporal sequence of NB delamination (top, early; bottom, late; see Table 1). n, number of clones observed; nd, not determined. See text for terminology of cell types.

Here, because it seems unlikely that NB 5-3 exhibits a unique sensitivity for Dil we conclude that apoptosis plays an important role in the elaboration of the NB 5-3 clone.

In 1 (of 22) preparation a clone consisting of only four undifferentiated cells located in the lateral cortex and associated with a large number of cell fragments was obtained from a precursor identified as NB5-3.

NB 6-2. NB 6-2 delaminates during S2 (stage 9; Table 1) to occupy a position ventral in the NB layer directly posterior to the parasegmental furrow where it exhibits a lens-like shape (Fig. 2).

The NB 6-2 lineage includes 8-16 interneurons located in the lateral cortex. Most of the interneurons form two distinct fascicles across the posterior commissure (6-2lcp1 and 6-2lcp2). In addition, there are small ipsilateral anterior extensions along the lateral border of the connective (6-2l; Figs. 9A and 10). In a few cases (3 of 11 samples) one or two pycnotic cells were detected at the lateral border of the nerve cord.

In one case the progenitor appeared to delaminate at mid stage 11. Despite this, the clone displayed the typical projection pattern and comprised 10 cells.

NB 7-2. NB 7-2 delaminates during S2 (Table 1). Until the end of stage 10, NB 7-2 is positioned immediately posterior and in the same dorsoventral position as NB 6-2. Possibly due to the delamination of NB 7-3 and the lateral shift of NB 1-2 during early stage 11, NB 7-2 is pushed more dorsally (Fig. 2).

The NB 7-2 lineage comprises 8-14 interneurons (mostly 12 cells) in the lateral cortex. They form two major fascicles, one traversing contralaterally across the posterior commissure (7-2lcp) and one ipsilateral fascicle (7-2l) extending posterior along the dorsal border of the connective thereby performing a characteristic turn from a lateral to a medial longitudinal route (Figs. 9B, 9C, and 10). Similar to the NB 6-2 clone, there are also short extensions growing anteriorly along the lateral border of the ipsilateral connective.

In 1 (of 13) sample the clone originated from a precursor delaminating at late stage 11 (S5). This clone comprised only eight cells but showed no alterations in the projection pattern.

NB 7-3. The neuroectodermal progenitor always divides once in the ectoderm to give rise to NB 7-3 and an epidermoblast. Delamination of NB 7-3 occurs during S4 (mid stage 10 to early stage 11, n = 5) or during S5 (late stage 11, n = 3; Table 1). By late stage 11 the clone consists of two cells in the ectoderm and the fairly small NB 7-3 positioned ventral and lateral to NB 7-2 in the neural layer (Fig. 2).

In late embryos the lineage comprises an epidermal subclone of four cells (nine cells in 1 of 17 cases) and the NB 7-3 subclone of only three to five neurons (mostly four) in the lateral cortex (7-3l). They form an interneuronal fascicle projecting contralaterally across the posterior commissure (Figs. 9D, 10). During stage 16 the dorsoposterior neuron in the cell cluster develops an ipsilateral posterior axon. At stage 17 this small projection ends near or inside the poste-
Embryonic CNS Lineages of D. melanogaster

FIG. 9. Photos of the lineages of intermediate NB 6-2, NB 7-2, and NB 7-3. Dorsal views (A, B, D) and lateral view (C; ventral to the bottom) of stage 17 nerve cords; anterior to the left. (A) NB 6-2 clone; neuropile stained with BP102 antibody. (B) Two NB 7-2 clones in neighboring neuromeres. (C) Lateral view of same preparation shown in B. (D) NB 7-3 clone and en-engrailed staining; about three cells in the clone express en; the dorsoposterior ipsilateral projecting motoneuron is unstained (arrowhead). White arrow indicates cell fragments. Bar = 10 μm.

FIG. 8. Photos of the lineages of intermediate NB 3-2, NB 4-2, and NB 5-3. Dorsal views (A, C, E, F) and lateral views (B, D; ventral to the bottom) of stage 17 nerve cords (except F, stage 16); anterior to the left. (A, B, same embryo) NB 3-2 clone in a thoracic and an abdominal neuromere; outgrowth of the motoneuronal projections (open arrows) precedes the interneuronal contralateral fascicle, which develops late in stage 16. (C) Two NB 4-2 clones double stained with α-eve antibody; the eve-expressing RP2 motoneuron (arrow) and the NB1-1 derivatives aCC and pCC (square bracket) are marked; note the nuclear eve staining in the DiI-labeled RP2 cells; arrowheads, RP2 projection terminating on dorsal muscles; open arrows, two or three additional motoprojections innervating ventral muscles. (D) NB 4-2 clone, lateral view. (E, F) NB 5-3 clones; white arrows indicate pycnotic cells in the clones. (E) Fully differentiated NB 5-3 clone (α-eve antibody staining; only EL neurons in focus). (F) The NB 5-3 clone is located anterior and dorsolateral inside the neural gooseberry expression domain. Except for one or two cells (open arrow), all NB 5-3 progeny can be stained with an α-gooseberry-proximal antibody. Bar = 10 μm, except in D, bar = 6 μm.

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Prior root of the intersegmental nerve or the segmental nerve; this fiber possibly represents a late differentiating motoneuron (7-3M). At least three of the 7-3I can be stained with an anti-Invected (en) antibody (Fig. 9D). They are part of the F group (Cui and Doe, 1992) of en-expressing neurons that also contains progeny of NB 1-2. Among the en-expressing medial and intermediate NBs (NB 6-1, 6-2, 7-1, 7-2, 7-3, and 1-2; Doe, 1992), only NB 1-2 and NB 7-3 produce a subset of neurons that maintain en expression at stage 17.

In the grasshopper CNS NB 7-3 is one of the latest NBs to delaminate (Doe and Goodman, 1985) and produces three or four ganglion mother cells (GMCs), with the first GMC giving rise to two neurons that synthesize the neurotransmitter serotonin (Taghert and Goodman, 1984). The immunofluorescent staining pattern of serotonin neurons in the Drosophila embryo and larva is very similar to that of the grasshopper (Lundell and Hirsh, 1994), i.e., their fibers fasciculate and project through the posterior commissure. Furthermore, the genes huckebein and engrailed have been shown to be coexpressed in the serotonin neurons and in NB 7-3, suggesting that NB 7-3 is the progenitor for these neurons also in Drosophila (Lundell et al., 1996). This is further supported by our data. As in the grasshopper, NB 7-3 is one of the latest neuroblasts.
to delaminate, and it gives rise to only two or three GMCs. Furthermore, the position in the cortex and the contralateral projection shown for the serotonin immunoreactive neurons in the Drosophila embryonic CNS (Lundell and Hirsh, 1994) corresponds to the location and projection pattern we describe here for the 7-3.

In most of the preparations we find labeled cell fragments at the lateral border of the ventral nerve cord (Fig. 9D) that presumably result from cell death within the NB7-3 lineage.

DISCUSSION

Assignment of the Lineages to Specific NBs

Prerequisite for the assignment of the distinct types of lineages to specific NBs was the identification of the labeled NBs in living embryos. Using the criteria described previously we achieved a high degree of reliability in NB identification as indicated by the distribution of lineage types obtained (Table 2) and by independent control experiments (Fig. 3). However, in contrast to the other 16 NBs, identification was problematic in the case of NB 7-1. This is most likely due to considerable positional variability of this NB around mid stage 11. Movements of NB 7-1 often seem to result in a partial overlap with NB 5-2, and thus to its repeated misidentification as NB 5-2. However, there are strong indications that we assigned the correct lineage to NB 7-1. On the one hand, we obtained a different type of lineage for NB 5-2 with high reproducibility, and the fact that all cells within this lineage express the gooseberry-proximal protein is consistent with previous reports (Zhang et al., 1994; Buentzow and Holmgren, 1995). On the other hand, the lineage we assigned to NB 7-1 comprises the CQ neurons at least two of which have been previously reported to originate from NB 7-1 (Broadus et al., 1995). Furthermore, the location of the clone in the posterior neuromere partially inside the posterior and medial portion of the gooseberry-proximal domain is consistent with its origin from NB 7-1, a medial posterior gooseberry-expressing NB (Doe, 1992; Zhang et al., 1994; Buentzow and Holmgren, 1995).

Assignment of Previously Identified Neurons to Particular Lineages

A number of cells in the embryonic Drosophila CNS are well known from previous studies. Most of them have been identified by their structural homology to cells in the grasshopper embryo (Thomas et al., 1984) and the expression profiles of certain genes are known for these cells (e.g., reviewed in Goodman and Doe, 1993). By double labeling with an \( \alpha\)-even-skipped, \( \alpha\)-engrailed, or \( \alpha\)-gooseberry-proximal antibody, our data confirm previous reports assigning the aCC, pCC neurons, and the abdominal A-SPG, B-SPG, and LV-SPG to the NB1-1 lineage (Thomas et al., 1984; Udolph et al., 1993; Broadus et al., 1995), the thoracic A-SPG to the NB 2-2 lineage (Bossing et al., 1996), the RP2 motoneuron to the NB 4-2 lineage (Thomas et al., 1984; Doe et al., 1988a,b; Patel et al., 1989a; Udolph et al., 1995; Chu-LaGraff et al., 1995), the medial CQ neurons to NB 7-1 (Broadus et al., 1995), a pair of serotoninergic neurons to NB 7-3 (Lundell and Hirsh, 1994; Lundell et al., 1996), and the vMP2 and dMP2 neurons representing the MP2 lineage (Thomas et al., 1984; Doe et al., 1988a; Spana et al., 1995).

Furthermore, we provide the first evidence that the identified RP1, RP3, RP4, and RP5 neurons (Goodman et al., 1984; Sink and Whittington, 1991) derive from NB 3-1; that the fpCC (Goodman and Doe, 1993; "candidate fpCC" in Broadus et al., 1995) and the remaining CQ neurons (Patel et al., 1989a; Broadus et al., 1995) also belong to the lineage of NB 7-1; and that NB 1-2 and NB 7-3 contribute four to six progeny to the PI group of engrailed expressing cells (Patel et al., 1989b; Cui and Doe, 1992). Future work will have to assign further molecular markers/gene expression patterns to the various lineages and their individual components to facilitate screening for mutant phenotypes and the identification of the genes controlling lineage development.

Distribution of Glia, Motoneurons, and Interneurons among Ventral NB Lineages

Fifteen of the 17 medial and intermediate NBs that derive from the ventral half of the neuroectoderm give rise to neuronal progeny exclusively and 2 give rise to neurons as well as glia. None of these progenitors is a pure glioblast. Only a few of the NBs signiﬁcantly differ by their mitotic activity (Table 3). Thus, the vast majority of the about 30 glial cells per hemineuromere (Ito et al., 1995) arise from progenitors of the dorsal half of the neuroectoderm.

In late embryos, nine of the lineages show interneuronal projections exclusively, and eight have interneuronal as well as motoneuronal fibers. In two of the latter lineages (NB 5-2 and NB 7-3), at stage 17 the motoneuronal projections still seem to be in an early phase of differentiation because they generally have grown toward the nerve root but not yet left the CNS. None of the lineages consists of motoneurons exclusively. Due to cell clustering and fasciculation of fibers we were not able to assign each motoneuronal and interneuronal projection to a certain cell body. Therefore, it is possible that some cells in the lineages are still lacking fibers in the embryo and start differentiating postembryonically.
N Bs segregating during S1–S3 give rise to clones comprising between 10 and more than 20 cells (Table 3). N Bs divide asymmetrically in a stem cell mode budding off a chain of ganglion mother cells that in turn divide once symmetrically to produce two postmitotic progeny (Bauer, 1904; Hartenstein et al., 1987). Thus, the number of embryonic divisions of the ventral S1–S3 N Bs range from 5 to 12. An exception is MP2, which delaminates like a NB during S1 but divides only once nearly symmetrically to produce the dmP2 and vMP2 neurons (Thomas et al., 1984; Doe et al., 1988a; Spana and Doe, 1995). Therefore, with respect to division pattern it resembles the behavior of midline progenitors (Klämbt et al., 1991; Bossing and Technau, 1994).

Ventral N Bs delaminating during S4 and S5 (NB 2-1, NB 5-1, and NB 7-3) give rise to clones comprising 2–10 cells corresponding to only one to five divisions (Table 3). Interestingly, all these N Bs have an epidermal sister clone (see Technau and Campos-Ortega, 1986). This indicates that their neuroectodermal progenitors divide and although one of the two daughter cells subsequently delaminates as NB, the other one always remains in the ectoderm as epidermblast. This is consistent with the view that the decision of the neuroectodermal cells to adopt the epidermal or the neural fate is mediated by cell–cell interactions and that within distinct neural competence groups of neuroectodermal cells (so-called proneural cell clusters) only one cell is singled out to become a neuroblast (reviewed in Campos-Ortega, 1993). According to this model, the neuroectodermal progenitors of NB 2-1, NB 5-1, and NB 7-3 divide in the ectoderm and the two progeny remain within the limits of the respective proneural cluster; here they participate in the cell interactions that determine one cell to delaminate as NB, the sibling and the other cells of the cluster develop as epidermblasst. Probably due to their previous division in the ectoderm, the size of the delaminating S4/S5 N Bs as well as the number of their progeny is smaller compared to earlier N Bs delaminating without a previous division.

Segmental Differences

As opposed to the situation in grasshopper embryos (Bate, 1976; Doe and Goodman, 1985) there are no apparent differences between thoracic and abdominal NB arrays in Drosophila embryos (Doe, 1992). However, three of the ventral N Bs (NB 1-1, NB 2-2, and NB 3-1) express segmental differences in their embryonic lineages (for the neuroglioblasts NB 1-1 and NB 2-2, see Udolph et al., 1993; Bossing et al., 1996). It is possible that other ventral NB lineages express segment-specific structural differences (e.g., in the dendritic branching pattern) that were beyond the level of resolution. Segmental specificity of NB 1-1 has been shown to be regulated by homeotic genes (Ubx and Abd-A) and to be determined in the neuroectoderm (Prokop and Technau, 1994). Further segment-specific features become obvious in the CNS during postembryonic stages (Valles and White, 1988; Truman and Bate, 1988; Prokop and Technau, 1991; Cantera and Nüssel, 1992). For example, whereas in the thorax most embryonic N Bs resume proliferation in the larva, this is not the case in the abdomen (except the most posterior neuromeres). Segment-specific commitment of precursors for postembryonic proliferation is already achieved in the early gastrula neuroectoderm (Prokop and Technau, 1991; A. Prokop, S. Bray, and G. Technau, unpublished data).

Spatial Relationships

The positions of the N Bs in the NB layer correlate with their site of origin in the neuroectoderm. For example, the medial N Bs derive from the ventralmost neuroectoderm and the intermediate N Bs from the ventrolateral neuroectoderm. No lateral row N Bs are obtained upon labeling of cells in these regions of the neuroectoderm (see Hartenstein et al., 1994; Udolph et al., 1995). Also, the dorsoventral (mediolateral) and anteroposterior location of the clones in the late embryonic neuromeres correspond to the positions of their parental N Bs. However, in some lineages, specific progeny cells migrate away from the main cluster (e.g., aCC/pCC, RP2, A-SPG, and B-SPG).

The segment-specific neuroglioblasts NB 1-1 and NB 2-2 are closely associated medial N Bs and have thus been exposed to similar positional cues in the neuroectoderm. They give rise to only one thoracic (NB 2-2) and three abdominal (NB 1-1) glial cells, whereas all other glia derive from the dorsal half of the neuroectoderm (H. Schmidt, T. Bossing, and G. Technau, unpublished data). Such a dorsoventral positional preference is not obvious with regard to the production of motoneurons: about 20 of 34 motoneurons (Sink and Whittington, 1991) derive from the ventral half of the neuroectoderm; 11–13 of these arise from medial N Bs (NB 1-1, NB 2-2, NB 3-1, NB 5-2, and NB 7-1), and 8–10 from intermediate N Bs (NB 3-2, NB 4-2, and NB 7-3). Furthermore, there is no strict correlation between the dorsoventral site of origin of progenitors giving rise to motoneurons and the muscle innervation pattern. Although most of the motoneurons derived from the medial and intermediate N Bs innervate ventral and ventrolateral muscles, some of them, the aCC, RP2, 3-2M ar, and 7-1M, innervate dorsal muscles. These cells derive from four different N Bs (medial NB 1-1 and NB 7-1 and intermediate NB 3-2 and NB 4-2). Interestingly, the same N Bs give rise to further motoneurons that innervate ventral and ventrolateral muscles.

Axon Projection Patterns

All the lineages form contralateral interneuronal projections, except those derived from NB 1-1 and MP2. Most of the motoneuronal axons project ipsilaterally (except in the NB 3-1 and NB 5-2 lineages). The contralateral projection patterns generally correlate with the anteroposterior segmental position of the N Bs (except for NB 1-2, NB 4-2, and NB 7-1). Contralateral fibers of neurons derived from anterior N Bs (NB 2-1, NB 2-2, NB 3-1, and NB 3-2) traverse
across the anterior commissure, those from posterior NBs (NB 5-1, NB 6-1, NB 6-2, NB 7-2, and NB 7-3) extend across the posterior commissure, and those from NBs in the middle of the neuromere (NB 4-1, NB 5-2, and NB 5-3) cross through both commissures.

Most of the clonal progeny cells are arranged as dense clusters. Generally, cells of these clusters form two or three distinct fascicles. In some clones one or a few larger cells occupy positions apart from the cluster, such as aCC, pCC, RP2, and the TB neuron. These cells, of which the aCC/pCC and the RP2 are known to appear first in the NB 1-1 and NB 4-2 lineages (Thomas et al., 1984; Patel et al., 1989a; Doe, 1992; Broadus et al., 1995), project along separate routes and do not or only partially fasciculate with the other members of their clones.

Motoneurons of the NB 3-2 and NB 4-2 lineages innervate muscles of their own segment as well as of the next posterior segment. In the lineages of NB 1-1, NB 1-2, MP2, NB 2-2, NB 4-1, NB 5-2, NB 6-1, and NB 7-2 we find interneurons projecting across neuromere borders (“projection interneurons”). In the late embryonic lineages of the other ventral NBs the interneuronal projections are within the limits of their neuromere; these may belong to “local interneurons” and/or interneurons that further differentiate into projection interneurons at later stages.

Expression of Segmentation Genes in the CNS Is Not Lineal

Double labeling with antibodies can be used to test whether gene expression patterns correspond to clonal boundaries. We used antibodies directed against the products of three different segmentation genes. Two of the medial and intermediate NBs (NB 1-2 and NB 7-3) were found to contribute to the population of CNS cells that express eve at late stage 17. Both lineages also comprise cells that do not express eve. The same is true for eve, which is expressed by only a few cells in the lineages of NB 1-1, NB 7-1, and NB 4-2 (Table 3; see Doe et al., 1988b). In the case of gsb-p we find all progeny expressing the gene in the NB 5-2 clone. This corresponds to the data of Buenzow and Holmgren (1995) using a different approach. These authors report gsb expression (between stages 10 and 15) in all progeny of 8 of 10 gsb-expressing NBs. Among these are the medial NB 5-1, NB 5-2, NB 6-1, and the intermediate NB 6-2, whereas they did not detect gsb-p expression in the progeny of NB 7-1 and could not assign a clone to NB 5-3, two further gsb-expressing NBs. We did not assay the lineages of NB 5-1, NB 6-1, and NB 6-2 for gsb expression. However, for NB 7-1 we find most of the progeny not expressing the gene and only a small subset located in the gsb expression domain. One of the NB 7-1 progeny, the fpCC neuron, has been previously reported to be gsb negative (Broadus et al., 1995). Also, for the NB 5-3 progeny our data suggest that a few of them do not express gsb. This, however, needs to be further substantiated. We did not test gsb expression at earlier stages.

Thus, in the late embryonic lineages of medial and intermediate NBs the expression of en and eve is not lineal, whereas gsb expression appears to be lineal in some, but not all, of the gsb-expressing NBs.

Variabilities

Although the stages of delamination of the various NBs in general corresponded to the pattern previously described (Hartenstein and Campos-Ortega, 1984; Doe, 1992), they often deviated from this pattern (Table 1). We cannot rule out that this variability is due to technical influences. However, we do not think the development of the embryos is generally faster or slower than unlabeled embryos. If so, one would expect all NBs of an embryo to be affected to the same extent; this is not the case. For example, we found low variability for NB 4-2, but high variability for NB 1-2 (Table 1). Furthermore, concomitant labelings of two or more neuroectodermal cells in the same embryo and comparison of their actual times of delamination with those expected from previous reports suggest that the observed variability is not solely due to general developmental retardation: In 31 of 89 embryos with up to four labeled NBs, only one cell was delayed or both were delayed to different degrees. On the other hand, it could be possible that the Dil affects the delamination time of the various NBs to different degrees. Interestingly, the variability in the delamination of NBs had no obvious effect on the composition of their lineages. This is in agreement with data showing that NB fate is specified early in the neuroectoderm (Chu-La Graff and Doe, 1993; Skeath et al., 1995).

With the exception of NB 7-1, variability of the positions of NBs upon delamination into the neural layer is rather low. The degree of positional variability differs between the individual NBs and correlates with the efficiency of their identification in vivo.Variability was also found to be low in the neuronal projection patterns.

Significant variation was observed in the number of cells for each type of lineage (Table 3; see Udolph et al., 1993, for NB 1-1). Partly this variability could be due to mistakes in counting tightly clustered cells, especially if the clonal size exceeds 10 cells. On the other hand, even the smallest lineages (e.g., NB 7-3 and NB 5-1) exhibit variations in their numbers. The same was found for the CNS midline lineages (Bossing and Technau, 1994). Therefore, variations in cell number seem to be an inherent feature of cell lineage. However, numbers of some neural types seem to be more fixed than others. For example, in all NB 1-1 lineages we found only one aCC and one pCC (see Udolph et al., 1993) and all NB 4-2 lineages consistently contained only one RP2. These cells derive from the first GMC in these lineages and are assumed to pioneer the first axonal fascicles (Thomas et al., 1984; Jacobs and Goodman, 1989). Variable numbers of clonal cells may be partly compensated by inter-
lineage cell communication or by apoptosis. Alternatively, it may not be necessary to compensate for variable cell numbers.

Programmed cell death has been reported to occur during neurogenesis in vertebrates as well as in invertebrates (Bate et al., 1981; Oppenheim, 1991; Truman et al., 1992; Zhou et al., 1995). According to recent estimations about 50% of all cells in the embryonic nerve cord of Drosophila are subjected to apoptosis (White et al., 1994). We find indications for apoptosis in the late embryonic lineages of the abdominal N B 3-1, N B 5-3, N B 6-2, and N B 7-3. Fragmented cells are removed from the CNS and engulfed by phagocytic hemocytes (Abrams et al., 1993; Sonnenfeld and Jacobs, 1995). Because this is likely to be a continuous process and we examined most of the lineages after mid stage 17, we are unable to estimate from the present data the contribution of apoptosis in the development of the various lineages.

**Prospects**

We describe here the complete embryonic lineages of 17 identified N Bs (medial and intermediate) that represent more than 50% of the entire population of N Bs per hemisegment and we discuss principle features expressed by these lineages. The lineages of the remaining N Bs are currently being investigated because the complete segmental set of lineages will provide deeper insight into general mechanisms of CNS lineage development. The identified wildtype lineages provide a foundation for the interpretation of mutant phenotypes, gene expression patterns and experimental manipulations (e.g., misexpression, cell transplantation, and cell ablation). Further investigations will have to link a variety of cell-specific molecular markers to the individual components of the respective lineages and determine their sequence of birth. The data will thus facilitate the clarification of the molecular genetic mechanisms leading to cell fate specification in the CNS. They may also form a basis for investigating the relationships between cell function and lineage.

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