The mechanisms leading to cell diversity in the CNS feature among the major unsolved problems in developmental biology. Approaching these complex mechanisms requires detailed information on normal development at a cellular level. This includes identification of the various neural cell types and clarification of their origin, their lineage-relationships and the dynamic aspects of their development like morphogenetic movements, division patterns and differentiation. Accordingly, considerable effort has been put into cell-lineage analysis in a large number of different organisms and a variety of technical approaches have been used.

The fruitfly *Drosophila* is especially well suited to investigations of the origin of cell diversity in a complex CNS using different approaches on the molecular, genetic and cellular level. However, while many candidate genes controlling cell fate in the CNS have been identified in *Drosophila*, it has been difficult to interpret expression patterns and mutant phenotypes due to lack of knowledge on the types and lineage relationships among cells in the wild-type CNS.

Several different strategies have been used so far to label *Drosophila* embryonic cells and to trace their fate. One is the generation of genetic mosaics in which the fate of genetically labelled cells is followed. This may be achieved by the induction of mitotic recombination (e.g. Stern, 1936; Garcia-Bellido et al., 1973), by chromosome loss (gynandromorph technique; for review see e.g. Janning, 1978) or by microsurgically mixing cells derived from genetically different individuals (e.g. Illmensee, 1978). Another approach makes use of the enzymatic lineage marker HRP, which may be directly injected into cells (Technau and Campos-Ortega, 1985), or cells from donors, which have been globally labelled with HRP, may be transplanted into unlabelled hosts (Technau, 1986). A huge variety of cell-specific markers has been generated using the enhancer-trap technique (O’Kane and Gehring, 1987) or by producing antibodies. Recently, photoactivation of a caged fluorescein (Mitchison, 1989) has been used to trace the development of epidermal progenitors (Vincent and O’Farrell, 1992). However, for lineage analysis, most of these techniques have some limitations. For example: the numbers of labelled progenitors are difficult to ascertain (genetic mosaics; dye injections); cells have to be punctured by a capillary (dye injections) or are removed from their original environment (transplantation of labelled cells); expression of cell-specific markers is transient and, in most cases, it is not clonally restricted (enhancer-trap lines; antibodies); and finally, the types (morphology) of progeny cells remain obscure because the marker is limited to cellular substructures (e.g. nucleus) or is weakly or inhomogenously expressed (genetic mosaics; most enhancer trap lines and antibodies, photoactivation of fluorescent dyes).

To overcome these limitations, we have developed a new method for lineage tracing in which individual cells are non-invasively labelled in their original positions with a lipophilic fluorescent tracer (DiI). Development of a labelled progenitor cell can be followed in vivo (cell divisions, morphogenetic movements and differentiation). Following photoconversion of the dye, fully differentiated clones can be analyzed in permanent preparations.

**SUMMARY**

We present a new method for marking single cells and tracing their development through embryogenesis. Cells are labelled with a lipophilic fluorescent tracer (DiI) in their normal positions without impaling their membranes. The dye does not diffuse between cells but is transferred to the progeny, disclosing their morphology in all detail. Behaviour of labelled cells can be observed in vivo (cell divisions, morphogenetic movements and differentiation). Following photoconversion of the dye, fully differentiated clones can be analyzed in permanent preparations.

We apply this method for cell lineage analysis of the embryonic *Drosophila* CNS. Here we describe the fate of the CNS midline cells. We present the complete lineages of these cells in the fully differentiated embryo and show that variability exists in segmental numbers of the midline progenitors as well as in the composition of their lineages.

Key words: cell lineage, CNS, midline, *Drosophila*, DiI, single cell labelling
review see Nambu et al., 1993). Many of the cells that derive from the ventral midline have been previously identified (e.g. Jacobs and Goodman, 1989) and their lineage relationships have been investigated by using enhancer-trap lines as cell-specific markers (Klämbt et al., 1991). However, as the applicability of enhancer trap lines for lineage analysis is limited and since cell morphologies are hardly detectable using these markers, the types of progeny of the midline cells and their lineage relationships have not yet been entirely clarified. Our approach has allowed us to uncover completely the lineages of the midline progenitors including the detailed morphologies of their progenies. We will also present information about the early dynamic behaviour of the midline cells until they delaminate from the ectoderm. Finally, we will demonstrate that variability exists in segmental numbers of the midline progenitors as well as in the composition of their lineages.

MATERIALS AND METHODS

Mounting embryos for labelling and in vivo inspection

Eggs were collected from the Oregon R wild-type strain. Embryos at the blastoderm stage were mechanically dechorionated, mounted with their ventral side down onto a coverslip coated with glue, desiccated for about 5-10 minutes and covered with fluorocarbon oil as described elsewhere (Prokop and Technau, 1993). While mounting the embryos, they were slightly pressed onto the coverslip so that the midline and several adjacent rows of neuroectodermal cells came to lie in one focal plane (Fig. 1). Preparations were transferred to an inverted microscope (Leica, Fluovert), equipped with fluorescence and bright-field optics and a videocamera (see below).

Labelling individual cells with DiI

As a non-toxic solvent for the DiI (Molecular Probes), we used vegetable oil (e.g. Soya oil). 0.75-1.0 mg DiI/ml oil was sonicated and ground with an angle of about 20-30° on a commercial capillary grinder to obtain an outer tip diameter of about 1 μm. The capillary was fixed with the ground side up to the holder of a manual micromanipulator (Leica). Pressure in the capillary was controlled by an air-filled single-micromanipulator (Leica). Pressure in the capillary and the individual cells could be easily identified (Fig. 2A). Within a few seconds, the dye diffused into the cell membrane leaving behind the non-fluorescent oil solvent. Only the cell that was first contacted by the drop was labelled. This could be confirmed by the excitation of the fluorescence (fluorescein filter set, 450-490 nm; Fig. 2C,D). Labelling was restricted to this cell and its progeny (Fig. 2D-F).

The midline cells were easily identifiable by their shape and position under the magnification used. Their position along the longitudinal axis was determined by counting the cells beginning at the posterior edge of the cephalic furrow. Cells were labelled 20-25 minutes after the onset of gastrulation (at 24°C; early stage 8; stages according to Campos-Ortega and Hartenstein, 1985), before they divided and delaminated from the ectoderm. We labelled up to four midline cells at different positions in the same embryo.

In vivo analysis

The early behaviour of the midline cells until their delamination from the ectoderm was continuously monitored by time-lapse videomicroscopy under normal transmitted light. An image (videocameras: Hamamatsu C2400-07 or C2400-77) was loaded on a time-lapse recorder (Panasonic AG 6720A) every three seconds, following integration of 8 frames and contrast enhancement by an image processor (Hamamatsu DVS 1000).

In vivo inspection of DiI-labelled cells was carried out at selected stages by the simultaneous use of fluorescent and transmitted light and documented by the vide system. By using a fluorescein filter set and appropriate light intensity (adjustable halogen lamp), prolonged excitation of the fluorescence does not seem to impair the development of the labelled cells. For inspection of fully differentiated clones at late stage 17, we switched to a rhodamin filter set, which elicits a very bright fluorescence (Fig. 2E,F). Alternatively, or in addition, endpoint analysis of clones was carried out in permanent whole-mount preparations following photoconversion of the dye.

Photoconversion of labelled clones for permanent whole-mount preparations

To obtain permanent whole-mount preparations with labelled clones in a fully differentiated state, we made use of the fact that DiI can be photoconverted into a dark brown precipitate (e.g. von Bartheld et al., 1990). When they reached the appropriate stage (late stage 17, first spontaneous muscle contractions), the embryos were processed under the microscope while still fixed on the coverslip. First, a capillary was pushed through the abdomen of the embryo, making holes on both lateral sides. After retraction of the capillary, a solution of 0.2 M KCl was injected with increasing pressure until most of the gut slipped out through the contralateral hole. The specimen was then fixed by injecting a mixture of 3.7% formaldehyde and 0.1% glutaraldehyde in
PBS (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.2). After 15 minutes, a solution of 1% milk powder (MP) in PBS was injected into the embryo (about the same amount as the fixative) and the embryo was incubated for about 10 minutes. The concentration of the fixative was further diluted by a second injection of MP solution (treatment with MP was found to reduce background staining). After about 30 minutes, most of this solution was removed and replaced by the injection of PBS. About 10 minutes later, the embryos were injected with diaminobenzidine solution (2-3 mg DAB in 100 mM Tris-HCl; pH, 7.4) and irradiated for 10-20 minutes with mercury light transmitted through a rhodamin filter set and a 63× or 100× oil immersion objective. Intensity of the brown reaction product is normally adequate shortly after the clone shows no more fluorescence. Embryos were then fixed according to Zalokar and Erk (1977) for 15 minutes, devitellinized mechanically, washed in PBT (0.33% Triton X-100 in PBS) for 2×10 minutes, dehydrated in ethanol, transferred into Araldite and embedded in capillaries with an inner diameter of 0.2 mm (see Prokop and Technau, 1993). Preparations can be inspected and documented from all sides by rotating the capillaries under the objective. If conservation of the peripheral nerves is not required, background staining may be reduced or completely abolished if photoconversion is applied to isolated CNS or to embryos after complete removal of the gut system.

**Immunohistochemistry**

Embryos of the transformant strain P[3,7sim/lacZ] (Nambu et al., 1991) were stained for β-galactosidase expression using a β-gal antibody (Dianova) or were double stained with an additional antibody against Engrailed (4D9; Patel et al., 1989). The staining procedure was performed as described elsewhere (Schmidt-Ott and Technau, 1992).

**RESULTS**

**The method**

The method, described above, essentially meets all require-
ments to be used for a detailed cell-lineage analysis in *Drosophila* (and presumably other species).

1. Cells are labelled individually. Labelling of a single cell can be directly monitored under the fluorescence microscope (Fig. 2A-D).

2. Cells are labelled in situ, i.e. in their original position. Since the cell to be labelled directly faces the microscopic lens, it can be unambiguously identified. Any cell at the external surface of the embryo can be marked. Its position can be determined with respect to morphological landmarks (e.g. cephalic furrow, ventral furrow) and to the known early gastrula fate map (Technau and Campos-Ortega, 1985; Technau, 1987).

3. The dye (DiI) solution is non-toxic. The solvent (vegetable oil) that we selected for DiI is not deleterious for the cells, as opposed to alcohol, which is normally used as a solvent for lipophilic dyes (Honig and Hume, 1986).

4. Cells are labelled in a non-invasive fashion. Contact of the dye solution with the external surface of the membrane is sufficient for the cell to become labelled. Due to its lipophilic nature, the dye invades and homogenously stains the entire cell membrane within a few seconds, leaving behind the droplet of oil solvent between the vitelline membrane and the epithelium. Transfer of the dye from the solvent to the cell is almost quantitative so that only the cell that is first brought into contact with the dye solution becomes labelled.

5. The dye does not diffuse between cells but is transferred to all embryonic progeny with neglectable dilution.

6. The dye intensely and homogeneously labels the entire cell membrane of the embryonic progeny cells, thus disclosing their detailed morphology at any stage of differentiation (Figs 2, 3-5).

7. Dynamic aspects of the development (cell divisions, morphogenetic movements, fibre growth) can be directly traced in vivo and documented by video- or laser scanning microscopy. Prolonged excitation of the fluorescence (up to 3 minutes) does not seem to interfere with normal development of the cells when using a fluorescein filter set and appropriate adjustment of light intensity. Also, fading of the dye is very low under these conditions. Due to the orientation of the embryo and the use of an inverted microscope, the labelled structures are close to the lens. This, as well as the use of oil immersion lenses (up to 100×), allows for high resolution. To address the position and types of labelled cells in the context of surrounding tissues more easily, fluorescent and transmitted light may be used simultaneously.

8. For inspection of the fully differentiated clone, one can switch to a rhodamin filter set which elicits a very bright fluorescence. Prolonged excitation under these conditions impairs the composition of fully differentiated clones can be analyzed in detail in permanent whole-mount preparations (Fig. 5).

9. The method works efficiently. 73% of the labelled midline progenitors resulted in labelled clones. For labellings in the ventral neurogenic ectoderm, we have gained an efficacy of 90%. Furthermore, it is possible to mark individually several different cells in the same embryo.

### The early development of the midline cells

At the blastoderm stage, the mesectodermal or midline progenitor cells are arranged as one row on either side separating the anlage of the mesoderm from the neurogenic ectoderm. During gastrulation, the mesoderm invaginates (forming the ventral furrow) and the two rows of mesectodermal cells become juxtaposed along the ventral midline (Poulson, 1950; Nambu et al., 1990; Klämbt et al., 1991). At this stage, these cells can be unambiguously identified in the living embryo due to their position and their characteristic shape. To disclose the dynamics of their early behaviour until they delaminate from the ectoderm, we continuously monitored the midline cells in vivo using time-lapse videomicroscopy.

About 25 minutes (at 24°C) after the onset of gastrulation, (a.o.g.) the peripheral diameter of the midline cells significantly increases (Fig. 3A). About 10 minutes later, they alternately rotate clockwise and counterclockwise (at least five times) for 3-4 minutes. These rotations are followed by their first postblastodermal division (Fig. 3B,C). This behaviour can also be observed for other dividing cells in the neurogenic ectoderm. Orientation of the mitotic spindle is in parallel to the epithelial layer but seems to be random within this plane. About 40 minutes a.o.g., the midline cells complete their mitosis and their daughter cells occupy the medial 2-3 rows of the ectoderm. In the course of further germ band elongation, they become stretched along the longitudinal axis forming a single row on either side in which sibling cells remain next to each other (Fig. 3C). At the end of stage 9, their basal part becomes progressively shifted interiorly, but they still maintain a prominent cytoplasmic extension to the periphery, which progressively stretches along the longitudinal axis until completion of germ band elongation (late stage 10; Fig. 3D; see also Wharton and Crews, 1993). Finally, during the second half of stage 11, all midline cells completely delaminate from the ectoderm. As a rule enlargement of cell diameters, division and delamination begin near the cephalic furrow and progress posteriorly. Further divisions among midline cells are only performed by the MNB and by progenitors that exceptionally give rise to larger compound clones (see below). In these cases, the second division takes place between stages 11 and 13 and the third division between stages 14 and 16.

Taken together, all midline progenitors divide while still in the ectoderm, and delamination is not completed before the end of stage 11. The sequence of early dynamic processes, including enlargement of cell diameters, rotatory movements, division and segregation, follows a precisely regulated temporal and spatial pattern (see also Foe, 1989).

### The lineages of the midline cells

Thoracic midline progenitors numbers 8-15 on either side (as counted from the cephalic furrow) were individually labelled about 35 times each (see Materials and methods). In total we analyzed 537 clones. These fall into five different classes (see Figs 4, 5).

1. **VUM neurons (Figs 4A; 5A)**

Clones of this class consist of cells known as ventral unpaired median (VUM) neurons (Goodman et al., 1984; Sink and Whittington, 1991). We obtained 215 clones of VUM neurons (40% of all clones). In 169 cases (79% of all VUM clones), the
Lineage analysis of the Drosophila CNS midline consisted of only two cells, one motoneuron and one interneuron. But we also gained VUM clones that contained four cells \( (n=32; \ 15\% \ of \ all \ VUM \ clones; \ Fig. \ 5A) \) or six cells \( (n=14; \ 6\% \ of \ all \ VUM \ clones). \)

At stage 17 the closely associated cell bodies of the VUM neurons are located medially in the ventral and posterior cortex region of the neuromere. Their fibers run tightly fasciculated dorsally towards the neuropile where they separate to form a motoneuronal projection bifurcating in the dorsal part of the anterior commissure and an interneuronal projection bifurcating in the ventral part of the posterior commissure. First, outgrowth of axonal processes was detected at stage 13. In stage 16, the VUM neurons appear to be shifted from a position close to the posterior commissure towards their final position in the most ventral cortex region.

In most instances \( (n=121) \), we were able to distinguish between two types of 2-cell VUM clones differing with respect to their motoneuronal projection: In one type \( (n=76; \ 63\% ; \ see \ Fig. \ 4A) \), the fiber on either side leaves the CNS through the posterior root of the anterior fascicle (intersegmental nerve) and ends in a dorsolateral to dorsal region. In the other type \( (n=45; \ 37\% ) \), the fiber leaves the CNS through the posterior fascicle (segmental nerve) and ends in a ventral-to-ventrolateral region of the embryo (nomenclature of nerve roots according to Thomas et al., 1984). The course of the interneuronal projection shows no significant variations.

In 28 cases of the 4-cell VUM clones, we examined the projection pattern in some detail. In most instances \( (n=22; \ 79\% ) \), we detected two bifurcated motoneuronal as well as two bifurcated interneuronal projections. The interneuronal projections left the posterior commissure tightly fasciculated and did not separate until close to their termination sites in the connectives. The motoneuronal projections in most cases \( (n=13) \) left the CNS separately through the segmental and intersegmental nerve (see Fig. 5A) to innervate muscles in the ventral-to-ventrolateral and dorsolateral-to-dorsal region, respectively. In 9 cases, the fibers left the CNS through the same fascicle only to end on muscles either in the ventral-to-ventrolateral region or in the dorsolateral-to-dorsal region. In most of these cases, we were able to detect separate endplates but not to identify the innervated muscles. In 6 cases \( (21\% ) \), we could only resolve one interneuronal and one motoneuronal projection. This could have been due to tight fasciculation of the projections or to an undifferentiated state of two neurons of the clone.

The projection pattern of the 6-cell VUM clones is essentially the same as for the 4-cell clones. In 7 instances \( (n=50\% ) \), we detected two interneuronal and two motoneuronal fibers on either side (in 2 cases the motoneuronal projections left the CNS through the same fascicle to innervate ventral muscles) and, in 7 instances, we detected only one of each projection.

(2) Midline glia (Figs 4B, 5D)
78 clones \( (14.5\% \ of \ all \ clones) \) consisted of glial cells previously described as midline glia (Jacobs and Goodman, 1989;
Klämbt and Goodman, 1991; Klämbt et al., 1991). In most cases, the clones were composed of 2 cells; in three cases, we found 3-4 cells and in five cases 1-2 cells. As cells of each clone were closely attached to each other and their nuclei were sometimes obscured, we were not always able to determine precisely the number of glial cells per clone. In 54 instances, we determined the position of the cells. At stage 17, they typically \((n=39)\) envelop the two commissures with one nucleus in a more ventral and the second nucleus in a more dorsal position between the commissures. In 9 cases the two cells

**Fig. 4.** Camera-lucida drawings of photoconverted preparations showing characteristic examples of the five types of midline clones at late stage 17. Each clone is presented in horizontal (left) and lateral view (right). (A) VUM clone consisting of one motoneuron (blue fibers) and one interneuron (red fibers); (B) pair of midline glia; (C) MP1 clone consisting of a pair of interneurons with ipsilateral projections; (D) MNB clone representing a cell cluster with short bilateral projections in the anterior and posterior commissure (blue) and a long bilateral fiber, which enters the intersegmental nerve (red); (E) UMI clone (the projections of the two interneurons are indicated in blue and red, respectively). Broken lines mark the outlines of the neuropil; the outline of the ventral nerve cord is marked by solid lines. In A-E, anterior is left and in the lateral views dorsal is up. a, anterior commissure; araf, anterior root of the anterior fascicle (intersegmental nerve); con, connective; dvc, dorsoventral channel, demarcating the neuromere border; p, posterior commissure; pf, posterior fascicle (segmental nerve), praf, posterior root of the anterior fascicle (intersegmental nerve).
were located dorsoposteriorly to the posterior commissure and in 4 cases dorsoanteriorly to the anterior commissure. We also obtained two clones consisting of two cells which were located in neighbouring neuromeres.

(3) MP1 neurons (Figs 4C; 5B,C)
79 clones (14.7% of all clones) consisted of a pair of interneurons previously described as midline precursor 1 (MP1) neurons (e.g. Goodman et al., 1984). At stage 17, the MP1 neurons reside slightly anterior and ventral to the posterior commissure in the corner formed by the connectives and the posterior commissure. Their ipsilateral projection bifurcates in an anterior and posterior branch, which runs within the medial sector of the connective. Since the branches at this stage still seemed to be growing, their length varied among the preparations. The posterior branch may span up to three neuromeres whereas the anterior branch may span up to two neuromeres.

At stage 13, the MP1 neurons are located dorsally in close neighbourhood to each other. At this stage, we could detect outgrowth of fibers. Bifurcation of the fibers occurred at the end of stage 14. During the condensation of the nerve cord (stage 16/17), the cell bodies of the MP1 become shifted more laterally to their final position.

(4) Cell cluster (MNB clone) (Figs 4D, 5E)
This type of clone was obtained in 71 instances (13.2% of all clones). The clones comprised 5-8 neurons, most of which consisted of 6 cells (n=35; 49%). Their cell bodies form a dense medial cluster ventrally in the posterior cortex of the neuromere. They send a fascicle dorsally towards the posterior commissure where it bends to run anteriorly along the midline to the anterior commissure. At the anterior edge of the anterior commissure, it splits into short bilaterally projecting fibers. Between the commissures, one fiber leaves the main bundle and bifurcates at the dorsoposterior rim of the anterior commissure to project on either side to the lateral area of the connective. In late embryos (end of stage 17), this fiber may have reached the border of the ventral nerve cord by projecting through the intersegmental nerve. In one case, it had even left the CNS to end near a ventrolateral muscle, suggesting that it represents a late differentiating motoneuronal projection. In a number of late stage 17 embryos, we observed a further projection which medially enters the posterior commissure and splits into short bilaterally projecting fibers similar to the anterior projection in the anterior commissure. We did not detect any differentiation of fibers before stage 16.

At stage 17, in addition to three of the VUM neurons, a cluster of about 6 small cells stains positive with the anti-engrailed antibody. Since the median neuroblasts (MNB) and its progeny are known to express engrailed (Patel et al., 1989; Doe, 1992) and because these cells are located in the same position as the cluster of cells described here, we believe that this type of clone derives from the MNB.

(5) Unpaired median interneurons (UMI) (Figs 4E, 5F)
We gained 75 clones (14% of all clones) consisting of two interneurons that have not been described before. We call these cells ‘unpaired median interneurons’ (UMI). Their cell bodies

---

Fig. 5. Photocverted preparations of different types of midline clones at stage 17. (A) VUM clone, (B,C; same preparation) two MP1 clones in consecutive segments, (D) midline glia, (E) MNB clone, (F) UMI clone; (A,B,E,F) horizontal views (anterior is left), (C,D) lateral views (dorsal is up). In A,E and F different focal planes were combined using the Photoshop program on a Macintosh computer. a, anterior commissure, i, interneuronal projection, m1, m2, motoneuronal projections, p, posterior commissure. Scale bar 8 μm in A,D,F, 10 μm in B, and 13 μm in C,E.
occupy a position dorsal to the VUM and MNB cells (see Fig. 7B,C). One of them projects medially towards the anterior region of the anterior commissure. Here it bifurcates and both branches enter the connective where they turn anteriorly. The fiber of the second UMI neuron splits in the posterior commissure to project bilaterally to the lateral part of the connective. Here it bifurcates again on either side into an anterior and a posterior branch. An additional small bilateral branch is formed in the anterior commissure.

We did not detect any axonal outgrowth of the UMI before stage 15.

Variabilities in the composition of the clones

In a number of cases (n=19; 3.5% of all clones), the composition of clones differed from the typical patterns described above in that cells normally belonging to different lineages appeared together.

In four cases, MP1 cells appeared together with UMI neurons. One of these clones consisted of 2 MP1 and 2 UMI neurons. Two clones consisted of 3-4 cells and only 1 UMI projection could be detected. One clone consisted of only 1 MP1 and 1 UMI cell.

There were six cases in which 2 MP1 neurons shared a common lineage with 2 midline glia.

Finally, in nine cases a VUM clone (1 motoneuron, 1 interneuron) was combined with a cluster of 6-7 cells, the position and projection pattern of which resembled the MNB clone.

Thus, midline progenitors also give rise to compound clones. These do not seem to represent a random mixture of clonal types, since only three of ten possible combinations among the five different classes of clones were observed. Similarly, the 4-cell and 6-cell variants of the VUM clonal type may be considered as compound clones comprising cells that normally are members of separate lineages derived from three different VUM progenitors (see below).

Segmental number and identity of the midline progenitors

Consecutive midline progenitors of about two segmental anlagen in the thoracic region were selected for our analysis (Fig. 6). Each of these progenitors were labelled an approximately equal number of times and they gave rise to the five different types of clones described above. Consequently, the ratios of the frequencies of the various clonal types (in % of all 537 clones analyzed) should correspond to the segmental number of progenitors giving rise to each particular type. According to this assumption, there are three progenitors per segment for the VUM clones (40% of all clones) and one progenitor for each of the other four clonal types (approx. 14% each for MP1, midline-glia, UMI and MNB). The existence of three VUM progenitors is further supported by the fact that we found two types of 2-cell VUM clones (with respect to the motoneuronal projection), one of which appeared twice as often as the other (see above).

Thus, our data suggests the existence of seven midline progenitors per segment. However, eight midline progenitors have been suggested by previous reports (e.g. Klämbt et al., 1991). We therefore counted the number of midline cells using the expression pattern of the segment polarity gene *engrailed* as a segmental marker. Embryos at stage 8 of the transformant strain P[3.7sim/lacZ], which yield midline-specific β-galactosidase expression in a pattern similar to the endogenous sim protein (Nambu et al., 1991, 1993), were double stained with antibodies against β-gal and engrailed (Fig. 7A). We examined 31 embryos and counted the number of midline progenitors in 121 parasegments between the first thoracic (T1) and the second abdominal segment (A2). The number of progenitors turned out to be variable: 8 cells were found in 55%, 7 in 31%, and six in 11% of all segments evaluated. In two exceptional cases (2%), we counted 9 and in one case (1%) even 10 cells. In total, this corresponds to an average of 7.5 midline progenitors per segment.

Variability in the number of midline cells per segment is

![Fig. 6. Distribution of clonal types derived from thoracic midline progenitors no. 8-15 (as counted from the cephalic furrow). Frequencies are given in % of total numbers of clones (n) obtained from the respective progenitors of both (left and right) sides.](image-url)
further corroborated by the following experiment. We simul- 
taneously labelled - in the same embryo - 4 midline progeni- 
tors in a 4-cell-distance from each other, i.e. progenitor no. X 
was labelled together with progenitor no. X+4 in correspond- 
ing positions on both sides of the midline. If all segments 
инvariably comprised 8 midline progenitors (4 on either side) 
and given that they are specified according to their anteropos- 
terior order in a segment, one would expect identical combi- 
nations of labelled clones to be obtained in neighbouring neu- 
eromes. We observed 11 embryos in which the progenitors 
gave rise to four labelled clones, two pairs in consecutive neu- 
eromes. However, in only two cases were the two pairs of 
clones identical: in one of these cases, a UMI and a midline 
glia clone occurred together in both neuromeres, in the other 
case, a UMI and a MNB clone.

Our assumption that there is only one midline progenitor per 
segment for the midline glia is in conflict with previous data 
suggesting the existence of three midline progenitors giving 
rise to three pairs of midline glia, the MGA, MGM and MGP 
(Jacobs and Goodman 1989; Klämbt and Goodman, 1991). To 
ascertain the number of midline glia per segment, we inspected 
embryos of the transformant P[3.7sim/ lacZ] strain (Nambu et 
al., 1991). The sim gene product is first detectable in all midline 
cells at the end of gastrulation (Crews et al., 1988; Fig. 7A). 
At later stages, their progeny vary considerably in their levels 
of sim expression (Fig. 7B,C). The MP1 neurons do not express 
sim, the MNB progeny and the VUMs express it at low levels 
and the midline glia show a high level of expression (Crews et 
al., 1988; Nambu et al., 1990). The VUM and UMI neurons 
and the progeny of the MNB exhibit a nuclear staining 
whereas, due to the strong expression, the cytoplasm is also 
stained in the midline glia. At stage 17 the numbers of labelled 
midline glia per neuromere varied between 2 and 4 cells. In a 
few exceptional cases, we even found 1 or 5 cells. On average, 
numbers were higher in the thorax compared to the abdomen. 
At stage 16, one or two further cells are labelled dorsally and 
posterior to the posterior commissure, a position which was 
previously described for the MGP cells (Jacobs and Goodman, 
1989; Klämbt and Goodman, 1991). In contrast to the more 
anteriorly located midline glia, these cells show no stained 
cytoplasmic processes. Since at the beginning of stage 17, they 
become shifted ventrally together with the MNB progeny and 
since they also stain positive with the anti-engrailed antibody, 
we believe that these cells belong to the MNB clone rather than 
being a subclass of midline glia. Thus, the average segmental 
number of midline glia labelled in this strain is higher (3-4 
midline glia, corresponding to 2 progenitors in most of the 
segments) than the number suggested by the frequencies of glia 
among the DiI-labelled midline clones (2 midline glia per 
segment, corresponding to 1 progenitor). For possible reasons 
for this discrepancy see Discussion.

As shown in Fig. 6, a progenitor labelled at a certain distance 
from the posterior edge of the cephalic furrow, e.g. progenitor 
no. 10, may give rise to any of the five types of midline clones. 
Such a high variability would be expected since the numbers of 
midline progenitors per segment (and the positions of 
segmental borders) are variable and since the cephalic furrow 
is a dynamic structure. However, the frequency with which a 
certain type of clone is produced clearly differs among con- 
secutive progenitors in a periodic manner. For example, the 
putative MNB clone was most frequently obtained from prog- 
enitors no. 10 and 14. The distribution of these peak values for 
the various clonal types allows deduction of the anterior- 
posterior sequence of the respective progenitors: Progenitors 
for the midline glia would be followed by the VUM progeni- 
tors and then by the UMI progenitor and the MNB. The relative 
position of the MP1 is not clear from our data. Considering the 
location of the MNB clone in the neuromere, the MNB seems 
to demarcate the posterior segment border (see also Klämbt et 
al., 1991).

However, for the following reasons, it is unlikely that posi- 
tional information is sufficient for the individual progenitors 
to become specified: (1) there are two files of progenitors on 
either side of the midline, which give rise to paired as well as 
unpaired cells, (2) the expression of the various clonal types 
seems to be independent of their side (left/right) of origin and 
(3) segmental numbers of the progenitors are variable. 
Therefore, in addition to positional information, interactions 
among the midline cells are likely to play an important role in 
their determination.

DISCUSSION

We have described a new method for labelling individual 
embryonic cells in Drosophila (and possibly other organisms) 
and directly tracing their development. The most significant 
features of this method are the following: cells are labelled 
non-invasively while remaining in their original position; the 
tracer is transferred to all embryonic progeny, disclosing their 
morphology in detail; the development of the labelled cell 
and its progeny can be observed in vivo and subsequently the com- 
position of the fully differentiated lineage can be analyzed in 
permanent whole-mount preparations. The technique works 
reliably and efficiently. Any cell in the peripheral cell layer of 
the embryo can be labelled. For example, the method allows 
tracing of the lineages of all CNS progenitor cells. Wild-type 
lineage data is a prerequisite for efficiently applying the 
genetic and experimental tools in Drosophila in an attempt to 
understand the mechanisms leading to cell fate specification in 
the CNS. A detailed map of molecular markers for individual 
neuroblasts and ganglion mother cells has been recently estab- 
lished (Doe, 1992). It is possible now to link complete lineages 
to specific neuroblasts in the map and to assay mutant pheno- 
types (Doe and Technau, 1993).

Here we used this method to trace the wild-type fate of the 
CNS midline progenitors in the Drosophila embryo. Because 
of their unique properties, Poulson (1950) called these cells 
mesectoderm. Discrete characteristics of these cells include the 
following: They are initially in contact with the mesodermal 
anelge and their shape differs from the other neuroectodermal 
cells. They form a distinct mitotic domain (Foe, 1989). 
Mutations in a number of genes selectively affect the differ- 
entiation of the midline cells (e.g. Rothberg et al., 1990; 
V. Nambu et al., 1991; Klämbt et al., 1991). They give rise to 
a variety of neuronal and glial cells, which have been shown 
to play a key role in the formation of the axonal scaffold, espe- 
cially of the commissural axon tracts that interconnect the two 
halves of the nerve cord (Jacobs and Goodman, 1989; Klämbt 
et al., 1991; Klämbt, 1993).

First descriptions on CNS midline progenitors and mor- 
phologies of their progeny came from studies on the grasshopper.
Among them were seven progenitors which were called midline precursors 1-7 (MP1-7) and one neuroblast, called median neuroblast (MNB). The unpaired precursors MP1,3-6 and MNB lie on the midline, the pair of MP2 lie near it. The MPs divide only once symmetrically, whereas the MNB divides asymmetrically according to a stem cell mode to produce more than 90 neurons (Bate, 1976; Bate and Grnnewald, 1981; Goodman and Spitzer, 1979; Thompson and Siegler, 1993).

In Drosophila, the existence of eight midline progenitors has been suggested by previous studies. Among them were the MNB, the MP1, 1-3 VUM progenitors and three glial progenitors. The MP1 and the glial progenitors were reported to divide once to give rise to the two MP1 neurons and three pairs of midline glia (MGA, MGM and MGP), respectively, whereas the other progenitors divide more than once, one to three of them giving rise to a group of six VUM neurons and their special glial support cells and one of them giving rise to the MNB and its support cells (Jacobs and Goodman, 1989; Klämbt et al., 1991). Thus, due to the limitations inherited with the markers used, it was not possible to clarify entirely the lineages of the midline progenitors and the morphology of cell types.

Using the DiI method, we obtained five different types of midline clones. One of these clonal types consisted of two bilaterally projecting interneurons (UMI) which have not previously been described in Drosophila. One of these cells resembles the H cell in the grasshopper (Siegler, personal communication), which derives from the MP3 (Goodman et al., 1981). However, the second MP3 cell in the grasshopper sends only one contralateral fiber anteriorly, whereas in Drosophila the second cell forms projections on either side. Furthermore, whereas in the grasshopper the morphology and survival of the MP3 progeny depend on their segmental position (Bate et al., 1981), we have no indication so far for different morphologies and/or survival of these cells among segments T1-A1 in Drosophila.

The MP1 clone consists of two ipsilaterally projecting interneurons as previously described for Drosophila as well as for grasshopper (Schistocerca) and moth (Manduca) (Thomas et al., 1984). In addition to the posterior directed projection observed at rather early stages (Thomas et al., 1984), we found a prominent anterior branch for both cells in the late embryo.

A further type of clone represents a cluster of 5-8 neurons. Most of these cells are interneurons; in late embryos one of the fibers on either side enters the intersegmental nerve and, thus, may represent a motoneuronal projection. These cells have not been previously described in Drosophila. Since this is the only clone that consistently comprises more than two cells, we suggest that this clone represents the progeny of the MNB. This is further substantiated by the fact that the position of the cluster coincides with the location of a cluster of engrailed expressing cells. The MNB and its progeny have been shown to express engrailed (Patel et al., 1989; Doe, 1992). In the grasshopper, the MNB gives rise to a large number of progeny (more than 90 in T3), which are known as dorsal unaired median (DUM) neurons, among them efferent as well as local and intersegmental interneurons (Thompson and Siegler, 1991). These cells assume a characteristic location on the midline dorsal and posteriorly in the segments and they form typical median neurites, which bifurcate and extend bilaterally (e.g. Goodman and Spitzer, 1979). Thus, with respect to the projection pattern there are some similarities with cells of the putative MNB clone in the late Drosophila embryo. These similarities are even more obvious for the VUM neurons in Drosophila. Accordingly, the VUM cells have been assumed to be homologous to grasshopper DUM neurons (Goodman et al., 1984). However, in Drosophila the VUMs do not derive from the putative MNB (see also Klämbt et al., 1991).

The DiI labellings reveal that the VUM clones typically consist of two cells, one motoneuron and one interneuron. Our data suggests the existence of three VUM progenitors. This is
consistent with the existence of six VUM neurons (Klämbt et al., 1991). However, we could not confirm the assumption of Klämbt et al. (1991) that the two glial support cells associated with the VUMs derive from the VUM progenitors. Instead these glial support cells derive from a lateral neuroblast and migrate towards the midline during early stage 12 (J. Urban, unpublished results).

The fifth type of clone obtained, consists of two glial cells, most of which reside between and enwrap the two commissures. Inspection of transformant sim/lacZ embryos at late stages revealed a variable number of 2-4 midline glia per thoracic neuromere. This is in conflict with previous reports describing the existence of three pairs of midline glia (MGA, MGM, MGP) derived from three glial progenitors in each segment (Jacobs and Goodman, 1989; Klämbt et al., 1991). There are some indications that those cells, previously described as MGP, are neuronal progeny derived from the putative MNB (see results). We therefore believe that there exist two progenitors for midline glia in most thoracic segments. However, the frequencies of clonal types obtained from the DiI labellings suggest the existence of only one glial progenitor among the midline cells. It is possible, that the presumed second glial progenitor resides apart from the midline in the neurogenic ectoderm and has therefore not been labelled in our experiments. Alternatively, cell death may lead to a reduced number of midline glia and might partly account for the DiI labellings (27%) which did not yield labelled progenies in the late embryo (compared to only 10% in the lateral neuroectoderm; see first chapter of results). Indeed, during stage 15 dying cells can be observed in the midline of the CNS (data not shown) by using acridine orange or toluidine blue as markers for programmed cell death (see also Abrams et al., 1993).

It is important to note that the system shows significant variability: a) The number of midline progenitors (sim/lacZ expressing cells) generally varies between 6 and 8 cells, with an average of 7.5 cells per segment. b) In a significant number of cases (12%) the composition of clones differed from the typical pattern in that they comprised additional cells either of the same type (4-cell and 6-cell VUM clones; 8.5% of all clones) or of different types (e.g. MP1/UM1; 3.5% of all clones). It is tempting to speculate that the generation of larger compound clones in the midline is a function of the number of progenitor available in a particular segment. In this way the variability in progenitor cell numbers may be compensated by variabilities in clone sizes to equip segments with an adequate final population of midline cells. Nevertheless, c), in the fully differentiated embryo, the segmental number of sim expressing midline glia was found to vary between 2 and 4 cells. Furthermore, variability of cell numbers in the late embryo has also been reported for the lineage of the neuroblast NB1-1 (Udolph et al., 1993). Thus, the widespread assumption of invariant lineages in the CNS of Drosophila has to be reconsidered.

Knowing the midline lineages and their variabilities, it is now possible to experimentally approach the mechanisms leading to their specification.

We thank Stephen Crews, Kei Ito, Christian Klämbt, Melody Siegler, and Paul Whittington for helpful discussions, Joachim Urban for critical comments on the manuscript and for unpublished information, Stephen Crews for the transformant strain P[3,7sim/lacZ], Kristina Hawthorne for correcting the English, and Ursula Böll for help with the photographs. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to G. M. T. (Te 130/1-4).

REFERENCES


(Accepted 8 April 1994)