Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets

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SUMMARY

An experimental analysis of neurogenesis requires a detailed understanding of wild-type neural development. Recent DiI cell lineage studies have begun to elucidate the family of neurons and glia produced by each *Drosophila* embryonic neural precursor (neuroblast). Here we use DiI labeling to extend and clarify previous studies, but our analysis differs from previous studies in four major features: we analyze and compare lineages of every known embryonic neuroblast; we use an in vivo landmark (*engrailed*-GFP) to increase the accuracy of neuroblast identification; we use confocal fluorescence and Nomarski microscopy to collect three-dimensional data in living embryos simultaneously for each DiI-labeled clone, the *engrailed*-GFP landmark, and the entire CNS and muscle target field (Nomarski images); and finally, we analyze clones very late in embryonic development, which reveals novel cell types and axon/dendrite complexity.

We identify the parental neuroblasts for all the cell types of the embryonic CNS: motoneurons, intersegmental interneurons, local interneurons, glia and neurosecretory cells (whose origins had never been determined). We identify muscle contacts for every thoracic and abdominal motoneuron at stage 17. We define the parental neuroblasts for neurons or glia expressing well-known molecular markers or neurotransmitters. We correlate *Drosophila* cell lineage data with information derived from other insects.

In addition, we make the following novel conclusions: (1) neuroblasts at similar dorsoventral positions, but not anteroposterior positions, often generate similar cell lineages, and (2) neuroblasts at similar dorsoventral positions often produce the same motoneuron subtype: ventral neuroblasts typically generate motoneurons with dorsal muscle targets, while dorsal neuroblasts produce motoneurons with ventral muscle targets.

Lineage data and movies can be found at http://www.biologists.com/Development/movies/dev8623.html and http://www.uoneuro.uoregon.edu/doelab/lineages.

Key words: *Drosophila*, Neuroblast, Lineage, Motoneuron, Axon projection, Neurosecretory cell, Glia

INTRODUCTION

Developmental neurobiologists working on insect model systems aim to understand how neurons within a relatively simple developing nervous system achieve cellular and synaptic complexity. In insects, we know that stem cells generating neurons and glia, neuroblasts (NBs), are specified by positional cues shortly after they are formed in the neuroectoderm (Doe, 1992; Skeath et al., 1995). Less is known about the mechanisms by which their daughter ganglion mother cells (GMCs) and granddaughter neurons and glia achieve their cell fates. Before an experimental or genetic analysis of CNS cell fate can be accurately interpreted, it is essential to understand the wild-type development of the cells in question and to develop molecular markers for different cell fates.

Over the last few years, lineage analysis has been done on a number of insects, particularly *Schistocerca* (grasshopper) and *Drosophila* embryos (Beer et al., 1987; Prokop and Technau, 1993; Buenzow and Holmgren, 1995). In *Schistocerca*, there are 30 embryonic NBs, and 10 NB cell lineages have been partially defined using a variety of lineage tracing techniques (Shepherd and Laurent, 1992; Condron and Zinn, 1994; Raper et al., 1984; Doe and Goodman, 1985; Bastiani et al., 1984; Leitch et al., 1992). Until recently, lineage analysis has not been possible in *Drosophila* because NBs are too small and inaccessible to be labeled with lineage tracers. The development of a method for in vivo DiI labeling of *Drosophila* neuroectodermal cells (Bossing and Technau, 1994) solved the labeling problem, and the development of a detailed NB map (Doe, 1992) allowed the labeled NBs to be individually identified. The combination of these advances has led to a series of NB cell lineage studies in both wild-type and mutant *Drosophila* embryos (Bossing and Technau, 1994; Bossing et al., 1995, 1996; Chu-LaGraff et al., 1995; Landgraf et al., 1997; Schmidt et al., 1997).

These recent *Drosophila* studies represent a major advance in our understanding of NB cell lineage in *Drosophila*, but as a group they suffer the following drawbacks: (1) each study examined only a subset of the total number of NBs, which limited the potential for a synthetic overview of neurogenesis (e.g. NB position vs. cell type produced), (2) each study...
followed NB cell lineages only until stage 15 or 16, which is too early to detect the differentiation of many motor and interneurons, (3) there are internal contradictions in NB identity and clonal composition between these studies, (4) relatively little attempt has been made to link NB cell lineage to gene expression patterns, which is an essential step in making cell lineage data useful in analyzing gene function, and (5) Drosophila NB lineages have not been compared to NB lineages in other insects.

To overcome these problems, we conducted a comprehensive analysis of every known NB cell lineage in the Drosophila embryo throughout the end of embryogenesis. We obtained cell lineage information for all embryonic NBs, revealing correlations between NB position and clonal composition, and between cell size and cell type; we analyzed the clones at late stage 17, revealing a great deal of motor and interneuron differentiation that had been previously missed; we attempt to resolve the existing conflicts in NB identification and clonal composition; we begin to link each NB cell lineage to the existing molecular markers expressed in the lineage; we compare NB cell lineage and cell morphology data between Drosophila and other insects, revealing interesting similarities and differences in the mode of neurogenesis.

MATERIALS AND METHODS

Fly stocks, Dil labeling and neuroblast identification

We delivered DiI (1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbo-cyanine perchlorate; Molecular Probes, Inc.) to neuroectodermal cells by the method of Bossing and Technau (1994), with the following modifications. All procedures were done at 16°C. Embryos were collected from females that were homozygous for a UAS-GFP insertion (a gift from Barry Dickson and Corey Goodman) crossed to males that carried a GAL 4 insert in the engrailed regulatory region (a gift from Andrea Brand). These engrailed-GFP embryos expressed GFP in the engrailed pattern by stage 10. NB injections and subsequent NB identifications were done on a Nikon Diaphot inverted microscope, equipped with a 100x oil immersion lens, bright-field and fluorescent optics; neutral density filters to attenuate the UV excitation (OD=3.0; Chroma Technologies), and a Hamamatsu CCD camera. We viewed GFP and DiI fluorochromes with a filter cube that allows only the longest wavelength subset of DiI fluorochromes to be excited while maximizing throughput of GFP excitation in the yellow range (Chroma Technologies); ND filters and a specialized filter set allowed us to view DiI-labeled NBs in GFP embryos with minimal phototoxicity. Embryos were labeled at stage 8. After 10 hours at 16°C, embryos developed to stage 11; at this stage, engrailed-GFP was clearly expressed and NBs could be identified using this in vivo positional marker.

Clonal analysis

37 hours AEL at 16°C, the embryo is well advanced into stage 17. Cuticle is present over all but a small area in the ventral thorax; we pressed that area to the surface of a Superfrost Plus (Fisher) glass slide that had been made into a dissection well, and dissected the embryo in insect saline. About half the embryos were subsequently fixed in 5% methanol-free formaldehyde (in PBS) for 5 minutes at 16°C, which allowed delicate neurites to be photographed by confocal microscopy with minimal membrane damage. Dissected embryos were imaged on a Biorad 1024 microscope, using a Leitz 50x water immersion lens, as 1.5 μm step z-series. Data were collected simultaneously at 568 nm excitation (for DiI) and at 488 nm (for engrailed-GFP) illumination. Each z-series was immediately rescanned using Nomarski optics to determine cellular positions within the CNS and identify motoneuronal target muscle(s). Cell and axon measurements were done with Biorad software calibrated to a stage micrometer. Biorad software was used to project each z-series to form two-dimensional images, which were assembled into figures using Photoshop (v 5.0) and Freehand (v 7.02) software. Biorad Lasersharp software was used to generate rotational movies from selected z-series data sets.

Web-site data

For each clone, we have web site links to the following data files: (1) Dil z-series; (2) Dil and engrailed-GFP double label z-series; (3) Dil z-series movie showing rotation around the y-axis; (4) Dil and engrailed-GFP double-label movie showing rotation around the y-axis. In addition, all the NB clone schematics will be provided as a single Freehand 7.0 file. The URL for access to all NB lineage data, plus the movies described above, can be found at http://www.biologists.com/Development/movies/dev8623.html and http://www.uoneuro.uoregon.edu/doelab/lineages.

Terminology

We will use the following terminology to describe the three dimensions of the developing CNS. We use medial, intermediate and lateral to define position along the x-axis of the embryo within the hemisegment (with medial being closest to the ventral midline); we use anterior and posterior to define position along the y-axis of the hemisegment; and we use dorsal and ventral to define position along the z-axis of the CNS (with ventral being most external and dorsal being most internal). In all figures, the ventral midline (the most medial position of the CNS) is indicated by a small white triangle, anterior is up.

RESULTS

We have identified the following cell types in our analysis: motoneurons, intersegmental interneurons, local interneurons, glia and neurosecretory cells. We define motoneurons as having axonal projections into the body wall musculature and large cell bodies (between 6.5 and 8.0 μm). We describe neuromuscular contacts that were consistently observed at stage 17 of embryogenesis; whether or not these contacts persist throughout larval development will require further study. We define intersegmental interneurons as neurons with axon projections that extend between segments within the CNS, as they have been defined in other insects (Burrows, 1996). We define local interneurons as neurons with axon projections that terminate within their segment of origin in the CNS. Local interneurons generally have smaller cell bodies (2.5-5.0 μm) compared to intersegmental interneurons (5.5-7.0 μm). Because the range of cell body sizes forms a continuum, however, we do not propose that cell function can be absolutely correlated with soma size. We define glia as cells with large cell outlines, diffuse cell borders, comparatively weak Dil labeling and positions consistent with previously described glia (Ito et al., 1995). Neurosecretory cells are generally defined as cells ‘that produce nonfocal release of various chemicals at sites other than the usual classical synapses’ (Burrows, 1996). We define neurosecretory cells as having projections into nerve roots that do not extend to the body wall muscles (except see MNB), and that correspond in size, position and morphology to previously described neurosecretory cells (Carr and Taghert, 1988a,b; Broadie et al., 1990; Tubbiz and Sylwester, 1990; Dircksen et al., 1991). Neurosecretory cells have medium-sized cell bodies
5.0-6.0 \mu\text{m}). Previous lineage studies have not identified any neurosecretory cells in the Drosophila CNS (Bossing et al., 1996; Schmidt et al., 1997).

**NB 1-1**

NB 1-1 was initially called NB 2-2 (Doe, 1992) but was subsequently renamed NB 1-1 by Broadus et al. (1995). NB 1-1 delaminates at S1 as the most anterior NB in the segment. Its first GMC generates the well-characterized aCC motoneuron and pCC intersegmental interneuron (Broadus et al., 1995). In addition, NB 1-1 generates a cluster of local interneurons, and the SPG-A and SPG-B glial cells in abdominal segments; in thoracic segments NB 1-1 generates another motoneuron, CoA (Cousin of aCC), but does not produce glia (Udolph et al., 1993; Broadus et al., 1995; Bossing et al., 1995, 1996). In the grasshopper Schistocerca, the first GMC from NB 1-1 also produces the aCC motoneuron and pCC intersegmental interneuron (Goodman et al., 1982); other progeny of NB 1-1 have not been described.

We generated 13 NB 1-1 lineages: 4 at stage 15, 4 at stage 16, and 5 at stage 17 (2 thoracic, 3 abdominal). The clone consists of an average of 14 cells throughout these stages of development. Our NB 1-1 clones are similar to those of previous studies (Table 3). [http://www.uoneuro.uoregon.edu/doelab/lineages/NB1-1.html]

(A) Motoneurons

In both thoracic and abdominal segments, NB 1-1 produces the aCC motoneuron. aCC is a large round cell (6.9 \mu\text{m} at stage 16; n=4) that enlarges with age (8.2 \mu\text{m} at stage 17; n=4). It sits at the dorsal surface of the CNS, just posterior to the junction of the posterior commissure and the longitudinal connective (Fig. NB1-1B, circled; inset). By stage 17, aCC extends an axon to the dorsal midline of the embryo, terminating at muscle 1 (Fig. NB1-1B). We frequently observe additional branches to neighboring muscles in the dorsal muscle group (e.g. muscles 2 and 9; see Fig. NB1-1 and Table 2). By late stage 16, aCC has a short contralaterally projecting neurite extending into the posterior commissure. In thoracic segments, a second motoneuron, CoA, lies posterior and lateral to pCC. It is oval (4.4×7.0 \mu\text{m}, with its long axis perpendicular to the midline) with an axon projecting ipsilaterally via S NB to muscles 12 and 13 (n=2, Fig. NB1-1A; Tables 2, 3). In addition, thoracic clones produce 2 or 3 large cell bodies just lateral to aCC/pCC (Fig. NB1-1A). Because they are large cells, we speculate that they are either late differentiating motoneurons or intersegmental interneurons.

(B) Interneurons and glia

There is one intersegmental interneuron in this lineage, the large round pCC (6.8 \mu\text{m} at stage 17; n=5). Its axon projects anteriorly in an ipsilateral medial fascicle for more than 2 segments by stage 17 (n=2, Fig. NB1-1B, circled, inset; and movies). In addition, there is a cluster of ~8 local interneurons that have loosely fasciculated posterior ipsilateral projections. Their cell bodies fall into two size populations: 50% are less than 4 \mu\text{m}, with the remainder being larger, 5.2 \mu\text{m} (n=72).

NB 1-1 generates glia in the abdomen only (Udolph et al., 1993 and this study). It generates a large (27×40 \mu\text{m}; n=3) dorsal subperineural glial cell that lies dorsal to aCC and pCC (dashed outlines in Fig. NB1-1B,C, inset). In addition, it generates a segmental nerve root glia (which may be the Segment Boundary Cell of Jacobs and Goodman (1989a,b); Fig. NB1-1B, inset). An almost identical nerve root glial cell is derived from NB 7-1 lineage (Fig. NB7-1), and both NBs 1-1 and 7-1 generate pioneering motoneurons that extend out the intersegmental nerve (ISN) in close contact with these nerve root glia (aCC and the Us, respectively). Whether there is a molecular or functional relationship between the motoneurons and the lineally related nerve root glia is an open question.

**NB 1-2**

NB 1-2 was originally called NB 1-1 (Doe, 1992) but was renamed NB 1-2 by Broadus et al. (1995). Bossing et al. (1996) describe the clone as consisting of 16-24 interneurons, including the TB neuron, which has a unique axon projection and a cell body apart from the clone. We generated 19 NB 1-2 clones: 1 at stage 14, 4 at stage 15, 8 at stage 16, and 6 at stage 17 (3 thoracic, 3 abdominal). The clone averaged 22 cells at stage 14 and 32 (thoracic) or 19 (abdominal) cells at stage 17 (Table 3). Our NB 1-2 clone is similar to previous studies, except we detect late-differentiating motoneurons not described before (Tables 2, 3). [http://www.uoneuro.uoregon.edu/doelab/lineages/NB1-2.html]
(A) Motoneurons
In the T3 segment, NB 1-2 generates the previously identified DC1 motoneuron (Kolodkin et al., 1993; Matthes et al., 1995) which has a large oval cell body (6x8.2 μm; n=2) located dorsally and medially; it projects ipsilateral posterior via SNb to muscle 31 in the adjacent posterior segment A1 (muscle 31 is present only in segment A1; Crossley, 1978; Hooper, 1986; Fig. NB1-2A,C, circles). In segment T2, there are 2-4 DC motoneurons that have large (8 μm, n=2) lateral cell body positions, but they also project ipsilateral posterior out the SNb to innervate muscle 33 and possibly all the mouthpart-associated muscles (muscles 32-36; Crossley, 1978; Hooper, 1986; Fig. NB1-2B, circles). NB 1-2 does not produce motoneurons in the abdominal segments.

(B) Interneurons
There are at least three intersegmental interneurons, including the unique ‘Torsten Bossing’ (TB) neuron (Bossing et al., 1996). TB is a large round cell (7.0 μm; n=17); prior to stage 14 it projects anterior ipsilaterally to the anterior commissure of its own segment, it then projects across the midline and extends anteriorly in the contralateral connective (Fig. NB1-2A, broken circle). By stage 17, the TB cell body has migrated medially, and produced a large axon extending anteriorly in the contralateral connective (Fig. NB1-2, and movies). The second and third intersegmental interneurons are large cells (6 μm; n=8) at the anteromedial edge of the clone. Their axons extend anteriorly in the ipsilateral connective (Fig. NB1-2F,G arrows). A fourth intersegmental inter-neuron is observed posterior to A3 (data not shown). In addition, there are approximately 10-15 local interneurons derived from this clone; about half are small (3.8 μm) and half are larger (5.1 μm; n=13). They extend neurites in the posterior commissure of their own segment and in the anterior commissure of the adjacent posterior segment before making complex arborizations, similar to that of NB 6-2 clones.

NB 1-3
This precursor has not been included in early NB maps (Doe, 1992; Broadus et al., 1995). It was first identified by Schmidt et al. (1997) as ‘NB 1-3,’ an extremely lateral huckebein-positive cell that produces glial cells and 3 motoneurons that project contralaterally into both the ISN and SN, and they observed one of these MNs to innervate muscle 14.
We have generated one clone similar to ‘NB 1-3’ (we usually did not label at extremely lateral positions), and we believe it produces some of the glia of the transverse nerve (TN), rather than motoneurons. The TN contains neurosecretory cells, motoneurons, mesodermally derived ‘DM cells’, glia and sensory axons (Fig. NB1-3, inset). In *Drosophila*, the origin of
the TN glia is unclear but, in *Manduca*, the TN glia (including the ‘strap cells’) develop from a lateral position similar to NB 1-3 (Carr and Taghert; 1988a,b). Our clone contains cells that closely match those described for *Manduca* TN glia, and hence we propose that they are *Drosophila* TN glia. We also detected a PNS subclone in this lineage (not shown). [http://www.neuro.uoregon.edu/doelab/lineages/NB1-3.html]

We labeled the mesodermal precursor of the Transverse Nerve DM cells and found that the lineage includes the muscle 6,7,12 and 13 progenitors as well (*n*=6, Fig. NB1-3).

**NB 2-1**
NB 2-1 delaminates at S4. It has previously been described as generating approximately 8 interneurons and an obligate subclone of approximately 4 epidermal cells, with a tangle of ipsilateral projections and a single interneuronal projection through the anterior commissure (Bossing et al., 1996). We generated 9 NB 2-1 clones, 3 in the thorax (that did not survive) and 6 in the abdomen; 2 were examined at stage 15 (containing 9 cells); 2 at stage16 (containing 13 cells), and 2 at stage 17 (containing 16 cells). We observed an epidermal clone in 2 out of 6 clones. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB2-1.html]

(A) Interneurons and glia
All of the cells are local interneurons with relatively large cell bodies at stage 15 (4.7 μm; *n*=10). By stage 17, about 25% were small (2.2 μm) with the rest remaining larger (4.0 μm; *n*=16). All interneurons project across the midline in a single posterior fascicle within the anterior commissure; they extend to the contralateral connective via the anterior commissure and then bifurcate. In both of the stage 17 clones, we observed a segmental nerve glial cell (data not shown).

**NB 2-2**
NB 2-2 delaminates at S2. It was initially called NB 2-3 (Doe,
but was subsequently renamed NB 2-2 by Broadus et al. (1995). This lineage has been described as containing 2 or 3 motoneurons projecting ipsilaterally out the SN and ramifying over the ventrolateral muscle group, 10-12 interneurons and the SPG-A glia in the thorax (Bossing et al., 1995, 1996; Udolph et al., 1993). Landgraf et al. (1997) identified the motoneurons from NB 2-2 as extending into SNa and innervating muscles 21 and 22. In *Schistocerca*, the first GMC of NB 2-2 generates the excitatory motoneurons, Df (fast) and Ds (slow), that innervate the simple coxal muscle 133a of the leg (Ball et al., 1985).

We generated 14 NB 2-2 clones: 3 at stage 15, 6 at stage 16, and 5 at stage 17 (2 thoracic, 3 abdominal). There are between 20 and 22 cells in the clone at all stages assayed. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB2-2.html]

(A) Motoneurons
In both abdominal and thoracic segments, we detect 3 or 4 motoneurons projecting ipsilaterally out the SNa to innervate muscles 21 and 22 (consistent with Landgraf et al., 1997 but unlike Bossing et al., 1995, 1996). In addition, we usually see a projection out the SNd, innervating muscle 17 (Fig. NB2-2G, Tables 2, 3). We also confirm the results of Sink and Whitington (1991a) showing that motoneurons projecting to muscles 21 and 22 have substantial dendritic arborizations in the ipsilateral longitudinal connectives (Fig. NB2-2 B,D small arrows), and that all abdominal NB 2-2 clones include these motoneurons. Thus, if these *Drosophila* motoneurons are homologs of the *Schistocerca* Df and Ds motoneurons, they either die or acquire functions independent of leg muscle innervation in abdominal segments.

(B) Interneurons and glia
Prior to stage 17, all interneurons project across the anterior commissure and extend slightly anteriorly in the contralateral connective. During stage 17, several develop intersegmental projections in both anterior and posterior directions (Fig. NB2-2 D,G), with the remainder maintaining local projections. Short ipsilateral projections were present in thoracic and abdominal clones. All of the interneurons of this clone are approximately the same size (5.1 μm; n=54). In thoracic clones, we observe a single, dorsally located subperineural glial cell, presumably SPG-A, similar to previous studies (Bossing et al., 1995, 1996).

### NB 2-3
NB 2-3 delaminates at S5. It was initially called NB 1-2 (Doe, 1992) but was subsequently renamed NB 2-3 by Broadus et al. (1995). Its lineage was not identified by Bossing et al. (1995, 1996) or Schmidt et al., (1997). We labeled NB 2-3 15 times, but only 6 thoracic clones developed. In abdominal segments, only a large labeled NB is detectable at stage 17, it either does not divide or all its progeny die. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB2-3.html]

(A) Motoneurons
The thoracic NB 2-3 generates three very large round cells (9.5 μm; n=7) at the dorsal side of the clone. The large size of these cells (characteristic of motoneurons) and their presence specifically in thoracic segments, suggests that they may be latent leg motoneurons that will extend axons during leg-disk eversion. Consistent with this proposal, Truman et al. (1993) observe that the thorax-specific leg motoneurons are born during embryonic development, but wait for leg imaginal disc eversion before pioneering leg innervation. Other adult leg motoneurons may derive from the NB 1-1 lineage, which generates 2 or 3 large axonless cells adjacent to aCC and pCC only in thoracic segments (Fig. NB1-1).

(B) Interneurons
In 5 of 6 clones, we observe 2-4 small interneurons (3.2 μm; n=6) at the ventral side of the clone; they project across the anterior commissure and bifurcate in the connective (Fig. NB2-3).

### NB 2-4
NB 2-4 delaminates during S4. It has been described by Schmidt et al. (1997) as producing 2 motoneurons that project contralaterally out the anterior root of the ISN, 7 or 8 interneurons with ipsilateral posterior projections and an obligate epidermal subclone. We generated 8 clones from NB 2-4, and scored 3 at stage 16 (1 thoracic, 2 abdominal; averaging 14 cells per clone) and 5 at stage 17 (2 thoracic, 3 abdominal; averaging 10 cells per clone). As expected from the
decline in cell numbers, we detected significant amounts of cell death in this clone (Fig. NB2-4, asterisks). We detected one motoneuron and a cluster of local interneurons; 2/8 clones had epidermal cells and 3/8 clones had a PNS subclone. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB2-4.html]

(A) Motoneurons
We find only one motoneuron to be derived from NB 2-4, contrary to the findings of Schmidt et al. (1997). It is mediumsized (6.4 μm; n=4), dorsal, and migrates medially in abdominal segments. It projects contralaterally via the anterior commissure to the posterior root of the ISN before joining the SNa and forming an unusually robust ‘ribbon-like’ synapse along the side of muscle 8 (Fig. NB2-4). This pattern of innervation is a novel finding in *Drosophila* embryos. However, a thin nerve branch, containing projections from the LBD and TA sensory cells, has been observed to connect SNa to ISN in third instar *Drosophila* larvae (H. Keshishian, unpublished results). It appears that the motoneuron derived from NB 2-4 extends to muscle 8 by traveling the same pathway and may be the *Drosophila* homolog of the leukokinin cell innervating muscle 8 in *Calliphora*, as reported by Cantera and Nassel (1992).

(B) Interneurons
There are ~8 local interneurons; half are small (3.1 μm; n=8) and half are larger (4.8 μm; n=7). We do not observe a posterior ipsilateral projection (Schmidt et al., 1997). Instead, we find a robust projection across the anterior commissure into the contralateral connective, which turns anteriorly and extends to the anterior segment border. This is identical to the NB 2-4 interneuronal projections determined by *eagle-kinesin-lacZ* staining (Higashijima et al., 1996). One interneuron migrates medially away from the clone, similar to the motoneuron. It appears to be a bipolar interneuron, previously unheard of in insect systems (Burrows, 1996). It can have one projection extending a short distance anteriorly (11 μm; n=2) and a second more robust projection extending posteriorly (32 μm; n=3) in the ipsilateral longitudinal connective (Fig. NB2-4 B,C (arrow)). The posterior projection frequently formed a loop at its terminus (Fig. NB2-4 C,D).

NB 2-5 delaminates as the most anterior-lateral S1 NB (Doe, 1992). Schmidt et al. (1997) describe this lineage as containing 13-18 neurons: 1 motoneuron, 2-5 contralaterally projecting interneurons, 2-5 ipsilaterally projecting interneurons and 2 glial cells. We generated 13 NB 2-5 clones, and scored 2 at stage 15 (1 thoracic, 1 abdominal), 6 at stage 16 (1 thoracic, 5 abdominal) and 5 at stage 17 (2 thoracic, 3 abdominal). There were 15-22 cells at stage 17 in all segments. Similar to Schmidt et al. (1997), we find that this clone generates a diverse array of cell types: glia, motoneurons, intersegmental interneurons, local interneurons and, in 3/13 cases, a PNS subclone (Table 1). [http://www.uoneuro.uoregon.edu/doelab/lineages/NB2-5.html]

(A) Motoneurons
We detect a single ovoid motoneuron (6.2×4.6 μm; n=6) that projects ipsilaterally into the SNd and makes a forked ending in the clefts between muscles 15, 16 and 17 (Fig. NB2-5C; Table 2).

(B) Interneurons and glia
There are an indeterminate number of intersegmental interneurons in this clone: we detect 3-6 contralateral anterior projections that extend all the way to the brain, and 2-4
ipsilateral anterior projections that extend only half as far. It is not clear if each projection derives from a distinct cell or if one cell has dual projections (Fig. NB2-5). At least two of the intersegmental interneuron cell bodies are dorsal and large (8.1 μm; n=12) dorsal cells. Burrows (1996) describes this type of intersegmental interneuron in other insects, but little is known about their function or lineage. Local interneurons extend to the anterior border in the ipsilateral longitudinal connective (Fig. NB2-5A,C, arrows).

We detected glia in only 3/13 clones; clones can contain both segmental nerve and peripheral nerve glia (Fig. NB2-5, inset).

**NB 3-1**

NB 3-1 delaminates at S3 in the medial column of NBs. Its lineage was first described by Bossing et al. (1996) as producing RP1, RP3, RP4 and RP5 motoneurons that project contrateralally out the SNb to innervate ventral muscles, as well as a group of local interneurons that project across the anterior commissure before bifurcating in the connective. The RP motoneurons are the most thoroughly studied cells of the *Drosophila* CNS (e.g. Halpern et al., 1991; Fernandes and Keshishian, 1996, 1998; Halfon et al., 1997; Halfon and Keshishian, 1998; Desai et al., 1996; Krueger et al., 1996; Cash et al., 1992; Broadie and Bate, 1993; Keshishian et al., 1995, 1996; Sink and Whittington, 1991a,b). Other insects have similar RP neurons (Thomas et al., 1984; Jacobs and Goodman, 1989b), but their parental NB has not been identified.

We generated 18 NB 3-1 clones, and scored one at stage 14, 4 at stage 15 (2 thoracic, 2 abdominal), 6 at stage 16 (2 thoracic and 4 abdominal) and 7 at stage 17 (2 thoracic, 5 abdominal). We see no segment-specific differences. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB3-1.html]

**A. Motoneurons**

The RP1, RP3, RP4 and RP5 motoneurons are all large cells (7.4x5.7 μm; n=27) that lie close to the midline, and span the distance between the anterior and posterior commissures (Fig. NB3-1, black circles). RP4 and RP1 are most dorsal; ventral and lateral to them is RP3, followed by RP5. The RP motoneurons project to the ventral muscles 12, 13, 14, 15, 28, 30, 6 and 7 in abdominal segments (Fig. NB3-1E-G), in
agreement with Sink and Whittington (1991a,b), who used Lucifer Yellow injections into RP cell bodies to conclude that RP1 innervates muscle 13, RP4 innervates muscles 13, RP3 innervates muscles 6 and 7, and RP5 innervates muscles 15, 16, 7, 6, 13 and 12. Our results, and those of Sink and Whittington (1991a,b), differ from those of Landgraf et al. (1997), who did DiI backfills from synaptic contacts and found that the RPs innervate only muscles 12, 13, 6 and 7. In addition, both Sink and Whittington (1991a,b) and this study observe RP dendrites projecting anteriorly in a medial fascicle of the contralateral connective (Fig. NB3-1 C,D single dashed arrow).

(B) Interneurons
We found the number of interneurons in this lineage to be highly variable; there can be as few as 2 or as many as 18. Bossing et al. (1996) speculated that cell death leads to the variability in the number of interneurons. We do not observe differences in cell death between small or large clones, and propose instead that the variable number of interneurons is due to differences in the time at which NB 3-1 stops dividing. There are about twice as many intersegmental interneurons as there are local interneurons; both project across the anterior commissure in three axon bundles and then extend in a lateral fascicle of the contralateral connective, with the local projections turning anteriorly and the intersegmental projections turning posteriorly (Fig. NB3-1C,D,E,G large arrows; Table 1). The majority of the interneurons are medium sized (4.8 μm; n=46), which may be the intersegmental interneurons, but there are always several small cells (3.0 μm; n=7), which may be local interneurons.
Table 1. Cells generated by each neuroblast in the thorax

<table>
<thead>
<tr>
<th>NB</th>
<th>MNs</th>
<th>IINs</th>
<th>LINs</th>
<th>NSCs</th>
<th>Glia</th>
<th>Additional notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>putative segment boundary cells; abdominal segments vary</td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>4</td>
<td>3</td>
<td>15</td>
<td>1</td>
<td>putative semaphorin* DC motorneurons in thorax only</td>
<td></td>
</tr>
<tr>
<td>“1-3”</td>
<td>6</td>
<td>generates presumptive glia of Transverse Nerve (strap cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-1</td>
<td>15</td>
<td>1</td>
<td>segmental nerve glia observed at stage 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-2</td>
<td>2</td>
<td>4</td>
<td>15</td>
<td>0</td>
<td>one glia in abdominal segments only</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>segmental nerve glia; motoneurons in the adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>unique innervation to muscle 8, via SNa.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-5</td>
<td>1</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>segmental nerve glia; interneurons extend to brain.</td>
<td></td>
</tr>
<tr>
<td>3-1</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>number of local interneurons varies significantly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-2</td>
<td>6</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-3</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>indistinguishable from NB 4-4 clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-5</td>
<td>1</td>
<td>6</td>
<td>20</td>
<td>1</td>
<td>segmental nerve glia; motoneuron in T1 only</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>0</td>
<td>8</td>
<td>25</td>
<td>1</td>
<td>one motoneuron of the Transverse Nerve in abdominal segments only</td>
<td></td>
</tr>
<tr>
<td>4-2</td>
<td>4</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-3</td>
<td>8</td>
<td>3</td>
<td>Bursicon cells of Transverse Nerve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-4</td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>indistinguishable from NB 3-4 clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-1</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-2</td>
<td>1</td>
<td>6</td>
<td>30</td>
<td>produces majority of posterior commissure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-3</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>motoneuron present in T1-A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-4</td>
<td>4</td>
<td>1</td>
<td>putative CCAP* cells of Transverse Nerve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-5</td>
<td>6</td>
<td>1</td>
<td>Va cell of Transverse Nerve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-6</td>
<td>15</td>
<td>5</td>
<td>lateral sub-perineurial glia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-1</td>
<td>1</td>
<td>3</td>
<td>25</td>
<td>motoneuron present in T1 only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-2</td>
<td>5</td>
<td>20</td>
<td>diverse array of interneurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-4</td>
<td>8</td>
<td>4</td>
<td>interneurons produced in thorax only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-1</td>
<td>6</td>
<td>30</td>
<td>1</td>
<td>U’s to ISN; putative SBC; largest clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-2</td>
<td>4</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-3</td>
<td>1</td>
<td>4</td>
<td>only serotonergic cells of thoracic and abdominal CNS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-4</td>
<td>15</td>
<td>6</td>
<td>large glial sub-clone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP1</td>
<td>2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP3</td>
<td>2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNB</td>
<td>2-6*</td>
<td>1-3*</td>
<td>Octopaminergic NSCs and GABA-ergic interneurons</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Per segment.

Table 2. Motoneuron lineage, axonal trajectory and targets

<table>
<thead>
<tr>
<th>MN identity</th>
<th>Axonal pathways</th>
<th>Muscle targets</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>midline crossing?</td>
<td>CNS exit route</td>
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<tr>
<td>aCC</td>
<td>no</td>
<td>AT-ISN</td>
</tr>
<tr>
<td>HK 1-4</td>
<td>no</td>
<td>PT-ISN</td>
</tr>
<tr>
<td>RP2</td>
<td>no</td>
<td>PT-ISN</td>
</tr>
<tr>
<td>U1-4</td>
<td>no</td>
<td>AT-ISN</td>
</tr>
<tr>
<td>CP</td>
<td>no</td>
<td>PT-ISN</td>
</tr>
<tr>
<td>MSII.2</td>
<td>no</td>
<td>PT-SNa</td>
</tr>
<tr>
<td>NAD</td>
<td>2-4</td>
<td>PT-SNa</td>
</tr>
<tr>
<td>HK 5,6</td>
<td>3-2</td>
<td>PT-SNa</td>
</tr>
<tr>
<td>KAD</td>
<td>5-3</td>
<td>PT-SNa</td>
</tr>
<tr>
<td>JTM</td>
<td>3-3</td>
<td>PT-SNa</td>
</tr>
<tr>
<td>RPI,3,4,5</td>
<td>3-1</td>
<td>AC</td>
</tr>
<tr>
<td>RPI,3,4,5</td>
<td>3-1</td>
<td>AC</td>
</tr>
<tr>
<td>AC</td>
<td>5-2</td>
<td>PC</td>
</tr>
<tr>
<td>U5,6</td>
<td>7-1</td>
<td>no</td>
</tr>
<tr>
<td>CoA</td>
<td>1-1</td>
<td>no</td>
</tr>
<tr>
<td>DC</td>
<td>1-2</td>
<td>no</td>
</tr>
<tr>
<td>CoR</td>
<td>4-2</td>
<td>no</td>
</tr>
<tr>
<td>MSI 3</td>
<td>2-2</td>
<td>no</td>
</tr>
<tr>
<td>REK</td>
<td>2-5</td>
<td>no</td>
</tr>
<tr>
<td>UT,8</td>
<td>7-1</td>
<td>no</td>
</tr>
<tr>
<td>EG 3</td>
<td>7-3</td>
<td>no</td>
</tr>
<tr>
<td>SM</td>
<td>4-1</td>
<td>bi-lateral</td>
</tr>
<tr>
<td>VUMs</td>
<td>MNB</td>
<td>bi-lateral</td>
</tr>
</tbody>
</table>

Abbreviations: Ab, Abdominal; adj ant, adjacent anterior segment; adj post, adjacent posterior segment; AT, anterior Tract; Ext, exterior; Int, interior; ISN, Intersegmental Nerve; PC, Posterior Commissure; PT, Posterior Tract; SN, Segmental Nerve; T, Thoracic; Ventrolat, Ventrolateral muscle group.
NB 3-2

NB 3-2 delaminates at S1 in the anteriormost row of the intermediate column. It later moves slightly posteriorly to become part of the row 3 NBs. Previous studies (Bossing et al., 1996; Landgraf et al., 1997) describe the clone as containing 10-18 cells: 3-6 motoneurons projecting to muscles 18, 11, 19 and 20, 23 and 24, plus a group of local interneurons with contralateral projections.

We generated 24 NB 3-2 clones, scoring 1 at stage 14 (abdominal), 5 at stage 15 (1 thoracic, 4 abdominal), 8 at stage 16 (2 thoracic, 6 abdominal), and 10 at stage 17 (4 thoracic and 6 abdominal). We find the clone to increase from an average of 15 cells at stage 15 ($n=5$) to 22 cells at stage 17 ($n=6$). There are no segment-specific variations in the clone. At stage 17, we observe 6 motoneurons and 12-16 local interneurons (Fig. NB3-2), with projections similar to those observed previously (Bossing et al., 1996; Landgraf et al., 1997). The NB 3-2 clone is almost a mirror image of the NB 4-2 clone beginning at stage 16 (Fig. NB3-2D and diagram): both generate at least 4 motoneurons that project into the ISN and the SN via separate trajectories, and both produce a large number of local interneurons that bifurcate in the contralateral longitudinal connective. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB3-2.html]

(A) Motoneurons

At stage 14, we observed a labeled NB surrounded by a cluster of 6 motoneurons that have begun to extend axons (Fig. NB3-2A); the clone does not appear to contain local interneurons, suggesting that motoneurons are born first in this lineage. All 6 motoneurons are round and increase in size from 4.9 (23 cells measured in 5 clones) to 7.0 $\mu$m (12 cells measured in 3 clones) between stage 15 and 17. At stage 16, two motoneurons are
positioned at the anterior ventral edge of the clone. These project via the SNa to muscles 4, 18 and 24. We cannot rule out the possibility that muscle 23 may be innervated by these cells as well, as observed by Landgraf et al. (1997). The other four motoneurons are dorsomedial in the clone; these project via the ISN to muscles 2, 10, 11, 19 and 20. Differences between our results and Landgraf et al. (1997) are that they propose that an ISN projection innervates muscle 18, which we do not observe, and that they do not observe a projection to muscles 2 or 10, which we see 100% of the time at stage 17 (Table 2).

Innervation of muscle 4 by SNa is a unique finding. Nevertheless, we see this projection consistently and believe that this innervation has been missed in previous studies for two reasons. First, these axons form delicate diffuse projections at muscle 4, making it easy to see how such innervation might be overlooked in light microscopic preparations of embryonic fillets. Secondly, the difficulty in assaying these endings is compounded by the complex projection of SNa beyond its major branch point (described at the motoraxon web site http://www.caltech.edu/~zinn/motoraxons/fma%20home%20page.html). We see a thin branch to muscle 4 as occurring at the external surface of the muscle (i.e., beneath the surface presented in dissection; see Fig. NB3-2 movies).

(A) Motoneurons
In 2 out of 3 stage 17 abdominal clones, we detect a single oval motoneuron (6.4×5 μm) positioned midway along the dorsoventral extent of the clone, but displaced slightly laterally and posteriorly (circled in Fig. NB3-3 C,D); it projects out the SNa and forms a robust ending on muscle 5. The NB 3-3-derived motoneuron forms only in abdominal segments, and its muscle 5 target also forms only in abdominal segments. Cash et al. (1992) and Landgraf et al. (1997) both describe muscles 5 and 8 as being innervated by a single motoneuron that exits the CNS via SNa, but did not identify the clonal origin of this motoneuron. The neuron that these groups describe apparently matches the motoneuron that we find in this lineage, both in the position of its cell body within the hemisegment and in the pattern of its neurite extensions (Fig. NB3-3 and Tables 2, 3).

(B) Interneurons
All of the interneurons are local, with a robust projection in three fascicles across the anterior commissure, before bifurcating at the contralateral longitudinal connective and extending to the anterior and posterior segment borders. By stage 17, half the cells are small (2.4 μm) and half are larger (4.0 μm; n=36).

NB 3-3
NB 3-3 delaminates at S4 in an intermediate column of NBs. The lineage was partially described by Higashijima et al. (1996) who showed that an eagle-kinesin-lacZ gene expressed in NB 3-3 revealed a cluster of interneurons that projected across the anterior commissure. These data were confirmed by DiI labeling of NB 3-3 (Schmidt et al., 1997), who document 10-13 interneurons with similar projections. Both studies show that the lateral even-skipped-positive (EL) cells come from this lineage, but neither study detected a motoneuron in the clone.

We generated 11 NB 3-3 clones: 2 at stage 15 (1 thoracic, 1 abdominal), 4 at stage 16 (2 thoracic, 2 abdominal) and 5 at stage 17 (2 thoracic, 3 abdominal). We found the clone to consist of 10-18 cells, containing the previously described local interneurons, as well as a single motoneuron that had not been seen before. Every clone contained DiI-labeled cellular debris associated with cell death (asterisks in Fig. NB3-3). We never observed an epidermal subclone. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB3-3.html]
NBs 3-4/4-4

NBs 3-4 and 4-4 are adjacent NBs that delaminate at S5 and S4, respectively. The lineage of NB 4-4 was first described as containing 8-11 interneurons with both ipsilateral and contralateral anterior projections and an obligate epidermal subclone. No lineage has been described for NB 3-4. We group them here because their clones appear to be indistinguishable. We cannot rule out the possibility that only one NB generates this clone, with the other NB generating a different clone that we have misidentified, never observed, or is lost through cell death prior to differentiating. NB 3-4/4-4 clones die with greater than normal frequency (50% survival compared to the usual 80% survival) and so we cannot rule the latter possibility out.

We generated 10 NB 3-4/4-4 clones that survived, with 1 scored at stage 15, 2 at stage 16, and 7 at stage 17; all were abdominal except 2 thoracic stage 17 clones. At stage 17 there are 10-18 neurons: one motoneuron, at least two intersegmental interneurons, and local interneurons. We found epidermal subclones in 50% of our lineages and PNS subclones in 10% of our lineages. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB3-4&4-4.html]

(A) Motoneurons

NB 3-4/4-4 produces a single small motoneuron (5.2 μm at stage 15) which is located at the posterior ventral boundary of the clone and projects into the anterior root of the ISN by stage 15. It exits the CNS via SNd to innervate muscles 15, 16 and 17 and possibly 14, 28 and 30 (Fig. NB3-4/4-4, single arrows). There is a small dendritic arborization in the ipsilateral longitudinal connective.

(B) Interneurons

At least two intersegmental interneurons form by stage 17, one extending anterior ipsilaterally and the other crossing the anterior commissure before extending anteriorly. Local interneurons share the same projection patterns, but do not extend beyond the segment border. The ipsilateral longitudinal connective has the intersegmental interneuronal axon in the medial fascicle and the local axon in the lateral one. In the contralateral longitudinal connective, the situation is reversed, with the local projection closer to the midline than the intersegmental one. Cell sizes are bimodal: most are large (5.2 μm; n=20), but 15% are smaller (3.2 μm; n=5).

NB 3-5

NB 3-5 delaminates at S1 in the anterior-most row of the lateral column. Schmidt et al. (1997) first described its lineage as containing 19-24 interneurons with both ipsilateral and...
### Table 3. Comparison of insect lineage studies

<table>
<thead>
<tr>
<th>NB</th>
<th>Drosophila lineage data</th>
<th>Motoneurons (muscle targets)</th>
<th>Data from other insects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bossing et al, 1996 stage 15</td>
<td>Schmidt et al, 1997 stage 16</td>
<td>This study stage 17</td>
</tr>
<tr>
<td>1-1</td>
<td>1 or 2 MNs 8-14 INs glia in A</td>
<td>NA</td>
<td>1 or 2 MNs pCC is an IIN 2 additional INs in T 6-10 LINs SPGs and Seg Glia</td>
</tr>
<tr>
<td>1-2</td>
<td>16-24 INs TB neuron</td>
<td>NA</td>
<td>1-4 MNs 3-4 INs, including TB 10-15 LINs</td>
</tr>
<tr>
<td>1-3</td>
<td>3 MNs additional glia</td>
<td>NA</td>
<td>likely Dm homolog strap cells and others.</td>
</tr>
<tr>
<td>2-1</td>
<td>8 INs Obligate epi subclone</td>
<td>NA</td>
<td>16 LINs segmental nerve glia epi subclone is not oblige</td>
</tr>
<tr>
<td>2-2</td>
<td>2 or 3 MNs 10-12 INs SPG-A in T</td>
<td>NA</td>
<td>2 or 3 MNs 2-4 INs 15 LINs SPC-A in T</td>
</tr>
<tr>
<td>2-3</td>
<td>NA</td>
<td>NA</td>
<td>putative leg MNs 3-5 LINs</td>
</tr>
<tr>
<td>2-4</td>
<td>2 MNs 7-10 INs</td>
<td>1 MN</td>
<td>1 MN (8) via novel synapse</td>
</tr>
<tr>
<td>2-5</td>
<td>NA</td>
<td>1 MN</td>
<td>1 MN (15,16,17)</td>
</tr>
<tr>
<td>3-1</td>
<td>4 MNs (RP1,3,4,5) 3-8 INs cell death</td>
<td>NA</td>
<td>4 MNs (RP1,3,4,5) 3-6 INs Variable # of LINs no cell death</td>
</tr>
<tr>
<td>3-2</td>
<td>3 or 4 MNs 7-14 INs</td>
<td>NA</td>
<td>6 MNs 10-16 LINs</td>
</tr>
<tr>
<td>3-3</td>
<td>10-13 INs, ELs, obligate epi subclone</td>
<td>NA</td>
<td>66% produce 1 MN 3 INs 4-10 LINs no epi subclone cell death</td>
</tr>
<tr>
<td>3-4/ 4-4</td>
<td>3-4/4-4 clones are indistinguishable 1 MN 2 INs 8-15 LINs</td>
<td>NA</td>
<td>3-4/4-4 clones are indistinguishable 1 MN 2 INs 8-15 LINs</td>
</tr>
<tr>
<td>3-5</td>
<td>19-24 INs</td>
<td>NA</td>
<td>The MN of the TN 12-15 INs 12-15 LINs 1 SN glial cell</td>
</tr>
<tr>
<td>4-1</td>
<td>12-18 INs</td>
<td>NA</td>
<td>likely TN (25)</td>
</tr>
<tr>
<td>4-2</td>
<td>3 MNs several INs</td>
<td>NA</td>
<td>4 MNs 20 LINs Occasional epi subclone.</td>
</tr>
</tbody>
</table>

**References:**

**Abbreviations:**
A: Abdominal; CCAP: Crustacean Cardioactive Peptide; Dm: Drosophila melanogaster; epi: epidermal; IIN: Intersegmental Interneuron; IN: Interneuron; ISN: Intersegmental Nerve; LIN: Local Interneuron; MN: Motoneuron; PNS: Peripheral Nervous System; SBC: Segment Boundary Cells; SN: Segmental Nerve; SPG: Sub-perineurial Glial cell; T: Thoracic; TN: Transverse Nerve
<table>
<thead>
<tr>
<th>NB</th>
<th>Drosophila lineage data</th>
<th>Motoneurons (muscle targets)</th>
<th>Data from other insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-3</td>
<td>NA</td>
<td>8-13 neurons include several MNs, obligate epi subclone, PNS subclone</td>
<td>Schistocerca: not homologous to Dm lineage; generates MNs, IINs and others(^b) 13,14,15</td>
</tr>
<tr>
<td>5-1</td>
<td>2-4 INs, obligate epi-subclone</td>
<td>6-9 LINS epi subclone is not obligate</td>
<td>Schistocerca: not homologous to Dm lineage; generates MNs, IINs and others(^b) 13,14,15</td>
</tr>
<tr>
<td>5-2</td>
<td>1 MN 16-25 INs</td>
<td>NA</td>
<td>Schistocerca: not homologous to Dm lineage; generates CI1 and CI3 (common inhibitory MNs) and IINs (GABA(^+)) 8,10,11</td>
</tr>
<tr>
<td>5-3</td>
<td>9-15 INs cell death</td>
<td>1 MN in T1-A1 3-5 IINs 5-8 axonless INs</td>
<td>Schistocerca: homologous to Dm lineage; smallest lineage generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>5-4</td>
<td>NA</td>
<td>2-4 putative neurosecretory cells(^d) 17,18</td>
<td>Schistocerca: homologous to Dm lineage; generates MNs, IINs and others(^b) 13,14,15</td>
</tr>
<tr>
<td>5-5</td>
<td>NA</td>
<td>10-14 cells in T 3-5 glia in all segments</td>
<td>Schistocerca: homologous to Dm lineage; smallest lineage generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>6-1</td>
<td>10-16 neurons epi subclone</td>
<td>1 MN in T1 only 3-5 IINs no epi subclone</td>
<td>Schistocerca: homologous to Dm lineage; smallest lineage generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>6-2</td>
<td>8-16 INs</td>
<td>NA</td>
<td>Schistocerca: homologous to Dm lineage; generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>7-1</td>
<td>3 MNs (the U’s) 13-19 INs</td>
<td>6 MNs -30 LINS putative SBCs</td>
<td>Schistocerca: homologous to Dm lineage; smallest lineage generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>7-2</td>
<td>8-14 INs</td>
<td>NA</td>
<td>Schistocerca: homologous to Dm lineage; smallest lineage generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>7-3</td>
<td>4-5 neurons, obligate epi-subclone</td>
<td>1 MN 4-5 LINS No obligate epi subclone</td>
<td>Schistocerca: homologous to Dm lineage; smallest lineage generates serotonergic cells 14,15,16</td>
</tr>
<tr>
<td>7-4</td>
<td>NA</td>
<td>8-12 INs 3-4 glia 8-15 Ins in T 5-6 glia fewer INs in abdomen</td>
<td>Schistocerca: homologous to Dm lineage 17</td>
</tr>
<tr>
<td>MP1</td>
<td>2 IINs per segment</td>
<td>NA</td>
<td>Schistocerca: homologous to Dm lineage 19</td>
</tr>
<tr>
<td>MP2</td>
<td>2 IINs</td>
<td>NA</td>
<td>Schistocerca: homologous to Dm lineage 19</td>
</tr>
<tr>
<td>MP3</td>
<td>2 IINs per segment</td>
<td>NA</td>
<td>Schistocerca: homologous to Dm lineage 19</td>
</tr>
<tr>
<td>MNB</td>
<td>5-8 neurons(^e)</td>
<td>1-3 neurosecretory cells and 2-6 interneurons</td>
<td>Schistocerca: homologous to Dm lineage 19, Periplaneta, Locusta: homologous to Dm lineage 20,21</td>
</tr>
</tbody>
</table>
contralateral anterior projections. We generated 15 NB 3-5 clones, scoring 4 at stage 15 (1 thoracic, 3 abdominal), 4 at stage 16 (3 thoracic, 1 abdominal) and 7 at stage 17 (5 thoracic, 2 abdominal). We detect 20-30 interneurons similar to those observed previously. In addition, we also observe a motoneuron (in segment T1 only) and a segmental nerve glial cell (in abdominal segments only). [http://www.uoneuro.uoregon.edu/deolab/lineages/NB3-5.html]

(A) Motoneurons
In the first thoracic segment (T1), this lineage contains a large motoneuron (7.2 μm) that projects ipsilaterally via the posterior root of the ISN and forms synapses on muscles 1, 3 and 9 (Fig. NB3-5). This motoneuron is not generated in any other thoracic or abdominal segments.

(B) Interneurons and glia
The majority of the interneurons are intersegmental, except in T1, where they are all local. They project across the anterior commissure in at least three separate fascicles and then defasciculate and form large, diffuse anterior projections in the contralateral connective (Fig. NB3-5B). In thoracic segments, there is an ipsilateral anterior projection that is only occasionally present in abdominal segments and there are almost twice as many cells as in abdominal segments. The first thoracic segment appears to generate only local interneurons with a similar projection pattern. Abdominal clones contain a nerve root glial cell that ensheathes the axons (Fig. NB3-5D and movies). This is similar to nerve root glia from NBs 2-1, 2-5 and 3-5, but different from the more rounded nerve root glia derived from NBs 1-1 and 7-1.

NB 4-1
NB 4-1 delaminates at S3 in the medial column. Bossing et al. (1996) described the lineage as containing 12-18 interneurons at stage 15, with posterior ipsilateral projections and contralateral projections in both anterior and posterior directions. In grasshopper, NB 4-1 generates intersegmental and local interneurons that project contralaterally in both anterior and posterior commissures; the interneurons are local, spiking and respond to sensory stimulation on the leg (Shepherd and Laurent, 1992; Leitch et al., 1992). We generated 14 NB 4-1 clones, scoring 3 at stage 15 (2 thoracic, 1 abdominal), 6 at stage 16 (3 thoracic, 3 abdominal) and 6 at stage 17 (3 thoracic, 3 abdominal). Our observations match previous descriptions through stage 15. At later stages, we find that interneurons increase in number to 25-30 and their projections become more complex. We also observe the production of the putative transverse nerve (TN) motoneuron. [http://www.uoneuro.uoregon.edu/deolab/lineages/NB4-1.html]

(A) Motoneurons
Abdominal clones produce the TN motoneuron. Thoracic clones do not have this motoneuron, nor do they have TNs; they are replaced by neurohaemal organs that appear to contain only neurosecretory cells (Taghert and Truman, 1982a,b;
The TN motoneuron is ovoid (6.5×4.2 μm; n=3) and dorsal in the CNS. It migrates medially and posteriorly between stage 16 and 17 (Fig. NB4-1F-H). Its axon is first detectable at stage 16 as it projects to the midline and, by stage 17, it bifurcates in the TN (Fig. NB4-1F-H, arrows) after traversing the median nerve (Fig. NB4-1F,G asterisks). The TN motoraxon in Manduca extends identically (Carr and Taghert, 1988a,b). Previous studies identify a motoneuron with similar morphology that forms a synapse on muscle 25 (Gorczyca et al., 1994; Landgraf et al., 1997; Thor and Thomas, 1997); we did not image the ending of the motoneuron in our clones. Bossing et al., (1996) did not observe this motoneuron in the NB 4-1 clone, presumably because they did not assay beyond stage 15.

(B) Interneurons
The interneurons are relatively large (6.0 μm; n=72 cells) and form a tightly packed cluster spanning the dorsoventral extent of the CNS. At least eight axons cross the anterior commissure, with two turning anteriorly and the rest posteriorly. There are three separate fascicles in the posterior commissure. The most anterior ends abruptly in the contralateral connective, the middle one projects anteriorly a short distance and the posterior one projects posteriorly a short distance. There are also ipsilateral projections extending anteriorly; a short projection in a lateral fascicle and a longer projection in a medial fascicle. These interneurons are quite similar to the interneurons derived from NB 4-1 in Schistocerca, except the Drosophila interneurons are not purely local and some form ipsilateral arborizations.

NB 4-2
NB 4-2 delaminates at S2 in the intermediate column. The first GMC (GMC 4-2a) generates the even-skipped-positive RP2 motoneuron and RP2 sib (Doe et al., 1988; Doe, 1992). DiI lineage analysis of NB 4-2 done by Chu-LaGraff et al. (1995) and Bossing et al. (1996) revealed 10-16 cells including the RP2 motoneuron, at least one ‘cousin of RP2’ (CoR) motoneuron, as well as a pool of local interneurons with contralateral projections. In Schistocerca, late-born progeny of NB 4-2 include a large population of intersegmental interneurons with ipsilateral projections (Shepherd and Laurent, 1992), which most closely resemble Drosophila NB 4-1 interneurons (see above). Schistocerca have an even-skipped-positive RP2 motoneuron with axon morphology similar to the Drosophila RP2 motoneuron (Goodman et al., 1984), but its parental NB has not been determined.

We generated 22 NB 4-2 clones, scoring 4 at stage 15 (2 abdominal, 2 thoracic), 6 at stage 16 (3 abdominal, 2 thoracic) and 12 at stage 17 (9 abdominal, 4 thoracic). Our results are similar to previous studies (Chu-LaGraff et al., 1995; Bossing et al., 1996), except we note more cells and additional axonal complexity at stage 17. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB4-2.html]
(A) Motoneurons
There are at least 4 motoneurons in this clone, and possibly an additional thoracic motoneuron that extends an axon late in stage 17 (Fig. NB4-2B). The RP2 motoneuron is a large round cell (7.4 μm; n=19) that migrates dorsally and medially from the clone. It lies at the extreme dorsal surface of the CNS above the junction of the anterior commissure and the longitudinal connective (Fig. NB4-2, white circles). The RP2 axon projects ipsilaterally via the posterior root of the ISN and travels in the ISN until forming an ending at muscle 2. It has branches at muscles 11 and 20 and at muscles 3 and 19 (Fig. NB4-2D, Table 2). In addition, there are three CoR motoneurons, which are also dorsally located, but lateral, posterior and smaller (6.9 μm; n=36) than RP2 (Fig. NB4-2, black circles). The CoRs project ipsilaterally and comprise the entirety of the SNC root innervating muscles 26, 27, and 29 (Fig. NB4-2D; Table 2). Landgraf et al. (1997) backfilled motoneurons from neuromuscular junctions and identified the CoR motoneurons as innervating targets that we believe to be innervated by the RP1, RP3, RP4 and RP5 motoneurons (derived from NB 3-1). However, they did backfill motoneurons innervating muscles 26, 27 and 29 matching the sizes, shapes and positions of the CoR motoneurons. Each of the motoneurons in this clone appears to be associated with a smaller cell, typified by the RP2/RP2sib pair of siblings.

(B) Interneurons
There are ~19 small (4.4 μm; n=126) interneurons by stage 17. Two or three interneurons have axon projections into anterior commissure with bifurcations in an intermediate fascicle of the contralateral connective. In addition, the teardrop-shaped RP2sib has a very short projection into the neuropil; the cell and its projection persist to stage 17 (Fig. NB4-2B,C,D, white broken circles). The majority of cells in this clone appear axonless and in close contact with the much larger motoneurons. This arrangement is consistent with findings in other systems, in which small, axonless local interneurons function to modify motoneuronal function (Pearson and Fourtner, 1975; Burrows, 1996). It is interesting to speculate that this one-to-one relationship is due to a sibling relationship between motoneurons and axonless interneurons, as appears to be the case for RP2/RP2sib.
(C) Other cells
In 5 of 15 late stage 16 and stage 17 clones, we observe a cluster of epidermal cells in close apposition to the RP2 motoneuronal fascicle (Fig. NB4-2, inset). It is interesting that these epidermal subclones are always in an identical position, in close contact with the RP2 axon, at about 50% of its trajectory. Migrating epithelial cells are not unknown (Englund et al., 1999); we propose that the neur ectodermal cluster(s) giving rise to the NB 4-2 (and NB 3-2) lineage generates an epithelial subclone prior to delaminating a NB and that this subclone then migrates to a reproducible lateral position. Alternatively, there may be a specialized mechanism for DiI transfer between these two cell types; this has never been observed in any other clone and we think it is unlikely. The functional significance of the epidermal subclone (e.g. in motoneuron pathfinding) remains to be determined.

NB 4-3
NB 4-3 delaminates at S5. Schmidt et al. (1997) described this lineage as containing 8-13 neurons, including many motoneurons projecting into the SN whose endings could not be identified. They also found an obligate epidermal clone and frequently observed a PNS clone. We generated 5 abdominal NB 4-3 clones, scoring 1 at stage 15, 2 at stage 16 and 2 at stage 17. We believe the cells previously identified as motoneurons (Schmidt et al., 1997) are actually neurosecretory cells of the TN. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB4-3.html]

(A) Neurosecretory cells and interneurons
There are 2 or 3 neurosecretory cells that project together out of the CNS, appearing to join the SN in the periphery. These cells are slightly smaller than most motoneurons (5.3 μm; n=4) and correlate well with the positions and projections of the Bursicon-containing B1, B2 and B3 neurosecretory cells in Manduca (Taghert and Truman 1982a,b; Carr and Taghert, 1988a,b; Broadie et al., 1990; Tublitz and Sylwester, 1990). There are an additional 10-12 small (3.1 μm; n=6) axonless interneurons at stage 17.

NB 4-4
See NB 3-4.

NB 5-1
NB 5-1 delaminates at S5 in the medial column. Bossing et al. (1996) described its lineage as containing 2-4 local interneurons that project in a single bundle across the posterior commissure, and a much larger obligate epidermal subclone. They observed that despite labeling large numbers of NB 5-1s, only 4 clones developed. In Schistocerca, Shepherd and Laurent (1992) showed that NB 5-1 produced 9 inter-segmental interneurons and 6 motoneurons, including the 2nd common inhibitory motoneuron (CI2) which provides inhibitory innervation to three muscles in the distal leg (Hale and Burrows, 1985; Wolf and Lang, 1994). They never observed local interneurons to
derive from this lineage. We too generated only 4 NB 5-1 clones, and scored 2 at stage 15 (1 abdominal, 1 indeterminate segment) and 2 at stage 17 (abdominal). It is possible that we labeled a larger number of NB 5-1 clones, but did not recognize them because an epidermal subclone obscured the neural components. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB5-1.html]

(A) Interneurons
At stage 17, we detect 6-9 local interneurons with a prominent projection across the posterior commissure, bifurcating at the contralateral connective, similar to that described by Bossing et al. (1996). Two cells are intermediate sized (5.5 \( \mu \)m; \( n=4 \)) and the remainder are the smallest cells observed in any lineage (2.2 \( \mu \)m; \( n=6 \)).

Because we did not assay NB 5-1 in the thorax, it is possible that thoracic NB 5-1 generates the *Drosophila* homolog of CI2 described in grasshopper.

**NB 5-2**

NB 5-2 delaminates at S1 in the medial column. Its lineage was described by Bossing et al. (1996) as containing 17-26 cells: a large group of interneurons with contralateral projections, and a motoneuron projecting contralaterally out the ISN, and occasionally reaching muscle 13. Subsequently, the motoneuron target was shown to be muscle 12 (Landgraf et al., 1997). We generated 24 NB 5-2 lineages, scoring 5 at stage 15 (3 thoracic, 2 abdominal), 8 at stage 16 (2 thoracic, 6 abdominal) and 11 at stage 17 (2 thoracic, 9 abdominal). By stage 17, the clone consists of 30-40 neurons in thoracic segments and 16-24 neurons in abdominal segments. It generates a motoneuron, several intersegmental interneurons and a large number of local interneurons (Tables 1, 3). [http://www.uoneuro.uoregon.edu/doelab/lineages/NB5-2.html]

(A) Motoneurons

The motoneuron is a large (6.5 \( \mu \)m; \( n=8 \)) cell and located 1-2 cell diameters from the dorsal surface of the clone (Fig. NB5-2D, white circle). It projects across the posterior commissure, turns posteriorly to exit the CNS in SNb, and forms a bifurcated ending on muscle 12. In one case, we also observed a branching projection from this motoneuron to muscle 5 (data not shown).

(B) Interneurons

There are 4 distinct intersegmental interneuronal projections. One crosses the anterior commissure, follows the median nerve for a short distance, and turns anteriorly in a medial fascicle of the contralateral connective. This projection is established by stage 15 and originates from the dorsalmost cell of the clone (4.8 \( \mu \)m; yellow circle in Fig. NB5-2D). Two other projections cross the anterior commissure, with one turning anteriorly and another turning posteriorly in a medial fascicle of the longitudinal connective. The fourth projection is across the posterior commissure with an anterior turn in a lateral fascicle of the contralateral connective. We also observed an ipsilateral anterior intersegmental projection in about 25% of the clones (Fig. NB5-2C, Table 1). There are a large number of local
interneurons, and most or all project across the posterior commissure and then turn anteriorly, forming an extensive arborizations (see Fig. NB5-2 movies). These interneurons constitute the majority of the posterior commissure at stage 17. The interneurons fall into two size classes: about half are medially located and medium sized (4.5 μm, including the intersegmental interneurons) and half are laterally located and very small (2.8 μm).

**NB 5-3**

NB 5-3 delaminates at S1 in the intermediate column. Its lineage was first described as containing 9-15 interneurons arranged in two clusters, with significant amounts of cell death associated with the clone (Bossing et al., 1996). Schmidt et al. (1997) amended these observations by reporting a motoneuron that projects out the SN with an unknown synaptic target. We generated 13 NB 5-3 clones, and scored 4 at stage 15 (3 thoracic, 1 abdominal), 3 at stage 16 (2 thoracic, 1 abdominal) and 6 at stage 17 (4 thoracic, 2 abdominal). Our results are similar to Schmidt et al. (1997). The clone consists of 5-13 cells, separated into medial and lateral clusters, containing a segment-specific motoneuron and a group of interneurons. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB5-3.html]

(A) Motoneurons

There is at least one motoneuron located laterally in the clone (4.6 μm; n=3). It projects ipsilaterally into the posterior root of the ISN, exits the CNS via SNa, and reaches the posterior group of SNa targets, including muscles 23, 24 and 18.

(B) Interneurons

There is a medial cluster of three unusually large (6.9 μm; n=6) interneurons that cross the posterior commissure and project anteriorly in a lateral fascicle of the connective. A second cluster lies more laterally, and also projects across the posterior commissure (in a more posterior fascicle) before turning anterior in a medial fascicle of the contralateral connective. All of the lateral cells were the same size (4.6 μm; n=16). In abdominal clones, there is also a branch from the lateral cluster that extends into the medial fascicle of the contralateral connective (Fig. NB5-3D). All of the cells in the NB 5-3 lineage have large cell bodies and the only projections that we observe at stage 17 are intersegmental, but we cannot rule out the possibility that some of these cells are local interneurons.

**NB 5-4**

NB 5-4 delaminates during S5 in an intermediate column. Schmidt et al. (1997) propose that NB 5-4 generates 5-9 neurons (thoracic), or 3 or 4 motoneurons (abdominal) that have an ipsilateral projection to an unknown target. We generated 5 clones from NB 5-4, and scored 2 at stage 16 (abdominal) and 3 at stage 17 (1 thoracic, 1 abdominal, and 1 undefined segment). We propose that the clone consists of 2 or 3 neurosecretory cells and abdomen-specific interneurons. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB5-4.html]

(A) Neurosecretory cells

In both *Schistocerca* and *Manduca*, abdominal segments contain crustacean cardioactive peptide (CCAP; also called CAP2A) -immunoreactive neurons with similar complex dendritic arborizations that project medially and dorsally before turning laterally and anastomosing with the TN (Broadie et al., 1990; Dircksen et al., 1991; reviewed in Burrows, 1996). Some confusion exists in the literature concerning the neuroanatomy of these cells. Some CCAP/CAP+ cells originate in the periphery and extend...
axon into the TN from positions outside the CNS (Taghert et al., 1988; Nassel, 1996, for review) whereas others arise from medial and posterolateral positions within the ganglion (Broadie et al., 1990; Tübli and Sylwester, 1990; Dirksen et al., 1991; Tübli and Loi, 1993; Loi and Tübli, 1993).

We observe that both thoracic and abdominal lineages contain 2 or 3 large cells (4.8×7.2 μm; n=4) that project towards the midline, bifurcate and extend bilaterally out the CNS in the TN (or possibly SNa); these cells also have a short projection into the ipsilateral connective (Fig. NB5-4). These cells match the cell body position and axon projection pattern of the CCAP/CAP+ neurosecretory cells.

(B) Interneurons
Abdominal clones contain an additional 2-4 smaller cells (4.5 μm; n=4) that only contain the local interneuronal projection described above (Fig. NB5-4).

**NB 5-5**
NB 5-5 delaminates at S5. Its lineage has not been described
Clonal analysis of *Drosophila* embryonic neuroblasts

in *Drosophila*, although we believe it produces the 'clone Y' of Schmidt et al. (1997) consisting of 6-9 interneurons and a motoneuron that exits the CNS via the TN. In *Schistocerca*, NB 5-5 generates the first and third common inhibitory motoneurons, CI1 and CI3 (Hale and Burrows, 1985), as well as intersegmental interneurons with GABA-like immunoreactivity (Burrows, 1996).

We generated four NB 5-5 clones, and assayed two at stage 15 and two at stage 17. Three of these were in thoracic segments and one was abdominal. There are 8-11 exceptionally large neurons (8.2 μm; *n*=8 cells) including local interneurons and neurosecretory cells, but not common inhibitory motoneurons nor the motoneuron of the TN. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB5-5.html]

(A) Neurosecretory Cells
Both thoracic and abdominal clones contain a ventral cell that migrates to the midline and has a rhomboidal-shape (10×7.5 μm at stage 17; *n*=2). It extends two bifurcating projections laterally before joining the TN nerve (Fig. NB5-5, broken circles; Table 3). This putative neurosecretory cell is similar to the description of the *Manduca* Va neurosecretory cell in shape and ganglionic position (O'Brien and Taghert, 1989; Carr and Taghert, 1988a,b). Its position is also similar to that of the medial CCAP-positive cell previously observed in *Drosophila* and *Manduca* (Broadie et al., 1990; Tublitz and Sylwester, 1990). Finally, we cannot completely rule out the possibility that this cell is a TN motoneuron, as postulated by Schmidt et al. (1997), because its mature endings may form postembryonically.

(B) Interneurons
Most of the clone consists of local interneurons that project either across the posterior commissure or posteriorly in the ipsilateral connective. Both interneuronal projections are at the dorsal surface of the CNS.

**NB 5-6**
NB 5-6 delaminates at S1 in the lateral column. Its lineage was described by Schmidt et al. (1997) as containing 10-14 local interneurons and a variable number of glial cells in thoracic segments, with fewer interneurons in abdominal segments. We generated 11 NB 5-6 clones, and scored 2 at stage 15 (1 thoracic, 1 abdominal), 4 at stage 16 (3 thoracic, 1 abdominal), and 5 at stage 17 (2 thoracic, 3 abdominal). We also observe both local interneurons and glia (Fig. NB5-6). [http://uoneuro.uoregon.edu/doelab/lineages/NB5-6.html]

(A) Interneurons and glia
The local interneurons have four distinct axon trajectories: (1) anterior in the ipsilateral connective, (2) briefly posterior in the ipsilateral connective before reversing and projecting anterior (see Fig. NB5-6C, asterisk and movies), (3) posterior in the ipsilateral connective, and (4) across the anterior commissure then extending anterior in the contralateral connective. Most interneurons are relatively large (5.2 μm; *n*=8) but 2 or 3 cells are lateral, ventral and smaller (3.2 μm; *n*=7). The stage 17 clone spans the lateral half of the hemisegment (see Fig. NB5-6).

We detect dorsal, medial and lateral subperineural glia with equal probabilities, but find it impossible to distinguish these cells except by position, which is greatly variable (Ito et al., 1995). We also observe medial cell body glia (Fig. NB5-6C) and occasionally see ventral subperineural glial cells (*n*=2).
NB 6-1
NB 6-1 delaminates at S3 in the medial column. Its lineage was described as containing 10-16 interneurons, and an occasional epidermal subclone (Bossing et al., 1996). We generated 30 NB 6-1 lineages, and scored 4 at stage 14 (all abdominal), 5 at stage 15 (1 thoracic, 4 abdominal), 10 at stage 16 (3 thoracic, 7 abdominal) and 11 at stage 17 (3 thoracic, 8 abdominal). Thoracic and abdominal clones contain the same average number of cells: 15 at stage 15 (n=4), 18 at stage 16 (n=8) and 26 at late stage 17 (n=7). The clone produces a segment-specific motoneuron, intersegmental interneurons and local interneurons. We never saw an epidermal subclone. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB6-1.html]

(A) Motoneurons
A motoneuron was only observed in the first thoracic segment (Fig. NB6-1E, single arrow). It projects out the posterior root of the ISN, not yet forming a synapse at the time that we assayed (mid-stage 17). One type of interneuron, with posterior contralateral projections (see below) is specifically absent in this segment (Fig. NB6-1E, double arrow).

(B) Interneurons
There are three distinct intersegmental interneuron projection patterns: (1) 3 or more axons project across the posterior commissure and then extend anteriorly; (2) 1 or 2 axons project across the posterior commissure but then extend posteriorly (these are absent in T1); and (3) 3 or more axons project posteriorly in the ipsilateral connective. In addition, there are local interneurons that project anteriorly and posteriorly in the ipsilateral connective (Fig. NB6-1D). In general, dorsal cells project ipsilaterally and ventral cells project contralaterally. The interneuronal projections form extremely complex endings in the CNS. The cells fall into two size categories: about one-third are large (5.1 μm; n=20), and two-thirds are small (3.0 μm; n=20).

NB 6-2
NB 6-2 delaminates at S2 in the intermediate column. Its lineage was described by Bossing et al. (1996) as containing 8-16 interneurons that project in two bundles across the posterior commissure. We generated 14 NB clones, and scored 4 at stage 15 (2 thoracic, 2
abdominal), 5 at stage 16 (4 thoracic, 1 abdominal), and 5 at stage 17 (2 thoracic, 3 abdominal). There is an average of 23 cells by stage 17 (n=4), including both intersegmental and local interneurons (Fig. NB6-2). [http://www.uoneuro.uoregon.edu/doelab/lineages/NB6-2.html]

(A) Interneurons
The two most medial cells of the clone are large, dorsal, engrailed-GFP-negative intersegmental interneurons (6.5 µm; n=16). They project across the posterior commissure in an anterior fascicle and then extend anteriorly. A second pair of very large cells (10×3.8 µm; n=14) are ventral and engrailed-GFP-positive; the more posterior of the pair is an intersegmental interneuron that projects across the posterior commissure in a posterior fascicle and then extends posteriorly. The more anterior of the pair is a local interneuron that we call the ‘comb cell’ because it has a distinctive short anterior ipsilateral projection with all of its synaptic contacts forming on the medial side of the process. It is remarkable that these two cells share a number of distinctive features (engrailed-GFP-positive, large oblong cell shape, ventral position), yet make such different projections (local ipsilateral versus intersegmental contralateral).

The remaining local interneurons are round and varied in size (2.0-6.5 µm; n=35). They project across the posterior commissure and extend anteriorly and posteriorly. The anterior projection ends in a complex meshwork similar to that observed in the NB 1-2 clone.

NB 6-4
NB 6-4 delaminates at S3 in the lateral column. Its lineage was first described by Schmidt et al. (1997) as producing 4-6 dorsally located interneurons, a medial cell body glia and the VUM support glia in thoracic segments. In abdominal segments, the same glial cells form but the interneuronal components are absent. Higashijima et al. (1996) identified similar NB 6-4 progeny using eagle-kinesin-lacZ transgenes expressed in NB 6-4 and, more recently, Akiyama-Oda et al. (1999) used this marker and others to show that the first daughter cell of thoracic NB 6-4 is a glial precursor (M1) that generates 3 medial glia, while the subsequent 2 or 3 progeny are GMCs that generate 4-6 interneurons. We generated 13 NB 6-4 clones, and scored 2 at stage 15 (1 thoracic, 1 abdominal), 4 at stage 16 (2 thoracic, 2 abdominal), and 7 at stage 17 (4 thoracic, 3 abdominal). We also observe both glia and interneurons in thoracic clones, and glia in abdominal clones. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB6-4.html]

(A) Interneurons
Thoracic clones contain 4-10 local interneurons. Three axons project in a posterior fascicle of the posterior commissure and appear to ‘arch anteriorly’ within the commissure. Arched commissural projections are also observed in clones derived from NBs 2-3, 5-2, 5-5, 6-2 and 7-3.

In addition, 2 or 3 small interneurons (3.8 µm; n=7) project posteriorly in a lateral fascicle of the ipsilateral connective. The remaining cells of the larger clones have no detectable projections. Abdominal clones contain 2 cells that may be interneurons with very short axons into the neuropil.

(B) Glia
In thoracic segments, we observe 2-4 lateral cell body glia, whereas previous researchers detect only medialmost cell body glia (MM-CBG, Higashijima et al., 1996; Schmidt et al., 1997; Akiyama-Oda et al., 1998, 1999). We observe putative MM-CBG in thoracic NB 5-6 clones (Fig. NB5-6C). The reason for the different glial positions compared to previous studies is unknown. In abdominal segments, we observe 1 or 2 subperineural glial cells, 1 or 2 cell body glia, and the 2 MM-CBG. This is similar to previous studies, although we see more cells, perhaps because we assay later in development.

NB 7-1
NB 7-1 delaminates at S1 in the posteriormost row of the medial column. Bossing et al. (1996) described the NB 7-1 lineage in Drosophila as consisting of 16-22 neurons, including the even-skipped-positive U motoneurons that innervate dorsal muscles and a group of interneurons. We generated 47 NB 7-1 clones, and assayed 5 at stage 15 (2 thoracic, 3 abdominal), 16 at stage 16 (5 thoracic, 11 abdominal), 22 at stage 17 (6 thoracic, 16 abdominal) and 5 were of indeterminate age. The NB 7-1 clone is the largest of all embryonic NB clones, generating 40 or more cells in thoracic segments. There is no segmental variation except there are always more cells in thoracic clones. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB7-1.html]

(A) Motoneurons
There are two classes of motoneurons: a group of 4-6 that we always observe, and a pair of motoneurons that are infrequently observed (4/47 clones). The group of 4-6 motoneurons are large cells (6.4 µm; n=24), positioned in the middle of the clone. They have complex ipsilateral projections: (1) out the anterior root of the ISN to muscles 2, 3, 4, 9, 10, 19, 20 and possibly 11 (dorsal muscle group), (2) into the SNb to muscles 6, 7 and possibly 13, and (3) into the SNd to muscles 15, 16 and 17 (Fig. NB7-1C,
We agree with Bossing et al. (1996) that these are the previously described U motoneurons (Sink and Whittington, 1991a,b; Goodman and Doe, 1993; Van Vactor et al., 1993; Chiba and Keshishian, 1996), and we support the results of Landgraf et al. (1977) that there are six U motoneurons. Remarkably, nearly half of the larval body wall muscles (13 of 30) receive innervation from the U motoneurons (Fig. NB7-1C, Table 2).

Larval muscle 9 is innervated by one of the U motoneurons and gives rise to the adult DLMa and DLMb muscles; in the adult, DLMs are innervated by a motoneuron called MN5. Because the U and MN5 motoneurons have the same CNS position, axonal morphology and muscle target (Ikeda and Koenig, 1988; Fernandes and Keshishian, 1998; this study), we suggest that an embryonic U motoneuron develops into the adult MN5 motoneuron, which would make it the first motoneuron to be identified throughout the *Drosophila* lifecycle.

The rare pair of motoneurons were only observed in abdominal segments; they lie at the anterior border of the clone and project out the posterior root of the ISN by stage 16. They were never seen fully extended to their target (Fig. NB7-1 inset).

(B) Interneurons and glia

There are only local interneurons in this clone. The previously identified ‘friend of pCC’ (fpCC) interneuron (Bossing et al., 1996; Broadus et al., 1995) is the most medial cell in the clone. It projects anteriorly in a medial fascicle of the ipsilateral...
Clonal analysis of *Drosophila* embryonic neuroblasts

connective, then across the anterior commissure and ultimately extends anteriorly in the contralateral connective (Fig. NB7-1, fpCC inset). Two other interneurons have a similar trajectory, except they take a more lateral fascicle in the ipsilateral connective before crossing the anterior commissure with the fpCC, and then one turns anterior and the other posterior. Finally, there are 2-6 axons that project across the posterior commissure before turning both anterior and posterior. The fpCC may act as a guidepost cell, because we observe interneuronal processes making intimate contact with this cell both dorsal and ventral to it (Fig. NB7-1, fpCC inset). All of the contralaterally projecting interneurons are large (5 \( \mu \text{m} \); \( n=90 \)), but there is also a population of small axonless local interneurons (2.8 \( \mu \text{m} \); \( n=36 \)) that may modulate U motoneuron function, as has been observed in other systems (Pearson and Fourtner, 1975).

A proximal nerve root glial cell is reproducibly observed (Fig. NB7-1A), similar to the nerve root glia cell derived from NB 1-1, and either could be a ‘segment boundary cell’ (Jacobs and Goodman, 1989a,b).

**NB 7-2**

NB 7-2 delaminates at S2 in the intermediate column. Bossing et al. (1996) described its lineage as containing 8-14 interneurons that project either contralaterally in the posterior commissure, or ipsilaterally posterior. We generated 8 NB 7-2 clones, and scored 3 at stage 16 (1 thoracic, 2 abdominal) and 5 at stage 17 (2 thoracic, 3 abdominal). We observe local and intersegmental interneurons in the clone (Fig. NB7-2C). [http://www.uoneuro.uoregon.edu/doelab/lineages/NB7-2.html]

**(A) Interneurons**

There are ~8 intersegmental interneurons with two distinct projection patterns: (1) ipsilateral posterior for at least three segments, and (2) contralateral across the posterior commissure and then posterior. The contralateral projection is shorter and contains fewer axons than the ipsilateral projection (Fig. NB7-2C). There are ~4 local interneurons that project anteriorly in both ipsilateral and contralateral connectives. The clone contains large oval presumptive intersegmental interneurons (7×4.2 \( \mu \text{m} \); \( n=16 \)), and smaller presumptive local interneurons (4.1 \( \mu \text{m} \); \( n=10 \)). NB 7-2 is the only lineage generating ipsilateral posterior intersegmental interneurons by stage 17.

**NB 7-3**

NB 7-3 delaminates at S5 in the posteriormost row of the intermediate column. The NB 7-3 clone was described by Bossing et al. (1996) as producing 3 interneurons projecting across the posterior commissure, a single motoneuron exiting via the SNd to an undefined muscle target and an obligate epidermal subclone of 4-9 cells. Higashijima et al. (1997) used an *eagle-kinesin-lacZ* transgene expressed in NB 7-3 to reveal 3 interneurons (termed EW1-3) and a motoneuron (termed GW). Finally, Lundell and Hirsh (1998) showed that the *huckebein* and *eagle-lacZ* genes are expressed in both NB 7-3 and the serotonergic neurons, providing the first evidence that NB 7-3 produces serotonergic neurons in *Drosophila*.

In *Schistocerca*, NB 7-3 produces only 6 neurons: the S1 and S2 serotonergic neurons from GMC-1, the S3 neuron (serotonergic in T1 only) and its sibling neuron from GMC-2, as well as two uncharacterized neurons from GMC-3 (Taghert and Goodman, 1984). The S1, S2 and S3 neurons are local interneurons that cross the posterior commissure and bifurcate in the contralateral connective (Taghert and Goodman, 1984). There is now lineage, morphological and biochemical homology between the EW1-3 neurons in *Drosophila* and the S1-3 neurons in *Schistocerca*.

We generated 11 NB 7-3 clones, and scored 3 clones at stage 15 (1 thoracic, 2 abdominal), 4 at stage 16 (2 thoracic, 2 abdominal) and 4 at stage 17 (2 thoracic, 2 abdominal). Every clone contains interneurons and a single motoneuron, and four clones generated epidermal subclones in addition to these neurons. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB7-3.html]
(A) Motoneurons
The relatively small GW motoneuron (5.3 μm; n=8) is positioned to the lateral side of the clone and projects out the posterior root of the ISN, joins the SNd and contacts muscles 15,16 and 17 from the external embryonic surface before forming a fork-like endings in the clefts that separate these three muscles (Fig. NB7-3B,C, Table 2).

(B) Interneurons and glia
We observe 3 local interneurons (EW1-3 or S1-3), slightly larger than the GW motoneuron, that project across the posterior commissure before forming large synaptic complexes spanning as much as 50 μm in the contralateral connective. Thoracic clones produce more complex arborizations than abdominal clones (Fig. NB7-3 B,C). In addition, there is always a small axonless cell (3.7 μm; n=11; Fig. NB7-3, asterisk).

NB 7-4
NB 7-4 delaminates at S1 in the posteriormost row of the lateral column. Schmidt et al. (1997) described the NB 7-4 lineage as consisting of 8-12 interneurons that cross the posterior commissure and then extend both anterior and posterior, and 3 or 4 glial cells (2 channel glia and a lateral subperineural glial cell). In Schistocerca, NB 7-4 generates the sibling intersegmental interneurons Q1/Q2, C/G and Q5/Q6. All cross the posterior commissure. Q1, Q2 and C then extend posteriorly while G, Q5 and Q6 extend anteriorly (Raper et al., 1983a,b). The C and G interneurons have been identified in the adult.
metathorax and are called IN 314 and IN 714, respectively (reviewed in Boyan and Ball, 1993).

We generated 3 NB 7-4 clones, all assayed at stage 17 (2 thoracic, 1 abdominal). We observe more neurons in thoracic segments (16-20 neurons, 4-5 glia) than in abdominal segments (12 neurons, 4 glia). Our results are essentially the same as previously reported (Schmidt et al., 1997), except that we detect more cells in each clone, presumably because we assay at a later stage of development. [http://www.uoneuro.uoregon.edu/doelab/lineages/NB7-4.html]

(A) Interneurons and glia
The intersegmental interneurons are similarly sized (4.8 μm; n=12) and project across the posterior commissure; putative homologs of Q1, Q2 and C project posteriorly, while the putative homologs of G, Q5 and Q6 project anteriorly. In addition, many local interneurons project across the posterior commissure and turn anteriorly in a medial fascicle of the connective (see Fig. NB7-4B, asterisk). Similar to Schmidt et al. (1997), we observe a variable number of glia, including channel glia, subperineural glia and cell body glia (Fig. NB7-4A,B)

Median neuroblast (MNB)
The MNB is an unpaired NB that forms at S4 from the mesectodermal cells along the ventral midline of the CNS. Klambt et al. (1991) analyzed enhancer trap lines expressed at the midline and concluded that the MNB and VUM precursors generate distinct lineages. Bossing and Technau (1994) reported that the MNB generates a clone of motoneurons with bifurcating bilateral projections and local interneurons; they also scored very similar unpaired midline clones that they assigned to ‘VUM precursors.’

All insect model systems have intensively studied Dorsal Unpaired Median (DUM) neurons that derive from the MNB (Plotnikova, 1969; Crossman et al., 1971; Hoyle, 1974, 1978; Hoyle and Dagan, 1978; Goodman and Spitzer, 1979; Thompson and Siegler, 1991, 1993; Condron et al., 1994; Campbell et al, 1995; Condron and Zinn, 1995). The DUM neurons include octopamine-positive neurosecretory cells that extend bifurcating axons to various (unidentified) muscle targets and GABA-positive interneurons (Goodman and Spitzer, 1979; Goodman et al., 1979; Condron et al., 1994; Condron and Zinn, 1995). In each of these insects, ventrally positioned cells with similar properties (VUMs) exist, but whether they arise from the MNB is unclear (Pflueger et al., 1993; K. Thompson, personal communication).

We generated 12 MNB clones, 2 at stage 15, 4 at stage 16 and 5 at stage 17; all were at the anterior edge of the engrailed stripe, included engrailed positive cells, and produced similar axonal projections. Our stage 15 clones contained 2 cells with projections similar to those observed by Bossing and Technau (1996); however, by stage 17, the clones were more complex and always contained more than two cells. We suggest that the ‘VUM neurons’ are early born progeny of the MNB, rather than a distinct cell lineage.

(A) Neurosecretory cells/motoneurons
This clone contains both large and small cells but, because their axons are tightly fasciculated as they enter the neuropil, it is
impossible to assign projections to individual cells. Presumably the larger cells (7.2×6.8 μm; n=3) are the motoneurons/neurosecretory cells that extend bilateral projections to body wall muscle targets. At stage 15, there is only one bifurcating projection to the posterior root of the ISN; by stage 17, we observed a robust projection to SNb (n=5) as well (Fig. MNB). The ISN projection always extends to the dorsal muscle group, but target selection appears to be random (consistent with a neurosecretory function). We never saw branches to SNa, SNc or SNd muscle targets (Fig. MNB).

(B) Interneurons

We observed 2-6 smaller cells (average diameter 4.2 μm, n=6) that we presume generate the interneuronal projection (Fig. MNB). The interneurons bifurcate dorsally and remain at an extremely dorsal position, extending to segment boundaries in both connectives.

MP1

MP1 (Midline Precursor 1) forms as an unpaired precursor from the mesectodermal cells along the ventral midline of the CNS. In *Drosophila*, Bossing and Technau (1994) describe this clone as consisting of two bilateral neurons at the midline, anterior to the posterior commissure. Each neuron sends a bifurcating axon anterior and posterior for two to three segments in a medial fascicle of the longitudinal connectives. In grasshoppers, the MP1 precursor is observed to divide once, producing two bilaterally symmetrical intersegmental interneurons; these neurons are involved in pioneering medial fascicles of the longitudinal connectives (Goodman and Spitzer, 1979; Goodman et al., 1979; Bate and Grunewald, 1981; Goodman et al., 1984).

We generated 10 MP1 clones, 4 at stage 15, 2 at stage 16 and 4 at stage 17 (Fig. MP1). Our results are in agreement with Bossing and Technau (1994), but includes a projection anteriorly from the posteriorly projecting axon fascicle at stage 17 (Fig. MP1, and diagram).

MP2

MP2 (Midline Precursor 2), despite its name, forms bilaterally as part of the medial column of S1 NBs in both *Drosophila* and grasshopper (Doe, 1992; Broadus et al., 1995; Broadus and Doe, 1995). The MP2 lineage was initially described in grasshopper (Bate and Grunewald, 1981; Goodman et al., 1984) as producing two intersegmental interneurons: vMP2, which projects anteriorly in a medial fascicle of the connective, and dMP2, which projects posteriorly in a different medial fascicle. The same lineage has been observed in *Drosophila* (Thomas et al., 1984; Spana et al., 1995; Bossing and Technau, 1996), and MP2 has been used to study how Numb, Notch and Sanpodo establish distinct sibling neuron fates (Spana et al., 1995; Spana and Doe, 1996; Skeath and Doe, 1998).

We generated 18 MP2 lineages, 6 at stage 15, 3 at stage 16 and 9 at stage 17; our results are in agreement with Bossing and Technau (1996), except that we find dMP2 and vMP2 to extend axons across the entire length of the CNS at stage 17, making the MP2 and NB 2-5 the only two precursors that generate intersegmental interneurons spanning the length of the CNS at embryonic stage 17.

MP3

MP3 (Midline Precursor 3) forms as an unpaired precursor from the mesectodermal cells along the ventral midline of the CNS. In grasshopper, MP3 generates the H-cell and Hsib (Goodman et al., 1979, 1981; Bate et al., 1981; Bate and Grunewald, 1981; Goodman et al., 1984). The H-cell is an
interneuron that generates complex arborizations on both sides of the midline, with a single robust projection connecting the two in a letter-H arrangement (in T3 and A1 segments); other segments have similar but simpler projection patterns. In *Drosophila*, Bossing and Technau (1994) describe a 2-cell midline clone (UMI; unpaired median interneurons) that contains a neuron resembling the H cell – the neuron projects into the posterior commissure, bifurcates and extends in both anterior and posterior directions on both sides of the midline – but because they saw no segmental variation they were reluctant to call it an MP3 homolog.

We generated 6 MP3 clones: 2 at stage 15, 1 at stage 16 and 3 at stage 17 (Fig. MP3). Our stage 15 clones are similar to those observed by Bossing and Technau (1996) but, by stage 17, the clones were more complex and clearly matched the segment-specific axon projections reported for the grasshopper MP3 neurons. In T3, the axon projections form an ‘H’ with bilateral projections in a medial fascicle of the longitudinal connectives for at least three segments, and additional bilateral projections in a lateral fascicle of the connective; the lateral projections are always asymmetric and the shorter projection branches dramatically (Fig. MP3). In A1, the clone has similar projections. A lower abdominal segment contained projections that extended bilaterally but lacked A/P extensions in the connectives. These segment-specific patterns are similar to the MP3 clone in grasshopper.

**DISCUSSION**

This study is the first to use a vital anatomical marker (*engrailed-GFP*) in combination with assaying live clones, which helps ensure accurate NB identification and minimizes fixation artifacts. It is also the first to assay cell fates as late as mid-stage 17, revealing previously unknown cell types and neuronal projections. We have identified all of the neural cell types generated by stage 17 from all 30 identified NBs in each embryonic hemisegment: 32 motoneurons (38 in the thorax), ~60 intersegmental interneurons, ~30 glial cells, 7 neurosecretory cells and ~250 local/axonless interneurons for a total of approximately 400 cells per abdominal hemisegment (and as many as 500 in thoracic hemisegments). This is the first study to identify the NBs that produce neurosecretory cells in *Drosophila*; a previous study interpreted these cells as being motoneurons (Schmidt et al., 1997).

**Neuroblasts at similar mediolateral positions often generate similar motoneuronal subtypes**

A number of studies have speculated on the mechanisms responsible for motoneuronal organization in the insect CNS (Siegler and Pousman, 1990a,b; Landgraf et al., 1997). Although motoneurons can arise from NBs in all regions of the CNS (anterior or posterior rows; medial, intermediate or lateral columns), we find that there is a correlation between the mediolateral position of a NB and the type of motoneuron it produces. Medial and intermediate column NBs produce all of the motoneurons that innervate dorsal muscle targets (i.e. all motoneurons projecting out the ISN). Intermediate column NBs usually generate motoneurons that innervate intermediate muscle groups (5 of the 7 NBs generating SNa, SNb and SNe are derived from intermediate column NBs). Conversely, lateral column NBs generate the majority of motoneurons.

![Fig. 1. Pairs of NBs at similar DV positions in the hemisegment generate similar lineages. Vertical line represents the midline, horizontal lines represent segmental boundaries. Similarly shaded NBs generate similar clones; unshaded NBs are provided for reference. Clones were found to be similar in numbers of cells, types of cells and axonal trajectories at stage 16.](image-url)
Regulation of NB clone size and segment-specific features

NBs born early usually generate more cells by stage 17 than do NBs born late. While some clones stop dividing prior to the end of embryogenesis (e.g. NB 3-1 and NB 7-3), most clones continued to enlarge for as long as we were able to examine them. The largest clone is derived from thoracic NB 7-1, which produces more than 40 cells. Generating 40 progeny requires at least 20 cell divisions, which is near the maximum possible divisions considering the reported NB cell cycle time of 40 minutes (Hartenstein et al., 1987); alternatively, some GMCs may divide more than once (as does the first progeny, M1, of thoracic NB 6-4; Akiyama-Oda et al., 1999). NBs that form at the same time as the thoracic NB 7-1, such as the abdominal NB 7-1 or any other S1 NBs, do not generate such large clones; these differences could be due to variation in cell cycle characteristics, cell death or timing of mitotic quiescence. The latter two mechanisms are clearly true for a few NBs (early quiescence of NBs 3-1 and 7-3; cell death in lineages of NBs 2-4, 3-3, and 3-4/4-4), but most NB clone size differences are likely to arise as a result of altered cell cycle characteristics. In the case of thoracic versus abdominal NBs with different clone sizes (e.g. NB 7-1), the variation in cell cycle characteristics is likely to be regulated by homeotic genes.

Birth-order of motoneurons, interneurons, and glia

In larger insects, it is common for motoneurons to be generated first, intersegmental interneurons next and local interneurons last (reviewed in Burrows, 1996). Our results in Drosophila support this general model, although there are exceptions. In many motoneuron-producing lineages, such as NB 3-2 and NB 7-1, we observe young clones (stage 14) containing only the parental NB and a group of differentiated motoneurons; i.e. motoneurons are born and extend axons before most other neurons are even born.

We also find the intersegmental interneurons are likely to be produced prior to local interneurons, because the overwhelming majority of late differentiating cells are local interneurons (although the time of differentiation may not always be correlated with the time of birth). Previous studies, and our data, suggest that NB clones contain a variable number of local interneurons (Bossing et al., 1996; Schmidt et al., 1997; this study). This is consistent with a model of neurogenensis in which NB lineages pause at a variable point at the end of embryonic development, during production of local interneurons; when the NB is reactivated during larval development, local interneuron production resumes (Truman et al., 1993; Booker and Truman, 1987). Truman et al. (1993) suggest that motoneurons and intersegmental interneurons are generated early in development because they are likely to be crucial for larval crawling and feeding behaviors, while local interneurons are primarily generated postembryonically because they integrate adult sensory inputs and/or modulate motoneuronal outputs.

Cell migration

Previous studies show that medial NBs remain at fixed positions, but there is variable movement of lateral NBs (Bossing et al., 1996; Schmidt et al., 1997), and our results confirm these findings. In addition, we observe neuron and glial migration, invariably in the medial direction (towards the ventral midline). Many NB clones contain one or two neurons that always migrate medially, away from the rest of the NB clone (NBs 1-2, 2-2, 2-4, 2-5, 3-4/4-4, 4-2, 5-2, 5-3, 5-5, 6-2 and 7-1). The medially displaced neurons are always intimately contacted by growth cones extending towards the midline from the more lateral body of the clone, and thus may act as ‘guidepost’ cells (Bentley and Caudy, 1983a,b). In addition, we observe medial migration of glia (e.g. the MM-CBG from the thoracic NB 5-6 and abdominal NB 6-4, or the longitudinal glia derived from the lateral GB; Fig. NB6-4 and data not shown).

Unique axonal arborizations

We have demonstrated a remarkable axonal complexity in these embryonic Drosophila lineages, yet there is clearly less complexity than that in other insect systems, even at the approximately the same developmental stage (Ho and Goodman, 1982; Raper et al., 1983a,b; Thomas et al., 1984). Some features do, however, stand out. Amos and Mesce (1994) have documented looping axons near the segmental border of the embryonic Manduca CNS. We also observe looping axons near the segmental border (from NB 1-2 and NB 6-2 lineages). To our knowledge, the unusual asymmetric arborizations of the comb cells (derived from NB 6-2 and NB 7-3) have not been
documented in other systems. These may represent modified loopings, as these also occur close to segmental boundaries.

**Transverse nerve components**

In *Manduca*, the TN consists of 1 motoneuron and 8-10 neurosecretory cells in every abdominal hemisegment (Taghert et al., 1988). Neural components can be classified into 3 groups: (1) a posterior, lateral cluster of 3 or 4 Bursicon* and CCAP/CAP* neurosecretory cells that constitute the well-described ‘B-cell pathway’ (Taghert et al., 1988; Broadie et al., 1990; Loi and Tublitz, 1993; Tublitz and Loi, 1993), (2) a medial neurosecretory cell (Va) that extends bifurcating processes into the TN (O’Brien and Taghert, 1989; Broadie et al., 1990; Dircksen et al., 1991; Loi and Tublitz, 1993; Tublitz and Loi, 1993), and (3) a motoneuron that projects posteriorly, bifurcates to extend out of the CNS in the TN and innervates spiracle muscles in abdominal segments. In addition to the neural components, there are also glial ‘strap cells’ which are essential for pioneering the TN in *Manduca* (Carr and Taghert, 1988a,b); the strap cells are joined by other migrating glia that together line the Transverse Nerve (Carr and Taghert, 1988a,b). Finally, there are mesodermally derived DM cells at the dorsal surface of the TN in both *Drosophila* and *Manduca*; these cells have both glial and neural characteristics (Loi and Tublitz, 1993; Chiang et al., 1994; Gorczyka et al., 1994).

We report here that all of the components of the TN in *Drosophila* appear to be analogous to those observed in *Manduca* embryos. (1) The B-cell pathway of neurosecretory cells is likely to be derived from NB 4-3 and NB 5-4, which generate the only cells in the *Drosophila* embryonic CNS that have trajectories similar to the unique *Manduca* B-cell like profiles. (2) NB 5-5 produces a horizontally bifurcating cell, one of only two in the CNS, which may be the homolog of the Va neurosecretory cell of *Manduca* (Carr and Taghert, 1988a,b; Broadie et al., 1990). (3) NB 4-1 produces the other horizontally bifurcating cell, which we believe likely to be the TN motoneuron; it extends posteriorly in the median nerve before dividing to send axons into both branches of the TN. Its muscle targets remain an open question, although several studies reveal a motoneuron matching this description that innervates abdominal muscle 25 (Gorczyca et al., 1994; Thor and Thomas, 1997; Landgraf et al., 1997). (4) We have observed one case of putative TN glia arising from NB 1-3. (5) We have observed mesoderm precursors that produce the DM cells, as well as some or all of muscles 6, 7, 12 and 13. Interestingly, all NBs giving rise to the neurosecretory cells of the TN (NBs 4-3, 5-4 and 5-5) form at a similar time and position within the neuroectoderm, suggesting a common patterned mechanism may be involved in the generation of neurosecretory cell lineages.

**Glia**

Schmidt et al. (1997) observe that the majority of identified glial cells are produced from the lateral NB lineages; our results are consistent with this observation. In addition, we find that NB 1-1 and NB 7-1 generate the segment boundary cells (SBCs) of the ISN described by Jacobs and Goodman (1989a,b). Interestingly, NB 1-1 and NB 7-1 also generate motoneurons that pioneer the ISN and come in close contact with the SBC glia, suggesting that SBC glia serve as pathfinding cues for lineally related neurons, similar to our observation that medially migrating neurons in many clones may act as pathfinding cues for lineally related neurons with medial projections. Alternatively, lineally related cells respond similarly to cues regulating both cell migration and growth cone guidance, thus leading to the correspondence between the position of lineally related migrating cells and subsequent axon projections. We find that NB 2-5 generated a previously unidentified segmental nerve root glia, but the motoneuron in this clone does not appear to contact this glia. We update the list of NBs that produce glia to include NBs 1-1, 1-3, 2-1, 2-2, 2-5, 3-5, 4-6, 4-71 and 7-4.

**A comparison of neuroblast lineage data**

The Technau laboratory has generated ~1000 DiI neuroblast labelings (Bossing et al., 1996; Schmidt et al., 1997) and we have generated ~700 (Chu-LaGraff et al., 1995; this study); we find the studies agree on 24 NBs, but have different interpretations of 4 NB lineages (1-3, 4-3, 4-5 and MNB) and we assign clones to 3 previously uncharacterized NBs (2-3, 3-4, and 5-5).

**New NB clones**

The Technau laboratory has not published lineages for NBs 2-3, 3-4 or 5-5, and they identify ‘Clone Y’ as developing from an unknown NB. We find the NB 2-3 lineage is superficially similar to NB 2-1, and does not develop in abdominal segments. The NB 3-4 lineage is essentially identical to the NB 4-4 lineage. Finally, the NB 5-5 lineage appears to be the ‘clone Y’ of Schmidt et al. (1997). This clone is similar to the thoracic NB 6-4 clone, but can be distinguished by the presence of the Va neurosecretory cell, some unusually large neurons, and only two engrammed-GFP* cells.

**Clone interpretation differences**

Both Schmidt et al. (1997) and our study identify clones from NBs 1-3, 4-3, 5-4, and the MNB but we differ on the interpretation of cell types contained within these clones. Schmidt et al. (1997) conclude that NB 1-3 produces motoneurons lacking neuromuscular junctions; we suggest that it generates transverse nerve (TN) glia. However, we labeled this cell only once, and recognize that a definitive resolution of the cell type of its progeny requires additional labelings. Schmidt et al. (1997) report that NB 4-3 generates several interneurons as well as motoneurons that innervate unknown targets via SNa; we conclude that it produces neurosecretory cells of the TN (probably the Bursicon* cells that have been thoroughly studied in *Manduca*). Schmidt et al. (1997) report that the NB 5-4 clone produces motoneurons that enter SNa and an obligate epidermal subclone; we believe that the described motoneurons are actually neurosecretory cells. Finally, Bossing and Technau (1994) report different lineages for the MNB and VUM cells, whereas we feel the MNB lineage includes the VUM cells.

**Conclusions**

A growing number of genes are known to be expressed in subsets of NBs, GMCs, neurons or glia within the CNS. Our comprehensive analysis of NB cell lineage should provide a foundation for the characterization of mutations in these genes. Mutations can be analyzed directly using DiI labeling, or by scoring for neural features that are specific for individual NBs.
It is important to note that although DiI-labeling is labor intensive, the information provided here is sufficiently detailed so that future experiments need not rely on DiI-labeling in order to examine questions of cell fate specification, axon pathfinding or synaptic target recognition. Using information on unique neural features derived from identified NBs (Table 4), mutations in a gene expressed in any of these NBs can be assayed for the given characteristic feature without having to do DiI labeling. Some examples are: (1) NB 1-2 produces motoneurons that innervate the mouth hook muscles and are the only semaphorin II-positive motoneurons; mutations in genes expressed in NB 1-2 can now be scored directly for defects in these semaphorin II-positive motoneurons. (2) NB 4-1 generates the single motoneuron of the TN nerve that provides unique innervation to muscle 25; mutations in genes expressed in NB 4-1 can be scored for defects in TN nerve formation or synaptic contacts on muscle 25. (3) NB 4-3 generates the Bursicon-positive neurosecretory cells; mutations in genes expressed in NB 4-3 can be scored for the normal development of neurosecretory cells/B-cell pathway. (4) NB 4-2 generates the even-skipped-positive RP2 motoneuron, as well as the entirety of SNC; mutations affecting the SNC can be scored for defects in the NB 4-2 lineage or, conversely, mutations in genes expressed in the NB 4-2 lineage can be scored for SNC defects. (5) NB 5-2 generates the majority of the posterior commissure; mutants with reduced posterior commissures can be scored for defects in NB 5-2 formation or cell lineage. This is not to say that these indirect markers of lineage integrity can be used to unequivocally define genetic defects at the single-cell level; instead we suggest that this type of data can be used to rapidly identify phenotypes which can be subsequently investigated by more precise cell lineage or single cell analysis, and may also suggest alternative models for phenotypes that might previously have been over-simplified.

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Table 4. Unique markers for neuroblast progeny

<table>
<thead>
<tr>
<th>NB</th>
<th>Identified cells</th>
<th>Muscle targets</th>
<th>Molecular markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>eve1 repo2 fasII3 BP1024 Other</td>
</tr>
<tr>
<td>1-1</td>
<td>aCC motoneuron</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>1-1</td>
<td>pCC interneuron</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1-1</td>
<td>CoA motoneuron</td>
<td>12,13</td>
<td>+</td>
</tr>
<tr>
<td>1-1</td>
<td>SPG-A,B glia (A only)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1-1</td>
<td>SBC (putative)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1-2</td>
<td>DC motoneurons (T only)</td>
<td>31-35</td>
<td>+</td>
</tr>
<tr>
<td>2-2</td>
<td>unique innervation (A and T)</td>
<td>21-23</td>
<td>+</td>
</tr>
<tr>
<td>2-2</td>
<td>SPG-A (T only)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>2-4</td>
<td>unique motoneuron</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>3-1</td>
<td>RP1,3,4,5</td>
<td>SNb</td>
<td>+</td>
</tr>
<tr>
<td>3-2</td>
<td>unique innervation (A only)</td>
<td>24,18,4</td>
<td>+</td>
</tr>
<tr>
<td>3-3</td>
<td>significant input to ISN</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>3-5</td>
<td>motoneuron (A only)</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>3-5</td>
<td>EL neurons</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>4-1</td>
<td>likely motoneuron of TN (A only)</td>
<td>25</td>
<td>+</td>
</tr>
<tr>
<td>4-2</td>
<td>RP2</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>4-2</td>
<td>RP2 sib</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>4-3</td>
<td>putative B1,B2,B3 cells</td>
<td>26,27,29</td>
<td>+</td>
</tr>
<tr>
<td>4-5</td>
<td>AC motoneuron</td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>4-5</td>
<td>neurosecretory cells of TN</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>5-7</td>
<td>putative neurosecretory cell</td>
<td>ISN</td>
<td>+</td>
</tr>
<tr>
<td>7-1</td>
<td>U MNS</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7-2</td>
<td>putative SBCs</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7-3</td>
<td>Only serotoninergic cells in CNS</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1-3</td>
<td>putative TN strap cells</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>MNB</td>
<td>VUMs</td>
<td>ISN and SNC targets</td>
<td></td>
</tr>
</tbody>
</table>


Abbreviations: A, Abdominal; ACC, anterior Corner Cell; CCAP, Crustacean Cardioactive Peptide; CoA, Cousin of aCC; Dm, Drosophila melanogaster; epi, epidermal; IIN, Intersegmental Interneuron; IN, Interneuron; ISN, Intersegmental Nerve; LIN, Local Interneuron; MN, Motoneuron; PNS, Peripheral Nervous System; SBC, Segment Boundary Cells; SN, Segmental Nerve; SPG, Sub-Perineurial Glial cell; T, Thoracic; TN, Transverse Nerve.

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