

Developmental architecture of adult-specific lineages in the ventral CNS of *Drosophila*

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Summary

In *Drosophila* most thoracic neuroblasts have two neurogenic periods: an initial brief period during embryogenesis and a second prolonged phase during larval growth. This study focuses on the adult-specific neurons that are born primarily during the second phase of neurogenesis. The fasciculated neurites arising from each cluster of adult-specific neurons express the cell-adhesion protein Neurotactin and they make a complex scaffold of neurite bundles within the thoracic neuropils. Using MARCM clones, we identified the 24 lineages that make up the scaffold of a thoracic hemineuromere. Unlike the early-born neurons that are strikingly diverse in both form and

function, the adult specific cells in a given lineage are remarkably similar and typically project to only one or two initial targets, which appear to be the bundled neurites from other lineages. Correlated changes in the contacts between the lineages in different segments suggest that these initial contacts have functional significance in terms of future synaptic partners. This paper provides an overall view of the initial connections that eventually lead to the complex connectivity of the bulk of the thoracic neurons.

Key words: Neurogenesis, Metamorphosis, Neuronal architecture

Introduction

Developing nervous systems face the challenge of generating a wide diversity of neuronal types and then establishing precise patterns of synaptic connectivity between these cells. Neuronal precursor cells in the CNS have a major influence on the properties of the neurons that they produce, but this influence can change through time. For example, in the vertebrate cerebral cortex (Desai and McConnell, 2000) and retina (Cepko et al., 1996) neuronal precursor cells initially have the competence to produce a variety of neuronal types but this competence becomes progressively reduced as proliferation progresses. This dependence of neuronal phenotypes on the properties of their precursor cell reaches its extreme in the developing CNS of insects. In these animals, the precursor cells [called neuroblasts (NBs)] are organized in a stereotyped array of rows and columns, with each stem cell having a unique identity as determined by position (Bate, 1976; Doe and Goodman, 1985) and gene expression (Doe, 1992). The neurons arising from each NB are highly diverse but unique to that stem cell (Bossing et al., 1996b; Schmidt et al., 1997; Schmid et al., 1999). The phenotypic diversity in the early progeny of each NB is specified by the sequential expression of the transcription factors Hunchback, Kruppel, PDM and Castor (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001), but fairly early in their lineages, the NBs lose the competence to respond to these factors (e.g. Pearson and Doe, 2003) and the diversity of cell types that they can produce becomes progressively restricted. Although the early diversity of neurons within these lineages has been extensively

studied, we know less about the phenotypes of later cells. At present, the entire set of neurons for only one lineage, the median lineage in grasshoppers, is known. After a highly diverse initial set of progeny, the later born cells are quite similar, being divided into two large classes of interneurons (Thompson and Siegler, 1991), an observation entirely consistent with the idea of restricted phenotypic diversity later in a lineage. The key issue here is whether this example is the rule or the exception.

In insects that have complete metamorphosis, like *Drosophila* and the moth *Manduca sexta*, the NBs generate an initial set of neurons that regulate larval behavior, but many then make a much larger set that have an adult-specific function (Booker and Truman, 1987; Truman and Bate, 1988; Prokop and Technau, 1991). These adult-specific neurons, most of which are born during larval life, extend a primary neurite into the neuropil but then arrest. As the larva grows, each NB accumulates a growing cluster of these arrested immature neurons until the onset of metamorphosis when these cells show intense sprouting as they find their adult synaptic targets. In this paper, we have focused on the adult-specific lineages in the ventral CNS. The fasciculated neurites arising from these lineages express the cell adhesion protein, Neurotactin (de la Escalera et al., 1990), and they make a complex scaffold of neurite bundles within the thoracic neuropils. Through the use of MARCM-based clones (Lee and Luo, 1999), we identified the 24 lineages that make up the scaffold of a thoracic hemineuromere. Unlike the early-born neurons that are strikingly diverse in both form and function, the later-born cells

in a given lineage are remarkably similar and typically project to only one or two primary targets, which appear to be the bundled neurites from other lineages. Correlated changes in these patterns of projection and contact between segmental neuromeres suggest that these initial contacts may denote future synaptic partners and functional relationships amongst the lineages. This paper provides an overall view of the initial connections that eventually lead to the complex connectivity of the bulk of the thoracic neurons. It establishes a developmental framework from which we will be able to understand the developmental rules that determine the synaptic connectivity of the adult CNS.

Materials and methods

Fly stocks

The MARCM technique was used, in which the FLP/FRT system induced clones that lacked GAL80, a suppressor of GAL4, to make *CD8::GFP*-labeled clones in an unlabeled background (Lee and Luo, 1999). The three GAL4 drivers used during the course of this work were *Elav*, *Actin* and *Tubulin*. The following genotypes were generated.

Elav based clones: *GAL4^{C155}, hsFLP; FRT^{42B}, tubP-GAL80/FRT^{42B}, UAS-mCD8::GFP*

Actin based clones: *hsFLP; FRT^{42B}, tubP-GAL80/FRT^{42B}, UAS-mCD8::GFP; ActinGAL4*

Tubulin-based clones: *hsFLP, tubP-GAL80, FRT^{19A}/FRT^{19A}, UAS-mCD8::GFP, tubP-GAL4*.

The *actinGAL4* stock was a gift from B. Edgar, all other stocks were obtained from the *Drosophila* stock center (Bloomington, Indiana).

Generation of MARCM clones

Two heat shock regimes were used to generate MARCM clones. In the early heat-shock regime, eggs were collected on grape juice plates for 2 hours, held for 3 hours (both at 25°C) and then incubated for 1 hour at 37°C. Hence, the embryos were heat shocked between 3 and 5 hours of embryogenesis. In the late heat-shock regime, eggs were collected for 2 hours, held for 5 hours (both at 25°C), and then incubated for 1 hour at 37°C. Embryos were therefore heat shocked between 5 and 7 hours of embryogenesis. Larvae were reared on standard cornmeal food at either 25°C or room temperature when precise staging was not a concern. Nervous systems were generally dissected from larvae that were in the late 3rd instar or during wandering.

Immunocytochemistry and in situ hybridization

Nervous systems were dissected from larvae and fixed in 3.7% buffered formaldehyde for about 1 hour at room temperature and then washed three times in PBS-TX [phosphate buffered saline (pH 7.8) with 1% Triton-X100]. Fixed samples were blocked in 2% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS-TX for 30 minutes and then incubated in various combinations of primary antibodies for 1 to 2 days at 4°C. In preparations examining the relationship of the *mCD8::GFP* labeled clones to the Neurotactin scaffold, the CNSs were incubated in a 1:50 dilution of an anti-Neurotactin monoclonal antibody (F4A; a generous gift from Dr M. Piovant) and 1:1000 dilution of a rabbit anti-*mCD8* (Caltag Laboratories, Burlingame, CA, USA) After washing out unbound primary antibodies, tissues were incubated overnight at 4°C in a 1:500 dilution of FITC conjugated donkey anti-rabbit IgG and Texas Red conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After repeated washes with PBS-TX, tissues were mounted on poly-lysine coated coverslips, dehydrated, cleared through xylene and mounted in DPX (Fluka, Bachs, Switzerland).

For diaminobenzidine (DAB)-stained preparations the tissue was fixed as above. The tissue was incubated in 2 N HCl in PBS for 30 minutes and washed in PBS-TX three times. Fixed samples were blocked in normal 2% horse serum (Vector Laboratories, Peterborough, UK) for 1 hour and then incubated in a 1:250 dilution of anti GFP (Roche Diagnostics, Lewes, UK) overnight at 4°C After washing out unbound primary antibody, tissues were incubated overnight at 4°C in a 1:500 dilution of biotinylated horse anti-mouse IgG. After washing the tissue was incubated in a 1% solution of an avidin-biotin complex (Vector Laboratories, Peterborough, UK) for 2 hours. The tissues were washed and the antibody binding revealed by incubation in a 3% solution of diaminobenzidine and hydrogen peroxide.

Microscopy and image processing

Fluorescently stained nervous systems were imaged at 60× using a BioRad MRC600 confocal microscope. *z*-stacks were collected with optical sections at 1.5 μm intervals. In collecting the *z*-stacks, the excitation wavelength was optimized for the respective fluorophore to avoid bleed-through.

Raw data stacks were imported into NIH Image (<http://rsb.info.nih.gov/nih-image/>). Where necessary, adjustment to contrast and brightness were made to the entire data stack. Some nervous systems had single clones or widely spaced clones so that each could be easily viewed without interference. In many cases, though, there were multiple clones in a region and these obscured details in projected or rotated images. In these stacks, we would select a particular clone and use the lasso tool to remove the stained processes and cell bodies from other clones. This procedure would be carried multiple times on the same data stack, in each case isolating a different clone. Using Image J (<http://rsb.info.nih.gov/ij/>), we then made merges of the whole clonal array and of the individual clones with the same Neurotactin scaffold. This allowed us to determine the relationship of the various clones to one another and to the Neurotactin scaffold.

The data in the paper are typically presented as ‘thick-section’ merges. We took 5-10 section portions of the Neurotactin stack and projected these as a two-dimensional image. The corresponding sections from the clone data stack were also projected as a flat image. The two projections were then combined in Photoshop (Adobe, San Jose, CA), with the Neurotactin image in red and the clone image overlaid in white.

Numbering of the lineages

We have been able to associate about half of the adult-specific lineages with their embryonic NB. We decided not to use the embryonic designations (e.g. 3-3 for the lineage from NB 3-3) for these lineages because we favored having a consistent nomenclature at this time, rather than one that was mixed. Generally the adult-specific lineages were numbered as they were identified and we have not tried to relate the numbering to either position or projection pattern.

Results

Neurotactin scaffold in the ventral CNS of larval *Drosophila*

The identification of the multicellular clones was facilitated by referencing each clone to Neurotactin-positive bundles in the ventral CNS. Neurotactin is a heterophilic cell adhesion protein and the fasciculated neurites of the immature neurons express it highly on their surface (Barthalay et al., 1990; de la Escalera et al., 1990). The Neurotactin staining revealed a complex scaffold of neurite bundles that included all of the arrested postembryonic neurons in the CNS. Nervous systems were double labeled so that we could identify which MARCM

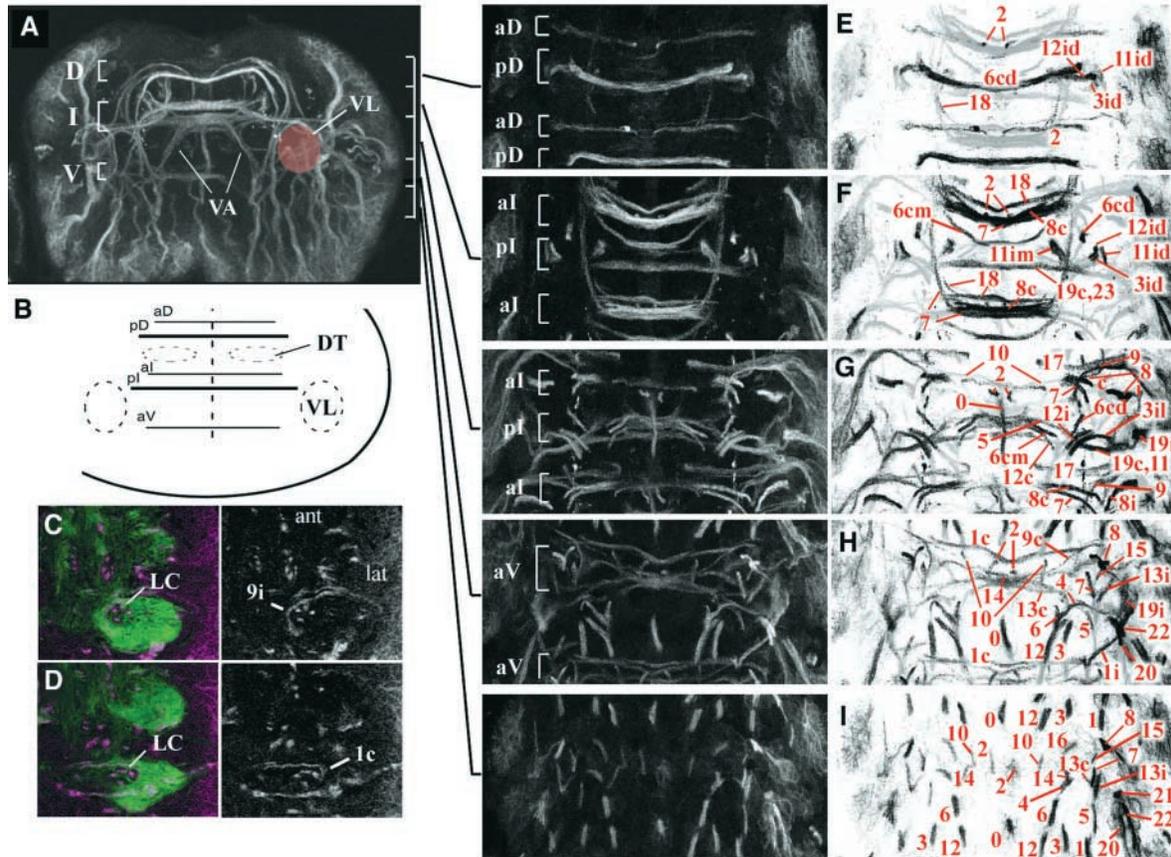


Fig. 1. Features of the Neurotactin-positive bundles in the thoracic CNS of larval *Drosophila*. (A) A thick transverse projection of the bundles in one thoracic neuromere. Brackets show the levels of the dorsal (D), intermediate (I) and ventral (V) commissures; VA, ventral arch; tinted region highlights the right ventrolateral (VL) neuropil. (B) Schematic representation of a cross section through the neuropil, showing positions of the commissures, ventrolateral neuropil and dorsal longitudinal tracts (DT). (C,D) Dorsal views of confocal sections through the VL neuropils in T2 and T3 of a nervous system immunostained for (left) Neurotactin (magenta) and with phalloidin (green). Phalloidin staining shows the fine fibrous neuropil characteristic of the VL neuropil, which contains a poorly stained core, the lateral cylinder (LC), that is bounded by the Neurotactin bundles 9i and 1c (right). Several bundles ascend through the cylinder. (E-I) Paired images of thick stack projections of the T2 and T3 neuropils from the levels indicated on (A). The lineage bundles are labeled on the negative version (right panel); the next ventral section is also included as a 'ghost' to aid in alignment of the bundles. Brackets show the anterior (aD) and posterior (pD) dorsal commissures, the anterior (aI) and posterior (pI) intermediate commissures, and the anterior ventral (aV) commissure.

clones were responsible for every component of the scaffold. The identity of the neurite bundles and the landmarks that are especially useful for their identification are summarized in Fig. 1. In cross-section, Neurotactin stained bundles cross the midline in ventral, intermediate and dorsal commissures (Fig. 1A). The intermediate commissure (Fig. 1F,G) contains the most bundles and is divided into anterior (aI) and posterior (pI) sections. The dorsal commissure (Fig. 1E) has a thick posterior component (pD) and a poorly developed anterior section (aD). Ventrally, there is only an anterior (aV) commissure (Fig. 1H). Another prominent neuropil landmark that we used is the 'ventral arch' (VA, Fig. 1A). It extends from selected ventral lineages up to the intermediate commissure. Three bundles make up the posterior ventral arch, whereas a single bundle forms the anterior ventral arch.

Over half of the lineages project their neurite bundles to an area of fine fibrous neuropil in the ventrolateral region of each thoracic neuromeres. Phalloidin staining shows a dense packing of fine processes in this neuropil (Fig. 1C,D). A prominent feature within this neuropil is a structure that we

called the 'lateral cylinder', which lacks fine processes. Neurotactin staining shows that the lateral cylinder is bounded on its dorsomedial side by bundle 9i and on its ventrolateral side by bundle 1c. It also contains 5 neurite bundles (7c, 8c, 8i, 15i, and 16i) that project dorsally through its core.

In the thoracic and A1 neuromeres, the postembryonic lineages are situated from the ventral midline around to the dorsolateral boundary of the cellular rind (Fig. 1A). The relative insertion points of the neurite bundles into the neuropil are invariant and can be used to identify the individual lineages. Fig. 1I shows a thick section projection of the bundles emerging from the lineages in the ventral region of the rind. The relative position of these lineages is schematically depicted in subsequent figures along with the remainder of the dorsal and lateral lineages that are missing from the section in Fig. 1I. For some of lineages, we have also included data from the subesophageal ganglion.

Characteristics of the segmental lineages

The following description is based on ~300 clones from

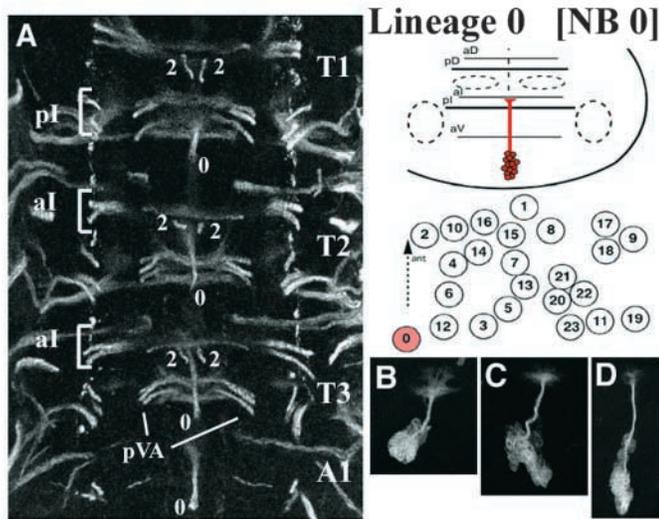


Fig. 2. Characteristics of median neuroblast lineage (lineage 0). (A) A thick section projection of confocal slices showing the Neurotactin scaffold between the ventral and intermediate commissures. The neurite bundle from lineage 0 projects to the level of the aI commissure up through T2, but is redirected to the pI commissure in T1. Posterior ventral arch (pVA) is labeled in T3 but is also present in T2 and T1. '0' identifies the ventral base of bundle 0 in each segment. (B-D) Ventral views of projections of full lineage 0 clones in the (B) S3, (C) T1 and (D) T3 neuromeres. Diagrams show the trajectory of the neurite bundle in the Neurotactin scaffold, and the position of the lineage 0 cluster relative to the other clusters in the hemisegmental array. Commissure abbreviations as in Fig. 1

individuals that carried Elav-GAL4 based MARCM clones and were double stained for Neurotactin (see Table S1 in the supplementary material). We analyzed an equivalent number of Elav clones that were either fluorescently marked but without Neurotactin labeling or were DAB-stained preparations. The variation in the number of times that we identified a particular lineage probably reflects the timing of when the neuroblast for the particular lineage started dividing in the embryo. In a very few cases (e.g. lineage 7 in T1), we know that the lineage is present despite our failure to recover clones in that segment, because we can identify the Neurotactin bundle corresponding to that of lineage 7 in that segment. A characteristic feature of each lineage is the pattern of projection of the bundle(s) of neurites that emerge from the cluster of immature neurons. We have given each of the 33 bundles at least a binary designation, including a number (indicating its lineage of origin) and a letter to indicate whether it projects ipsilateral (i) or contralateral (c). In the cases in which two bundles terminate on the same side of the midline, we added a second letter to denote dorsal (d), middle (m), ventral (v) or lateral (l) trajectories. Where we use merged thick sections to illustrate the relationship of a clone to the Neurotactin scaffold, the dorsal-most section is displayed at the top. We omitted the binary designation in labeling the figures in cases in which a lineage has only a single bundle. Additional information on each lineage is available at <http://depts.washington.edu/nbatlas/>.

Lineage 0

Lineage 0 (Fig. 2) is an Engrailed positive lineage produced by the median unpaired neuroblast (NB 0). A lineage 0 cell cluster is located at the ventral midline of segments S3 through A1. In T2, the neurites coming from the cluster form a single bundle (0) that projects anterodorsally to the midpoint of the aI commissure where the processes then splay out at about the level of the paired 2i bundles (Fig. 2A,D). An identical projection pattern is seen for the 0 bundle from the T3 and A1 clusters, but in T1 the bundle does not project anteriorly and terminates at the level of the pI commissure (Fig. 2A,C). In S3, the 0 bundle also projects to the pI commissure but some fibers turn anteriorly to form a more complex terminal projection (Fig. 2B).

Lineage 1

The cell cluster for lineage 1 (Fig. 3) is located ventrolaterally along the anterior border of the T1-

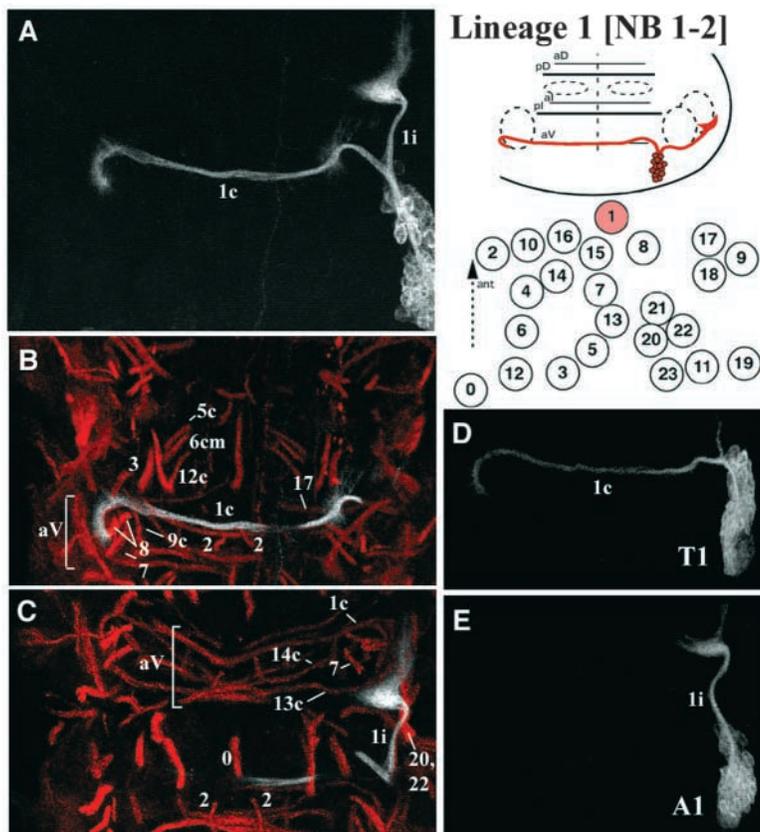


Fig. 3. Characteristics of lineage 1 (NB1-2). (A) Dorsal view of the projection of a lineage 1 clone in T3. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 1 to features of the Neurotactin scaffold (red) in the ventral neuropil. The contralateral bundle (1c) projects to the contralateral ventrolateral neuropil, whereas the ipsilateral bundle (1i) projects to the ventrolateral neuropil in T2. Progressively ventral sections. (D) Lineage 1 in T1 lacks the anterior li bundle. (E) Lineage 1 in A1 lacks the 1c bundle. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

A1 neuromeres. Lineage 1 is Engrailed positive, which identifies it as belonging to NB 1-2 (Broadus and Doe, 1995). Bundle 1c also has the projection pattern seen for some of the local interneurons generated by this NB (Schmid et al., 1999). In T3, the cell cluster produces two neurite bundles (Fig. 3A). The contralateral bundle (1c) loops dorsally over the bundle 17i and projects across the midline as the most anterior bundle in the aV commissure. It then curves around the anteriolateral border of the lateral cylinder in the ventrolateral neuropil. Lineage 1 is the only lineage that has a major projection to the ventral neuropil in an adjacent segment. The ipsilateral bundle (1i) projects anteriorly to the next ventrolateral neuropil, where it splays out in a ventral domain posterolateral to the lateral cylinder. Along its anterior path, bundle 1i curves around the ascending neurite bundles from lineages 20, 21 and 22.

In T2, lineage 1 has an anatomy that is identical to that seen in T3. In T1, however, the lineage has bundle 1c but the ipsilateral bundle (1i) is missing (Fig. 3D). By contrast, in A1, the ipsilateral bundle is present, while the contralateral bundle is absent (Fig. 3E). Lineage 1, therefore, appears to be comprised almost exclusively of neurons projecting to thoracic sensory neuropils, most likely related to the legs.

Lineage 2

The cell cluster from lineage 2 is located near the midline on the anterior margin of the neuromere (Fig. 1I). Fig. 4 shows two lineage 2 clones that were hit in the same neuromeres. In Actin-based clones, lineage 2 is associated with a cluster of larval neurons that lack efferents and have a simple contralateral projection similar to that described for the embryonic progeny of NB 2-1 (Schmid et al., 1999). Based on the morphology of its larval siblings and its position in the neuromeres, we have ascribed it to NB 2-1. Lineage 2 clusters are found only in segments T1 to T3 and their projection pattern is identical in all segments. A single neurite bundle (2i) projects dorsally from the cluster and then turns sharply lateral when reaching the dorsal surface of the neuropil. Although the processes do not cross the midline, they make up the majority of the aD commissure.

The 2i bundles are important landmarks in the Neurotactin scaffold. Within the aV commissure, the dorsally projecting 2i bundles divide the commissural bundles with the bundles from lineage 1 being anterior and those from lineages 13 and 14 being posterior (Fig. 1H, Fig. 4D). For the aI commissure, the lineage 18 bundles cross anterior to the 2i bundles, whereas lineages 7 and 8 cross behind them (Fig. 1F, Fig. 4C).

Lineage 3

Lineage 3 is an Engrailed-positive cluster situated on the posterior border of the neuromere just lateral to cluster 12 (Fig. 1I) in S3 through A1. Actin clones of lineage 3 include at least four motoneurons (the 'U' motoneurons) that project out the ipsilateral segmental nerve (Fig. 5F), showing that this cluster is produced by NB 7-1

Lineage 2 [NB 2-1]

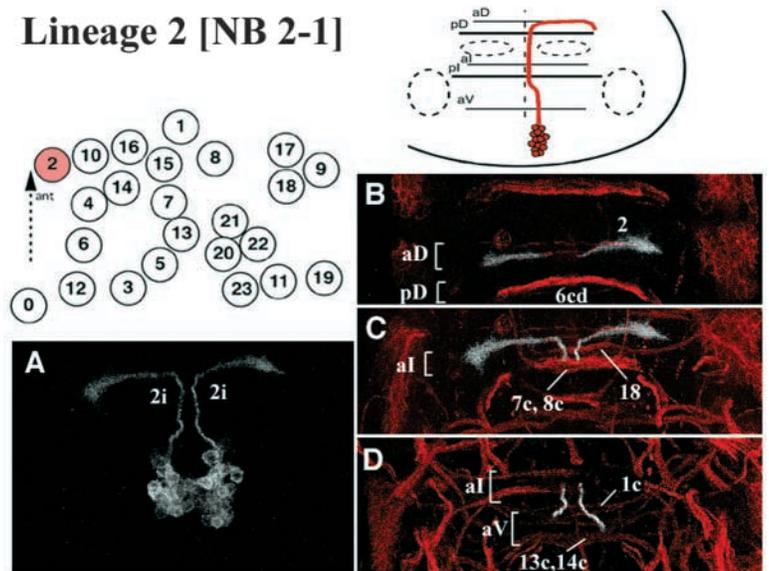


Fig. 4. Characteristics of lineage 2 (NB 2-1). (A) Ventral view of the projection of paired lineage 2 clones in the T2. (B-D) Thick section projections showing the relationship of the clone to (B) dorsal, (C) intermediate and (D) ventral features of the Neurotactin scaffold (red). Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

Lineage 3 [NB 7-1]

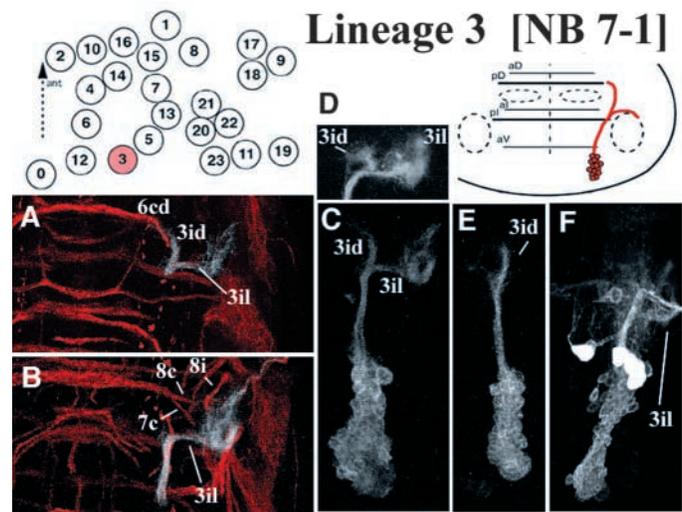


Fig. 5. Characteristics of lineage 3 (NB 7-1). (A,B) Thick section projections showing the relationship of the neurite bundles of lineage 3 clone from C to (A) dorsal and (B) ventral features of the Neurotactin scaffold (red). (C) Ventral view of the projection of a lineage 3 clones in the T3. (D) The intermediate to dorsal region of lineage 3 in T2, showing the expanded 3id bundle. (E) A1 version of lineage 3 lacking the 3il bundle. (F) An Actin-GAL4 based MARCM clone of lineage 3 showing larval neurons as well as the adult-specific cells. The neurons with bright cell bodies are four of the 'U' motoneurons made by NB 7-1. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

(Landgraf et al., 1997). In neuromere T3, a single neurite bundle (3i) projects dorsally from the cluster but then splits into a dorsal (3id) and a lateral (3il) bundle at the level of the pI

commissure (Fig. 5A-C). The 3id bundle continues dorsally and terminates next to bundle 6cd as the latter bends medially to form the pD commissure (Fig. 5A). The 3il bundle extends laterally and spreads anteriorly over the dorsal region of the ventrolateral neuropil (Fig. 5B).

In A1, lineage 3 lacks the 3il bundle and only bundle 3id is present (Fig. 5E) and it ends next to bundle 6cd as in T3. In segments T1 and T2, the termination of bundle 3id is more diffuse than seen in the posterior neuromeres (Fig. 5D). This altered terminal projection is associated with the presence of bundles 11id and 12id, which are confined to the T1 and T2 neuromeres.

Lineage 4

The lineage 4 cell cluster is situated near the midline, just posterior to cluster 10. It is present in segments T1 to T3 but it is not found in the subsophageal or abdominal neuromeres. Actin-based clones containing the larval siblings of lineage 4 include the motoneurons RP1,3,4,5 (data not shown), which identify this lineage as being from NB 3-1 (Landgraf et al., 1997). This adult-specific lineage produces a single neurite bundle (Fig. 6) that projects laterally along the ventral surface of the neuropil and terminates in the ventrolateral neuropil posterior to the lateral cylinder (Fig. 6B).

Lineage 5

Lineage 5 has a neuronal cluster situated in the ventrolateral region of the neuromere and is found in segments S3 to T3. The cluster produces a single neurite bundle (5c) that projects medially along the ventral neuropil and then bends dorsally as the anterior-most bundle of the posterior ventral arch (along with 6cm and 12c; Fig. 7B,C). The bundle broadens into a flattened projection that covers most of the anterior half of the pI commissure. Small neurites extend anteriorly on either side of the commissure (Fig. 8A,B). The lineage located in S3 has a more pronounced anterior projection when compared with its thoracic counterparts (not shown).

Lineage 6

Lineage 6 is a medial lineage situated just anterior to lineage 12 (Fig. 1I) and is found in segments S3 to A1. It is Engrailed negative and, hence, is from a NB row anterior to row 6. Based on its medial position and the large number of cells that it contributes to the posterior commissure (see Schmid et al., 1999), we have assigned this lineage to NB 5-2. In the thorax, the lineage 6 cluster produces two neurite bundles, 6cm and 6cd, both of which project to contralateral neuropil (Fig. 8A). Bundle 6cm is the middle bundle of the posterior ventral arch (Fig. 8D,E); it crosses the midline in the pI commissure and then projects anteriorly in a dorsal longitudinal tract (Fig. 8C). Bundle 6cd extends more dorsally and then crosses the midline as the major bundle of the pD commissure (Fig. 8B). The neurites extend to the lateral border of the pD commissure and then dip ventrally before they terminate at the level of the 6ci bundle (Fig. 8B,C).

The projection pattern of lineage 6 is the same in all the thoracic neuromeres, although there is a reduction in the

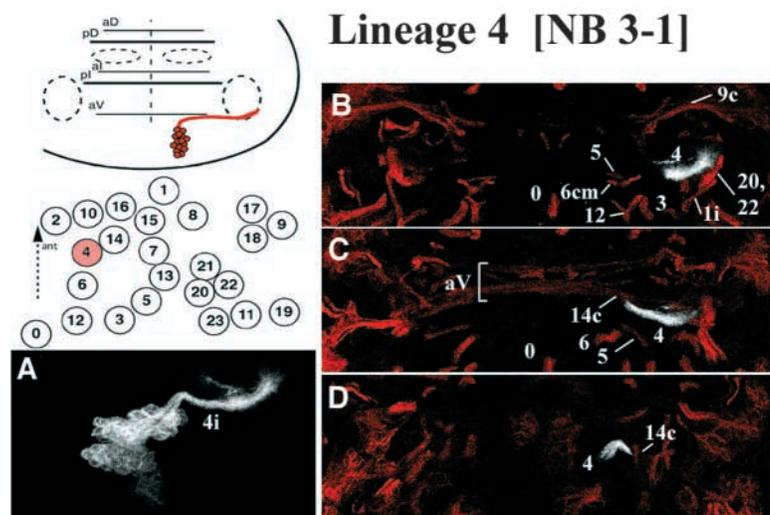


Fig. 6. Characteristics of lineage 4 (NB 3-1). (A) Ventral view of the projection of a lineage 4 clone. (B-D) Thick section projections showing the relationship of the neurite bundle from lineage 4 to features of the Neurotactin scaffold (red) in the ventral neuropil. (B-D) Progressively ventral slices. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

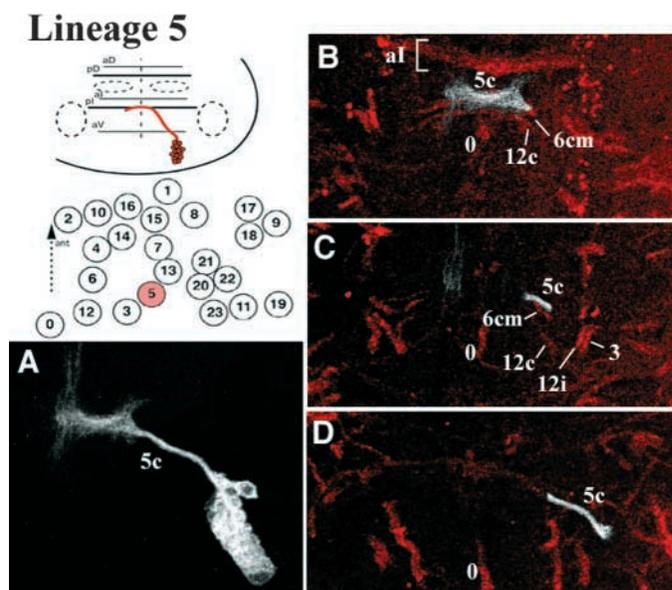


Fig. 7. Characteristics of lineage 5. (A) Ventral view of the projection of a lineage 5 MARCM clone in T1. (B-D) Thick section projections showing the relationship of the neurite bundle from lineage 5 to features of the Neurotactin scaffold (red) in intermediate and ventral neuropils. (B-D) Progressively ventral slices. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

number of fibers in the 6cd bundle in T1. The A1 version of lineage 6 also makes a slightly smaller 6cd bundle, but its 6cm bundle is dramatically reduced (Fig. 8A,D,E) and was missing in some nervous systems. Two neurite bundles are also found in the S3 version of lineage 6, and in this case, the 6cm bundle is also greatly reduced relative to bundle 6cd (data not shown).

Lineage 6 [NB 5-2]

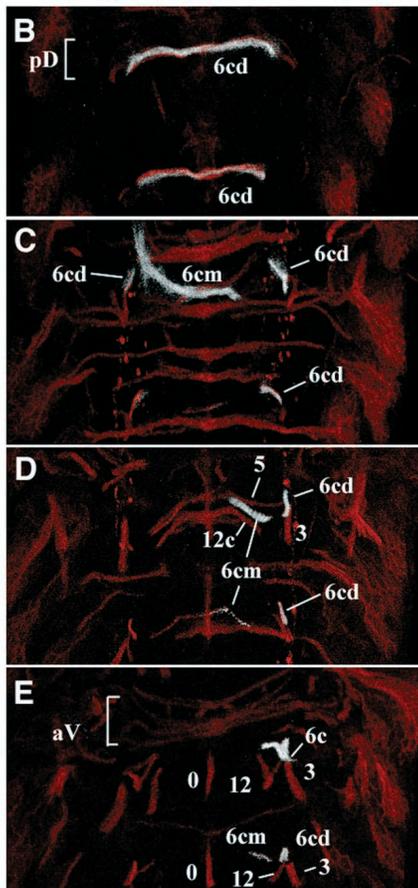
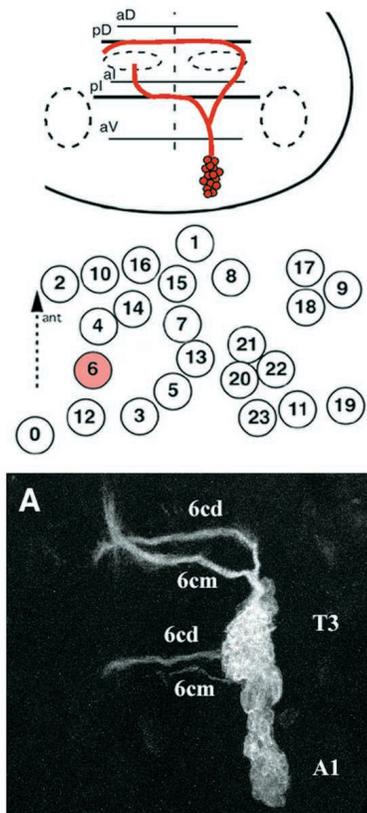
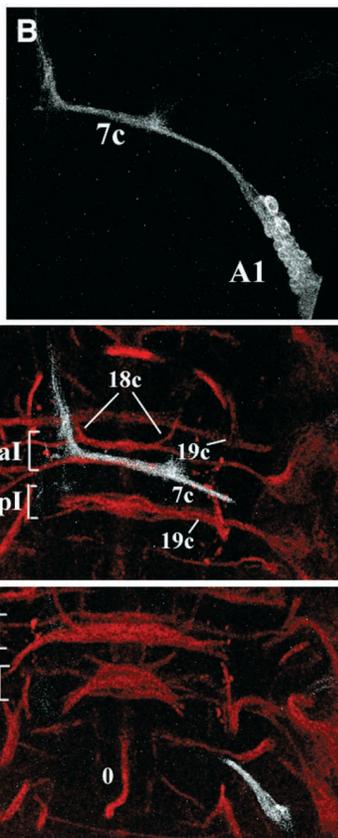
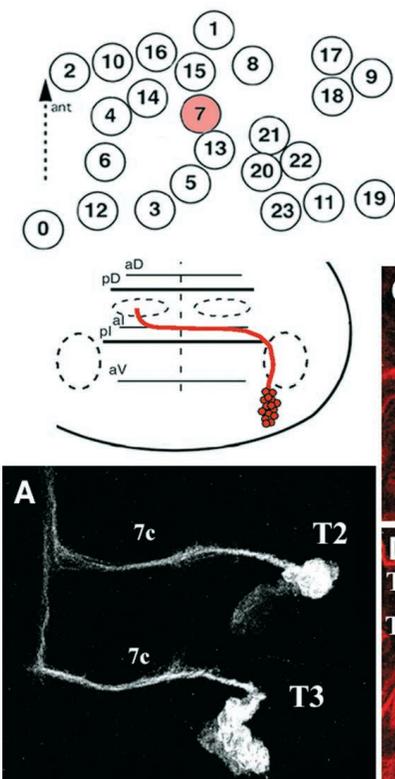


Fig. 8. Characteristics of lineage 6 (NB 5-2).

(A) Ventral view of the projection of lineage 6 MARCM clones in T3 and A1. (B-E) Thick section projections showing the relationship of the neurite bundles from lineage 6 to features of the Neurotactin scaffold (red). The relationship of the bundles to structures in the (B) dorsal, (C,D) intermediate and (E) ventral neuropils. In the thorax, bundle 6cm forms the middle bundle of the posterior ventral arch and then projects anteriorly in a dorsal tract; bundle 6cd forms the pD commissure. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

Lineage 7 [NB 3-3]



Lineage 7

Lineage 7 is a ventrolateral cluster in the anterior half of the hemineuromere and is surrounded by clusters 13, 14 and 15. It is found in segments T1 to A1 (Fig. 9). The projection pattern of the neurons in lineage 7 is identical to the interneurons that are produced by NB 3-3 during embryogenesis (Schmid et al., 1999), and we have ascribed this lineage to that NB. Fig. 9B-D shows an example of lineage 7 from A1 but the same projection pattern is seen in the thoracic neuromeres (Fig. 9A). The cluster produces a single neurite bundle (7c) that extends across the midline as a bundle of the aI commissure (Fig. 9C). In thoracic neuromeres, the bundle from lineages 7 and 8 make up the portion of the aI commissure located posterior to the ascending 2i bundles (e.g. Fig. 1F, Fig. 10B). Both lineage 2 and lineage 8 are absent from A1 so the only components of the aI commissure that remain are the bundles from lineages 7 and 18 (Fig. 9C). After crossing the midline, bundle 7c extends anteriorly in a dorsal tract. We did not recover a clone of lineage 7 in segment T1, but Neurotactin stained nervous systems show that lineage 7 is present in T1 and also projects towards more anterior segments. We could not tell with certainty whether lineage 7 is present in S3.

Lineage 8

Lineage 8 is a ventrolateral cluster situated just anterior to the lineage 15 cluster in the anterior half of the hemineuromere (Fig. 11). It is present only in segments T1 through T3. The neurite bundle leaving the cell cluster immediately splits into two bundles (8c and 8i) both of which extend

Fig. 9. Characteristics of lineage 7 (NB 3-3).

(A) Ventral view of the projection of lineage 7 MARCM clones from the T2 and T3 neuromeres. (B) Lineage 7 clone from A1. (C,D) Thick section projections showing the relationship of the neurite bundle from lineage 7 to features of the Neurotactin scaffold (red) in the (C) intermediate and (D) ventral neuropils. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

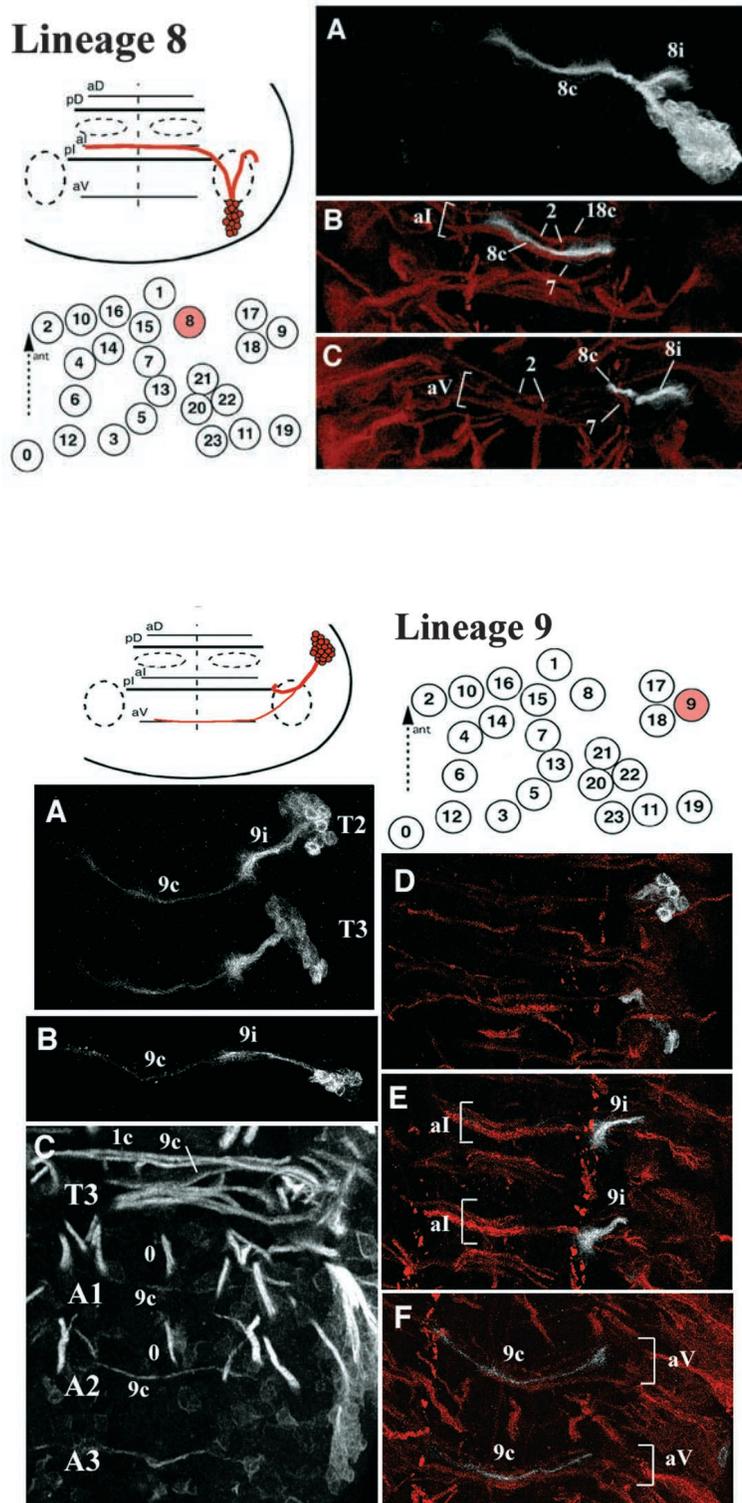


Fig. 11. Characteristics of lineage 9. (A) Ventral view of the projection of MARCM clones of lineage 9 in the T2 and T3 neuromeres. (B) Lineage 9 clone from A1. (C) A thick section projection of confocal slices showing the ventral commissures in T3-A3. In T3, the bundle is the thinnest of the four paired bundles that constitute the aV commissure; it is the only aV bundle that is found in the abdominal segments. (D-F) Thick section projections showing the relationship of the neurite bundles from lineage 9 to features of the Neurotactin scaffold (red) in intermediate (D,E) and ventral (F) neuropils. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

Fig. 10. Characteristics of lineage 8. (A) Ventral view of the projection of a lineage 8 clone. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 8 to features of the Neurotactin scaffold (red) in (B) intermediate and (C) ventral neuropils. Bundle 8c is one of the 3 paired bundles that constitute the aI commissure; bundle 8i projects to the most dorsal regions of the ventrolateral neuropil. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

dorsally through the lateral cylinder (Fig. 10A-C). Bundle 8c exits the cylinder dorsal to bundle 9i, extends across the midline as part of the aI tract just posterior to the ascending 2i bundles (Fig. 10B), and terminates shortly after crossing the midline. The ipsilateral bundle 8i extends dorsally to about the level of the intermediate commissure and then bends lateroventrally into the dorsal region of the ventrolateral neuropil, just anterior to the lateral cylinder (Fig. 10C). The bundle also contains a single peripheral axon that continues out of the CNS. The projections are similar in all three thoracic neuromeres.

Lineage 9

This lineage is typically the most dorsal cluster in the anterior half of the hemineuromere. The neurites from lineage 9 cluster to form a robust ipsilateral (bundle 9i) and a sparse contralateral projection (9c) (Fig. 11A). The neurites in the 9i bundle extend ventrally to just below the intermediate commissure where they then curve posteriorly to form the dorsomedial boundary of the lateral cylinder (Fig. 11C,G; Fig. 11E). The thin 9c bundle projects down to the level of the ventral commissure where it enters the commissure at an anterior level with the 1c bundle but then crosses over to the more posterior commissural bundles (13c and 14c) prior to reaching the midline (Fig. 11F). The bundle then ends prior to reaching the lateral neuropil.

In T1 lineage 9 has an additional, thin contralateral bundle that extends across the aI commissure anterior to the lineage 2 bundles (not shown). This second contralateral projection was seen in 4 of 6 T1 lineage 9 clones but was absent from the 11 clones recovered from segments T2 and T3. We have not managed to identify this lineage in the subesophageal neuromeres. The size of lineage 9 is greatly reduced in A1 (Fig. 11B) with a very small 9i bundle. The 9c bundle is also reduced to only a few neurites and is the last vestige of the ventral commissure bundles persisting in the abdomen. Lineage 9 is also found in the abdominal neuromeres posterior to A1 (Truman and Bate, 1988). Lineage 9 is the largest one and the only one of the three that we could identify with certainty.

Lineage 10

The cell cluster for lineage 10 is positioned just lateral to lineage 2 along the anterior border of the hemineuromere (Fig. 11). A single neurite bundle (10c)

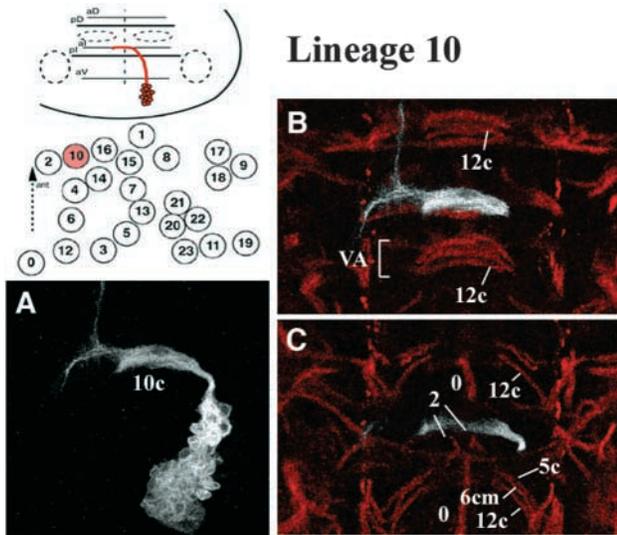


Fig. 12. Characteristics of lineage 10. (A) Ventral view of the projection of a lineage 10 clone. (B,C) Thick section projections showing the relationship of the neurite bundle from lineage 10 to features of the Neurotactin scaffold (red) in (B) intermediate and (C) ventral neuropils. Bundle 10c is the only bundle of the anterior ventral arch. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1; VA, ventral arch.

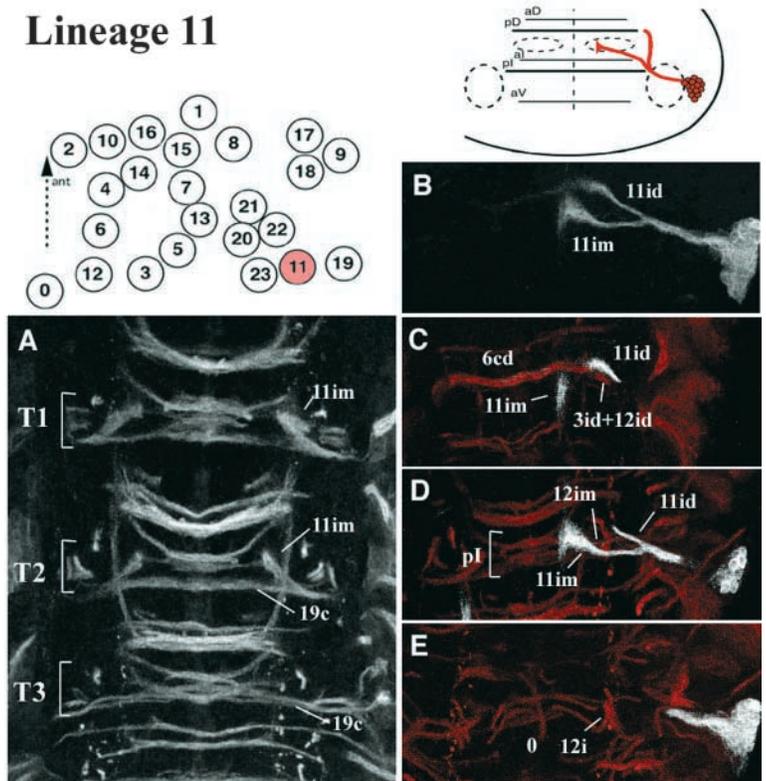
extends dorsally from the cluster, forming the anterior ventral arch, and then widens out towards the midline to form the floor of the al commissure (Fig. 12A-C). Most neurites terminate soon after crossing the midline but a few extend laterally and then turn either anteriorly or posteriorly through an intermediate level of the neuropil. We obtained only one example of a lineage 10 clone, which was in segment T2 (Fig. 12B). The similarity of the Neurotactin projection for this lineage in all of the thoracic segments, however, gives us confidence that this projection pattern is also seen in segments T1 and T3. Lineage 10 is not found outside of the thorax.

Lineage 11

Lineage 11 is an Engrailed-positive cluster situated in the lateral region of the hemineurone. Fig. 13B-E shows an example of lineage 11 in T2. A single neurite bundle projects dorsomedially from the neuronal cluster and runs beside bundle 19c towards the intermediate commissure. Bundle 11 splits before reaching the commissure, with one branch (11im)

Fig. 13. Characteristics of lineage 11. (A) A thick section projection of confocal slices showing a ventral view of the Neurotactin scaffold at the level of the intermediate commissures; brackets identify the pI commissures. The stubby projection of neurite bundle 11im is present in T1 and T2 but absent from T3. (B) Ventral view of the projection a MARCM clone of lineage 11. (C-E) Thick section projections showing the relationship of the neurite bundles from lineage 11 to features of the Neurotactin scaffold (red) in (C) dorsal and (D,E) intermediate neuropils. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

Lineage 11



extending dorsomedially and ending well short of the midline in a spray of arbor oriented along the anteroposterior axis (Fig. 14C,D). The other bundle (11id) extends dorsally and ends near the termination of bundle 3id at the point where bundle 6cd bends medially to form the pD commissure (Fig. 13C). A small wisp of arbor occasionally extends into the pD commissure.

Clones of lineage 11 were found in T2 and T1 but not in T3. Examination of the Neurotactin-stained scaffold at the level of the intermediate commissure shows the truncated anteromedial projections of bundle 11im in both T1 and T2 but this projection is absent from T3 (Fig. 13A). T3 also lacks the Neurotactin bundle that corresponds to bundle 11id. In addition, T3 is has one less Engrailed-positive cluster when compared with T1 and T2 (data not shown), and the missing cluster is at the location of lineage 11. Consequently, we have concluded that lineage 11 is truly missing from segment T3, rather than being altered into producing neurons with different projection patterns.

Lineage 12

This Engrailed positive lineage is just lateral to the median lineage (Fig. 11) and is found in S3-A1. Actin-GAL4 based MARCM clones show that this lineage has no motoneurons associated with it and the larval interneurons neurons have projections patterns that match the embryonic progeny of NB 6-1. The most complex projection pattern is seen for the lineage 12 clusters in T1 and T2 (Fig. 14B-F). The neurite bundle projects dorsally from the cluster and separates into contralateral (12c) and ipsilateral (12i) bundles (Fig. 14F). Bundle 12c is the most posterior bundle of the posterior ventral arch (which also contains bundles 6cm and 5c), and, after crossing the midline in the pI commissure, the bundle dips

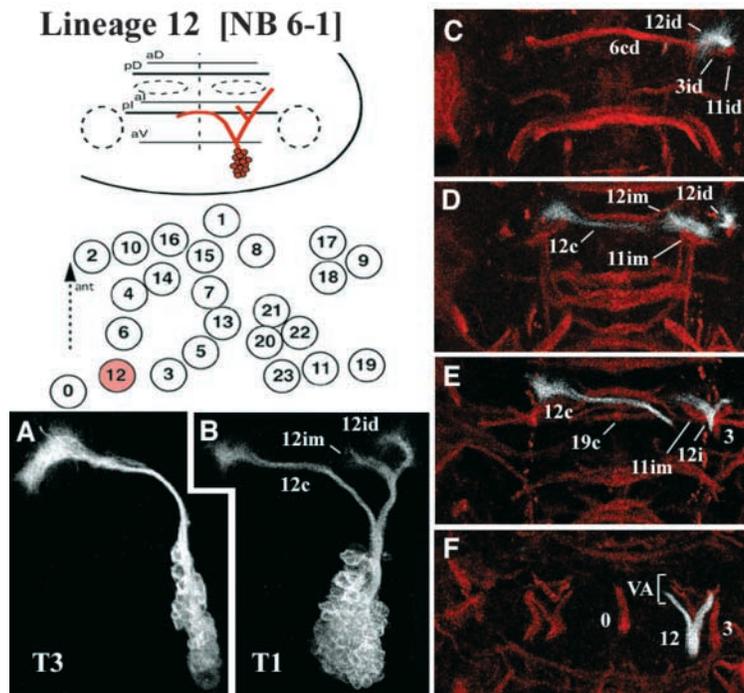


Fig. 14. Characteristics of lineage 12 (NB 6-1). (A) Ventral view of the projection of a MARCM clone of lineage 12 in T3; the 12i bundles are absent. (B) Lineage 12 clone from T1. (C-F) Thick section projections showing the relationship of the neurite bundle from lineage 12 to features of the Neurotactin scaffold (red) in (C) dorsal, (D,E) intermediate and (F) ventral neuropils. Bundle 12c is the posterior bundle of the posterior ventral arch; the 12i bundle bifurcates with sub-bundles going to intermediate (12im) and dorsal (12id) positions. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

slightly ventral and terminates in a compact spray of arbor. As it projects dorsally, the 12i branch further divides into middle (12im) and dorsal (12id) bundles at about the level of the intermediate commissure (Fig. 14E). The 12im bundle runs along side the 11im bundle from lineage 11 and terminates along with this bundle at about the same level of the intermediate neuropil (Fig. 14D). The 12id bundle continues to the dorsal-most region of the neuropil where it ends along with the 3id and 11id bundles (Fig. 14C).

In terms of its segmental morphology, lineage 12 is the most variable of all of the ventral lineages. We have examined 24 lineage 12 clones ranging from S3 to A1. In A1 ($n=2$), the neurons form a very thin 12c bundle and extend a 12i bundle that terminates in mid-neuropil, ventral to the normal bifurcation site. In T3 ($n=6$), half the lineages showed only the 12c bundle (Fig. 14A), whereas the other half also had a 12i bundle but one that terminated in intermediate neuropil as in A1. This lack of the dorsal projections of the 12i bundle is significant because T3 lacks lineage 11, which produces the bundles that the 12i sub-bundles contact in the intermediate and dorsal neuropils. In T2 ($n=7$), four of the clones showed the typical three bundles, but the remaining three had bundle 12c and 12id, which extended into its normal site in the dorsal neuropil but they lacked 12im. In T1, five out of six clones had the three bundles, with the remaining one showing a 12id, but not the 12im bundle. The lineage 12 cluster in S3 ($n=3$) completely lacks the contralateral 12c bundle and retains only bundle 12id.

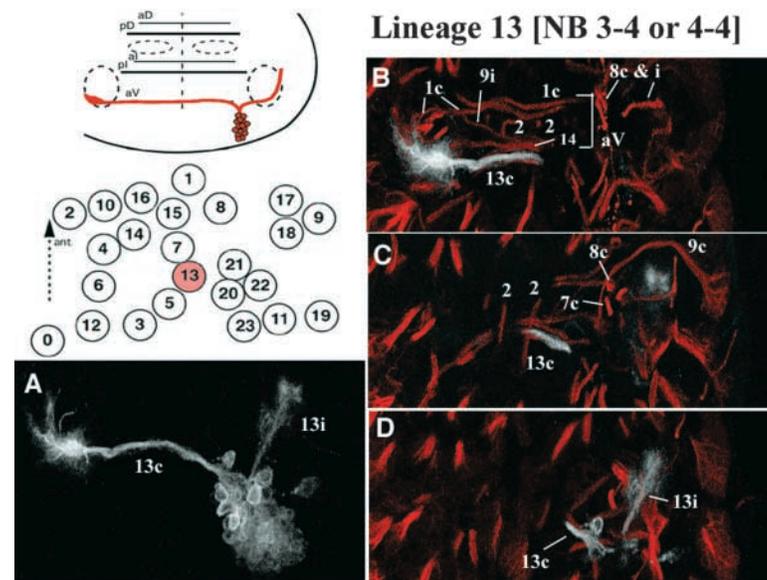


Fig. 15. Characteristics of lineage 13 (NB 3-4 or 4-4). (A) Ventral view of the projection of a MARCM clone of lineage 13. (B-D) Thick section projections showing the relationship of the neurite bundles from lineage 13 to features of the Neurotactin scaffold (red) in ventral neuropil. (B-D) Successively ventral sections. Bundle 13c is one of four paired bundles in the aV tract; bundle 13i projects to the anterior ventrolateral neuropil. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

Lineage 13

The cell cluster for this lineage is situated in the ventrolateral region of the hemineuromere between lineages 7 and 5 (Fig. 11). Larval neurons associated with the adult-specific lineage include an ipsilaterally projecting motoneuron and local interneurons with projections either ipsilaterally or contralaterally through the anterior commissure (data not shown). These are characteristic of either NB 3-4 or NB 4-4 [indistinguishable by Schmid et al. (Schmid et al., 1999)]. The adult-specific cluster produces two neurite bundles (Fig. 15). The contralateral projecting bundle (13c) contributes the most posterior bundle of the ventral commissure. It projects to the ventrolateral neuropil and arborizes around the posterolateral border of the lateral core (Fig. 15B). The 13i bundle projects dorsally and terminates in the dorsal region of the ventrolateral neuropil, just lateral to the lateral cylinder (Fig. 15C). Lineage 13 is found only in the thoracic neuromeres and projection pattern is similar in each.

Lineage 14

The cluster for this lineage is situated just lateral to the lineage 4 cluster. Its single bundle of neurites (14c) contributes to the ventral commissure and is situated anterior to the 13c bundles and immediately posterior to the ascending bundles from lineage 2

(Fig. 16B). The bundle projects into the ventrolateral neuropil and splays out medial to the lateral cylinder in apparent contact with neurites from bundle 9i (Fig. 16B,C). The projection patterns are similar in all three thoracic neuromeres and the cluster is found only in the thoracic neuromeres. Lineage 14 is the most medial cluster to project through the anterior commissure to the ventrolateral neuropil. This fits the anatomy of the cluster of local interneurons that arise from NB 4-1 in the locust (Shepherd and Laurent, 1992). Similar neurons are made by NB 4-1 in *Drosophila* (Schmid et al., 1999).

Lineage 15

Lineage 15 is unique in that it appears to be composed entirely of motoneurons (Fig. 17). The neurites from this cluster of about 30 neurons form a single bundle (15i) that projects dorsally through the lateral cylinder and then bends laterally where the bundle partially defasciculates and produces some short diffuse processes (Fig. 17B) before recollecting together in a compact bundle that leaves the CNS through the nerve leading to the leg imaginal discs (Fig. 17C). In the early 3rd instar lineage 15 has a neuroblast associated with it. By the mid-to-late 3rd instar, however, the NB is no longer evident. This is the only lineage that appears to lose its neuroblast prior to the start of metamorphosis. Lineage 15 is found only in the thoracic neuromeres and the projection pattern is similar in each.

Lineage 16

The cell cluster for lineage 16 is situated along the anterior border of the hemineuromere, just lateral to cluster 10 (Fig. 11). The neurite bundle (16i) projects dorsally from the cell cluster, through the lateral cylinder between bundles 8c and 8i (Fig. 18D), and into the ventrolateral neuropil, where it makes a short, compact projection after it emerges from the cylinder (Fig. 18B,C). Its termination is close to area where lineage 15 showed its partial defasciculation. This lineage is found only in the thoracic neuromeres and has a similar projection in each.

Lineage 17

This lineage is located dorsolaterally along the boundary between neuromeres. It is Engrailed negative, so we have placed it with the anterior lineages. Its neuroblast, along with those from lineages 18 and 9, make up the dorsal lateral group of neuroblasts originally called the triplet (Truman and Bate, 1988). Fig. 19A-C shows lineage 17 in segment A1. The neurite bundle (17i) projects into the ipsilateral ventromedial neuropil, but then loops dorsally and reflects back at the location of the aI commissure. We recovered only one clone of lineage 17 in the thorax (Fig. 19D; T2). It shows the same projection pattern as its abdominal counterpart except that loop is more pronounced (also Fig. 19E). The 17i loops are present in T2, T3, and A1 but missing from T1 (Fig. 19F). We have concluded that this lineage is missing from T1, but in the absence of an independent marker, such as the Engrailed staining we used for lineage 11, we cannot be completely confident that the lineage is absent rather than merely lacking its Neurotactin bundle.

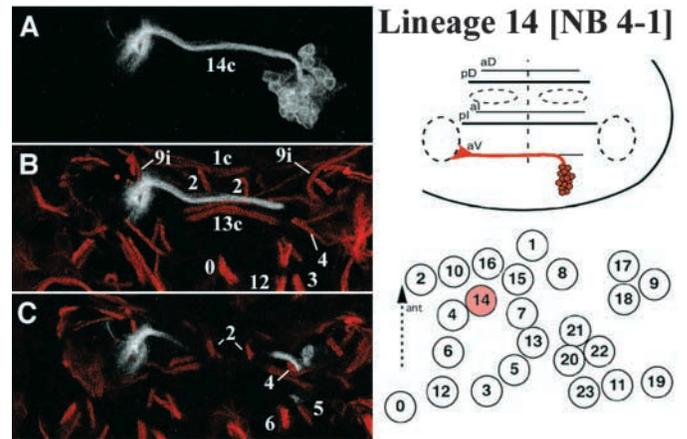


Fig. 16. Characteristics of lineage 14 (NB 4-1). (A) Ventral view of the projection of a MARCM clone of lineage 14. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 14 to features of the Neurotactin scaffold (red) in ventral neuropil. Successively ventral sections. Bundle 14c is one of four paired bundles in the aV tract. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

Lineage 18

The cluster for lineage 18 is in the dorsolateral region of the hemineuromere. The Neurotactin staining clearly showed that there was a lateral lineage whose neurite bundle made up the region of the aI commissure anterior to the 2i bundles (Fig. 20C). After crossing the midline, the bundle turns anteriorly and inserts into a dorsal tract. We found only a single clone of this lineage (Fig. 20A, from T2) in a CNS that was not double-labeled for Neurotactin. Its anatomy and general location in the

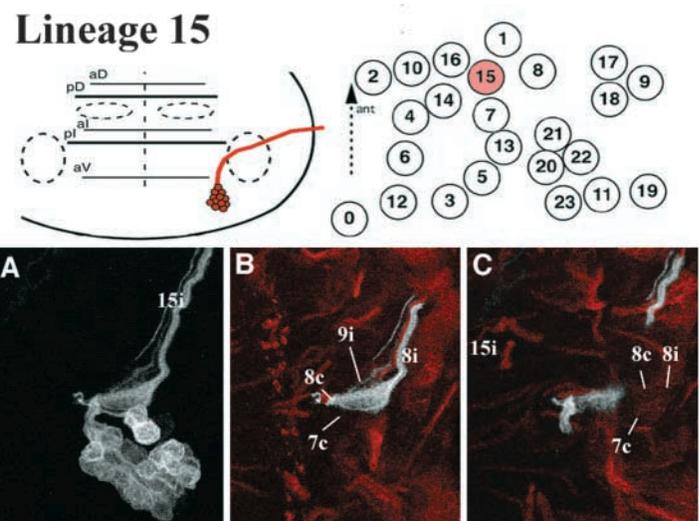


Fig. 17. Characteristics of lineage 15. (A) Ventral view of the projection of a MARCM clone of lineage 15. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 15 to features of the Neurotactin scaffold (red) in (B) intermediate and (C) ventral neuropils. All of the bundle 15 axons project into the periphery. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

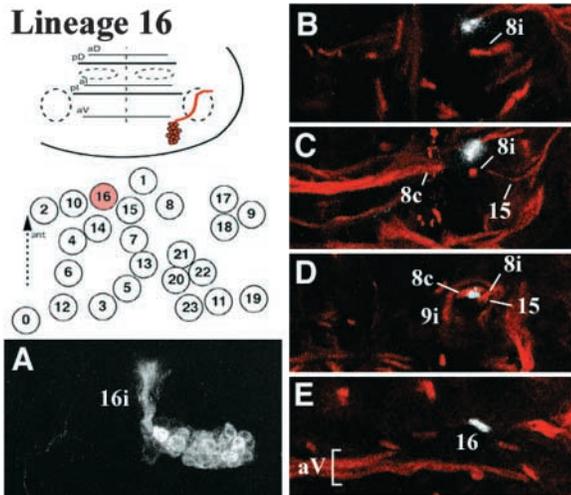


Fig. 18. Characteristics of lineage 16. (A) Ventral view of the projection of a MARCM clone of lineage 16. (B-E) Thick section projections showing the relationship of the neurite bundles from lineage 16 to features of the Neurotactin scaffold (red) in intermediate (B,C) and ventral (D,E) neuropils. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

neuromeres perfectly fit the prediction for this lineage based on the Neurotactin scaffold. The 0m bundle from the median lineage terminates where the neurite bundle (18c) from lineage 18 crosses the midline.

Both horizontal (Fig. 20C) and mid-sagittal (Fig. 20B) sections show that the 18c bundle is missing in T1 but present

in T2 through A1. As with lineage 17, we assume that this cluster is missing from T1 but cannot be entirely confident of its absence without an independent marker.

Lineage 19

The cell cluster for this lineage is situated dorsolaterally at the posterior border of the hemineuromere. From its position and the expression of Engrailed in the lineage we have attributed it to NB 7-4. The cluster gives rise to two neurite bundles (Fig. 21B-E). The contralateral bundle (19c) extends across the midline in the pI commissure and then bends dorsally to extend anteriorly in a dorsal longitudinal tract (Fig. 21C,D). A few neurites can also be seen to extend anteriorly from the bundle at other levels along the pI commissure. The ipsilateral bundle (19i) rapidly splays out into a diffuse projection just lateral of the lateral cylinder (Fig. 21E; bundles 8c, 8i and 7c ascend through the center of the lateral cylinder). Preparations with double clones show that bundle 19i terminates in the near vicinity of the fuzzy arbor from the lineage 15 motoneurons.

Lineage 19 is present in segments T1-A1. The lineage in T3 is identical to that in T2 but in T1 the 19c bundle is reduced to only a few fibers while the 19i bundle retains the pattern seen in more posterior thoracic lineages (Fig. 21A). In A1, by contrast, the 19c bundle is well developed but the 19i bundle is missing (data not shown).

Lineage 20

Lineage 20 is a ventrolateral lineage that includes two motor axons (Fig. 22). Based on its position, the presence of multiple efferents and similarity of larval cells to those described by Schmid et al. (Schmid et al., 1999), we have assigned the cluster to NB 5-4. Its neurite bundle from the adult-specific cluster comes together with that from lineages 21 and 22 to make a short, dorsally projecting tract. A landmark associated with this tract is the ascending 1i bundle from the next posterior segment that curves around it (Fig. 22C). Immediately after passing bundle 1i, the 20i bundle bends anterolaterally and the fibers splay out to fill in the ventral neuropil posterolateral to the lateral cylinder (Fig. 22B,C). Lineage 20 has a similar projection pattern in all thoracic segments. It is missing from both the subesophageal and abdominal neuromeres.

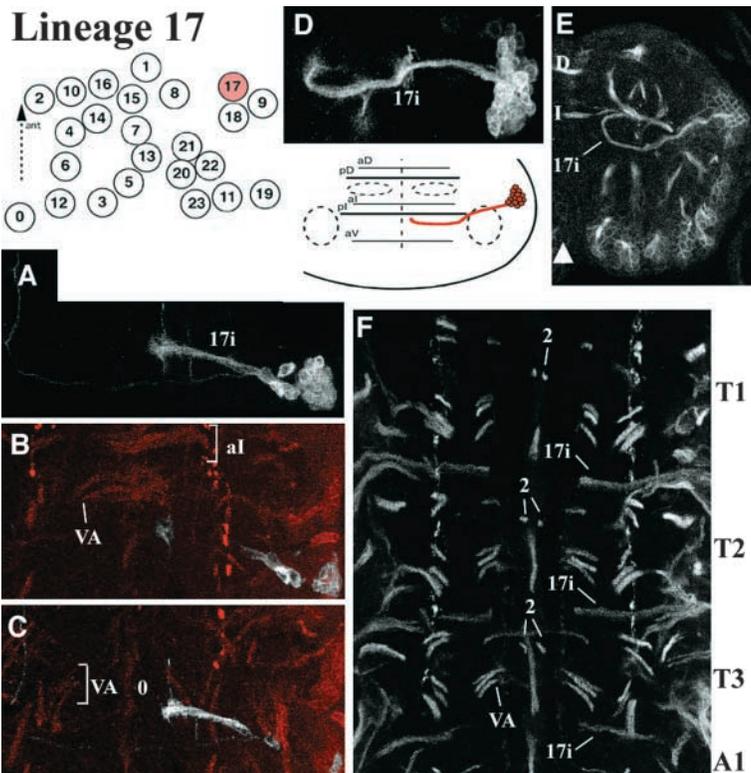
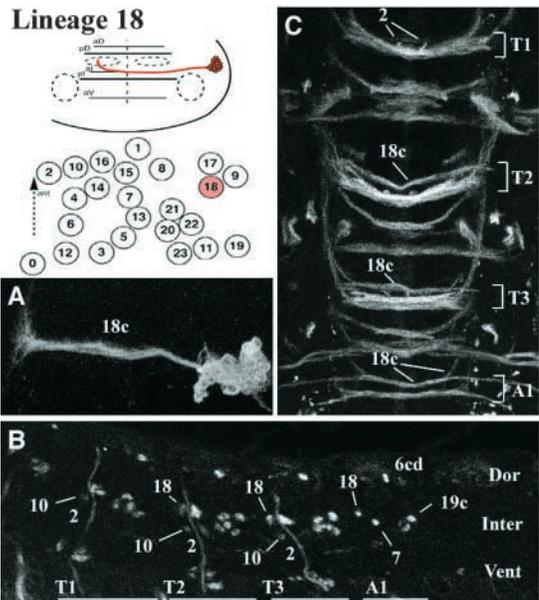


Fig. 19. Characteristics of lineage 17. (A) Ventral view of the projection of a MARCM clone of lineage 17 in A1. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 17 to features of the Neurotactin scaffold (red) in intermediate neuropil (successively ventral sections). (D) Ventral projection of lineage 17 clone in T2. In both A1 and the thoracic neuromeres, the neurite bundle abruptly hooks dorsally before it reaches the midline. (E) A thick transverse projection of the right hemineuropil showing the characteristic recurved bundle from lineage 17. Arrowhead denotes the midline. (F) A ventral view of a thick section of the Neurotactin scaffold showing a bundle 17 in A1, T3 and T2 but missing from T1. Numbers identify neurite bundles from other lineages; VA, ventral arch. Diagrams as in Fig. 2. D, dorsal commissure; I, intermediate commissures.

Fig. 20. Characteristics of lineage 18. (A) Ventral view of the projection of a MARCM clone of lineage 18 in T2. (B) A mid-sagittal section through the dorsal (Dor), intermediate (Inter) and ventral (Vent) commissural bundles in T1-A1. The neurite bundles for lineages 18 and 10 make up the region of the aI commissure immediately anterior to the ascending bundles from lineage 2. Bundle 18 is absent from T1 whereas bundle 10 is absent in A1. (C) A ventral view of a thick section of the Neurotactin scaffold at the level of the intermediate commissures. There is no lineage 18 bundle in the aI commissure in T1, whereas it is present in the corresponding commissure in posterior neuromeres (brackets). Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2.



Lineage 21

The cluster for this lineage is in the ventrolateral region of the hemineuropil just anterior to the clusters for lineages 20 and 22 (Fig. 11). The adult-specific cluster is confined to the thoracic neuromeres and the projection pattern appears identical in all of these segments. As described above, its neurite bundle collects with the bundles from lineages 20 and 22 and projects past bundle 1i (Fig. 23C). The neurites then project anteromedially and terminate just posterior to the lateral cylinder (Fig. 23B,C).

Lineage 22

This cluster is situated between lineages 20 and 21 (Fig. 11). The trajectory of its neurite bundle is very similar to that of lineage 20 and terminates in the same region of the ventrolateral neuropil (Fig. 24B). It is sometimes associated with a single motoneuron (Fig. 24A). It is present only in the thoracic neuromeres.

Lineage 23

This is a small, Engrailed-positive cluster in the ventrolateral region of the neuromeres. The neurons of the cluster extend a single bundle of neurites (23c) dorsally to the level of the pI commissure (Fig. 25B) and insert into the commissure at the posterior border (Fig. 25C) along with the contralateral bundle from lineage 19. The neurites terminate soon after crossing the midline. We have had only two clones that contained this lineage, but the Neurotactin staining suggests that the projection pattern is the same for this clone in all of the segments in which it resides (T1-A1). This was the only lineage that we found that also had a glial cell as part of the clone (Fig. 25).

Interrelationship of the projection patterns of lineages

The bundles of neurites from each of the lineages typically project to one or two primary targets. The various termination points do not correspond to any obvious glial marker and, indeed, we suspect that the targets may be bundles from other lineages. We have numerous examples in which the neurites from one bundle contact (e.g. Fig. 26A) or terminate in close proximity (e.g. Fig. 26B) to the termination sites from other bundles. The pattern of spatial

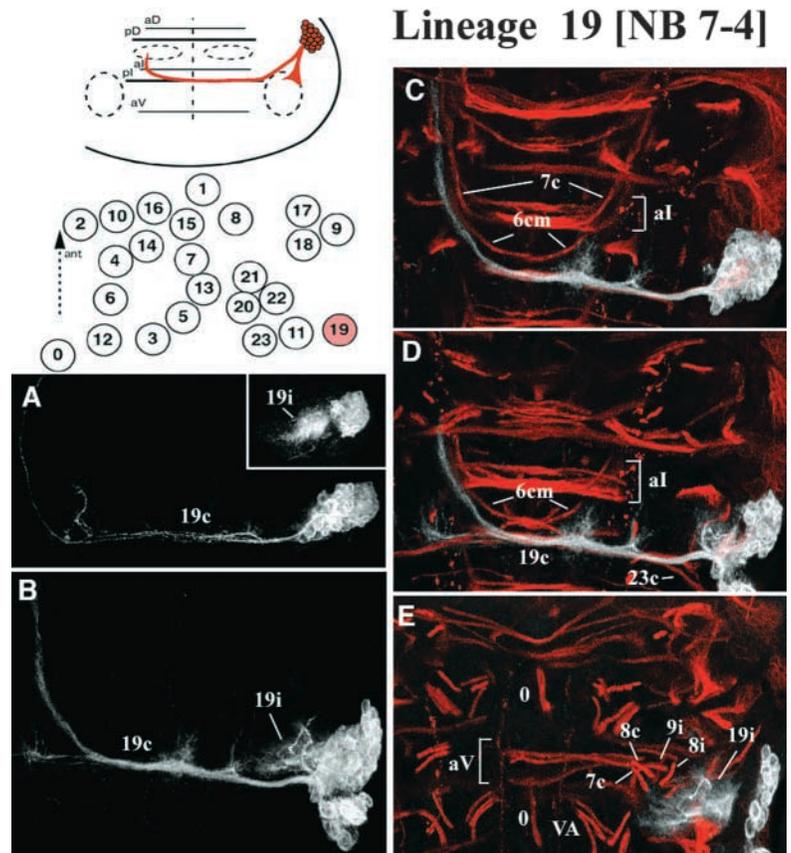


Fig. 21. Characteristics of lineage 19 (NB 7-4). (A) Ventral view of the projection of a lineage 19 MARCM clones from T1; inset is a more ventral slice showing bundle 19i. (B) Lineage 19 from T3 showing the contralateral (19c) and ipsilateral (19i) bundles. (C-E) Thick section projections showing the relationship of the neurite bundles from lineage 19 to features of the Neurotactin scaffold (red) in intermediate (C,D) and ventral (E) neuropils. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

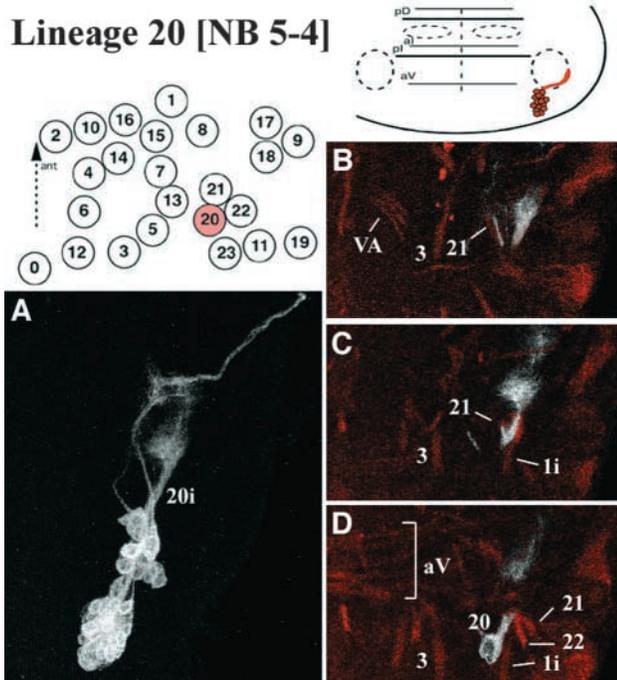


Fig. 22. Characteristics of lineage 20 (NB 5-4). (A) Ventral view of the projection of a MARCM clone of lineage 20. (B-D) Thick section projections showing the relationship of the neurite bundles from lineage 20 to features of the Neurotactin scaffold (red) in intermediate (B) and ventral (C,D) neuropil. Bundle 20 converges with bundles 22 and 21, and projects along the lateral edge of the ventrolateral neuropil. It also contains 2 axons that project to the periphery. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1; VA, ventral arch.

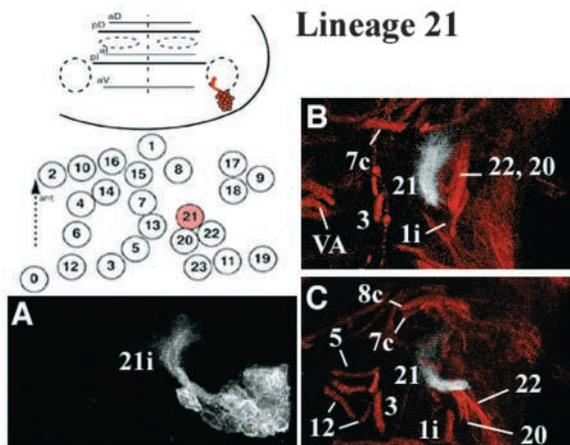


Fig. 23. Characteristics of lineage 21. (A) Ventral view of the projection of a MARCM clone of lineage 21. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 21 to features of the Neurotactin scaffold (red) in ventral neuropil. (B,C) Progressively more ventral regions of bundle 21. Bundle 21 converges with bundles 20 and 22 but arcs medially, when compared with the lateral arc of the other two bundles. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. VA, ventral arch. Commissure abbreviations as in Fig. 1.

overlap of lineage bundles within the T2 neuromere is summarized in Fig. 26C. The segmental variation in these connections is then shown in Fig. 27. This pattern suggests that there are lineage-wide rules for building much of the nervous system. This proposition is considered below.

Discussion

In insect embryos, the vast majority of neurons in each segmental ganglion arise from 30 paired and one unpaired neuroblasts. In basal insect groups, these segmental NBs show a single neurogenic period, each producing all of its progeny during embryogenesis (Shepherd and Bate, 1990; Truman and Ball, 1998). In insects with complete metamorphosis, however, most of the segmental NBs in the thorax have two neurogenic periods, involving a relatively brief phase of neurogenesis during embryonic development followed by a much more prolonged phase during larval life (Booker and Truman, 1987; Truman and Bate, 1988; Prokop and Technau, 1991). Mapping of postembryonic NBs in the thoracic neuromeres of *Drosophila* larvae indicated that 23 out of the 31 segmental NBs showed this second, larval phase of neurogenesis (Truman and Bate, 1988). The count from the present study is that there are 24 such clusters per hemisegment.

The MARCM clones analyzed in this study were induced early in embryogenesis, and should include both the embryonic and postembryonic progeny from a given neuroblast. This, indeed, was seen when Actin-GAL4 or tub-GAL4 was used as a driver to make the MARCM clones (e.g. Fig. 5F). The diversity of morphologies and strength of GFP expression in

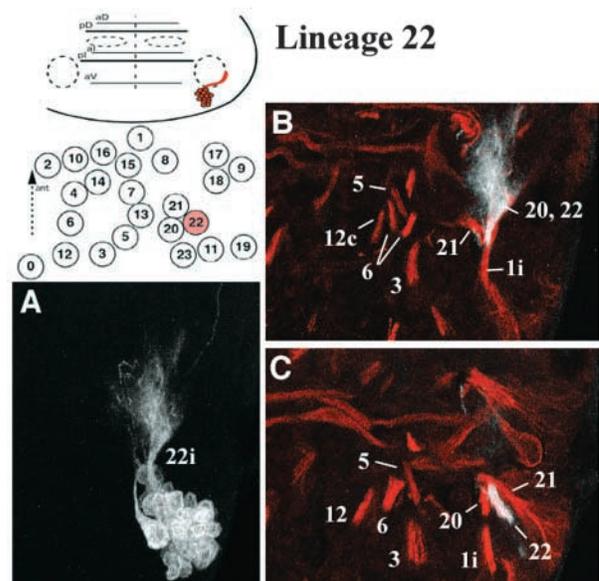


Fig. 24. Characteristics of lineage 22. (A) Ventral view of the projection of a MARCM clone of lineage 22. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 22 to features of the Neurotactin scaffold (red) in ventral neuropil. Bundle 22 converges with bundles 20 and 21, and projects with 20 along the lateral edge of the ventrolateral neuropil. It also contains at least one motor axon. Numbers identify neurite bundles from other lineages. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

the larval neurons, however, sometimes obscured some of the neurites arising from the associated adult-specific cluster. When we generated similar clones using the purported pan-neuronal driver line, *elav* [C155] (Lin and Goodman, 1994), the fully differentiated larval neurons in the clones typically failed to show GFP expression but expression was strong in the arrested, adult-specific cells. Although we do not know the reason that mature larval neurons fail to express under these conditions, *elav*-based clones were invaluable for determining the exact projection patterns of the clusters of adult-specific neurons and how each contributed to the overall Neurotactin scaffold. Having established the morphology of the adult-specific region of the lineage, we could then return to MARCM clones generated using tub-GAL4 and Actin-GAL4 drivers to associate the neurons of adult-specific clusters with their larval siblings. As the larval progeny of all of the embryonic neuroblasts have been described (Bossing et al., 1996b; Schmidt et al., 1997; Schmid et al., 1999), the larval neurons aided us in identifying the embryonic neuroblast responsible for many of the adult-specific clusters.

The early neurons generated by a given NB typically show a great diversity in terms of their type and their axonal projections (e.g. Ishiki et al., 2001; Pearson and Doe, 2003). Indeed, the projection patterns of the daughter cells can change dramatically from one GMC to the next [e.g. for the early neurons of the median neuroblast lineage, see Goodman and Spitzer (Goodman and Spitzer, 1979)]. Later born cells, though, appear to be much more similar in their morphologies, transmitters and functions (Shepherd and Laurent, 1992; Witten and Truman, 1991; Burrows and Siegler, 1982). The present study shows that the similarity in late-born progeny is a general rule for all lineages. Although each NB may show a high degree of diversity in the first few neurons that it produces, the vast majority of their progeny are similar in their pathfinding decisions, with typically only one or two initial targets for the neurites that leave a cluster. Indeed, we find only 33 major projection patterns for the thousands of neurons that are born within a thoracic hemineuromere.

The diversity of phenotypes in the early born cells of a lineage is accomplished through the sequential expression of a series of transcription factors (*hunchback*, *kruppel*, *pdm* and *castor*) that are passed from the NB to successive GMCs (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001). This molecular specification of unique identities imposed by the neuroblast on the first few neurons in a lineage appears to be lacking in the later born neurons, all of which express *grainyhead* (Brody and Odenwald, 2000). We suspect that the transition from uniquely specified GMCs to ones that express the same transcription factor marks the transition from generating unique individual neurons to generating neuronal classes. For the latter cells,

interaction with other neurons, rather than factors supplied by their NB, may then be essential for establishing identity within their neuronal class. It should be noted that the transition between uniquely identified neurons to neuronal classes does not necessarily lie at the dividing line between the embryonic and postembryonic phases of proliferation. By feeding larvae on diet containing bromodeoxyuridine (BUdR) from the time

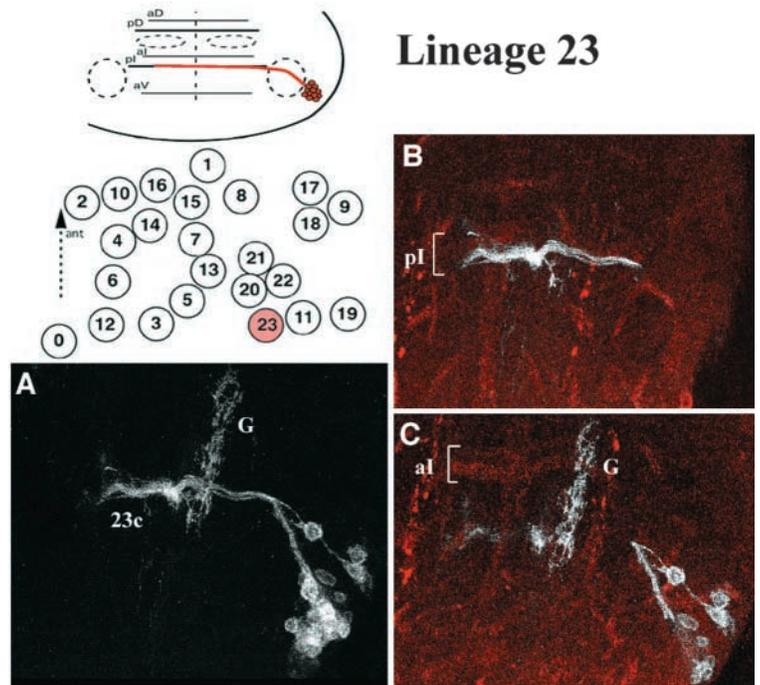


Fig. 25. Characteristics of lineage 23. (A) Ventral view of the projection of a MARCM clone of lineage 23. (B,C) Thick section projections showing the relationship of the neurite bundles from lineage 23 to features of the Neurotactin scaffold (red) in intermediate (B) and ventral (C) neuropils. Bundle 23c converges with bundles 19c (not shown) to form a posterior segment of the pI commissure. The MARCM clone for lineage 23 has a glial cell (G) associated with it. Diagrams as in Fig. 2. Commissure abbreviations as in Fig. 1.

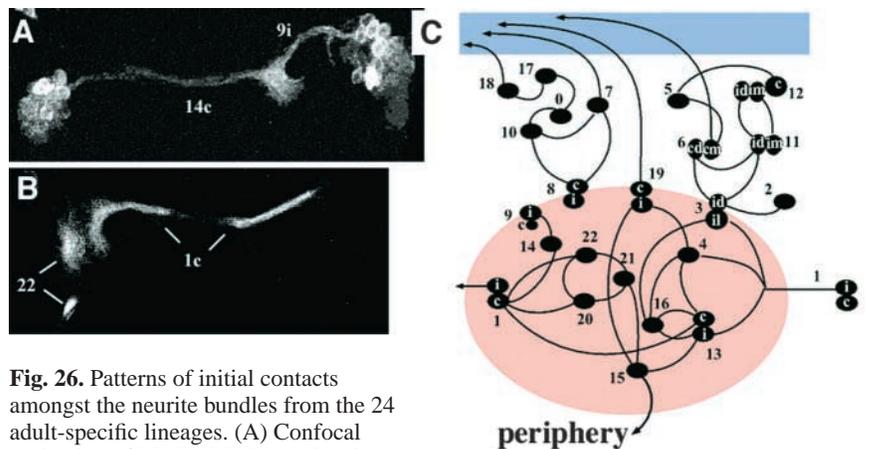


Fig. 26. Patterns of initial contacts amongst the neurite bundles from the 24 adult-specific lineages. (A) Confocal projection of MARCM clones showing contact between the 14c and 9i bundles. (B) Confocal optical section showing the neurites in bundle 1c terminating adjacent to those in bundle 22. (C) Schematic summary of the initial contacts made by the lineages in T2. Bundles within the pink circle terminate in the ventrolateral neuropil, those in the blue stripe project in dorsal tracts.

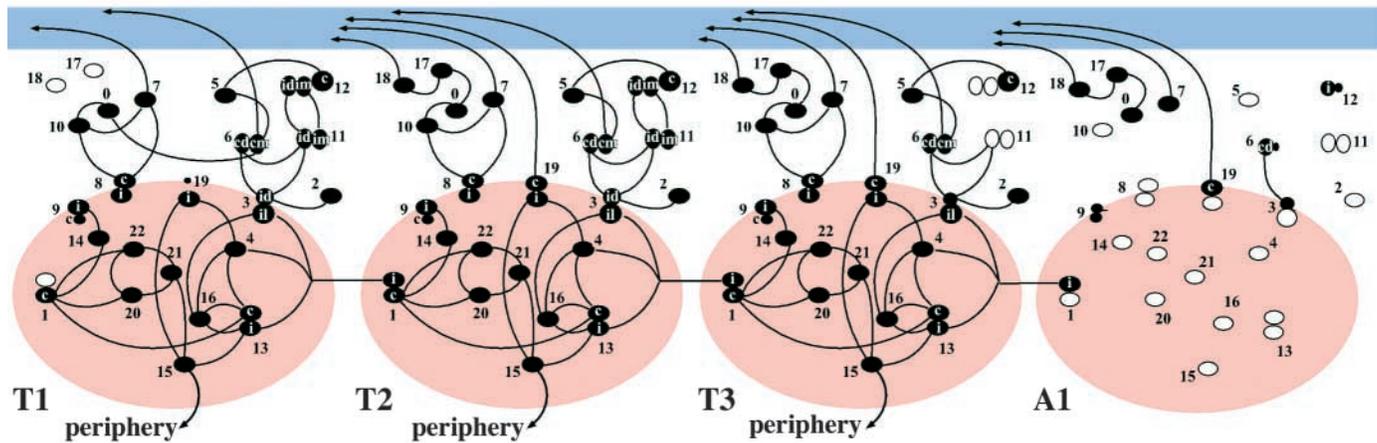


Fig. 27. Segment-specific patterns of initial contacts amongst the neurite bundles from the adult-specific lineages. The full complement of bundles is evident in T2. White ovals indicate which lineages or bundles are missing in other segments. Designations as in Fig. 26.

of hatching, we have labeled all of the neurons that are born during larval growth. Analysis of *Elav*-based MARCM clones in these larvae showed some lineages in which some of the developmentally arrested neurons were unlabeled and, hence, were born prior to hatching. These were always the neurons in the clone that were nearest the neuropil (i.e., the oldest cells) (J.W.T. and D.W.W., unpublished). Hence, the NBs do not necessarily stop dividing after they make the neurons that will be used in the larva, and they may depend on an extrinsic signal to terminate their embryonic phase of neurogenesis. These embryonically born cells may serve as pioneers to guide the growth of postembryonic members of their lineage.

An interesting feature of the adult-specific neurons is that each extends an initial neurite to a lineage-specific location but then their development stalls until pupariation. As illustrated in the developing hippocampus (Bagri et al., 2003), a developing neuron often sends out a single, unbranched process with a growth cone to navigate to an initial target, followed by interstitial sprouting then enables interactions with secondary targets. Contact with the initial target may persist or it may be lost through stereotyped pruning but connections with final targets are often then refined through local cell-cell interactions. In the adult-specific neurons in *Drosophila*, the period of developmental arrest separates axon pathfinding and contact with the initial target from the phase of interstitial sprouting to secondary targets. This arrest is terminated at the start of metamorphosis, when the neurons show a profuse sprouting, accompanied by the appearance of the *broad-Z3* transcription factor (B. Zhou, D.W.W., J.W.T. and L. M. Riddiford, unpublished), and the onset of nitric oxide (NO) sensitivity (S. Gibbs, D. Currie and J.W.T., unpublished). The latter observation is especially interesting because studies on other insect neurons show that the onset of NO sensitivity occurs as a neuron shifts from pathfinding to interacting with its synaptic targets (Truman et al., 1996; Ball and Truman, 1998; Gibbs and Truman, 1998). The appearance of NO sensitivity at the termination of arrest suggests that the neurons have switched into a new developmental mode in which interactions with future synaptic partners become of prime importance.

Hence, the larval CNS just prior to metamorphosis gives us an unprecedented snap-shot of neuronal development.

Thousands of neurons are arrested at their initial targets awaiting the hormonal signals that will initiate secondary sprouting. This probably represents a watershed in the development of the CNS. Up to this point in development, the identity of the neurons and their growth decisions may have been relatively 'hard-wired' by genetic information supplied by the NB and the ganglion mother cell. After this point, interactions with their primary and secondary targets probably dominate in shaping the final phenotypes of the cells.

Segmental variation and its functional implications

The map of initial contacts depicted in Fig. 26C is undoubtedly not a complete description of all of these contacts. In addition, at this time we cannot know the polarity of the contacts, i.e. who will be presynaptic and who will be postsynaptic. Nevertheless, this map probably provides a broad overview of the first step in establishing the connectivity for the bulk of the thoracic neurons. These initial contacts acquire some functional importance when we consider the segmental variation in their pattern (Fig. 27). The patterns in neuromeres T1, T3 and A1 are compared with the situation in T2, as this is the only segment that possesses the full complement of 24 postembryonic lineages. Importantly, many of the segmental changes involve coordinated changes in the lineages that project to the same region of the neuropil. The most obvious example involves the lineages associated with the ventrolateral neuropil. These include the motor lineage (lineage 15) that makes exclusively motoneurons and projects to a leg imaginal disc. Lineage 15 is confined to the thoracic neuromeres as are nine other lineages that send their neurite bundles exclusively to the ventrolateral neuropil. With one exception, these lineages show no obvious variation in their projection patterns between the three thoracic neuromeres. The only lineage that shows a variable projection pattern is lineage 1, which also has initial targets in two adjacent neuromeres. Accordingly this lineage retains its homosegmental projection (bundle 1c) in T1 but it lacks the 1i bundle (i.e. no bundle projects to the SEG). All of the lineages that project to the ventrolateral neuropil are absent from A1, with again the exception of lineage 1. The lineage 1 neurons arising in A1, though, all project to the T3 neuropil (via bundle 1i) and the homosegmental 1c bundle is missing. Our identification of the lineages in the subesophageal

neuromeres is not complete but it appears that most, if not all, of these lineages are also lacking from the SEG. Apart from lineages that project exclusively to the ventrolateral neuropil, there are a few lineages, like lineages 3 and 19 that have one bundle projecting to this neuropil and another projecting into more dorsal regions. This is especially interesting in the case of lineage 19 because its 19i bundle makes contact with the expanded area of the lineage 15 bundle and therefore may represent premotor interneurons. These ventrolateral projections, though, are missing in the A1 version of lineages 3 and 19 (see Fig. 5C for lineage 3). The uniformity of projection patterns within the thorax and their absence outside of this region of the body suggests that all of the lineages that project to ventrolateral neuropil make neurons involved with the sensory or motor requirements of the legs. This functional interpretation is supported by the fact that lineage 14 is one of the above lineages and its proposed homologues in grasshoppers (from NB 4-1) process input from leg mechanosensory hairs (Shepherd and Laurent, 1992) and integrate locomotor reflexes of the leg (reviewed by Burrows and Newland, 1997).

Although the ventrolateral projections are relatively stable within the thoracic neuromeres, projections to intermediate and dorsal neuropils show striking segmental variation. For example, lineage 11 is absent from T3 and the two lineages that send neurite bundles that terminate next to those of lineage 11 in more anterior segments, have these bundles reduced (bundle 3id in Fig. 5) or missing altogether (the 12im and 12id bundles of lineage 12, Fig. 14) in this segment (Fig. 27). T1 also has its unique set of changes. In T2 and posterior, the 0 bundle from the median NB projects to the aI commissure and appears to terminate between bundle 10c (ventral to it) and bundle 18c (dorsal). The 18c bundle is missing in T1 and we see that bundle 0 is redirected to the pI commissure (Fig. 2). T1 also shows a marked reduction in the number of bundles that project to anterior neuromeres; bundle 18c is missing and bundle 19c is greatly reduced to only a few fibers. Thus, the neurons in the 18c and 19c bundles may be involved in coordination within the thorax rather than taking information to higher centers in the head. We do not find obvious glial structures at the sites where the neurite bundles terminate. The correlated loss of converging bundles (such as seen for 12id, 3id and 11id in T3), suggest that the initial targets for the neurites in a bundle from one lineage may be bundles from other lineages. The map in Fig. 26C is the first attempt to identify the lineage-level rules that are used for establishing the initial connectivity map in the thoracic CNS. Whether these initial contacts are maintained and how they relate to secondary targets remains to be determined.

Our preliminary observations of embryonic induced single and double cell clones in lineage 6 show that in single neuron clones there is a single neurite that is either in the 6cm or 6cd bundle. By contrast, two neuron clones (arising from a GMC) show a neurite in both bundles. This suggests that the two bundles are built up by each GMC producing two daughters, one that chooses one pathway and one that chooses the other. While it obviously needs to be tested, we expect that this pattern will hold for all of the lineages that have bundles projecting to two initial targets. Interestingly, in the cases in which one bundle is lost in a given segment (1i in T1, 12id and im, and 3id in T3; and 19i and 1c in A1) the cell cluster in that

segment is markedly smaller than in other segments. A possible mechanism to explain the segmental difference is that cell death shapes the projection pattern by having the inappropriate daughter cell die after its birth. Studies of the median lineage in grasshopper embryos show the importance of divergent sibling fates and cell death in shaping features of that lineage (Thompson and Siegler, 1993; Jia and Siegler, 2002).

Conclusions

The results from this study have developmental, behavioral and evolutionary implications. Previous studies on the ventral ganglia (e.g. Broadus and Doe, 1995) and the brain (Urbach and Technau, 2003) show that the neuroblasts express a striking diversity of transcription factors and signaling molecules. Some of these molecules are involved in the establishment of the unique identity of the neuroblasts (e.g. Bhat, 1996) and their early-born progeny (Isshiki et al., 2001). Others, though, may function later in directing patterns of connectivity (e.g. Bossing et al., 1996a). It has been difficult to determine the latter, however, because projection patterns and potential targets were unknown for the vast majority of neurons in the lineage. Our study indicates that the first step in establishing the extreme complexity of CNS connections involves a rather simple set of rules, with the bulk of the neurons of a given lineage following one or two projection paths. At this time, we do not know if the 33 different projection trajectories that we see in the thoracic neuropil are the product of just 33 individual neurons per hemineuromere that pioneer the track for the rest of their lineage or if all of the adult-specific neurons follow the same set of cues to their initial targets. Irrespective of how they navigate their path, the initial connectivity patterns (Fig. 26C) suggest that neurons in one lineage use other lineages as their targets. This information should help us understand the roles of patterning genes such as *wingless* and *hedgehog* in establishing connectivity and neuronal properties within the CNS.

The elegant studies of the neural circuitry underlying sensory to motor coordination in the legs of grasshoppers (reviewed by Burrows and Newland, 1997) showed that functionally related neurons were clustered, and some, indeed are siblings that come from the same neuroblast (Shepherd and Laurent, 1992). The uniformity of initial projections that we see within each of the adult-specific lineages leads us to speculate that each neuroblast is devoted to making a very small number of functional neuronal types, with the noted exceptions of the early-born cells that have unique identities sculpted by the expression of *hunchback*, *kruppel*, etc. Changes in specific behavioral functions between species might then be reflected in selective alterations in the particular lineages whose neurons participated in that behavior. One possible illustration of this is in the shift from primitively wingless insects to those that can fly was accompanied with marked increase in neuronal progeny in only 14 out of the 31 thoracic lineages (Truman and Ball, 1998). Indeed, the later born neurons in some subsets of lineages may co-evolve because these cells are functionally connected. Although there has been only minor differences in the neuroblast arrays when one compares grasshoppers to *Drosophila* (Broadus and Doe, 1995), some of the neuroblasts have changed the blend of transcription factors that they express. It will be interesting to determine if these changes do indeed reflect a change in

identity of the neuroblast or whether it reflects an alteration in instructions as to how these neurons should connect.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/131/20/5167/DC1>

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