Development 129, 5181-5193 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV7996

Midline lineages in grasshopper produce neuronal siblings with asymmetric expression of Engrailed

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Accepted 6 August 2002

SUMMARY

The median neuroblast lineage of grasshopper has provided a model for the development of differing neuronal types within the insect central nervous system. According to the prevailing model, neurons of different types are produced in sequence. Contrary to this, we show that each ganglion mother cell from the median neuroblast produces two neurons of asymmetric type: one is Engrailed positive (of interneuronal fate); and one is Engrailed negative (of efferent fate). The mature neuronal population, however, results from differential neuronal death. This yields many interneurons and relatively few efferent neurons. Also contrary to previous reports, we find no evidence for glial production by the median neuroblast. We discuss evidence that neuronal lineages typically produce asymmetric progeny, an outcome that has important developmental and evolutionary implications.

Key words: Engrailed, MP, MNB, Lineage, Cell death, Asymmetry

INTRODUCTION

Homeodomain proteins function in the development and maintenance of neuronal phenotype, in addition to their many other roles in the determination of body pattern. In adult grasshopper, the homeodomain protein Engrailed (En) is expressed in the interneurons but not the efferent neurons within the dorsal unpaired median (DUM) groups of neurons, which arise embryonically from the median neuroblasts (MNBs) (Siegler and Pankhaniya, 1997). The interneurons and the efferents of the DUM group have somata in one tight cluster, but their primary neurites and axonal branches follow very different pathways within the CNS (Thompson and Siegler, 1991; Thompson and Siegler, 1993). Together, these observations prompted our hypothesis that En regulated aspects of neuronal pathfinding and neuronal morphology. Indeed, in *Drosophila* embryos, the En-positive CNS neurons are interneurons, whereas efferent neurons are En negative (Siegler and Jia, 1999). Moreover, genetic manipulation of *engrailed* expression altered the expression of neuronal cell adhesion molecules, and at the same time resulted in profound disruptions of normal neuronal morphology in the CNS. Studies in cockroach also implicate En in pathfinding choices made by sensory neurons (Marie et al., 2000).

The MNB lineage of grasshopper has provided a long-standing model for the development of large NB lineages containing differing neuronal types within the insect CNS. Perhaps more so than within any other NB lineage, individual neurons and small neuronal groups of like type have been broadly investigated, not only from a developmental perspective, but also in morphological, biochemical and behavioral studies of the adult. The present study focuses on the mechanisms by which the MNB gives rise to different neuronal types across the lineage.

The prevailing lineage model is a ‘sequential’ one, whereby the ganglion mother cell (GMC) divisions produce sibling pairs first of efferent neurons, then pairs of interneurons, either local interneurons or intersegmental interneurons (Goodman et al., 1980) (for reviews, see Burrows, 1996; Truman et al., 1993). The morphology of neurons in the MNB (or DUM) groups during embryogenesis and in adulthood is consistent with this model (Thompson and Siegler, 1991; Thompson and Siegler, 1993). However, En-positive neurons are produced from the MNB early in the lineage (Condron and Zinn, 1994; Condron et al., 1994). Engrailed expression is specific to interneurons (Siegler and Pankhaniya, 1997), and we were thus led to question the validity of the sequential model. We traced MNB and midline precursor (MP) lineages across embryogenesis in grasshopper, with the aim of discovering the order in which neurons of different types were produced, and what role En might have in this process.

Our results confirm some earlier findings, but at the same time lead to a significant revision in understanding of the MNB lineage and its progeny. We show that, as in *Drosophila*, the MNB in grasshopper does not produce midline glia, contrary to previous reports (Condron and Zinn, 1994; Condron et al., 1994). More importantly, we show that the MNB lineage is asymmetric in its production of neuronal progeny. Throughout the lineage, each GMC generates two nonidentical siblings: one En-positive and one En-negative neuron. A proportion of each neuronal type dies during the course of embryogenesis, but death is most pronounced among the En-negative efferent

neurons. Thus, differential cell death, rather than a timed lineage sequence that produces different neuronal types, accounts for the significantly smaller number of efferent neurons versus interneurons in the final neuronal populations.

We argue that the continued production of neurons of two essentially different types within a lineage, followed by selective death of some neurons, is normal across insect neuronal lineages. This provides a flexible and responsive mechanism whereby neuronal populations can be tailored to match segmental diversity across the insect body plan, without reconfiguring individual neuroblast lineages. A similar mechanism would likewise provide a ready means of matching neuronal populations in the CNS with changes in peripheral body form across the course of insect evolution, while not altering the central mechanism of neuronal production. This view is concordant with numerous findings across a diversity of organisms, where divisions of precursors yield progeny of asymmetric type, followed by selective cell death.

MATERIALS AND METHODS

Animals

Grasshoppers *Schistocerca americana* were reared in a laboratory colony. Females lay eggs in clutches, each containing 100-200 eggs. Eggs developed at 33°C, embryonic development thus taking 20 days (100%) and the basis for 'percent' staging of embryos (Bentley et al., 1979). The percentage scale is referenced to the morphology of the third thoracic (T3) segment. Embryos nearby in a clutch develop in synchrony. For bromodeoxyuridine (BrdU) injections, several eggs of a clutch were dissected for staging, and nearby undissected eggs were used for injection. Embryos were injected in ovo with 1 μl of 10 mM BrdU and allowed to develop a further 5%-15%. For routine immunohistochemistry or after BrdU injection and incubation, embryos were removed from the egg, staged, dissected in saline (Siegler and Pankhaniya, 1997) and fixed in 3.6% paraformaldehyde containing 35.5 mM sucrose.

Antibodies

Primary antibodies were obtained from the following sources: anti-En/Inv (MAb 4D9) and anti-BrdU (MAb G3G4) from the Developmental Studies Hybridoma Bank; MAb 8B7 (unidentified cytoplasmic epitope) and anti-Lachesin from M. Bastiani (U. Utah); and anti-Annulin (MAb 7H7) from D. Bentley (UC Berkeley). Commercial antibodies were: anti-BrdU (CalTag); rabbit anti-HRP-fluorescein isothiocyanate (FITC) #323-095-021 or lissamine rhodamine sulfonyl chloride (LRSC) #123-085-021; goat anti-mouse-indodicarbocyanine (Cy5) #115-175-003 or FITC #115-095-003; and goat anti rabbit-FITC #111-095-003 (Jackson ImmunoResearch).

Immunohistochemistry

Standard protocols were used for immunohistochemistry. Tissue was blocked in 5% normal goat serum (NGS) in saline, incubated in primary plus NGS overnight at 4°C, washed and incubated with secondary antibody preadsorbed against macerated fixed embryos. For triple-labeling with propidium iodide (PI), anti-En (4D9) and HRP Ab, the steps after fixation were: wash, incubate in 1 μg/ml PI for 2 hours at room temperature (RT), wash, block in NGS and incubate in 1:1 MAb4D9 in saline plus NGS overnight at 4°C; wash, block in NGS and incubate in goat anti-mouse Cy5 secondary for 2 hours at RT; wash, block and incubate in rabbit anti-HRP-FITC overnight at 4°C, wash and mount. For En/anti-HRP Ab double-labeling, PI was omitted. Tissue was mounted in SlowFade or ProLong (Molecular Probes).

One concern with En/BrdU/anti-HRP Ab triple-labeling was that only same species primaries (mouse monoclonals) were available for En and BrdU detection, and both are nuclear labels. Thus, for En/BrdU/anti-HRP Ab triple-labeling, additional steps were included. After incubation in 1:1 4D9, block and Cy5 secondary incubation, tissue was again fixed in 3.6% paraformaldehyde for 1 hour. The next steps were: wash and incubate in 2.5 N HCl in saline for 30 minutes to cleave DNA; wash, block and incubate in anti-BrdU mouse MAb; block, wash and incubate in goat anti-mouse FITC secondary; wash and incubate in anti-HRP-LRSC; wash and mount. The second fixation eliminated cross-reactivity. In all preparations, scored independently by both investigators, all four of the possible combinations of BrdU (positive or negative) and En (positive or negative) were found, indicating that false positives and false negatives had been eliminated in the procedure.

Microscopy

Confocal microscopy used a BioRad 1024 laser scanning confocal microscope and Zeiss compound microscope. Over 300 preparations were examined. A *z*-series of images was collected using simultaneous or sequential laser line scanning as appropriate for the fluorophore combination. For triple-labeling with LRSC or PI, FITC and Cy5, sequential scanning was used to avoid mixing of the LRSC (or PI) and FITC signal. Additionally in some cases, one channel was set with a transmitted light detector to record a confocal DIC image. Four sweeps were averaged for each step. Digital files were analyzed frame-by-frame, each frame being one step in a *z*-series. Scoring of cells for the presence or absence of a particular marker (e.g. BrdU, En, HRP) was done independently for each detection channel.

RESULTS

The CNS comprises individual neuromeres, repeating units along the anteroposterior body axis that are each associated with their respective body segments. The neuronal complement of each neuromere arises from MPs, which each divide once giving rise to two neurons, and from neuroblasts (NBs), which divide as stem cells over extended periods of embryonic development. NB divisions produce a series of GMCs, each of which then divides to yield two ganglion cells (GCs), which differentiate as neurons. The NBs including the MNB contribute substantial numbers of neurons to each neuromere, whereas the fewer MP progeny produce the early scaffold of the CNS. The neuromeres posterior to T3 are delayed sequentially by 1% development (Doe and Goodman, 1985). Thus, in a 28% embryo, the first and second abdominal neuromeres (A1, A2), are '27% neuromeres' and '26% neuromeres', respectively.

MPs 4-6 and MNB are separate neuronal progenitors

The progression of neuromere development is seen by labeling with an anti-HRP antibody (HRP-labeling) (Fig. 1A). The antibody recognizes a carbohydrate epitope present in many membrane proteins of insects (Snow et al., 1987). Neuronal labeling is readily distinguished from non-neuronal (glial) labeling. Neuronal labeling is distinctly punctate, whereas glial labeling is diffuse and weakly above background. Scattered, punctate HRP-labeling appears on developing neurons shortly after MP progeny or GCs are produced by division. Within a 1% developmental interval, the label is sufficiently strong to define the circumference of neuronal somata. As growth cones, neurites and axons arise, they also express the HRP label. This neuronal labeling persists, making it a robust marker of neuronal phenotype.

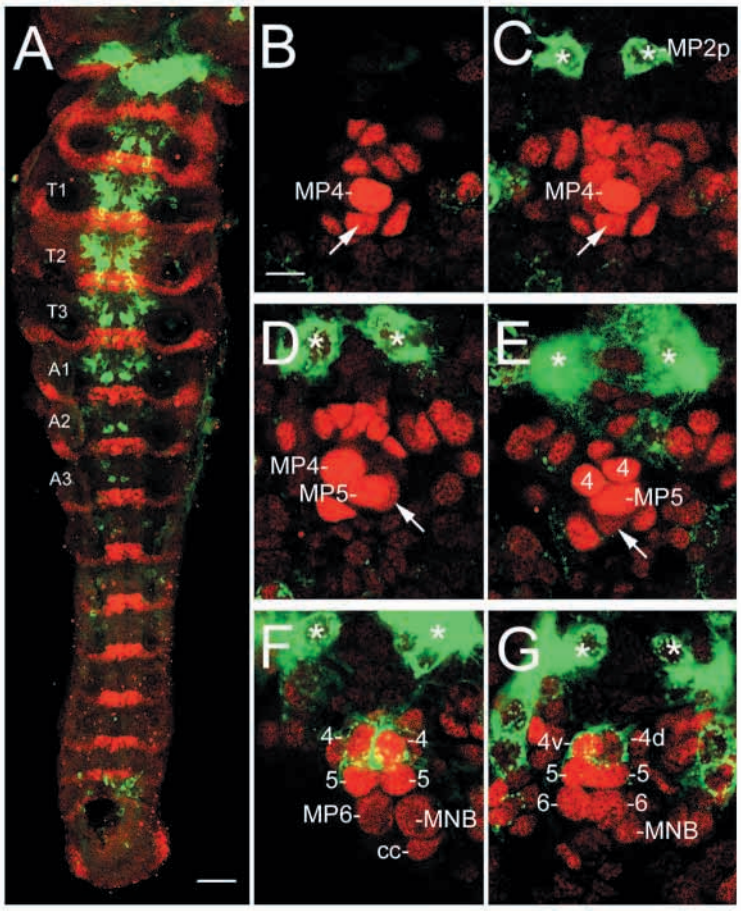


Fig. 1. Midline precursors MP4, MP5 and MP6, and the MNB originate sequentially from the En-positive midline regions. Labeling was by anti-En (red) and anti-HRP (green). (A) 28% embryo shows the pattern of En expression and the progression of neuronal differentiation across neuromeres. Neuromeres posterior to T3 are successively younger by about 1%. (B-G) Sequence of MP and MNB differentiation in 25-29% neuromeres. Stars indicate MP2 progeny. (B,C) 25% neuromere with MP4 and incipient MP5. (B) A relatively dorsal scan; (C) The scan in B together with underlying En-positive region. MP4 is the largest and most strongly En-positive cell at the midline. The incipient MP5 (arrow) is just posterior to MP4. (D) 26% neuromere. MP4 and MP5 express high levels of En. The incipient MP6 (arrow) lies ventral to MP5. (E) 27% neuromere. MP4 has divided to form two progeny (4, 4), anterior to MP5. (F) 28% neuromere. MP4 progeny (4, 4) have strong HRP label, indicating neuronal differentiation and one of the two siblings (on left) has decreased En expression. MP5 has divided to give rise to two progeny (5, 5). MP6 and the MNB with associated cap cell (cc) are side-by-side and posterior in the cluster. (G) 29% neuromere. MP6 has divided to give rise to two progeny (6, 6). One of the two MP4 progeny (4d) is more dorsal than its sibling and has decreased En expression. MP5 progeny (5, 5) are beginning neuronal differentiation judged by HRP-labeling, but MP6 progeny (6, 6) are yet unlabeled. The MNB is ventral to MP6 progeny. Scale bar: 100 μm in A; 15 μm in B-G.

Embryonic segments have an anterior En-negative region and a posterior En-positive region (Fig. 1A). The MNB, three of the MPs (MP4, MP5 and MP6) and NBs of rows 6 and 7 arise from the En-positive region. By 25% neuromere development, MP4 is apparent near the posterior of the En-positive region (Fig. 1B,C). MP4 is larger in diameter, more dorsal and has more intense En expression than surrounding cells at the midline. At 25%, the incipient MP5 is just posterior to MP4, and by 26% MP5 is equal to MP4 in size and En expression (Fig. 1B-D). By 27%, MP4 has divided to form two progeny, both of which are initially En positive, and by 28% MP5 has likewise divided (Fig. 1E,F). By 28%, the two MP4 progeny show the strong, punctate HRP-labeling characteristic of neuronal identity, whereas in the two MP5 progeny HRP-labeling is barely detectable (Fig. 1F). By 29%, one of the two MP4 sibling neurons now has a conspicuously reduced level of En expression (Fig. 1G). MP6 and the MNB differentiate from 26% onwards. At 26% the incipient MP6 is just ventral to MP5, but by 28% comes to lie posterior to the two MP5 progeny, and by 29% has itself divided to form two progeny, both of which are also initially En-positive (Fig. 1D-G). Differentiation of the MNB from the neurectoderm has a time course similar to that of MP6, and at 28% lies next to MP6, but is somewhat more ventral and is associated with its glial cap cell (Fig. 1F).

By 31% neuromere development, MP6 has divided to produce two progeny (Fig. 2A). At this time, the MP5 progeny, as well as the earlier-differentiating MP4 progeny, show strong, punctate HRP-labeling, whereas in the two MP6 progeny HRP-labeling is yet barely detectable (Fig. 2A,B). Stereotypic changes occur in the relative positions of the MP progeny. Initially, pairs of siblings lie side by side (e.g. Fig. 1E,F and Fig. 2A), but as neuronal differentiation proceeds, one of each pair comes to lie dorsal to the other (Fig. 2A,B,D). In addition by 31%, the MNB has produced at least three GMCs, which lie in a stack above it, the first born being the most dorsal (Fig. 2I). Glial nuclei are already in place at the circumference of the MNB (Fig. 2C). Sometime after 31%, and occasionally as late as 32%, the first GMC divides to produce the first pair of GCs. In the example shown in Fig. 2A,B, division of the first GMC is under way, but not complete. The incipient GC siblings have strong Lachesin surrounds, particularly at the central face between them, and the condensed chromosomes are positioned at their opposite poles.

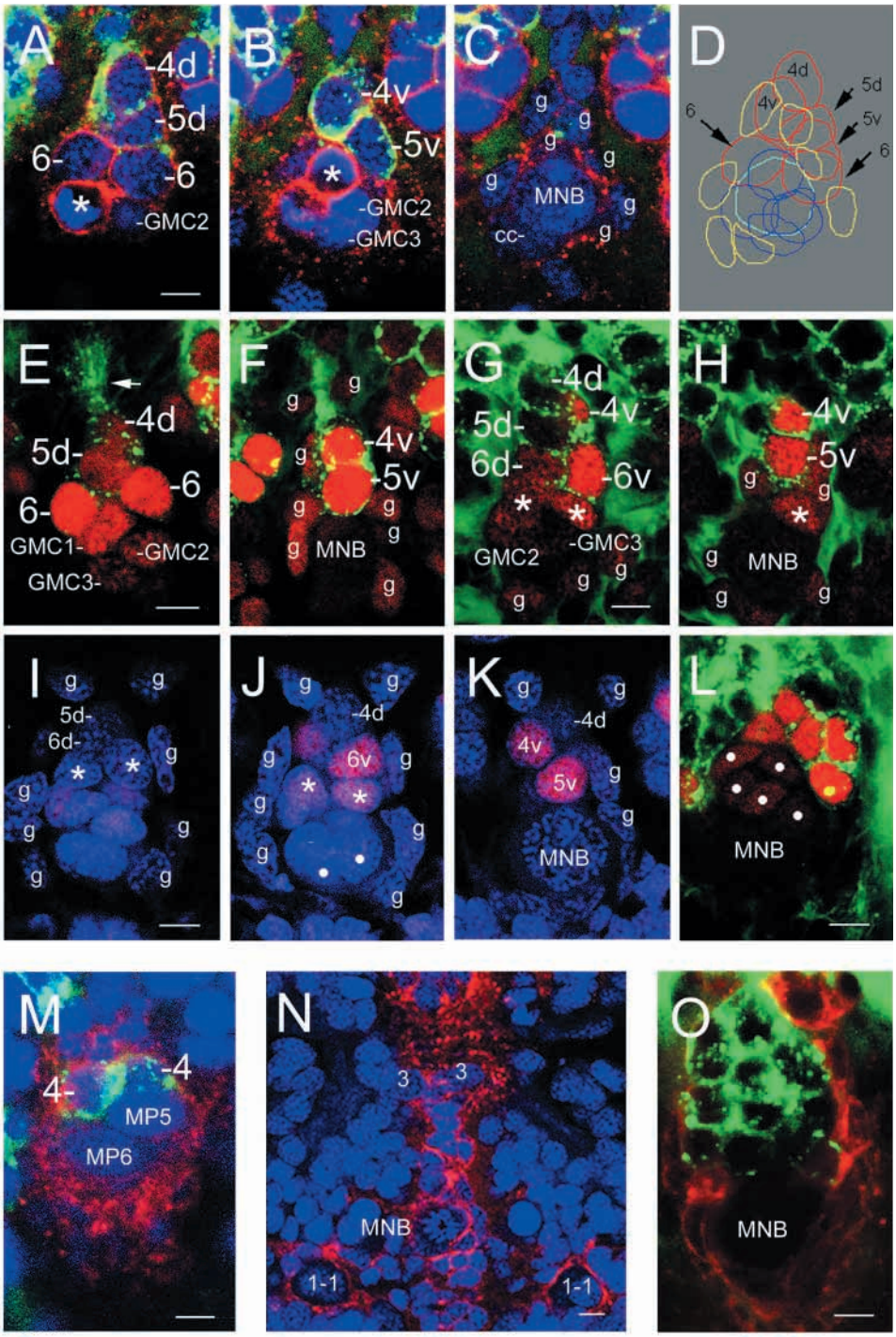
En expression is regulated during maturation of MP4-6 progeny and in MNB and its progeny

After each MP4-6 division, the two progeny initially have equally strong En expression, but this is maintained in only one sibling. A difference in En expression in the two MP4 progeny, born ~27%, is just detectable at 28% (Fig. 1F) and is pronounced by 31% (compare 4d, 4v in Fig. 2E,F). By 32%, one sibling has no detectable level of En, whereas the other maintains a high En level (compare 4d, 4v in Fig. 2G,H). The two MP4 progeny thus differentiate as one En-positive and one En-negative neuron between 27% and 32%, i.e. during a 4-5% developmental interval. The sibling pairs from MP5 and MP6

differentiate likewise, over similar intervals (Fig. 2E-H). This process is completed successively later in the MP5 and the MP6 siblings, reflecting the later times of MP5 and MP6 division (see Fig. 4A). Downregulation of En expression in one neuron of each pair occurs as the somata shift from a side-by-side, to a dorsoventral orientation. The sibling that maintains a high level of En expression invariably comes to lie more ventrally and the sibling that downregulates En expression, more dorsally. A difference in orientation is evident before differentiation of the En-positive and En-negative fates is complete (Fig. 2E,F).

The MNB is En-positive initially (Fig. 1F). However, En expression is never as strong as it is in MPs 4-6, and by 29% the MNB has a background level of En (31% is shown in Fig. 2F). By contrast, MNB GMCs become En positive as they mature. The GMCs form an ordered stack or column, with the newest GMC adjacent to the MNB. When GMCs first arise from the MNB they are En-negative, but gradually become strongly En-positive, expression being highest in the oldest GMC (e.g. GMC1 in Fig. 2E). En upregulation across the maturing GMCs is seen throughout development, as shown in a 36% neuromere (Fig. 2I-K) and a 55% neuromere (Fig. 2L).

The MPs 4-6 and the MNB differentiate within a glial surround, which arises early in midline development. At 27.5%, prior to differentiation of the MNB, glia surround the cluster containing the two MP4 progeny, MP5 and MP6 (Fig. 2M). Slightly later, the MNB and its earliest progeny are also encompassed by glial cells (31.5% is shown in Fig. 2N; Fig. 3F). En-positive glial nuclei are evident at the circumference of the MNB, for example by the time it produces its first GMCs (shown at 32% and 33% in Fig. 2F-H). The glial surround persists throughout development, as shown, for example, in a 43% neuromere (Fig. 2O).



Development of type-specific midline neurite bundles

The midline fiber tract comprises two separate bundles, starting with the outgrowth of growth cones from the two MP4 progeny. By ~31-32%, only shortly after MP6 divides, both of the MP4 neurons have well-established neurites and growth cones oriented anteriorly (Fig. 3A-C). The two neurites can be traced individually to the cell bodies of the two MP4 progeny, one of which (4v) is more ventral (and remains En positive), and one of which (4d) is more dorsal (and becomes En negative) (Fig. 3B,C). The neurite of the En-positive sibling (4v) branches first, at the posterior midline. In 31-32% neuromeres, HRP-labeled filopodia of the 4v sibling contact growth cones of lateral neurons that are growing towards the midline (Fig. 3A,C). This is the first indication of the ventral region of the midline fiber tract, and of the incipient posterior commissure (PC). The ventral region of the midline fiber tract eventually includes all En-positive neuronal progeny of the

Fig. 2. Differentiation of the MP4-6 and MNB lineages in the presence of a glial surround. (A-D) 31% neuromere. MP4-6 progeny, MNB progeny, the MNB, and glial nuclei are shown in dorsal, intermediate and ventral levels of a confocal series (A-C). Labeling is by propidium iodide (blue) to reveal nuclei, anti-HRP (green) and anti-Lachesin (red). A schematic shows projected outlines of midline neuronal progeny and of glial nuclei (D). One sibling each (4d, 5d) of the MP4 and MP5 progeny is dorsal and anterior to MP6 progeny (6, 6) (A). The other two (4v, 5v) are ventrally adjacent to their sibling partners (B). The four MP4 and MP5 progeny have punctate HRP labeling (A,B). The more ventral siblings will remain En positive, and mature slightly before their more dorsal siblings, and have more conspicuous HRP labeling. MP6 progeny (6, 6) are apposed with Lachesin label between, and have the first signs of HRP labeling (A). The MNB (C) has produced three GMCs (A,B). The first GMC has produced two GCs, which are apposed and surrounded by Lachesin label (stars, A,B). The second and third GMCs (GMC2, GMC3) are more ventral (B), but dorsal to the MNB (C). The MNB is surrounded by glia (g), including the cap cell (cc) (C). Relative positions of cells of A-C are shown outlined (D). (E-H) 32% (E,F) and 33% neuromeres (G,H). En is downregulated in one sibling each of the MP4, MP5 and MP6 progeny, whereas En is upregulated in GMCs from the MNB. Labeling was by anti-En (red) and anti-HRP (green). In a 32% neuromere, one sibling each (4d, 5d) of the MP4 and MP5 progeny is dorsal, and anterior to MP6 progeny (6, 6) (E). The other two (4v, 5v) are ventrally adjacent to their sibling neurons (F). The more dorsal neurons (4d, 5d) have lowered En expression (E) but the more ventral siblings (4v, 5v) maintain high levels (F). MP4 and MP5 progeny have punctate HRP labeling (E,F), and growth cones of the MP4 progeny (4d, 4v) are evident at the anterior of the group (arrow, E). MP6 progeny (6, 6) show the first signs of HRP-labeling and have slight differences in En, with one (right) having the lower level (E). The first three GMCs of the MNB lineage are posterior to MP6 progeny and also relatively dorsal (E). GMC1 is about to divide, evinced by chromosomal shape, and has the highest level of En of the GMCs (E). The younger GMCs (GMC2, GMC3) each have yet lower levels of En. The MNB is ventral to the GMCs (F). En-positive glia nuclei (g) surround the MNB (F). In a 33% neuromere, the six MP progeny have punctate HRP labeling, and En downregulation is evident in the more dorsal neuron of each sibling pair (G,H). The 4d neuron has attained its final En-negative state, the 5d neuron has marginal En expression and the 6d neuron has conspicuously lower En expression than does its 6v sibling. The ventral siblings, 4v, 5v and 6v, maintain a high level of En expression (G,H). Of the MNB progeny, GMC1 has divided to produce two En-positive GCs (star), and the younger GMCs (GMC2, GMC3) are also En-positive (G). Glia nuclei (g) surround the MNB (H). (I-L) En is upregulated as GMCs mature during early (I-K) and late (L) embryogenesis. Labeling is by anti-En (red) and propidium iodide (blue) or anti-HRP (green). In a 36% neuromere, the dorsal siblings (4d, 5d, 6d) are En negative (I,J) but the ventral siblings (4v, 5v, 6v) continue to express En (J,K). Of the MNB progeny, two GCs more dorsal are En-negative (star, I), whereas their more ventral sibling GCs are En positive (star, J). Six GMCs (two marked with white dots) show variable levels of En expression (I,J) with the oldest and most dorsal GMCs having the highest En expression (I). MNB is ventral to its GMC progeny and is En negative, whereas surrounding glial nuclei (g) are En-positive (K). In a 55% neuromere, upregulation of En is evident in GMCs (white dots) in the MNB lineage (L). The MNB, which is more ventral to the GMCs, is En negative. (M-O) Glia surround the MP lineages and the MNB lineages. Labeling was by anti-Annulin (red), anti-HRP (green) and propidium iodide (blue). In a 27.5% neuromere, Annulin, a glial marker, surrounds the MP4 neurons (4, 4), MP5 and MP6 (M). In a 31.5% neuromere, Annulin labeling is extensive, and surrounds the dividing MNB (N). Other NBs and their associated GMCs are also surrounded by Annulin, only a few of which are seen at this dorsoventral level [e.g. NB 1-1 clusters; MP3 neurons (3, 3)]. In a 43% neuromere, the Annulin surround includes the MNB group as well as its mature neuronal progeny (O). Scale bars: 10 μm.

MPs 4-6, and of the MNB. Thus, the En-positive MP4 sibling pioneers the PC, as well as a distinct midline bundle. A few percent later, the 4d sibling is the first midline neuron to reach the anterior commissure (AC) and then to branch bilaterally. It thus pioneers the dorsal region of the midline tract, which eventually includes all En-negative progeny of the MPs 4-6, and of the MNB. The incipient AC is already delineated at the midline (Fig. 3A), so the 4d sibling does not pioneer the AC.

We found that anti-HRP and MAb 8B7 labeling reveal different neuronal features. MAb 8B7 was used extensively in earlier descriptions of the MNB group (Condron and Zinn, 1994; Condron et al., 1994), and thus provides important points of reference. The 8B7-label reveals the core of individual neurites, but strong, well-delineated 8B7-label is transient on somata. Labeling of somata by 8B7 is faint in the first stages of neuronal differentiation, at the time when punctate HRP-label first appears, and is highest during the early stages of neurite extension. For example, 8B7-label is stronger on the somata of MP progeny that have neurites (4d, 4v) than on younger progeny (e.g. 5d) (Fig. 3A,B), and is pronounced on the soma of a lateral neuron that also has strong 8B7-labeling of its relatively undeveloped neurite (Fig. 3D). Neurons with longer primary neurites or axons, i.e. having extended a distance at least two to three times the measure of the somata diameter, have only faint 8B7-label on their somata, but sustain the label on neurites and axons (Fig. 3E, lower panel). By contrast, HRP-label is apparent throughout these neurons (Fig. 3E, upper panel).

The restricted nature of the 8B7-label was useful in tracing further development at the midline (Fig. 3G-N). By 35-36% the six MP 4-6 progeny have mature levels of En expression. The three En-negative somata have assumed relatively dorsal positions and the MP4 sibling (4d), at least, has bilateral branches at the AC (Fig. 3G). The three En-positive somata remain relatively ventral, and two or three siblings typically have bilateral branches at the PC (Fig. 3H). The bundle containing the En-negative neurons is separate from that containing the En-positive neurons, and typically is immediately dorsal to it. Occasionally, however, the bundles are offset laterally, making their separate identities clear (as shown in a 38% neuromere, Fig. 3I,J). By 38%, all six MP neurons have branched in their respective commissures, and additional midline neuronal progeny have been contributed from the MNB lineage.

At later stages, the axons of the MP neurons could be traced past the edges of their respective commissures (data not shown). Two of the En-negative efferent neurons had bilateral branches that grow into a small anterior tract that exits nerve 1 (N1). They appear to be equivalent to the two DUM1 efferent neurons in the adult (Campbell et al., 1995). The third En-negative efferent neuron had bilateral branches that grew towards lateral nerves 3-5, and thus might be equivalent to any

one of several adult DUM neurons. The three En-positive interneurons had bilateral branches that join an ascending longitudinal tract. They thus prefigure the morphology of adult DUM interneurons (Siegler and Pankhaniya, 1997; Thompson and Siegler, 1991). The six MP4-6 neurons remained closely associated with the MNB progeny (Fig. 2G-K, Fig. 3G-K).

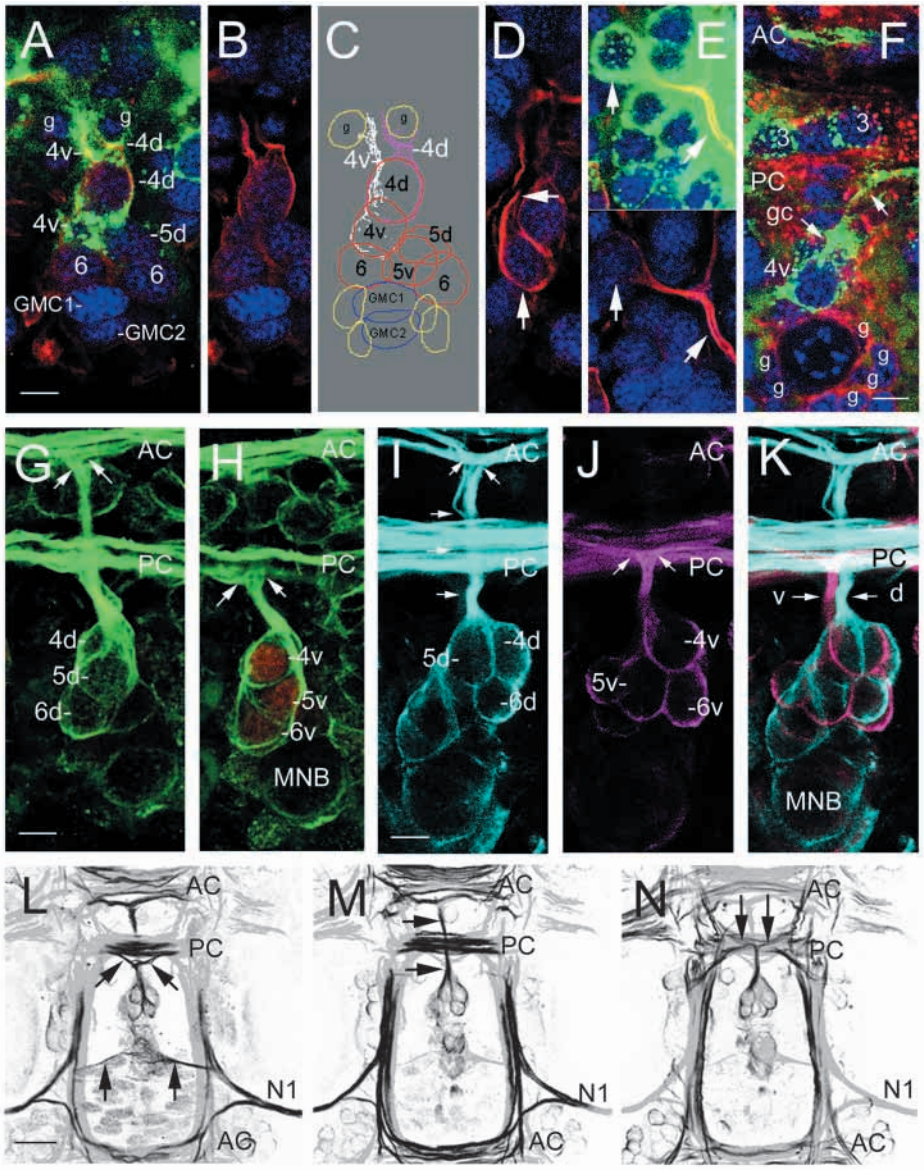


Fig. 3. Development of midline bundles. (A-E) 32% neuromere. Labeling is by propidium iodide (blue), anti-HRP (green) and anti-8B7 (red). Two midline growth cones extend anteriorly between two glia (g), one growth cone from 4v (left) and one from 4d (right) (A-C). Continuous and strong 8B7 label occurs at the core of the growth cones and around cell bodies, whereas HRP-label also marks the entirety of the growth cones from the 4d and 4v neurons (A,B). 8B7 label is faint and discontinuous on neuron 5d and HRP label is only weakly developed (A,B), as is also true for sibling neuron 5v (ventral to plane of scan). 8B7 labeling of 4d and 4v neurons was traced through all sections as shown on the schematic (C). 8B7 labeling of the 4v neurite is traced in white (left) and labeling of the 4d neurite is traced in magenta (right). The MNB and the MNB-associated glia are ventral to the plane of section. A lateral neuron has strong 8B7 label around its cell body (vertical arrow) and at the core of its relatively short growth cone (horizontal arrow) (D). HRP label (not shown) was comparable to that of the 4d neuron in A. Another and older lateral neuron has a well developed primary neurite (arrows), shown in both upper and lower panels (E). Upper panel shows all three labels used; lower panel shows confocal channels for anti-8B7 and PI labels only. 8B7 label is strong within the core of the neurite, but barely evident around the cell body (upward arrows). HRP label is strongly maintained on both the cell body and the primary neurite. (F) 31.5% neuromere. The midline fiber tract is just forming. The 4v neuron has an anteriorly extended growth cone (gc), with broad lamellipodium and filopodia. Its trajectory is towards other growth cones (upward oblique arrow) from lateral neurons that pioneer the posterior commissure (PC). HRP-label also shows part of the incipient anterior commissure (AC) and progeny of MP3 (3, 3). Annulin labeling reveals the glial surround; some glial nuclei (g) are indicated. Labeling is by propidium iodide (blue), anti-HRP (green) and anti-Annulin (red). (G-K) Neurites of MP4-6 progeny comprise two bundles, one dorsal, which branches at the AC, and one ventral, which branches at the PC. Panels show dorsal neurons (G,I), ventral neurons (H,J) or both (K). In a 35.5% neuromere (G,H), the three dorsal MP neurons are En-negative, and have midline neurites that cross the PC and extend anteriorly to branch bilaterally (oblique arrows) at the AC (G). The three ventral MP neurons are En positive, and have midline neurites that branch bilaterally (oblique arrows) at the PC (H). Labeling is by anti-8B7 (green) and anti-En (red). In a 38% neuromere (I-K), the dorsal and ventral bundles also have different mediolateral positions. The 8B7 label is shown by turquoise in the more dorsal panel (I) and by magenta in the more ventral panel (J), or combined (K). In the more dorsal panel (I), neurites (horizontal arrows) of the dorsal neurons (4d, 5d, 6d) extend to the AC, where they branch bilaterally (oblique arrows). In the more ventral panel (J), neurites of the ventral neurons (4v, 5v, 6v) extend only to the PC, where they branch bilaterally (oblique arrows). In both panels, the more posterior cells are MNB progeny. (L-N) The median nerve develops separately. Labeling was by anti-8B7. All panels show the encompassing neuronal framework in flat gray; each panel also shows in black the 8B7 labeling at only one dorsoventral level. In a 38% neuromere, the most dorsal panel shows the developing median nerve (L). The Y-shaped figure at the anterior (oblique arrows) is formed by neurites of spiracle motoneurons. The neurites meet at the midline just posterior to the PC and travel posteriorly in a bundle that is dorsal to, and separate from, the midline fiber tract. The horizontal neurites at the posterior (upward vertical arrows) comprise the peripheral portions of the incipient median nerve. The neurites have reached the midline, and in later stages will grow anteriorly to meet the neurites of the spiracle motoneurons and form the median nerve. Two successively more ventral panels show the dorsal bundle of the midline fiber tract (horizontal arrows) (M), and the ventral bundle of the midline fiber tract with bilateral branches in the PC (downward vertical arrows) (N). Scale bar: 10 μm in A-K, 20 μm in L-N.

Thus, what is commonly referred to as the ‘DUM group’ or the ‘MNB group’ of the adult also includes the MP4-6 progeny.

The median nerve, which has a portion of its path along the midline, is developing at the same time. At 38%, the peripheral and central portions of the incipient median nerve have not yet joined. The neurons that form the distal portion of the nerve grow inwards from the periphery, but have not yet met at the midline (vertical arrows, Fig. 3L). The central region arises from paired neurons of bilateral origin, including the spiracle motoneurons. Their axons have grown to the midline, dorsal to the PC, and are extending posteriorly (oblique arrows, Fig. 3L). A ‘Y’ shape is thus formed just posterior to the PC, which superficially resembles the bilateral PC branching of interneurons from MP4-6s and the MNB (Fig. 3M,N). The median nerve is quite separate in development, however. By 43% the proximal and distal portion of the median nerve have joined, and a portion of the median nerve traverses the cluster of neuronal somata produced by the MNB (data not shown). This morphology is evident also in adulthood (Thompson and Siegler, 1991).

The two midline bundles were traced to their mature form (data not shown). The bundle pioneered by 4v ultimately becomes the posterior DUM tract (PDT) of the mature ganglion, whereas the bundle pioneered by 4d ultimately becomes the superficial DUM tract (SDT) and/or deep DUM tract (DDT). The PDT comprises En-positive DUM interneurons, and the SDT/DDT comprises the En-negative DUM efferents (Thompson and Siegler, 1991).

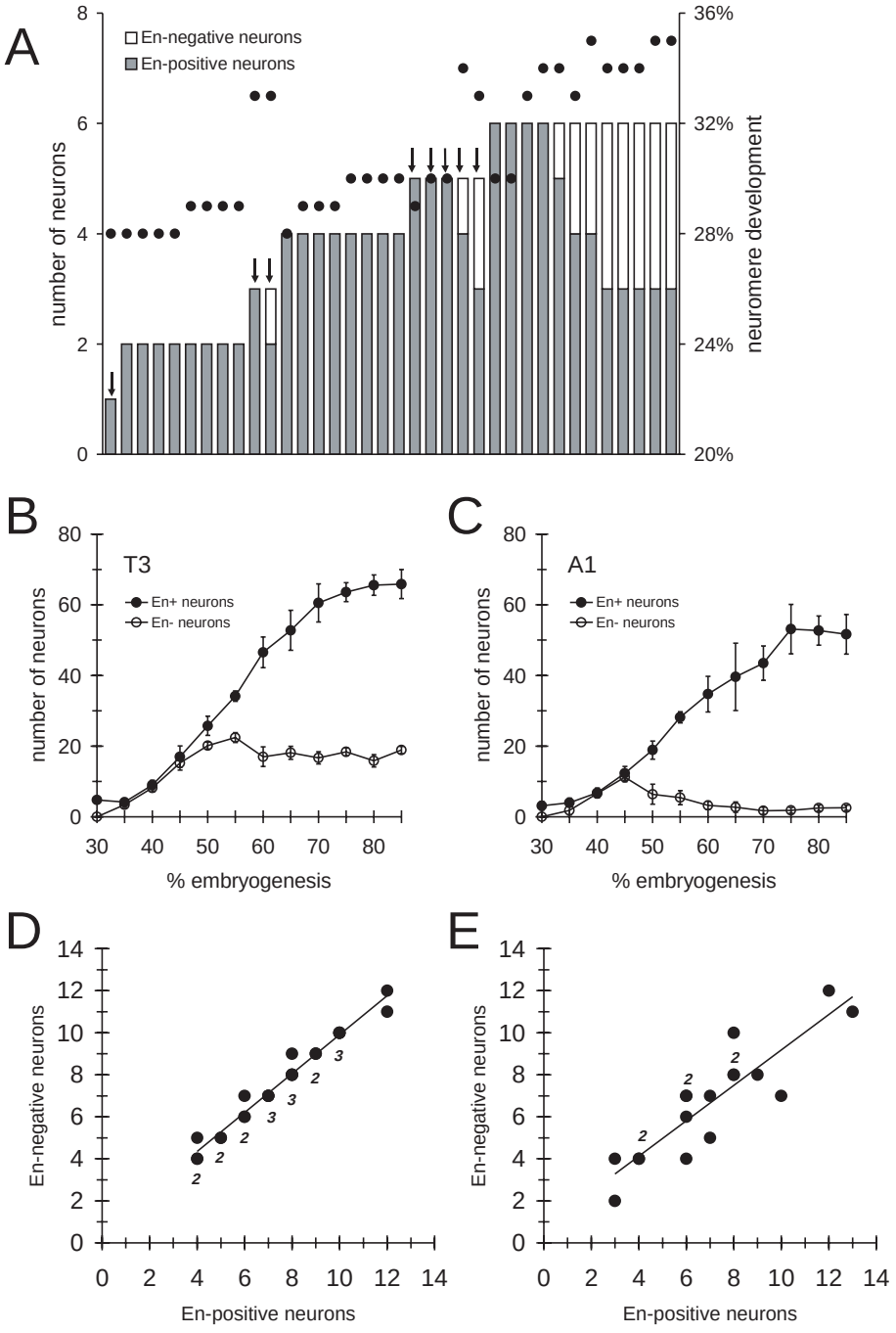


Fig. 4. Midline precursors and GMCs each produce one En-positive and one En-negative neuron. (A) En expression in neurons from MP4, MP5 and MP6, in 28% to 35% neuromeres (*n*=36). Vertical bars show the number of neurons (left *y*-axis) in individual neuromeres as the sum of neurons with no detectable En (white) and at least some En expression (gray). Downward arrows indicate neuromeres having one additional MP sibling that had not yet differentiated neuronal markers (not included in bar total). Chart bars are rank-ordered, first by the total neurons present, and then by the number of En-positive neurons. Filled circles above each bar indicate percent development of neuromere in which neurons were tallied (right *y*-axis). (B,C) Population curves for midline neurons in T3 (B) and A1 (C) neuromeres, including neurons from MP4-6 and MNB lineages. En-positive neurons (black circles) and En-negative neurons (white circles) are equal in number through 45% embryogenesis. After 45%, the number of En-positive neurons continues to increase, but the number of En-negative neurons remains steady or decreases. (D,E) Embryos injected with BrdU at 35% (D) or 55% (E) were allowed to develop a further 48 hours (see text). Data obtained from T3 and A1 neuromeres were pooled, as there was no population difference. For injections at 35%, *n*=22 neuromeres counted, of which 11 were T3, and 11 were A1. For injections at 55%, *n*=17 neuromeres counted, of which 10 were T3, and 7 were A1. BrdU-labeled neurons were counted by type, and the number of En-positive neurons in each neuromere (*x*-axis) was plotted against the number of En-negative neurons in the same individual neuromere (*y*-axis). Where more than one neuromere had identical counts of the two types, the points are annotated by that number.

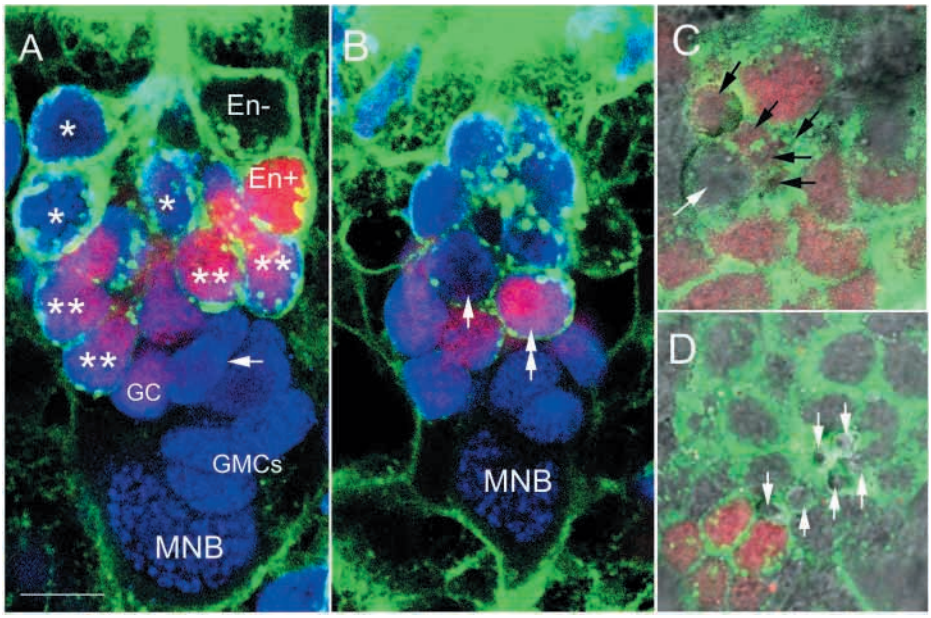


Fig. 5. Birth and death of En-positive and En-negative neurons. (A,B) Birth of neurons. The embryo was injected with BrdU at 35% and dissected after a further 48 hours (~45% embryogenesis). Labeling was by anti-HRP (green), anti-BrdU (blue) and anti-En (red). The MNB, GMCs, GCs and the neurons born during the injection period are BrdU positive. In A, a T3 neuromere, examples of BrdU-positive neurons that have downregulated En (En-negative) are marked by single stars; those that have some level of En are marked by double stars. Two neurons born prior to BrdU injection are also marked: one En negative (En–) and the other En positive (En+). The horizontal arrow indicates a dividing GMC. In B, an A1 neuromere, sibling BrdU-positive neurons are marked: one is En negative (single arrow, left); the other is En positive (double arrow, right). (C,D) Death of neurons. Pyknotic profiles shown in DIC images, with superimposed labeling from anti-HRP (green) and anti-En (red). (C) Pyknotic profiles at 70% in T3 (En positive, black arrows; En negative, white arrow); (D) pyknotic profiles at 55% in A1 (En negative, white arrows). Scale bar: 20 μm.

The MNB lineage: each GMC division yields one En-positive and one En-negative neuron

During the early development of the MNB lineages, up to 50% in T3 and 45% in A1 and posterior, newly differentiated neurons included both En-positive neurons (interneuronal fate) and En-negative neurons (efferent fate) in about equal numbers. This outcome was not expected: by the sequential model, the early part of the lineages should be composed of En-negative neurons alone. We therefore hypothesized that, as in the MP4-6 lineages, neurons of two types arise from each GMC division. We tested this hypothesis, first, by counting En-positive and En-negative neurons throughout development and, second, by labeling with BrdU to trace the birth of sibling neurons.

Evidence from population counts

En-positive and En-negative neurons increased in an essentially 1:1 fashion, through 45% embryogenesis. At 40%, neuronal counts (mean±s.d.) were 9.0±1.3 (En-positive) versus 8.2±1.1 (En-negative) in T3, and 6.8±3.7 (En-positive) versus 6.6±1.3 (En-negative) in A1. At 45%, counts were 17.0±3.0 (En-positive) versus 15.0±2.0 (En-negative) in T3, and 12.3±1.9 (En-positive) versus 11.2±1.3 (En-negative) in A1 (Fig. 4B,C). From 50% onwards, however, the numbers diverged conspicuously, to yield midline populations containing many more En-positive neurons than En-negative neurons. By 55% development, the populations have the mature number of En-negative efferent neurons. The first embryonic molt, from 45% to 55% embryogenesis, yields substantial cell death, particularly in abdominal neuromeres (Thompson and Siegler, 1993). Counts after 45% therefore do not measure the absolute numbers of the two neuronal types produced. However, taken together with the BrdU data (below), these data allow a minimal estimate for the extent of cell death among the neurons of efferent fate.

Evidence from BrdU labeling

To test our hypothesis directly that each GMC division gives rise to one sibling with an En-negative efferent fate and one sibling with an En-positive interneuronal fate, embryos were injected with BrdU. Forty-eight hours later, embryos were triple-labeled to assess BrdU incorporation, neuronal differentiation, and En expression (Fig. 5A,B). We sampled injection times across embryogenesis, but focused on two: 35% and 55%. By about 35%, the first GCs are produced. By 55%, the groups contain the mature number of En-negative (efferent) neurons, but less than the mature number of En-positive neurons (Fig. 4B,C). By the sequential model, 55% would be well past the time that efferent neurons are produced. Embryos injected at 35% matured to 43-44%, thus prior to the onset of neuronal cell death. Embryos injected at 55% matured to 63-65%, at or shortly before the time the MNB dies. For early and late intervals alike, we tallied En-positive and En-negative neurons (HRP labeled) that were born after BrdU injection (BrdU positive).

En-positive neurons and En-negative neurons labeled with BrdU were essentially identical in number, during both intervals, irrespective of the total number of neurons labeled. For injection at 35%, the number of BrdU-labeled neurons (mean±s.d.) was 7.5±2.4 (En positive) versus 7.6±2.3 (En negative) (Fig. 4D). For injection at 55%, the number of BrdU-labeled neurons was 7.1±2.9 (En positive) versus 6.7±2.7 (En negative) (Fig. 4E). Counts made from a sampling of other time points, and including other neuromeres (e.g. T2 and A2), likewise showed that equal numbers of En-positive and En-negative neurons were born from the MNB lineages.

The numbers of BrdU-labeled En-negative versus En-positive neurons in individual preparations were strongly correlated. The coefficient of correlation was 0.99 for T3 and A1 together, and 0.99 and 0.98, respectively, for T3 and A1 separately, for injections at 35% (Fig. 4D). The coefficient of correlation was 0.88 for T3 and A1 together, and 0.93 and 0.90, respectively, for T3 and A1 separately, for injections at 55%

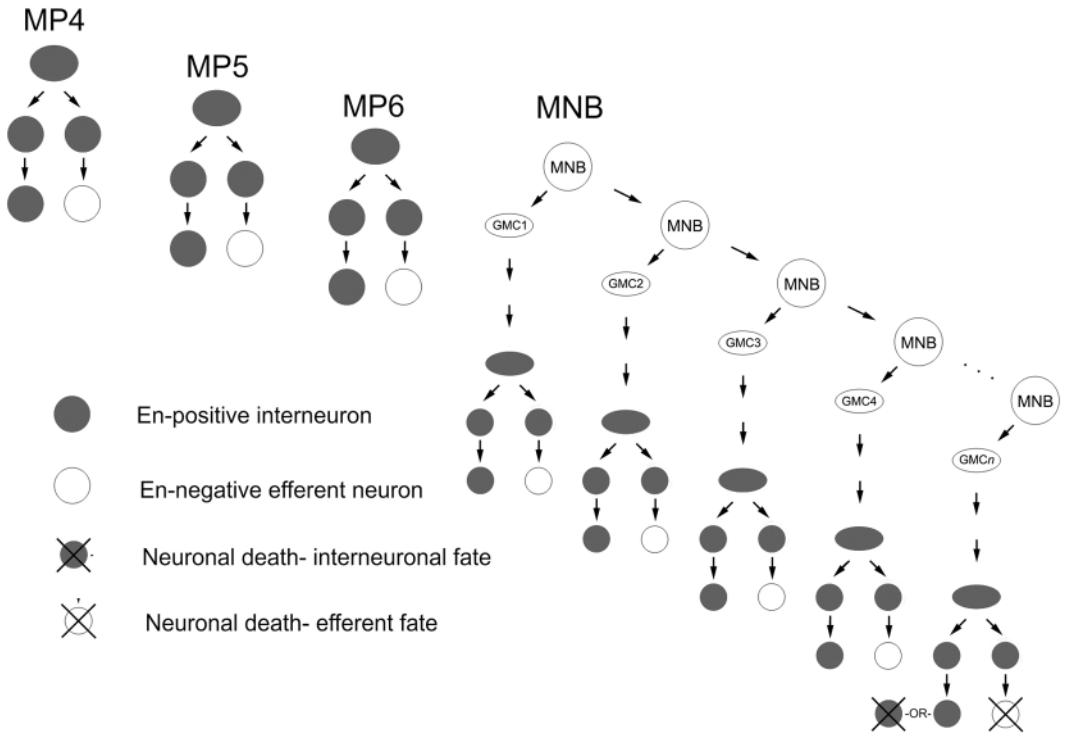


Fig. 6. Summary of MP4, MP5, MP6 and MNB lineages.

(Fig. 4E). All labeled T3 and A1 neuromeres were included in these data, and confirmed the prediction that equal numbers of the two neuronal types would be labeled irrespective of the total number of BrdU-labeled neurons. The average number of BrdU-labeled neurons in total (En-positive plus En-negative) was similar for the two intervals, 15.5±4.6 and 13.8±5.4 (35% and 55% injections, respectively). The range in number of BrdU-labeled neurons was eight to 24 neurons and five to 24 neurons (35% and 55% injections, respectively). Some neuromeres counted had complete labeling of the lineage (the MNB and all GMCs and GCs having BrdU labeling), whereas others had less complete labeling (some GCs and/or GMCs did not have BrdU labeling). Incomplete labeling would have occurred if BrdU was diluted away from the injection site, for example.

For injections at 35% embryogenesis, we also counted midline neurons that were BrdU-negative after the 48 hour interval. The numbers of each type were (mean±s.d.) 4.3±0.7 (En-positive) versus 4.3±0.7 (En-negative), or a total of 8.6±1.4 neurons. The sample comprised 24 neuromeres, 12 from T3, and 12 from A1. In 22 of the neuromeres, the counts of En-positive neurons/En-negative neurons respectively were 3/3, 4/4 or 5/5. In two cases, these in A1, values were 4/5, possibly reflecting the slightly later onset of HRP-label in the En-positive neurons. Three of the neuronal pairs could be identified from position and soma diameters as the MP4-6 progeny, whereas the additional one or two pairs are neurons produced by the MNB lineage.

Although each GMC division produces two neuronal progeny that are of unlike type, near the end of embryogenesis the midline groups contain greatly more En-positive neurons than En-negative neurons (Fig. 4B,C). These counts allow a minimal estimate of the extent of cell death, at least among En-negative progeny of efferent fate. In T3, counts at 85% embryogenesis show about 65 En-positive versus 19 En-negative neurons, or roughly 45 fewer En-negative neurons. In A1, counts at 85% embryogenesis show about 52 En-positive versus three En-negative neurons, or roughly 50 fewer En-negative neurons. From the disparity of numbers it is clear that cell death is crucial in shaping the mature populations. It can be inferred that the total number of progeny produced in the lineages are at least twice the number of surviving En-positive progeny. Thus, in T3 at least 35% (45/130) of the neurons, and in A1 about 50% (50/104) of the neurons produced in the MNB population die across embryonic development. This is an underestimate to the extent that En-positive neurons also die during these times.

From 50% onwards, pyknotic profiles had dense HRP-label, indicating neuronal differentiation prior to death. Some profiles were En-negative at the core, whereas others were En positive, confirming that neurons of both fates are removed from the population (Fig. 5C,D). It was previously assumed that all pyknotic profiles late in the lineage were cells of an interneuronal fate, in accordance with the sequential lineage model (Thompson and Siegler, 1993). In light of present data, this appears to be incorrect. These events are further documented in a related study (M. V. S. S. and X. X. J., unpublished). No evidence was found for cell death among GMCs or GCs, as identified by a lack of HRP labeling, reinforcing the view that some degree of neuronal differentiation precedes cell death.

DISCUSSION

MP4, MP 5 and MP6 and the MNB are separate neuronal progenitors

The midline precursors MP4, MP5 and MP6, and then the

MNB, arise in close temporal succession from 24-28% of neuromere development. In the sequence of origin, the three MPs each divide once, and the two progeny of each differentiate as neurons. Punctate HRP-label, a reliable marker of neuronal differentiation, appears in MP4 progeny by 28%, and in MP6 progeny by 31%. The MNB arises at the same time that MP6 enlarges (28%), and then divides in stem cell fashion typical of other NBs. The MNB produces its first GMC at 29%, but it is not until 36% that the two progeny of this first GMC begin neuronal differentiation. Thus, a 5% gap in the production of midline neurons occurs between 31% (neuronal differentiation of MP6 progeny) and 36% (neuronal differentiation of first MNB progeny).

Our results are consistent with the first description of the four separate progenitors (Bate and Grunewald, 1981). This contrasts with the view that the MP5 and MP6 progeny, and probably also the MP4 progeny, are the earliest neurons produced by the MNB (Condron and Zinn, 1994). It has also been reported that the MNB produces neurons from 28-33% and after 40%, and that during the intervening 7% gap, the MNB produces midline glia (Condron and Zinn, 1994; Condron et al., 1994). The 5% gap in differentiation that we observe is roughly similar to this in timing and extent, but we find a different cause: timing of neuronal differentiation among progeny of the different precursors.

To understand how our own findings might be reconciled with earlier reports, we ultimately focused on methodological issues. One point is readily resolved. The slight difference in the timing of the 'gap' in neuronal production can be ascribed to differences in the antibodies used to label neurons. The anti-HRP Ab detects new neurons at an earlier stage of differentiation than does MAb 8B7, the latter having been used in prior studies. A second point, that of the neuronal or glial identity of MNB progeny, can also be resolved by considering methodology, but requires more discussion. In an earlier study, midline lineages were assessed by injecting the putative MNB with tracer at 28-29% neuromere development, and then inspecting labeled progeny after a culture period of 72 hours (Condron and Zinn, 1994). While it might seem that such a direct approach would give a more accurate picture than that afforded from the close study of labeled, fixed preparations, two concerns are key. One is the identity of the injected progenitor. The other is the timing of glial development.

The 28% time point is important because the MNB and MP6 are then adjacent, and of similar size. In fixed, antibody-labeled embryos, the two can be readily distinguished from each other by their intensities of En expression and by their positions relative to differentiating MP4 and MP5 progeny, but use of live embryos does not afford such markings. Thus, it is entirely plausible that in the prior lineage studies, the MNB was injected with tracer in some 28-29% embryos, whereas MP6 was injected in others. Indeed, these earlier data show two outcomes of injection. In one outcome, a single pair of neurons of unlike type contained the injected label. Both neurons bifurcated at the midline, but at separate anteroposterior positions, this being consistent with the morphological differences we see between the sibling MP6 progeny. Unintended injection of MP6, instead of the MNB, thus could have lead to the conclusion that these siblings arose early from the MNB, prior to a purported period of glial production. In the other outcome, a small group of GMCs or neuronal progeny contained the injected label, this consistent with an injection of the MNB and with the lineal development that we report.

Do the MNBs produce glia?

The timing of midline glial development is also important in assessing our results relative to prior studies. We find that the MNB is surrounded by glia by (at least) 28%, and the glial sheath is intimately associated with the MNB by (at least) 34%. By an earlier view, the glia are produced from 33-40%, and a glial sheath surrounds the MNB after 35% (Condron and Zinn, 1994). In support of these observations, injection of the MNB at 34% labels the midline glial sheath only (or the sheath and neurons, depending on length of the subsequent period of culture). We suggest the possibility that tracer entered the already present glial sheath, which would necessarily be penetrated by an injection microelectrode before it reached the MNB. This would label glial cells directly, rather than via lineal conveyance from the MNB. Inadvertent introduction of tracers into the glial surround, instead of or in addition to injection of the MNB, might well be difficult to avoid, given their intimate association.

Our data do not support the idea that En is involved in switching MNB progeny from a neuronal fate to a glial fate. When En-antisense nucleotides were injected into the MNB, the results were taken as evidence that, by blocking En expression, the MNB was caused to produce extra neuronal progeny at the expense of glial progeny (Condron et al., 1994). A key methodological point is that neuronal somata were counted by using 8B7-label. Such counts would underestimate the normal population, however, because strong 8B7-label is transient in neuronal somata, as discussed in the Results. Indeed, by using the more robust HRP-label we counted about twice the number of neurons than were found by using the 8B7-label in normal embryos at the same stages (Condron and Zinn, 1994; Condron et al., 1994). Moreover, our neuronal counts in normals were virtually identical to those reported for the antisense-injected preparations. This suggests that neuronal number was not altered by the antisense manipulation, rather that the number of 8B7-labeled somata was increased. Midline neurons in the injected preparations also had truncated neurites, and it is plausible that, absent normal neurite elongation, the 8B7 epitope accumulated at the somata. Normally, 8B7-label on somata wanes as neurites extend, as shown in the present study. En-antisense nucleotides, introduced into the neuronal lineage via injection of the MNB, might well disturb development of the En-positive GMCs and neuronal progeny. A direct effect on En levels in the MNB is unlikely, however, because (as we show) En expression in the MNB is negligible by the time it is generating progeny. En-antisense injections also resulted in a failure of normal glial development, this interpreted as the result of switching the MNB fate to favor neuronal production. If En-antisense nucleotides were inadvertently introduced into the already present glial surround, this might well yield abnormal glial development as the glia themselves are En-positive.

We find no evidence that the MNB produces glial progeny, but the possibility cannot be formally excluded that at an initial division, a precursor of the MNB gives rises to the MNB and to a glioblast. In *Drosophila*, this pattern is seen in a limited number of NBs (or neuroglioblasts) (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). However, there is

no evidence from *Drosophila* that the MNB produces glia. Instead, the midline glia arise from separate glial progenitors (Jacobs and Goodman, 1989; Klämbt et al., 1991; Klämbt et al., 1996). Our evidence from grasshopper is consistent with this scheme, reinforcing the view that grasshopper and *Drosophila* share essential features of embryonic CNS development.

En expression and asymmetric fate in MP and MNB lineages

Progeny of the MPs4-6 are of asymmetric type. This pattern is again consistent with what we know of midline development in *Drosophila*. Three so-called VUM neurons in each embryonic segment are En-negative efferent neurons, whereas three presumed siblings are En-positive, and of interneuronal fate (Siegler and Jia, 1999).

The two types in grasshopper come to occupy respectively more dorsal, or more ventral somata positions, trace separate midline bundles, and branch bilaterally at separate anteroposterior locations. The three En-positive interneurons pioneer the ventral region of the midline fiber tract, and bifurcate at the PC. The three En-negative efferents pioneer the dorsal region of the midline fiber tract, and grow anteriorly past the PC to bifurcate at the AC. It was earlier reported that the two MP4 progeny were of like morphology, and pioneered the dorsal region of the median fiber tract, branching at the AC, whereas the two MP6 progeny (also of like morphology) pioneered the ventral region of the median fiber tract, and branched at the PC (Goodman et al., 1981), but this appears not to be the case. Sibling progeny of the MNB lineage likewise are of asymmetric type. This pattern of sibling pairs does not, however, reflect the final composition of the MNB group. Substantial cell death ultimately removes neurons of both types, though predominantly those of En-negative fate. In abdominal neuromeres, the three surviving efferent neurons (so-called DUM neurons) are almost certainly the three efferent progeny of MPs4-6, and given this, it appears that all efferent progeny generated by MNB abdominal lineages ultimately die. Pyknotic profiles were surrounded by robust HRP-label, indicating that death occurred only after neuronal differentiation had begun. The timing of these events suggests that neuronal cell death occurs in some En-negative neurons before their axons exit the CNS.

En expression levels change predictably during midline development. The MPs4-6 have the highest level of En expression just before division. A high level of En expression is maintained in one MP sibling, while in the other MP sibling En is reduced to undetectable levels after 1-2% of development. The MNB expresses En at its earliest stage, but never at the levels seen in the MPs. En expression wanes to background levels by the time the MNB is producing GMCs. The GMCs are En-negative as they arise from the MNB but gradually develop En expression, which is strongest just before a particular GMC divides to produce two GCs. En expression could regulate the expression of cell adhesion molecules in the GMCs as it does in neurons (Siegler and Jia, 1999), perhaps to allow the most mature GMC to separate from the GMC column and undergo division. Regulation of En expression in GMC sibling progeny is similar to that in the MP4-6 progeny. Although MPs are sometimes referred to as neuroblasts, our findings emphasize the similarity between MPs and GMCs, a similarity that may be important in understanding the contrasting modes of asymmetric division between neuroblasts, which act as stem cells, and other CNS neuronal precursors, which undergo terminal divisions.

Differential En expression would be critical for the differentiation of asymmetric neuronal types in the MP4-6 and MNB lineages. The gradual loss of En in one member of a sibling pair coincides with the beginning of neuronal differentiation, and is evident well before growth cone formation. Differential En expression could then contribute to the different pathway choices made by the neurons (Marie et al., 2000). In *Drosophila*, En regulates the expression of neuronal cell adhesion molecules, and misexpression of En in postmitotic neurons grossly alters neuronal morphology (Siegler and Jia, 1999). As in grasshopper, En expression occurs in interneurons, but not in efferent neurons, further evidence for a role of En in the differentiation of neuronal type (Siegler and Pankhaniya, 1997; Siegler and Jia, 1999; Siegler et al., 2001). The question remains unanswered as to the means by which local or intersegmental interneuronal fate is determined. En expression differs considerably among the mature interneurons, and may contribute to pathway choice (Siegler and Pankhaniya, 1997). A successive expression of several transcription factors could confer a more specific ordering of phenotype (Isshiki et al., 2001).

A revision of midline lineages – developmental and evolutionary implications

The MNB is one of the longest-lived NBs in grasshopper and produces one of the largest neuronal lineages. The MNB lineage was among the first of the NB lineages to be studied in detail, and in sum the morphological, electrophysiological and biochemical properties of its embryonic and adult neurons are more thoroughly known than for other lineage groups in insects. The MNB lineage of grasshopper has served as a basis for understanding how neuronal types are produced broadly across lineages of the insect CNS (Burrows, 1996; Truman et al., 1993).

The sequential model had seemed plausible, given that sibling neurons were assumed to remain in proximity (Goodman and Spitzer, 1979; Goodman et al., 1980). However, it is now clear that asymmetric neuronal siblings from GMC and MP divisions are not positioned according to birth order as previously thought, rather their somata come to occupy relatively dorsal or ventral locations according to neuronal type. Their primary neurites are wholly separated by neuronal type, tracing different midline tracts and different commissures. Thus, neuronal morphology and position are not good indicators of birth order within a lineage.

Our findings lead to a revised account of the MPs4-6 and MNB lineages (Fig. 6). Evidence has long been available from grasshopper, and then from *Drosophila*, that sibling neurons of apparently unlike, or asymmetric type are produced from MPs1-3 and from the first GMCs of some NB lineages (Doe et al., 1988a; Doe et al., 1988b; Goodman et al., 1981; Kuwada and Goodman, 1985). And, at least one postembryonic lineage in *Manduca* produces siblings of two types (Witten and Truman, 1991). These findings have not been incorporated into a more general model of neuronal lineages, however; possibly for two reasons. In contrast to other MPs, MPs4-6 were thought each to produce progeny of like type (Goodman et al., 1981).

And, in the few NB lineages with early progeny of apparently asymmetric type, the range of neuronal types produced across the lineage is unknown. Without this larger framework, it is hard to judge the significance of any phenotypic difference. Differences between siblings might be ascribed to a particular variation between neurons of the same basic type or they might, instead, typify an essential asymmetry that is maintained across a lineage. The neuronal progeny of embryonic NB lineages in *Drosophila* are now known in some detail, and it is striking that two-thirds of lineages contain interneurons as well as either motoneurons, or neurosecretory cells of an efferent type (Schmid et al., 1999). Interneurons predominate in the extant lineages, but this is not surprising in light of the extensive cell death we find among neurons of efferent fate, and suspect the same to be true of *Drosophila*. Of the remaining lineages in *Drosophila*, many generate interneurons having two distinctly different axonal projections, either anterior or posterior, or ipsilateral or contralateral. Both of these dichotomies could represent an essential asymmetry in lineages that do not produce efferent neurons; the MP2 siblings, for example, are asymmetric in that they have either an anterior or a posterior interneuronal projection (Spana et al., 1995).

We suggest that the production of paired siblings of asymmetric neuronal type is fundamental to insect neuronal lineages in the CNS. This view is concordant with the asymmetry by which sense organs are generated in the insect PNS. Furthermore, it parallels the essential role of asymmetry in the unfolding of cellular and neuronal type in *C. elegans*, and in the vertebrate CNS, as reviewed by Lu et al. (Lu et al., 2000) and references therein.

This idea has important implications for understanding and interpreting developmental and evolutionary events. During development, neuromeres across the CNS come to differ in size and composition, although each neuromere arises from a similar array of neuronal precursors. The means by which segmental specificity is achieved are largely unknown. In the MNB lineages, most interneurons survive, while only a minor faction of efferent neurons survive (this differing between thoracic and abdominal neuromeres). In effect, the lineages produce a large population of one neuronal type (interneurons), at the expense of overproducing another (efferent neurons).

Across insect evolution, the body appendages and their attendant sensory and motor apparati have been exquisitely formed and varied to match environmental needs. The first line of encounter, and thus the driving force for selection, is outward body form. The overproduction of central neurons, both of interneurons that integrate peripheral sensory input, and of efferent neurons that guide an outward motor response has a cost, but also a greater benefit. It confers considerable latitude to the CNS in matching outward demands, without necessitating fundamental changes in the lineal mechanisms by which different neuronal types are produced. Such a strategy offers simplicity and flexibility, providing a means by which neuronal populations in the insect CNS might be tailored to the wide diversity of peripheral structures across body segments, as well as tailored to the considerable diversity of body form and function that has emerged across the course of insect evolution.

Research was supported by NIH grants R01 NS32684, NSF grant IBN-9601385 and NSF/ Emory matching grant BIR-943734.

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