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Pattern of Expression of Engrailed in Relation to Gamma-Aminobutyric Acid Immunoreactivity in the Central Nervous System of the Adult Grasshopper

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ABSTRACT

Engrailed (En) protein expression in neurons of the mesothoracic and metathoracic ganglia of the adult grasshopper, *Schistocerca americana*, was examined by immunohistochemistry. Each neuromere had a dorsally located cluster of En-positive neurons within the dorsal unpaired median (DUM) group, comprising one cluster in the mesothoracic ganglion (T2) and four clusters in the metathoracic ganglion, one for each component neuromere (T3, A1, A2, A3). Ventrally, En-positive neurons occurred in the posterior one-third of each neuromere. In T2 and T3, three ventral groups of neurons were labeled bilaterally. In the abdominal neuromeres, many fewer ventral neurons were En-positive. These also were bilaterally symmetrical, but did not occur in patterns that allowed assignment of homology with the T2 and T3 groups. Altogether, En-positive neurons comprised roughly 10% of the ganglionic populations. In the bilateral groups, as in the DUM groups, En expression was restricted to interneurons, consistent with the suggestion that En expression contributes to some aspect of interneuronal phenotype. En-positive neurons in the DUM groups also expressed gamma-aminobutyric acid (GABA) immunoreactivity. Further study showed that all neurons in one En-positive bilateral group and some neurons in another bilateral group were GABA immunoreactive, but that neurons in a third bilateral group were En-positive only. Additionally, several discrete clusters of neurons were GABA-immunoreactive but En-negative. A provisional morphological scheme is presented, which relates the several neuronal clusters to their likely neuroblasts of origin, as a basis for further research into the composition of neuronal lineages. J. Comp. Neurol. 440:85–96, 2001.

Indexing terms: ***Schistocerca americana*; metathoracic ganglion; mesothoracic ganglion; homeodomain; neuroblast; neuronal lineage**

Homeobox genes function in the development and maintenance of neuronal phenotype, in addition to their many other roles in the determination of body pattern. In the adult grasshopper, the homeodomain protein Engrailed (En) is expressed differentially in interneurons but not in efferents 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 have somata in one tight cluster, but their primary neurites and axonal branches trace quite different pathways within the central nervous system (CNS; Thompson and Siegler, 1991, 1993). This observation led us to consider that Engrailed genes might regulate some aspect of pathfinding, and thus neuronal morphology. This hypothesis was tested in *Drosophila* (Siegler and Jia, 1999). It was shown that the En-positive neurons are interneurons and that efferent neurons do not express En. When *engrailed* expression was increased or decreased, certain cell adhesion molecules were altered in expression, consistent with a negative regulation by En. In addition, neuronal morphology

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was disturbed greatly, thus showing a role for En in determining neuronal shape. The normal pattern of differential expression of En and of the cell adhesion molecules was likewise consistent with such functions.

A possible second function for En involves the regulation of neurotransmitter phenotype. Other homeobox genes have been shown to play such a role in neuronal development (Tissier-Seta et al., 1993; Valarché et al., 1993; Jin et al. 1994; Lundgren et al., 1995; Zellmer et al., 1995; Lundell et al., 1996). In the adult DUM group, En is expressed together with the neurotransmitter gamma-aminobutyric acid (GABA). Coexpression occurs in the interneurons of the group, whereas neither En nor GABA is expressed in the efferent neurons of the DUM group (Siegler and Pankhaniya, 1997). However, we noted in passing that many neurons outside the DUM group also expressed En and GABA. These additional observations raised a series of questions: How widespread is neuronal En expression within the ganglia? Does En expression correlate with neuronal type and with GABA neurotransmitter identity? Are En-expressing and GABA-expressing neurons grouped in a fashion that might give clues as to their developmental origins from particular neuroblasts?

The present study had four specific aims. The first two related to the possible roles of En in determining cell shape and neurotransmitter phenotype. One aim was to map the remaining En-positive neurons across several neuromeres in the grasshopper and to provide evidence as to their neuronal type. Another aim was to determine the extent to which there was a positive association between En and GABA expression in neurons other than those in the DUM groups. The next two aims related to the lineal associations of identifiable groups of neurons that expressed En and GABA. One aim was to assess the variability of neuronal number in groups of neurons, especially the DUM groups (which originate embryonically from MNBs). This would provide an indication of the degree to which individual neuronal lineages varied in size between conspecifics, and therein, segment by segment. In particular, we investigated whether the number of so-called dorsal midline neurons (DUM neurons) varied because of differences in lineage sizes, or because the somata of the MNB lineages might also occur ventrally and thus be identified as "ventral unpaired median neurons" (VUMs). The other aim was to provide anatomical and histochemical data that would ultimately aid in the mapping neurons of different phenotypes to particular neuroblasts of origin. These data would provide a basis of reference for our ongoing work on the embryonic development of neuronal lineages in the grasshopper. They would also allow comparison with results from related work in other insects in which the lineal origins and possible homologies among neurons are being examined.

Abbreviations

A1–A3	abdominal neuromeres 1–3
CI	common inhibitor motor neuron
DUM	dorsal unpaired median
En	Engrailed protein, recognized by MAb 4D9
GMC	ganglion mother cell
LAG	lateral anterior group
LDG	lateral dorsal group
LVG	lateral ventral group
ML	midline group of local interneurons
MPG	median posterior group
MNB	median neuroblast
MVG	median ventral group
N1	nerve 1
N3	nerve 3
N4	nerve 4
N5	nerve 5
N6	nerve 6
NB	neuroblast
PVC	posterior ventral commissure
SMC	supramedian commissure
T2	second thoracic neuromere (mesothoracic ganglion)
T3	third thoracic neuromere

MATERIALS AND METHODS

Grasshoppers, *Schistocerca americana*, were obtained from our laboratory colony. Mesothoracic and metathoracic adult ganglia were dissected from cold-anesthetized insects in TES-buffered saline (TBS), pH 7.2, containing the following: 150 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 mM TES (pK_a = 7.4, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; Sigma, St. Louis, MO). Insects were killed by freezing. Ganglia were fixed in 4% paraformaldehyde in TBS containing 90 mM sucrose for 2 hours at 4°C. Following a brief wash in TBT (TBS with 0.5% Triton X-100, v/v), ganglia were dehydrated to absolute alcohol in a graded series to remove fat and permeabilize the tissue. Ganglia were rehydrated to 30% ETOH, then transferred to TBT.

The monoclonal antibody, 4D9, raised against the *invected* (*inv*) gene product of *Drosophila*, recognizes En proteins in a wide range of animals, including the grasshopper (Patel et al., 1989). Hybridoma supernatant was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). For light microscope immunohistochemistry, ganglia were incubated in TBT plus 5% normal horse serum (NHS; Vector Laboratories, Burlingame, CA) for 30 minutes and then placed in a 1:1 dilution of 4D9 in TBT plus 5% NHS for 18–20 hours. Ganglia were washed in TBT and then incubated in TBT plus 5% NHS for 30 minutes. Thereafter, they were incubated for 20–22 hours in a 1:400 dilution of preadsorbed secondary antibody, biotinylated horse anti-mouse IgG (Vectastain ABC Kit, Vector) in TBT plus 5% NHS. For preadsorption of the secondary antibody, preadsorption ganglia were fixed, dehydrated then rehydrated, and placed in a 1:100 dilution of the secondary antibody in TBT plus 5% NHS. Preadsorption ganglia were macerated and the mixture was held at 4°C for 22 hours with agitation. The mixture was centrifuged at low speed and the supernatant was diluted as described above. After washing, ganglia were incubated in a preformed complex of avidin and biotinylated horseradish peroxidase (HRP) from a Vectastain ABC Kit (Vector). They were processed following the manufacturer's instructions by using 3'3-diaminobenzidine tetrahydrochloride (DAB, Sigma) and nickel enhancement. Ganglia were dehydrated in ETOH and cleared in methyl salicylate. Control ganglia, processed as above but omitting either the primary or the secondary antibody, showed only faint, nonspecific background darkening. A modification of Timm's silver reaction was used to reveal octopamine-containing neurons in whole mount preparations (Siegler et al., 1991). Double-labeled preparations were processed first for the Timm's silver reaction, then for En immunohistochemistry as described above. Selected ganglia were

embedded in Eponate (Ted Pella, Redding, CA) and sectioned at a thickness of 8 μm.

In double-labeling experiments to reveal En and GABA immunoreactivity, tissue was fixed in 3.6% paraformaldehyde in TBS and processed for confocal microscopy by sequential labeling for En and GABA immunoreactivity. 4D9 was used at 1:1 and the second antibody was used at 1:100 (Cy-5-conjugated goat anti-mouse IgG; no. 115-175-003, Jackson Immunoresearch, West Grove, PA), with prior blocking with NHS and intervening washes as described above. Immunohistochemistry for GABA used the primary antibody at 1:300 (rabbit anti-GABA; no. A-2052, Sigma) and the second antibody at 1:400 (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG; no. 111-095-003, Jackson Immunoresearch), again with intervening washes. After processing, ganglia were mounted in SloFade mounting medium (Molecular Probes, Eugene, OR). Control ganglia, processed as above but omitting either the primary or the secondary antibodies, or both, showed only faint, nonspecific background fluorescence when illuminated during confocal microscopy.

For retrograde labeling, connectives were backfilled by using a 4% solution of biocytin, as described elsewhere for cobalt chloride (Siegler and Pousman, 1990). After diffusion of biocytin and fixation, ganglia were incubated in a 1:200 solution of streptavidin HRP in TBT, washed, and developed as described above for light microscope immunohistochemistry.

For light microscopy, photographs were taken by using differential interference contrast (DIC) optics on a Zeiss Axiophot microscope (Zeiss, Thornwood, NY). Color 35-mm photographic slides were scanned onto a Kodak Photo CD (Rochester, NY). The images were cropped, arranged as figures, and converted to gray scale images by using PhotoShop®. To count the numbers of labeled neurons and measure their soma diameters, ganglia were viewed under a Zeiss compound microscope equipped with a camera lucida. Drawings were made at 100× and 400× of the dorsal and ventral cortices. For confocal microscopy, ganglia were scanned by using a BioRad 1024 laser scanning confocal microscope (Hercules, CA) with a Zeiss compound microscope. A Z-series of images was collected for each preparation at a Z step of 1.0 μm. Four sweeps were averaged for each step by use of simultaneous scanning. Projected images were compiled by using BioRad processing software. Confocal and light microscopy digital image files were printed with a Codonics (Middleburg Heights, OH) dye-sublimation printer.

RESULTS

En protein expression is stereotyped in adult CNS

En immunoreactivity was examined in neurons of segmental ganglia of the adult ventral nerve cord: the mesothoracic ganglion, which is the second thoracic (T2) neuromere, and the metathoracic ganglion, which comprises the third thoracic neuromere (T3) and the first through third abdominal (A1–A3) neuromeres. The pattern of En immunoreactivity was similar qualitatively in all preparations examined (n = 126). To provide further evidence for stereotypy, eight preparations were examined in detail by making camera lucida drawings of En-positive somata (Fig. 1, see also Fig. 3). Counts of the number of labeled neurons (mean and SD) within each neuromere were summed (dorsal plus ventral) or separated by position (dorsal or ventral) within the cortex (columns A-C, Table 1).

Pattern in the mesothoracic ganglion

In the mesothoracic ganglion, 164 neurons on average are En-positive (column B, Table 1). These are grouped loosely within the posterior region of the cortex and labeled somata are scattered among those that are unlabeled (Figs. 1a,b). The most obvious En-positive group occurs posteriorly at the midline of the ganglion and contains about 64 somata of small diameter (open arrows, Figs. 1c-e, 2). These are interneurons of the DUM group and they are ventral to the En-negative somata of efferent DUM neurons (short black arrows indicate efferents in Fig. 2). Three other groups are referred to as "A", "B," and "C" groups. The A group is close to the ventral surface and anterior to the DUM group of the ganglion (open arrowheads in Figs. 1c-e, 2a). The A group contains about 20 neurons, some of which are arrayed in bilateral pairs. The somata of the A group are near the somata of the common inhibitory (CI) motor neurons (compare Figs. 2a,b). The bilateral B group is lateral and anterior to the DUM group and contains about 35 somata within each half of the ganglion (vertical arrows, Figs. 1c-e). The B group is adjacent to the larger somata of three or so En-negative motor neurons. The small C group in each half of the ganglion typically contains three neurons with small diameter cell bodies (black arrowheads, Figs. 1c-e).

Pattern in the metathoracic ganglion

In the metathoracic ganglion, the most obvious groups of En-positive somata lie along the dorsal midline in the DUM groups (Figs. 3a,d,e). The DUM group in T3 contains about 70 En-positive neurons, in A1 about 50–60, and in A2 about 20 (column B, Table 1). These are interneurons (Siegler and Pankhaniya, 1997). In the T3 DUM group, they are adjacent to or intermingled with about 20 larger En-negative somata of efferent neurons (Fig. 3d). In the A1–A3 DUM groups, the En-positive somata comprise most of the group, there being only three larger En-negative somata of efferent neurons (A1 is shown in Fig. 3e).

The metathoracic ganglion has numerous En-positive neurons. Apart from those in the dorsal DUM group, all are in the ventral cortex (Figs. 3b,c). The T3 neuromere has three ventral groups of En-positive neurons that are comparable in size and relative position to groups in the T2 neuromere. The A group at the midline contains 10–20 loosely grouped En-positive somata (open arrowheads, Figs. 3f,g). These are scattered near the larger En-negative somata of CI motor neurons, two of which are indicated (CI, Figs. 3f,g). The bilateral B group is posterolateral to the CIs and contains about 35 somata on each half of the ganglion (vertical arrows, Figs. 3f,g). The tiny bilateral C group in each half contains about three neurons with small diameter somata (black arrowheads, Figs. 3f,g). The ventral regions of the A1 and A2 neuromeres contain many fewer En-positive neurons than does T3 (column B, Table 1). A small group of En-positive somata occurs at the ventral midline of A1, A2, and A3. The somata are probably progeny of the MNB, but En-positive somata are otherwise scattered, with no groupings comparable to those of T2 and T3 (Figs. 3c,f,g).

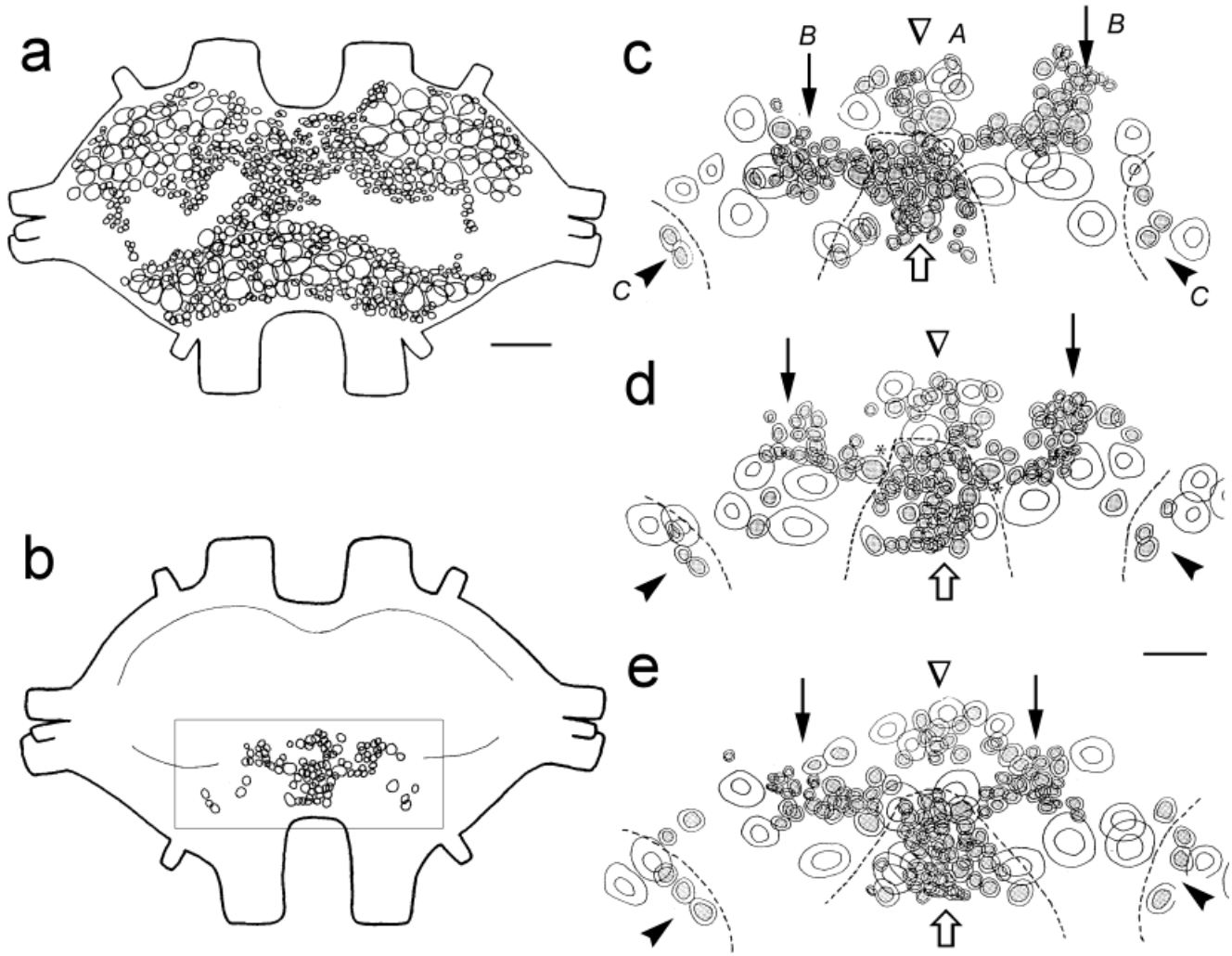

Fig. 1. Engrailed (En) immunoreactivity in the mesothoracic ganglion, illustrated by camera lucida drawings. **a:** Neuronal somata of the ventral ganglionic cortex, drawn from toluidine blue-stained whole-mount preparations (Altman and Bell, 1973). **b:** Somata of neurons containing En-positive nuclei, including all planes of focus in the ganglion. The labeled neurons occur only within the posterior one-third of the ganglionic cortex. **c-e:** Enlarged views of the posterior one-third of the mesothoracic ganglion from a region comparable to the rectangle indicated in b. The views show cell body outlines and nuclei of En-positive neurons and of selected unlabeled neurons with large-diameter somata. The nuclei of En-positive neurons are stippled. Dotted lines at the posterior midline of c-e demarcate a group of somata at the posterior of the ganglion (open arrows) that are more dorsal than the other labeled neurons and contain dorsal unpaired median (DUM) neurons, neuronal progeny of the median neuroblast (MNB). Dotted lines at the lateral edges demarcate the most lateral group of labeled neurons (C group, black arrowheads). A labeled group at the midline anterior to the MNB progeny contains bilaterally paired somata (A group, open arrowheads). A bilaterally symmetrical group of neurons (B group, vertical arrows) lies lateral to the midline groups. Scale bars = 200 μm in a,b, 100 μ in c-e.

Variability among preparations was most obvious along the midline of the metathoracic ganglion. For example, in the A2 neuromere, some preparations had few En-positive somata at the ventral midline (Fig. 3f), whereas others had a distinct cluster (Fig. 3g). Although some variability can be attributed to counting error and to experimental variation, ganglia clearly differ in the dorsoventral distribution of En-positive somata. Column D in Table 1 shows the percentage values of the SD divided by the mean for each set of counts. The counts of dorsal and ventral En-positive neurons considered separately (dorsal or ventral) were more variable than were counts of the En-positive populations as a whole (dorsal plus ventral). Variability was most pronounced for the abdominal neuromeres. In A1, the SDs were 14% and 12% for the dorsal and ventral counts, respectively, but only 3% of the mean for the population as a whole. In A2, the SDs were 23% and 25% of the mean for the dorsal and ventral counts respectively, but only 9% for the total.

For individual preparations, the number of En-positive neurons in the ventral cortex were related inversely to the number in the dorsal cortex (Fig. 4a), suggesting that neurons vary in soma position dorsoventrally. A likely source of variability is in the positions of the DUM groups, which shift during embryogenesis (Thompson and Siegler, 1993). The maximum number of dorsally located En-

TABLE 1. Number of En-Positive Neurons by Ganglion and Dorsoventral Location

Column	A Location of En-positive neurons	B Mean counted	C SD	D SD/mean (variability)	E Range counted	F Approximate number by type[1]	G Labeled population (%)[2]
Mesothoracic ganglion							
T2 (n = 7)	Dorsal plus ventral	164	12	7%	150–179		100.0
	Dorsal	64	4.1	6%	57–69	69 unpaired	42.1
	Ventral	100	12.3	12%	88–117	48 bilateral pairs	29.0
Metathoracic ganglion							
T3 (n = 8)	Dorsal plus ventral	166	7.8	5%	156–174		100.0
	Dorsal	70	3.9	6%	64–75	75 unpaired	45.2
	Ventral	96	6.9	7%	87–104	46 bilateral pairs	27.4
A1 (n = 8)	Dorsal plus ventral	104	2.8	3%	100–109		100.0
	Dorsal	50	7.1	14%	39–60	60 unpaired	57.7
	Ventral	54	6.5	12%	45–63	22 bilateral pairs	21.2
A2 (n = 8)	Dorsal plus ventral	46	4.2	9%	43–55		100.0
	Dorsal	18	4.2	23%	12–23	23 unpaired	50.0
	Ventral	28	6.9	25%	21–40	12 bilateral pairs	25.0

[1]Number of unpaired dorsal neurons is assumed to be equal to the highest value counted in the range, whereas the total of population is assumed to be equal to the mean value.
[2]Percentages for bilateral pairs are values for half of a neuromere.

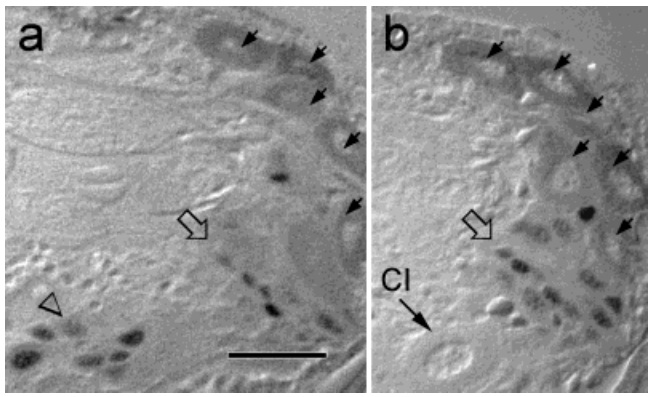

Fig. 2. Engrailed (En)-positive neurons at the midline of the mesothoracic ganglion. **a,b:** Two adjacent longitudinal sections, cropped to show the posterior of the ganglion. At the top of each panel is the dorsal surface of the ganglion, at the right edge of each panel is the posterior margin of the ganglion, and at the bottom is the ventral cortex of the ganglion. Four types of cell bodies are apparent. An open arrow indicates small somata that have En-positive nuclei and lie within the posterior midline dorsal unpaired median (DUM) group. Dorsal to them are the En-negative somata of efferent DUM neurons (short oblique arrows). Prior to En immunohistochemistry, the ganglion was processed for the Timm's silver reaction, to label the cytoplasm of octopamine-containing efferent DUM neurons. In a, an open arrowhead indicates En-positive somata that are part of the more anterior midline A group. In b, the large unlabeled cell body of a common inhibitory (CI) motor neuron is indicated. Scale bar = 100 μm.

positive somata in T3 and A1 counted in any preparation (column E, Table 1) matched the estimated number of interneurons within the DUM groups of these neuromeres (Thompson and Siegler, 1991, 1993). We suggest that preparations with the lower numbers of dorsal En-positive neurons have somata shifted to the ventral midline. The variable dorsoventral distribution of DUM efferents supports this idea (Fig. 4b). Silver staining reveals about 20 efferent neurons in the T3 DUM group and three efferent neurons in A1 and A2, with their positions more variable in A1 and A2 than in T3. Of the preparations examined for the T3 neuromere (n = 21), most had 18–21 dorsal somata and no ventral somata. In A1, the distribution of somata was divided about equally among the four possibilities, ranging from all dorsal to all ventral somata (n = 19). In A2, the most commonly observed distribution was that of one dorsal and two ventral somata (about 40% of cases, n = 14).

Evidence for type-specific expression

En immunoreactivity occurs only within the smaller-diameter interneuronal somata of T3 and A1 DUM groups (Figs. 3d,e; Siegler and Pankhaniya, 1997). The En-positive somata in the bilateral groups are also relatively small in diameter compared with somata of efferent DUM neurons. We can also identify other motor neurons based on their size and position, and these are invariably En-negative. In T3, these include the two pairs of CIs that have cell bodies at the ventral midline (CI, Figs. 3f,g; open arrowheads, Fig. 5) and at least three pairs of excitatory motor neurons (oblique arrows, Fig. 5a). No En-positive neurons occur in the lateral regions of the T2 and T3 neuromeres (Figs. 1b, 3c), where somata of leg and wing motor neurons are located (Siegler and Pousman, 1990). Conversely, the most conspicuous clusters of En-positive somata occur in the same positions as do somata of interneurons and have somata of similar diameters (compare Figs. 5b,c). These data provide strong evidence that all En-positive neurons are interneurons.

Overlap with GABA immunoreactivity

In the DUM groups, all En-positive interneurons are immunoreactive for the neurotransmitter GABA (Siegler and Pankhaniya, 1997). To determine whether this is true also for other En-positive neurons, ganglia were doubled labeled using an anti-GABA antiserum along with the En antibody. The major groups of GABA-immunoreactive somata have been described (Watson, 1986). In the ventral cortex, these are the lateral ventral group (LVG), the medial ventral group (MVG), and the medial posterior group (MPG), all shown in Figure 6. In addition, not shown in Figure 6, two other groups of GABA-positive somata are anterior and lateral to the LVG, the lateral anterior group (LAG) and the lateral dorsal group (LDG; Fig. 7b).

The large MPG region of GABA-positive somata (Watson, 1986) includes the only bilateral neurons that are En-positive. The GABA-positive neurons in this region lie in two rows across the ganglion (Fig. 6a). GABA-positive clusters in the more anterior row are indicated by

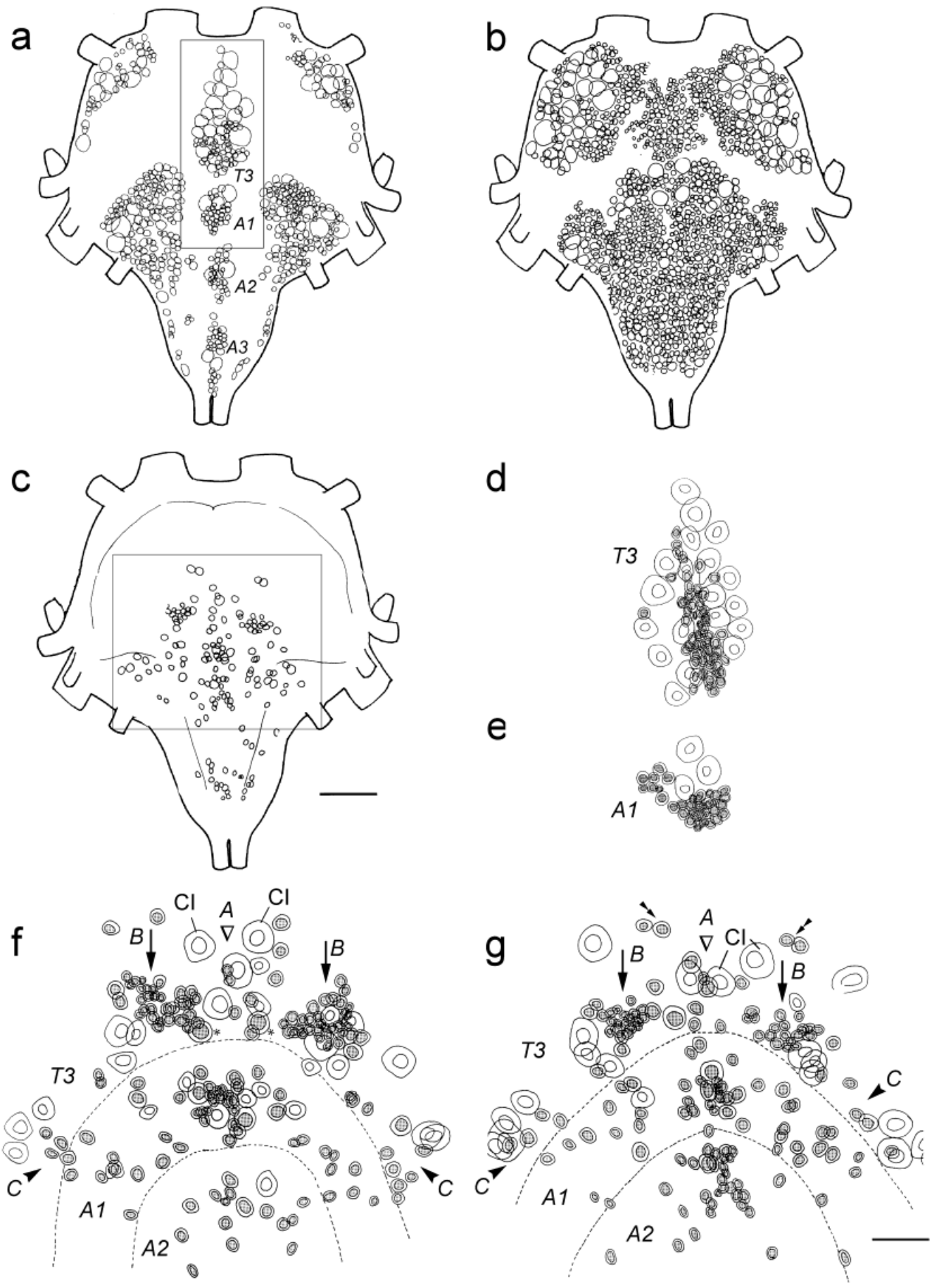

Figure 3

downward vertical or oblique arrows and include neurons of the A and B groups. The A group is heterogeneous, containing some neurons that are En positive and GABA-positive (horizontal arrows, Fig. 6b) and some neurons that are En-positive only (asterisks, Fig. 6a,b). The midline region posterior to the A group also contains En-positive nuclei of glia (oblique upward arrows, Fig. 6b). The B group contains neurons that are both En- and GABA-positive (Figs. 6a,c). Lateral to the B group is a third group in the anterior row, about equal in size to the B group, that contains GABA-positive but En-negative neurons (oblique downward arrows, Figs. 6a,c). The three groups are close to the somata of the GABA-positive CIs (white dots).

Clusters of GABA-positive somata in the more posterior of the two rows are indicated by upward vertical or oblique arrows (Fig. 6a). From the midline outward these include a small bilateral group close to the midline (long vertical upward arrows), a large bilateral group medial to the C group (short vertical upward arrows), and a small bilateral group lateral to the C group (oblique upward arrows). All are En-negative. Neurons of the C group are GABA-negative but En-positive (upward oblique arrowheads, Fig. 6a), but lie along the posterior row of GABA-positive clusters.

The T3 neuromere of the metathoracic ganglion contains clusters of GABA-positive neurons that are comparable to those of the mesothoracic ganglion. In the abdominal neuromeres, however, somata of En-positive GABA-positive neurons are scattered in the cortex (not shown) and, as such, they are not matched easily to the T2 or T3 neuronal groups.

DISCUSSION

Cell type specificity of En expression

From earlier work on the grasshopper, we hypothesized that the differential expression of En is instrumental in the development and maintenance of neuronal shape, by mediating the differential expression of cell adhesion or cell-affinity molecules (Siegler and Pankhaniya, 1997). Indeed, in *Drosophila*, we identified two adhesion molecules that are downregulated by En, Connectin and Neuroglian, neither of which normally is expressed in En-positive interneurons (Siegler and Jia, 1999). In the grasshopper, En continues to be expressed in the adult CNS. Based on earlier and present data, we suggest that En expression continues to function in maintaining adult neuronal phenotype.

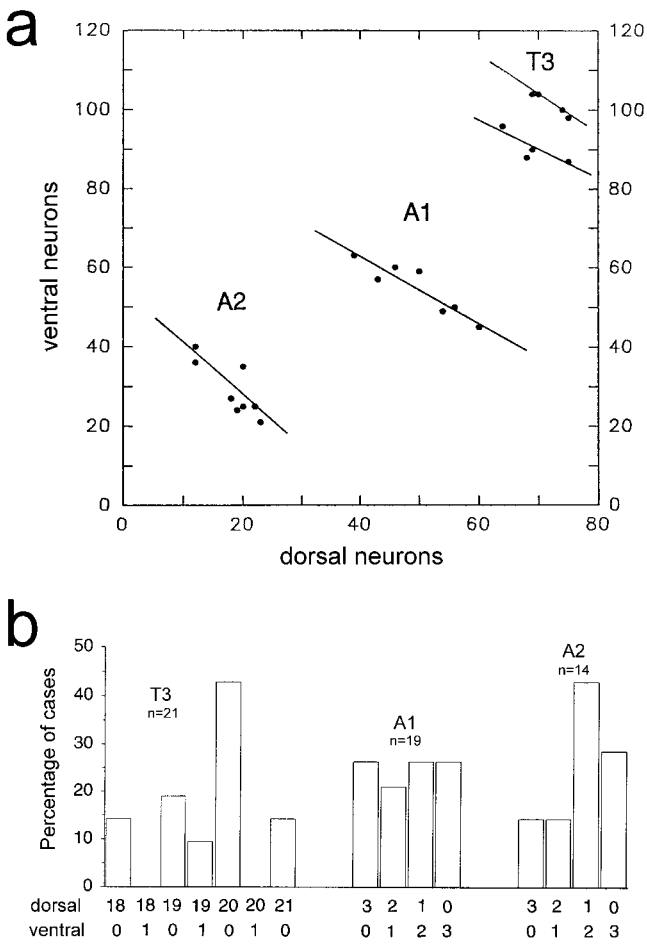

Fig. 4. Variable dorsoventral distribution of somata. **a:** The somata of Engrailed (En)-positive neurons are distributed variably between the dorsal and ventral cortex. For each of eight preparations, the number of En-positive nuclei in the ventral cortex is plotted against the number of En-positive nuclei in the dorsal cortex. For the T3 neuromere, two groups represent the results from two labeling runs. The upper first-order linear regression line has a slope of -1.0 ventral/dorsal and the lower first-order linear regression line has a slope of -0.74 ventral/dorsal. For the A1 and the A2 neuromeres, first-order linear regression lines plotted a slope of -0.84 ventral/dorsal and -1.36 ventral/dorsal, respectively. **b:** The somata of En-negative efferent dorsal unpaired median (DUM) neurons are distributed variably between the dorsal and ventral cortex in T3, A1, and A2 neuromeres. Neurons were stained by the Timm's silver reaction. For each neuromere, the preparations with the indicated number of dorsal and ventral somata are plotted as a percentage of the number of preparations examined.

Fig. 3. Engrailed (En) immunoreactivity in the metathoracic ganglion, illustrated by camera lucida drawings. **a:** Neuronal somata of the dorsal ganglionic cortex, drawn from toluidine blue-stained whole-mount preparation. The dorsal unpaired median (DUM) group in each neuromere lies at the midline, adjacent to the neuromere label. **b:** Neuronal somata of the ventral ganglionic cortex, drawn from toluidine blue-stained whole-mount preparation. **c:** Somata of neurons containing En-positive nuclei that lie within the ventral cortex. The labeled neurons are scattered widely in the ventral cortex, but in T3 occur predominantly within the posterior one-third of the neuromere. **d,e:** Enlarged views of the DUM groups of T3 and A1, showing cell body outlines and nuclei from a region comparable to the rectangle indicated in a. The nuclei of En-positive neurons are stippled. **f,g:** Enlarged views of the ventral cortex of the metathoracic ganglion, from a region comparable to the rectangle indicated in c, in two preparations. The nuclei of En-positive neurons are stippled. The dashed lines indicate the boundaries between the T3, A1, and A2 neuromeres. In T3, two somata of common inhibitor motor neurons (CI) are indicated. Three groups of En-positive somata are indicated with an open arrowhead (A group), vertical arrows (B group), and a black arrowhead (C group). Other labeled neurons occur as pairs but are not assigned readily to groups, e.g., a pair of relatively large cell bodies near the midline in T3 (asterisks in f) and two pairs of cell bodies anterior and lateral to the inhibitory motor neurons (double arrowheads in g). The A1 and A2 neuromeres each have a small group of labeled neurons at the midline, but other labeled nuclei are scattered and do not fall into obvious groups. A3 (not shown) is similar to A1 and A2 in this respect. Scale bars = 200 μm in a-c, 100 μm in d-g.

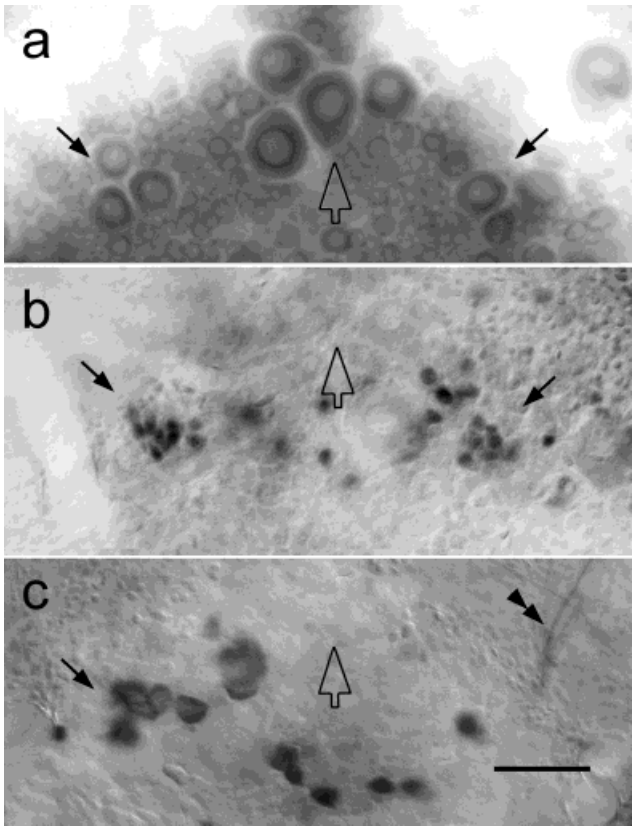

Fig. 5. Evidence that Engrailed (En) is expressed in interneurons but not in motor neurons. The ventral midline region of the T3 neuromere is shown, viewed with anterior at the top. **a:** Toluidine blue staining. Four large somata at the midline belong to two pairs of inhibitory motor neurons (open arrow; also indicates inhibitory motor neurons in b,c). Two or three medium-sized lateral somata (oblique arrows) on each half of the ganglion are those of excitatory motor neurons to coxal muscles (Siegler and Pousman, 1990). **b:** En immunoreactivity occurs predominantly in nuclei of small somata clustered around the lateral motor neurons (oblique arrows). **c:** Somata of intersegmental interneurons, labeled retrogradely with biocytin from a contralateral descending intersegmental connective, occur in the same regions as do En-positive nuclei. Most biocytin-labeled somata occur in the lateral region that contains En-positive nuclei (oblique arrow), contralateral to the labeled connective, with a scattering of labeled somata at the midline. Only a few of the labeled descending axons are seen in this plane of focus (double arrowhead). Scale bar = 100 μm.

En expression within bilateral groups is restricted to interneurons, as is the case for neurons within the DUM groups. Identified efferent neurons within the DUM group (Siegler and Pankhaniya, 1997) or motor neurons adjacent to the bilateral groups described in the present study do not express En. In embryonic *Drosophila*, all En-positive neurons in the CNS are interneurons (Siegler and Jia, 1999). The present results are also consistent with this division by neuronal type.

Nonetheless, differential expression of En cannot alone or uniquely determine the choice between an interneuronal or efferent neuronal fate, given that only a fraction of interneurons express En. The number of En-positive neurons is essentially the same in the T2 and T3 neuromeres, i.e., about 164 neurons. A1 and A2 contain about 100 and 50 En-positive neurons, respectively. T2 is estimated to contain about 2,000 neurons. The T3 neuromere of the metathoracic ganglion probably contains a like number. The En-positive neurons account for about 8% of the neurons in T2 and T3. No counts are available of the neuronal number for the A1–A3 neuromeres, but the unfused abdominal neuromeres each contain about 400 neurons. Based on relative size, we estimate that A1 contains about 800 neurons and that A2 contains about 480 neurons. Therefore, En-positive neurons would account for about 13% and 9.5% of the respective neuronal populations. The En-positive neurons would comprise a somewhat larger percentage of interneurons altogether because 10–15% of the total neurons in any neuromere are motor neurons, which are En-negative.

En expression likely confers some shared morphological properties, this accomplished by guiding the selection of particular morphological pathways during development through the differential expression of certain cell adhesion molecules (Siegler and Jia, 1999). The En-positive interneurons do share some morphological features, based on our results from the grasshopper here and elsewhere (Siegler and Pankhaniya, 1997) and on results of our study of En-positive neurons in *Drosophila* (Siegler and Jia, 1999). En-positive neurons either have primary neurites that branch with bilateral symmetry upon bifurcation at the midline (DUM interneurons) or primary neurites that traverse the midline and give rise to contralateral axons (other interneurons). In either case, the primary neurites traverse a posterior commissure of the neuromere. The DUM interneurons have their primary neurites within the posteriorly located supramedian commissure (SMC). The small lateral C group of En-positive interneurons, which are similar in all respects to serotonin-positive interneurons (see below), have their primary neurites within the posterior ventral commissure (PVC; Tyrer et al., 1984). The other En-positive interneurons also traverse the midline in one or more of the posterior commissures. In embryonic *Drosophila*, En-positive neurons (interneurons) traverse the midline likewise only within the posterior commissure. The intersegmental interneurons in the grasshopper and *Drosophila* also appear to share the feature of having anteriorly directed axons, a possibility that deserves further investigation. Nonetheless, it is likely that En is only one of several regulatory molecules that, by means of specific patterned expression among the population of interneurons, serve to direct the expression of particular combinations of cell adhesion and guidance molecules and thus ultimately to determine neuronal morphology. We expect, therefore, that other regulatory molecules singly or in combination will be correlated uniquely with other interneuronal types.

Whether En expression also has a role in determining neurotransmitter phenotype is an open topic. In the DUM groups of grasshopper, only the neurons that are En-positive also express GABA (Siegler and Pankhaniya, 1997). The B group, like the DUM group, is En- and GABA-positive. The C group is GABA-negative and, as discussed below, is likely to be immunoreactive for serotonin. The A group contains neurons that are GABA-negative, but are also unlikely to be positive for serotonin. Moreover, only a subset of neurons that are GABA-positive are also En-positive. Thus, En expression may be necessary, although not sufficient, for the development of neurotransmitter phenotypes only in particular subsets of

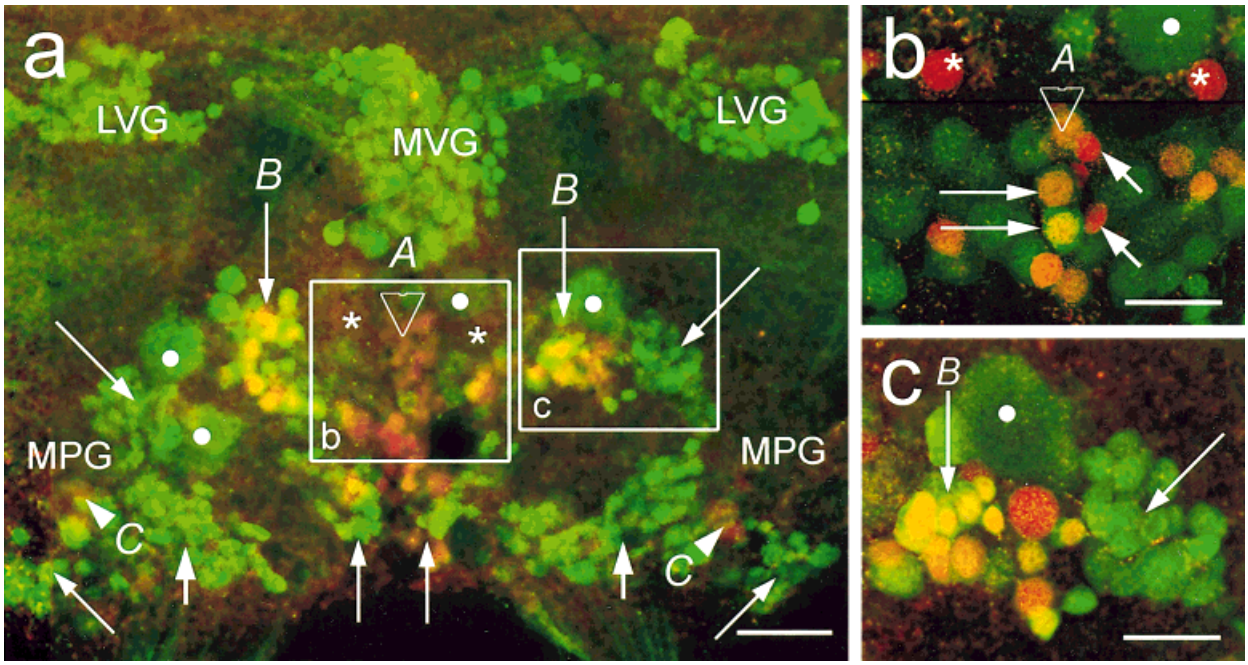

Fig. 6. Comparison of Engrailed (En) and gamma-aminobutyric acid (GABA) immunoreactivity. Confocal images of the ventral posterior region of the mesothoracic ganglion double labeled for En (red) and GABA (green). The dorsal unpaired median (DUM) group is not shown because it lies dorsal to the plane of the scans. **a:** The major groups of GABA-positive somata are the medial ventral group (MVG), the lateral ventral group (LVG), and the medial posterior group (MPG) (Watson, 1986). The identified A, B, and C groups of En-positive neurons are so labeled. C group neurons are GABA-negative. Within the MPG, clusters of GABA-positive neurons fall into an anterior row (clusters indicated by downward vertical or downward oblique arrows) and a posterior row (clusters indicated by upward vertical or upward oblique arrows). Common inhibitor motor neurons (CIs) are marked by white dots. Asterisks indicate the positions of two neurons depicted in b. This image is a projection of a Z-series taken with 10-μm steps, encompassing an 80-μm thickness. The left edge of part a includes an added small vertical strip (montage) obtained from a separate image file of an overlapping adjacent region, projected as for the main part of the image. See text for further description. **b:** Enlarged view of the A group region, corresponding to the "b" rectangle indicated in part a. In this case, the image from a single confocal section is shown above and below the black line, each chosen from one comprising the projected image of a, to show the labeled nuclei most clearly. Labeling is heterogeneous. The region includes neurons that are En-positive only (asterisks), neurons that are En-positive and GABA-positive (horizontal arrows), and En-positive glia (upward oblique arrows), as well as several neurons that are GABA-positive only (not indicated individually). **c:** Enlarged view of the B group region, corresponding to the "c" rectangle indicated in part a. A single confocal section is shown. The B group (left, downward arrow) contains neurons that are En- and GABA-positive. The B group is medial to a cluster of neurons that are GABA-positive but En-negative (downward oblique arrow) and posterior to the soma of a CI (white dot). Scale bars = 100 μm in a, 50 μ in b,c.

neurons, as is true for the NB 7-3 progeny in *Drosophila* (Lundell et al., 1996). Similarly, in *C. elegans*, another homeodomain protein, UNC-30, is necessary for neurotransmitter regulation only in a subset of GABA-expressing neurons (Jin et al., 1994).

Segmental differences in neuronal number and type

We also consider the variability and segment-specificity in the groups of neurons identified here. Assessing such is complicated by the fact that En-positive somata vary in dorsoventral position, particularly at the midline within the "DUM" groups of the abdominal neuromeres, which arise embryonically from MNBs. The neuronal somata that lie along the posterior edge of a neuromere shift in position during embryonic fusion of the metathoracic ganglion. As the neuropilar core of the neuromeres enlarges, somata that are adjacent initially could come to lie variably within the dorsal or ventral cortex. The A1 neuromere is separate from the A2 and A3 neuromeres until about stage 65% of embryonic development, when the interganglionic connectives shorten and the gaps are closed between A1, A2, and A3. The T3 and A1 neuromeres fuse earlier, around stage 50%, perhaps accounting for the smaller variability of soma position in the T3 neuromere. Thus, "DUM" neurons in abdominal neuromeres may have either dorsal or ventral somata, as shown here (giving rise instead to "VUM" neurons or ventral unpaired median neurons). Differences in soma position are a minor feature of neuronal morphology (Siegler and Pousman, 1990) and do not alter the functional properties of neurons, because synaptic connections occur only in the inner, neuropilar core of the ganglion. For example, neurites of identified "DUM" and "VUM" efferent neurons branch similarly within the neuropilar core (Pflüger and Watson, 1988).

In analyzing our numerical data, we started from the assumption that the largest number of dorsal En-positive neurons counted in any preparation was equivalent to the minimum contribution of the MNB to the interneuronal population in each neuromere (highest count of dorsal neurons, column E, Table 1). This value was subtracted from the mean total of En-positive neurons (dorsal plus ventral, column B, Table 1) and divided by two, to esti-

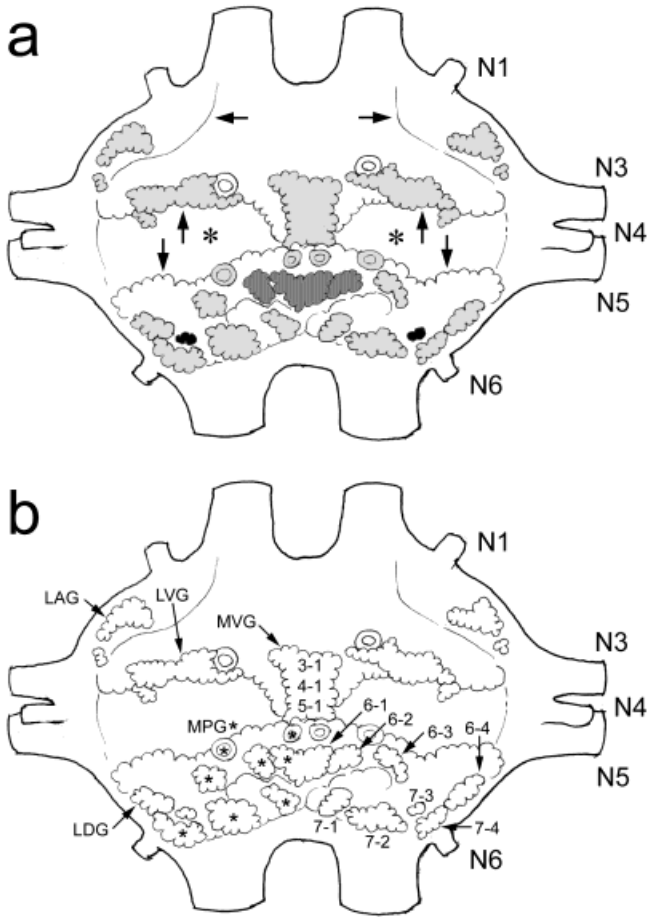

Fig. 7. Schematic summary of labeled neuronal clusters and provisional assignment of their originating neuroblasts, shown for the adult mesothoracic ganglion, viewed ventrally. **a,b:** The individual clusters are outlined. Thin lines inside the boundary of the ganglion trace the approximate border of the neuropil (horizontal arrows, a). Thicker lines contiguous with several of the labeled groups trace the extent of all neuronal somata seen in the ventral cortex of the ganglion (vertical arrows, a). A large area, comprising roughly the central third of each half-ganglion, contains no neuronal somata, having the ganglionic sheath closely apposed to the neuropilar core of the ganglion (asterisks, a). In a, labeled clusters and a few individual neurons are coded as follows: light gray, the cluster contains Engrailed (En)-negative, gamma-aminobutyric acid (GABA)-positive neurons; dark gray, the cluster contains predominantly En-positive, GABA-positive neurons; black, the cluster contains En-positive and GABA-negative neurons. For other abbreviations, see list. b: On the left, clusters are labeled according to Watson (1986). The MPG contains several clusters, each indicated by an asterisk. On the right, several clusters are labeled according to their originating neuroblasts, based on available data in the literature and on our further provisional assignments of identity, as explained in the text. For other abbreviations, see list.

mate the number of bilaterally paired neurons within a hemineuromere (number of bilateral pairs, column F, Table 1). By using this method, bilateral groups are inferred to contribute about 45 pairs of interneurons to the En-positive population in T2 and T3 neuromeres, 22 pairs in the A1 neuromere, and 12 pairs in the A2 neuromere.

Differences in the numbers of En-positive interneurons among the different neuromeres can be accounted for by at least three factors: differences in neuroblasts longevity, differences in neuroblast division rate, and differential cell death. The neuroblasts of T2 and T3 are longer-lived during embryogenesis than are neuroblasts of the abdominal neuromeres and they divide at a higher rate (Shepherd and Bate, 1990). The MNB, which gives rise to the DUM group, dies in T3 stages 75–80% of embryogenesis, in A1 between stages between 60–65%, and in A2–A4 between stages 60–65% (Thompson and Siegler, 1993; Siegler, unpublished observation). This would partially account for the differences in neuronal number. The MNB proliferation rate is initially similar in T2, T3, and in A1–A4. However, after stage 45% embryogenesis, the proliferation rate in A2–A4 drops to about half that in T2–A1 (Siegler, unpublished observation), accounting for further differences in group sizes. Cell death also contributes significantly to the smaller size of the DUM neuron population in A1 as compared with T3 (Thompson and Siegler, 1993) and cell death is more widespread generally in the abdominal neuromeres than in the thoracic neuromeres (Goodman and Bate, 1981). Whether there are also segmental differences in lineal cell fate cannot be assessed at present. Nonetheless, a comparison of the mature pattern of En expression described here with the En pattern in the developing embryo provides a basis at least for determining the potential contribution of differential proliferation and differential cell death to segmental differences in the complement of neuronal types.

Provisional assignment of groups by embryonic origin as a framework for further research

The distribution of En-positive interneurons into a limited number of groups in the adult and their morphological relationship to GABA-expressing neurons prompts us to consider their possible neuroblasts of origin, thus providing a provisional frame of reference for our continuing investigations of neuronal type in embryonic development. Figure 7a recapitulates the distribution of En-positive and GABA-positive groups in schematic form, leaving aside the MNB group, which already has been reported in detail. Figure 7b depicts our provisional view of the originating neuroblasts, also taking into account data available from other studies. The position of En-positive neurons within a ganglion and their placement relative to the clusters of GABA-expressing neurons suggest that the majority of En-positive neurons originate from neuroblasts within rows 6 and 7, the two most posterior rows of the embryonic neuroblast array. Although NB 1-2 (or NB 1-1) gives rise to progeny that initially express En in the embryo (Condron et al., 1994; Broadus and Doe, 1995), no neurons of comparable position were found in the adult. All of the En-positive neurons have somata posterior to the MVG of GABA-positive neurons (Watson, 1986), the MVG comprising progeny of NB 3-1, NB 4-1, and NB 5-1 (Shepherd and Laurent, 1992). The more posterior GABA-positive neurons are contained within the large MPG and contiguous LDG (Watson, 1986), which spans from the midline laterally to the area between nerve 6 (N6) and the outer edge of the posterior connective and is known to contain at least three separate embryonic groups (O'Dell and Watkins, 1988). With the exception of the three pairs of CIs, all of the GABA-positive neurons are interneurons. The disposition of En-positive neurons relative to the MPG and the LDG, and

the clustering of GABA-positive neurons, indicates that the clusters arise from row 6 and row 7 neuroblasts (Fig. 7).

Row 7 neuroblasts, along with the MNB, comprise the most posterior neuroblasts in each neuromere (Bate, 1976; Doe and Goodman, 1985). These neuroblasts arise from an En-positive strip of neurectoderm. However, within row 7, only the progeny of NB 7-3 (or perhaps NB 7-2) stably maintain En expression in the embryo (Condron et al., 1994). These En-positive neurons express serotonin (Condron et al., 1994) and comprise an unusually small lineage (6–10 neurons) that is also attributed to NB 7-3 (Taghert and Goodman, 1984). A small group of serotonin-immunoreactive interneurons have been described in the adult grasshopper (Tyrer et al., 1984), with somata in the same position as those of the tiny lateral C group consisting of two to three En-positive neurons, providing strong evidence that these indeed comprise NB 7-3 progeny. Comparable neurons that are both serotonin- and En-positive occur in *Drosophila* (Lundell et al., 1996), also deriving from NB 7-3 (Bossing et al., 1996; Schmidt et al., 1997).

Using the C group (NB 7-3 group) as a landmark (Figs. 6a, 7b), we suggest that the clusters of GABA-positive somata medial to the C group, which are En-negative, originate from NB 7-1 and NB 7-2. The more lateral cluster of GABA-positive and En-negative neurons may originate from NB 7-4. GABA-positive clusters also have been described in *Manduca*, grasshopper, and several other insects using nomenclature from *Manduca* (Witten and Truman, 1998). Based on soma position, we suggest that the posterior portions of the N and M groups of *Manduca* are equivalent to the more medial clusters delineated in this study (putative NB 7-1 and NB 7-2 progeny), whereas part of the T group corresponds to putative NB 7-4 progeny.

Row 6 neuroblasts likewise derive from En-positive neurectoderm. NB 6-1 and NB 6-2 give rise to En-positive neurons in the early embryo (Condron et al., 1994). Based on relative position, we suggest that the En-positive B group originates from NB 6-2, whereas the heterogeneous A group originates from NB 6-1. In the adult, interneurons of both groups have primary neurites that cross the midline in one of the posterior commissures, as seen in dye backfillings. Homologous interneurons may occur in *Drosophila*. In *Drosophila*, NB 6-1 and NB 6-2 both give rise to interneurons with primary neurites that extend contralaterally across the midline in a portion of the embryonic posterior commissure (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). Also in *Drosophila*, subsets of En-positive interneurons are similar in soma position and morphology to the NB 6-1 and NB 6-2 progeny. The B group and a part of the heterogeneous A group in grasshopper may also be comparable to the anterior portions of the N and M GABA-positive groups of *Manduca* and other insects (Witten and Truman, 1998). We identify two other distinct groups of interneuronal somata that are more lateral, and that are also GABA-positive but En-negative. Based on their relative positions and several landmarks, we suggest that the more medial group, which is part of the MPG, originates from NB 6-3, whereas the other, more lateral group originates from NB 6-4 and is equivalent to the LDG.

In the embryo, a few En-positive neurons derive from neuroblasts that are not themselves En-positive, i.e., neuroblasts anterior to row 6 (Condron et al., 1994). Progeny of these could contribute to the heterogeneous A group, which appears to contain some neurons that are GABA-positive, but others that are not (Fig. 6b). In *Drosophila*, the NH neurons lie anterior to the En-positive strip of neurons and express Invected but not En (Siegler and Jia, 1999). *Drosophila* has two *engrailed-like* genes, as compared with the single En gene in the grasshopper (Patel et al., 1989). The NH neurons of *Drosophila* are GABA-negative (Siegler and Jia, unpublished observation), as are some neurons in the A group of the grasshopper. No En-positive neurons occur anterior to the A group (this, including putative NB 6-1 progeny). We do not have sufficient landmarks to suggest which neuroblasts originate the more anterior GABA-positive groups of the LAG and the LVG. However, our data are consistent with the report that the MVG comprises progeny of the bilateral NB 3-1, NB 4-1, and NB 5-1 (Shepherd and Laurent, 1992).

In giving a provisional view of the relationship between certain neuronal groups in the adult and their embryonic neuroblasts of origin, it is important to emphasize that labeling for En or GABA immunoreactivity does not necessarily reveal the entire set of progeny of any given neuroblast. It is entirely possible that lineages of bilateral neuroblasts include neurons of unlike phenotypes. This is the case for the MNB lineage in the grasshopper (Siegler and Pankhaniya, 1997) and for several neuroblast lineages in *Drosophila* (Schmid et al., 1999). A likely instance is apparent already: We have suggested that one cluster of GABA-positive neurons originates from NB 7-1. In every T2 ganglion examined, this cluster was medial and slightly posterior to a number of large-diameter GABA-negative somata (Fig. 7b). These large somata are identical in position to that of the four ipsilateral motor neurons that innerve the dorsal longitudinal muscles of the T3 segment (Siegler and Pousman, 1990). Interestingly, in *Drosophila*, NB 7-1 gives rise not only to a cluster of interneurons, but also to motor neurons that ultimately innervate the adult dorsal longitudinal muscles (Schmid et al., 1999).

In another provisional assignment of neuroblast origin, we have suggested that another cluster of GABA-positive interneurons originates from NB 6-3. In comparing this interneuronal cluster among several different preparations, we find that the position of the cluster varies together with the position of at least one and usually two of the CI somata, these also being GABA-positive (Figs. 6a, 7b). It has been reported previously that two of the three CIs derive from NB 5-5 in the grasshopper embryo (Wolf and Lang, 1994). We raise the possibility that, additionally or instead, CIs derive from NB 6-3. NB 6-3 is immediately adjacent to NB 5-5 in the thoracic neuroblast arrays, but NB 5-5 does not occur in abdominal neuromeres (Doe and Goodman, 1985). Although the abdominal neuromeres lack NB 5-5, they do contain at least two and perhaps three peripheral inhibitors (Watson and Pflüger, 1987). By contrast, the holometabolous insects, including *Drosophila* and *Manduca*, lack peripheral inhibitors in any neuromere (Witten and Truman, 1998). *Drosophila* also lacks NB 6-3 (Bossing et al., 1996; Schmidt et al., 1997).

In the grasshopper and other larger insects, the segmental neuroblast arrays have clear homologies with those of *Drosophila*. The larger insects have much extended times of embryonic (and postembryonic) develop-

ment, as well as larger and generally more accessible neurons. Therefore, they can offer a degree of temporal resolution for developmental events and often a level of morphological analysis not readily available for studies of *Drosophila*. In contrast, *Drosophila* is preeminent as a subject for the genetic and molecular analysis of neuronal development. Comparisons among the species will continue to be informative in integrating these levels of analysis and will prove necessary for understanding how, and perhaps why, neuronal complements have been altered across evolution.

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