Neuron, Vol. 13, 541–554, September, 1994, Copyright © 1994 by Cell Press

engrailed Controls Glial/Neuronal Cell Fate Decisions at the Midline of the Central Nervous System

Barry G. Condron,* Nipam H. Patel,† and Kai Zinn*
*Division of Biology
California Institute of Technology
Pasadena, California 91125
†Division of Embryology
Carnegie Institution of Washington
Baltimore, Maryland 21210

Summary

The molecular mechanisms involved in glial/neuronal fate decisions during embryonic development are largely unknown. Here we show that the segment-polarity gene *engrailed*, which encodes a homeodomain protein, controls these decisions within an insect CNS lineage. The grasshopper median neuroblast (MNB) generates both neurons and midline glia in distinct temporal phases. *engrailed* expression in MNB progeny can be inhibited by injection of antisense oligodeoxynucleotides into the MNB nucleus. This produces a phenotype in which the midline glia do not develop and extra midline neurons are generated. In the absence of *engrailed* function, midline glial precursors are apparently converted into neuronal precursors. Thus, *engrailed* is required for execution of the decision between the glial and neuronal fates.

Introduction

During the development of the CNS in insect embryos, neuroblasts (NBs) delaminate from a sheet of neuroectodermal cells and divide asymmetrically to produce lineages of ganglion mother cells (GMCs). Each GMC divides once symmetrically to produce 2 neurons. These neurons extend growth cones along stereotyped pathways, forming a ladder-like array with paired longitudinal tracts and two commissural tracts (anterior and posterior) per neuromere (reviewed by Goodman and Doe, 1993). Drosophila and grasshopper NBs have also been found to produce glial progeny (Udolph et al., 1993; Condron and Zinn, 1994), suggesting that some insect NBs are analogous to multipotent neural progenitor cells in vertebrates.

Segmentation genes control metamerization of the Drosophila embryo and are later reexpressed in subsets of cells in the CNS. The functions of some of these genes during CNS development have been analyzed using clonal analysis, conditional mutations, and transgenes that are expressed during segmentation but not in the CNS (Lawrence and Johnston, 1984; Doe et al., 1988a, 1988b; Duffy et al., 1991; Chu-LaGraff and Doe, 1993). These studies suggested that segmentation genes also control CNS cell fates. However, because of the small size and close packing of neuronal cell bodies and processes in the embryonic Drosophila CNS, the phenotypes of mutant embryos are difficult to analyze. Only a few CNS axonal pathways can be selectively visualized by filling individual neurons with dye or by staining with currently available antibodies.

We chose to examine the functions of segmentation genes during CNS development by perturbing gene expression within specific cell lineages in grasshopper (Schistocerca americana) embryos. The grasshopper embryo provides an ideal system in which to study neural development because its neurons have large and accessible cell bodies, and the trajectories of CNS axons can be clearly visualized. Most identified neurons in the Drosophila CNS were first characterized as homologs of previously identified grasshopper neurons (Thomas et al., 1984; Jacobs and Goodman, 1989b; reviewed in Goodman and Doe, 1993), so results obtained in the grasshopper embryo are directly relevant to an understanding of CNS development in the fly.

In this study we examine the roles of the *engrailed* gene during the development of a specific CNS lineage and define its expression patterns within a number of other lineages. *engrailed* encodes a homeodomain protein (denoted here as "engrailed"). It is a member of the segment-polarity class of segmentation genes and controls the identity of cells within the posterior compartment of the segment (reviewed by Martinez-Arias, 1993). There is only one *engrailed*-like gene in the grasshopper genome (Patel et al., 1989a). Here we show that engrailed is initially expressed in a specific subset of NBs in the grasshopper CNS. Later, it is localized to a small number of neurons, some of which arise from NBs that do not themselves express engrailed. The expression patterns suggest that *engrailed* may specify NB identity as well as directly determine the fates of NB progeny.

In this paper, we focus on the functions of *engrailed* within the lineage produced by the unpaired median neuroblast (MNB), which is a multipotent progenitor cell. In grasshopper embryos, the MNB gives rise to the midline glia and to the majority of midline neurons. Glia and neurons are generated from the MNB in distinct temporal phases (Condron and Zinn, 1994). All MNB progeny transiently express engrailed at birth, but engrailed expression is stably maintained only in a subset of neuronal progeny. We find that when engrailed expression is inhibited in the MNB lineage, the midline glia fail to develop and many extra neurons are added to the MNB cluster. The loss of *engrailed* function appears to convert glial precursors into neuronal GMCs. Thus, *engrailed* is required for the acquisition of glial fates within this lineage.

Results

The MNB Lineage: Development and Engrailed Expression Pattern

The early development of the grasshopper MNB lineage has been described in detail (Bate and Grune-

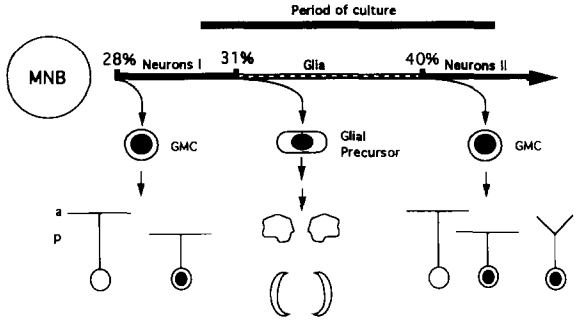


Figure 1. Schematic Diagram of the Development of the MNB Lineage

The developmental percentages during which neuronal and glial progeny are produced are indicated (the distances along the line are not to scale). Grasshopper embryos hatch after 20 days (100% of embryonic development), and there is an anterior to posterior developmental gradient of about 1% per segment. In this paper, we analyzed segments T3–A3 and have normalized all cited developmental percentages (except where noted) to those of the T3 segment. Black nuclei indicate engrailed expression. The positions of the commissures are indicated (a, p), relative to glial positions and to representative axonal trajectories of neuronal progeny. Irregular shapes are axonal glia, and crescent shapes are MNB sheath glia. After 40%, many more neurons are added to thoracic MNB clusters, and there are 80–90 progeny at hatching (Thompson and Siegler, 1991). (From Condron and Zinn, 1994.)

wald, 1981; Goodman, 1982; Condron and Zinn, 1994) and is summarized in Figure 1. The first 3–4 divisions of the MNB (28%–30% of development, as normalized to the T3 segment; see Figure 1 legend) produce neuronal GMCs. Each of these GMCs divides once to produce 2 pioneer neurons. The axons of the pioneer neurons extend anteriorly along the midline and bifurcate in the anterior and posterior commissures. The next 8–10 MNB divisions (31%–40%) generate midline glial precursors. The glial precursors probably divide a number of times, producing groups of axonal and sheath glia that enwrap the commissural tracts and ensheath the cluster of MNB progeny neurons. All midline glia that can be visualized with a monoclonal antibody (MAb) against the annulin protein (Bastiani et al., 1992; Singer et al., 1992) are derived from the MNB, and there are approximately 31 glia per segment. At about 40%, the MNB again begins producing neuronal GMCs. These divide to produce midline neurons whose axons follow several different bifurcating pathways.

Engrailed Is Expressed in a Complex Pattern within the MNB Lineage

The MNB arises from within the neuroepithelial engrailed stripe (Figure 2A). It is initally engrailed positive, but we cannot visualize engrailed in the MNB nucleus after 28% (Figures 2H and 2I). It is possible, however, that the MNB continues to express engrailed at a lower level than can be detected by antibody staining. Both glial and neuronal MNB progeny transiently express engrailed shortly after birth (Figure 2H), but expression is then switched off in glia and in some neurons (Condron and Zinn, 1994). Engrailed is stably maintained only in a subset of neuronal MNB progeny (Figure 2I).

The early development of the MNB lineage (prior to 45%) is almost identical in each segment, but later in development differences emerge between abdominal and thoracic segments. Abdominal MNB lineages are truncated earlier than those of thoracic MNBs, and some abdominal MNB progeny appear to die during embryonic development. Many new engrailed-positive neurons are added to thoracic MNB clusters after 50% (Figures 2J and 2K), and these neurons continue to express engrailed until at least 80% of development. At 80%, there are >50 engrailed-positive neurons in each thoracic MNB cluster, about 6 in A2–A6 abdominal clusters, and 2 in the A8 cluster.

Engrailed Expression Patterns within Lateral NB Lineages

The pattern of engrailed expression within the MNB lineage suggests that it may be required for the initial determination of the fates of glial and neuronal MNB progeny. Later in development, it could also determine the identities of engrailed-expressing neurons. To evaluate whether engrailed is likely to have similar functions in other lineages and to establish a complete map of engrailed expression within the CNS, we examined engrailed staining patterns within segmental ganglia at a number of developmental stages.

We find that all NBs which delaminate from the region contained within the neuroepithelial engrailed stripe initially express engrailed. These include all row 6 and 7 NBs and NB 1.2 (Figure 2A). Row 6 and 7 NBs also express engrailed in Drosophila embryos (Doe, 1992; Gutjahr et al., 1993). GMCs and neurons arising from engrailed-positive NBs also stain for engrailed, and in a segment at 34% of development, there are at least 80 engrailed-expressing cells. Many of these, however, turn off engrailed later in development. NB 7.1 and some of the other row 7 NBs do not produce any neurons that stably maintain engrailed expression.

Analysis of the groups of CNS neurons that express engrailed in a stable manner defines several different temporal patterns of expression within NB lineages. First, engrailed-positive neurons can be generated from engrailed-positive NBs. The EC cells are progeny of NB 6.1 or 6.2. Two EC cells appear by 34% (Figures 2E and 2F), and these are later joined by a third (Figure 2G). The serotonergic neurons stably express engrailed and are progeny of NB 7.2 or 7.3 (Taghert and Goodman, 1984). Serotonin and engrailed can be visualized in these cells after they extend processes (Figures 2J and 2K).

Engrailed-positive neurons can also be generated from engrailed-negative NBs and GMCs. The IC neurons, which are located between the commissures, are progeny of an engrailed-positive GMC. This GMC, however, probably arises from the engrailed-negative

NB 4.1 (Figures 2B–2D). The LE neuron (Figures 2B and 2F) is derived from an engrailed-negative GMC that may be a daughter of NB 5.3. All of the engrailed-positive neurons present in a 52% A3 ganglion are depicted in the diagram of Figure 2L. The IC and EC neurons, among others, continue to express engrailed until at least 80% of development. Engrailed-expressing neurons are also present in adult ganglia, but we do not know whether they correspond to the cell groups observed at 80%. The stable expression of engrailed in specific subsets of neurons, especially those derived from engrailed-negative NBs, suggests that it may be directly involved in the determination of neuronal cell fates and could be essential for maintenance of the phenotypes of mature neurons.

Inhibition of Engrailed Expression in the MNB Lineage by Antisense Oligodeoxynucleotide Injection

We inhibited engrailed expression within the MNB lineage by injection of antisense oligodeoxynucleotides (ODNs) into the MNB nucleus. To do this, we adapted methods developed in mammalian cell culture systems (Kleuss et al., 1991, 1992, 1993). These studies demonstrated that expression of specific G protein α, β, and γ subunits in rat GH3 cells could be inhibited by nuclear antisense ODN injection and that inhibition persisted through at least 2 rounds of cell division.

To evaluate the effectiveness of these techniques in grasshopper embryo cultures, we initially tested the stability and segregation of fluorescein-labeled 33-mers complementary to *engrailed* (Patel et al., 1989a) and to control mRNA sequences (see Experimental Procedures for details). When these ODNs were injected into MNB nuclei and the embryos cultured for 72 hr, as described by Myers and Bastiani (1993), a pattern of labeled progeny cells was observed. The pattern of labeled cells containing fluorescein-conjugated control ODN (Figure 3B) was quite similar to the normal arrangement of MNB progeny visualized using the coinjected rhodamine-dextran lineage tracer (Figure 3A). When fluorescent anti-engrailed ODN was injected, a large number of labeled progeny were also observed, but they formed a disorganized cluster. No labeled cells were observed at the positions of the commissures (Figure 3C; see below for discussion of phenotype). These data indicate that ODNs can be stably maintained in the lineage during the 10 or more cell divisions that take place in culture.

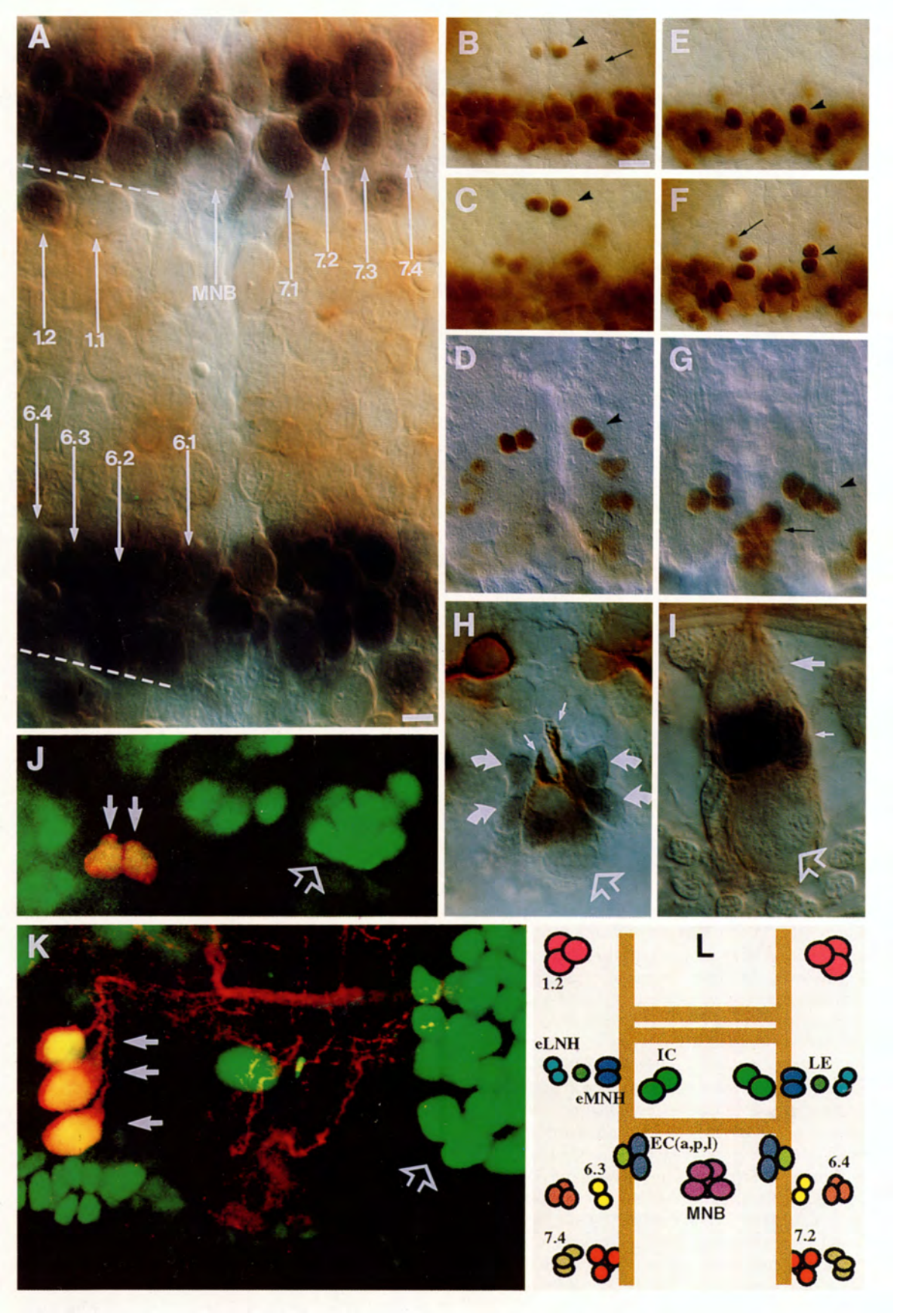
We obtained a quantitative estimate of the extent of inhibition of engrailed expression due to antisense *engrailed* ODN injection by measuring engrailed levels in a large number of injected lineages, using confocal microscopy. Mixtures of antisense or control ODNs were injected into MNB nuclei at 30%–33% (see Experimental Procedures for details). After culturing for 72 hr, the embryos were stained for engrailed using FITC-conjugated secondary antibody, and the fluorescence patterns were visualized by confocal microscopy. An average of 3 MNBs per embryo were injected, and 38 embryos were stained for engrailed after culturing. Of the 114 injected MNBs, 104 survived and produced lineages (63 control and 41 antisense). One pair of embryos produced in this experiment is shown in Figures 3D and 3E. Three MNBs in adjacent segments were injected in each of these embryos. engrailed expression in the IC, EC, and other lineages (see Figure 2L) is visible in both panels (green nuclei). At the end of the culture period (equivalent to 43%–44% of in vivo development), engrailed is expressed in a subset of neuronal MNB progeny. In each of the MNB lineages injected with control ODNs (Figure 3D), a cluster of bright yellow engrailed-expressing nuclei is visualized. The lineages injected with antisense *engrailed* ODNs display markedly reduced engrailed staining within the MNB cluster (Figure 3E).

To quantitate engrailed expression in these injected lineages, the fluorescein (engrailed) signal from each embryo was stored as a confocal *z*-series, and the total fluorescence in the MNB cluster in injected segments was measured and compared with total fluorescence in an adjacent (uninjected) engrailed-expressing cell group, the EC cells. A contour plot of these data for an antisense and a control lineage is shown in Figure 4A. The volumes within the borders of the MNB and EC clusters (representing summed fluorescence intensity) were determined, and a ratio of these volumes was calculated for each of the 104 injected segments. These ratios are plotted in Figure 4B. Control-injected lineages display a broad distribution of expression ratios centered around 3-4. There is a developmental gradient of about 1% per segment, and individual embryos develop at slightly different rates, so the numbers of transiently and stably engrailed-expressing progeny will vary among injected clusters.

The distribution of expression ratios in antisense-injected lineages is quite different ($p = 10^{-15}$; Student's t test), with the largest group of lineages displaying a ratio between 0 and 1 (Figure 4B). The average ratios are plotted in Figure 4C, which shows that antisense injection reduced total engrailed expression in the MNB cluster after 72 hr of culture by 5.4-fold relative to control-injected clusters. Expression ratios in control-injected segments are very similar to those observed in uncultured 44% segments, indicating that culturing and injection do not strongly affect engrailed expression (Figure 4C).

Inhibition of Engrailed Expression Alters Midline Cell Fates

An MNB injected with lineage tracer and/or control ODNs at 30% of development produces the full complement of midline glia, as well as 4–6 midline neurons, during the culture period (see Figure 1). The precursors of axonal glia migrate anteriorly after their birth, and their progeny take up positions around the anterior and posterior commissures (Condron and Zinn, 1994). The cell bodies of midline neurons, how-


A
B
C
D
E
F
G
H
I
J
K
L
MNB
7.1
7.2
7.3
7.4
1.2
1.1
6.4
6.3
6.2
6.1
eLNH
eMNH
IC
LE
EC(a,p,l)
6.3
6.4
MNB
7.4
7.2

ever, remain within the MNB cluster. In control-injected lineages, glia anterior to the MNB cluster can be visualized using tracer dye (Figures 3A and 3D) or fluorescent control ODN (Figure 3B).

In antisense *engrailed*-injected lineages, dye-filled progeny (or progeny labeled with fluorescent antisense ODN) do not migrate anteriorly along the midline. Instead, all of the progeny cells remain posterior to the posterior commissure (Figures 3C and 3E). To determine whether the midline glia are absent in antisense-injected lineages, we stained the embryos for the midline glial marker annulin (Bastiani et al., 1992), using FITC-conjugated secondary antibody, and simultaneously visualized rhodamine tracer in the MNB progeny. In segments where the MNB was injected with control ODNs, bright annulin staining is observed along the midline (green and yellow), and all of the annulin-expressing cells also contain the red rhodamine tracer (Figure 3J). In antisense-injected segments, no annulin expression at the midline is observed, and rhodamine tracer is confined to the position of the MNB cluster (Figure 3K). These data suggest that midline glia are not produced in MNB lineages in which engrailed expression is inhibited.

The absence of midline glia in antisense *engrailed*-injected lineages might indicate that the normally engrailed-positive glial precursors die when engrailed expression is inhibited. Alternatively, the glial precursors might be converted to neuronal GMCs, which are the other class of immediate daughters of the MNB. To examine this question, we visualized midline neurons within control and antisense-injected MNB clusters using the 8B7 MAb, which recognizes a cytoplasmic epitope in neuronal cell bodies and axons. Figure 3F shows a control-injected segment stained for 8B7 antigen (green). The MNB cluster neurons (yellow) are labeled with the coinjected rhodamine tracer, indicating that at least some of them were generated after injection. Glial progeny labeled with tracer dye (red) are visualized along the midline. A larger number of 8B7-positive, dye-labeled cells (yellow) are observed within an antisense-injected cluster (Figure 3G). Also note the absence of dye-labeled glial progeny anterior to the cluster in this segment.

We also examined midline neurons using another neuronal marker, the cell surface carbohydrate epitope recognized by anti-horseradish peroxidase (HRP) antisera (Jan and Jan, 1982; Snow et al., 1987; Katz et al., 1988; Desai et al., 1994). The control MNB cluster of Figure 3H displays only faint anti-HRP staining. In contrast, many anti-HRP-positive cells that also contain rhodamine tracer (yellow) are visualized in the antisense-injected cluster of Figure 3I. Dye-labeled glial progeny (red) are present along the midline in Figure 3H, but not in Figure 3I.

The neurons in antisense-injected MNB clusters are round and form loosely organized groups that extend dorsally and laterally beyond the normal boundaries of the clusters. In Figure 5, the morphologies of these cells are visualized with Nomarski optics using three different markers: biotin tracer dye, 8B7 antigen, and anti-HRP antisera. The neurons often appear to burst out of one side of the cluster (probably because of the absence of the glial sheath) and pile up dorsally. Their cell bodies stain brightly for 8B7 antigen, which becomes largely restricted to processes in normal neurons after they extend growth cones (compare the antisense-injected and uninjected clusters in Figure 5B). Most of the neurons in antisense-injected clusters do not have processes, although a few dye-labeled axons are usually observed in antisense-injected segments (see Figures 3E, 3G, and 3I).

To estimate the number of extra neurons produced as a consequence of inhibition of engrailed expression, we counted 8B7-positive cells in antisense and control-injected clusters, using preparations like those of Figure 5. At the end of the culture period, there were 10.2 $\pm$ 2.7 (n = 32) 8B7-positive neurons in control-injected MNB clusters, which corresponds approximately to the number seen at 43% of in vivo

Figure 2. Expression of Engrailed within Grasshopper Neuroblast Lineages

Engrailed expression is visualized with MAb 4D9, using HRP (B–G; brown staining) or HRP-Ni (A, H, I; black staining) immunohistochemistry and Nomarski optics, or using FITC-conjugated secondary antibody and confocal microscopy (J–K; green cells).

(A) NB pattern in a 37% segment. Engrailed-expressing NBs are indicated, as well as NB 1.1 and the MNB. Dashed lines are segment boundaries.

(B–D) The IC lineage, in 34% (B), 37% (C), and 49% (D) segments. (B) is prior to division of the GMCs (arrowhead). In (C) and (D) the neurons are indicated (arrowheads). The LE neuron is also visible in (B; arrow).

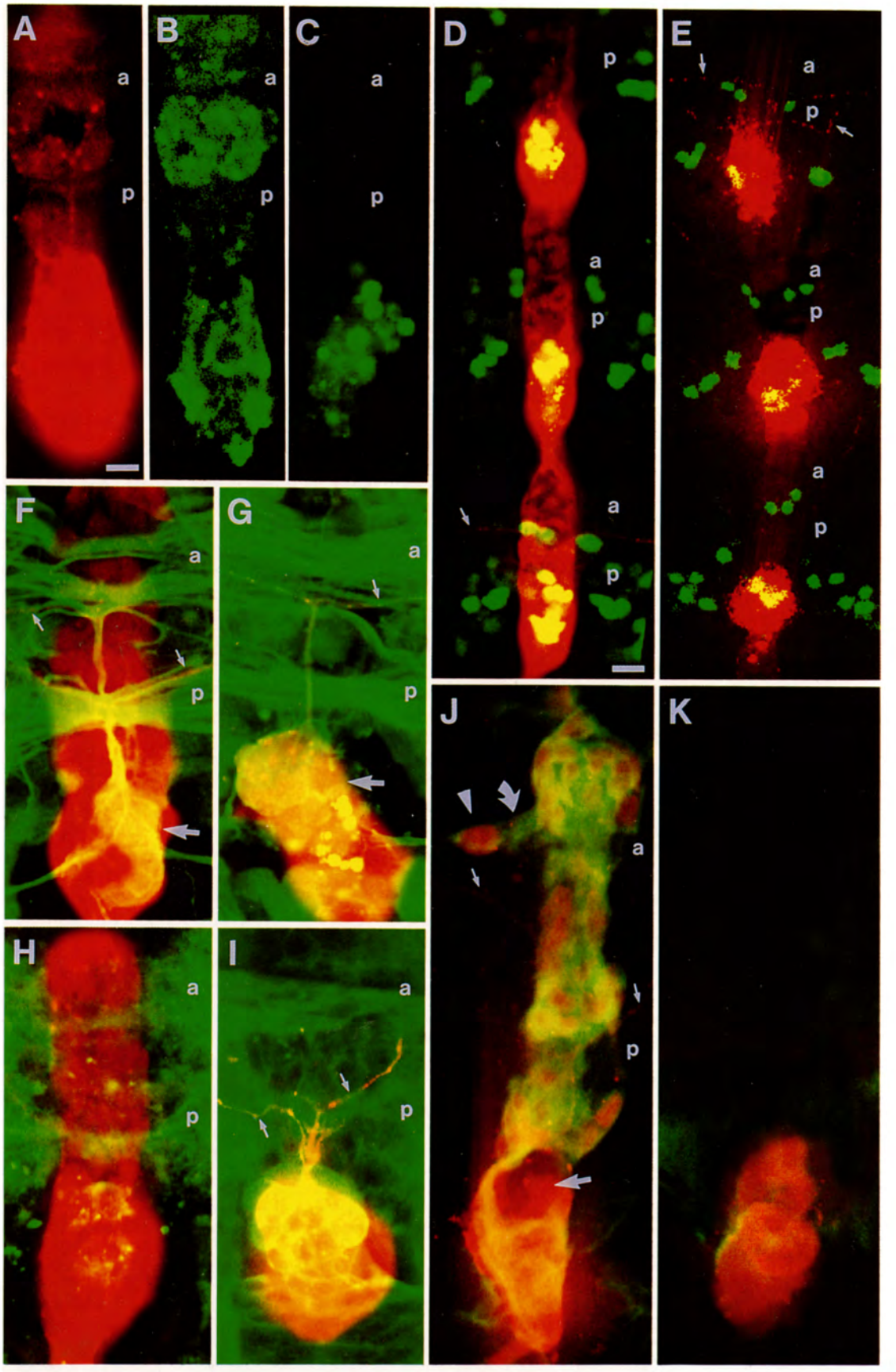
(E–G) The EC lineage, at 33% (E), 34% (F), and 49% (G). (B)/(F) and (D)/(G) are photographs of the same ganglion, but in different focal planes. In (E) the GMC has not divided, whereas in (F) 2 EC neurons (a and p; arrowhead) are present; they are joined by ECl in (G). The LE neuron is indicated in (F), and the MNB progeny in (G) (arrows).

(H–I) The MNB lineage, at 31% (H) and 45% (I). (H) is double stained with MAb 8B7 (brown), and 2 pioneer growth cones are visible (small arrows). Engrailed-expressing glial precursors (curved arrows) are lateral to the neurons. The MNB (open arrow) does not express engrailed. In (I), engrailed-positive (small arrow) and negative (large arrow) neuronal MNB progeny are indicated, as is the MNB (open arrow).

(J–K) The serotonergic lineage is visualized using a rabbit antibody against serotonin (red). In (J) (41%) 2 serotonin-positive, engrailed-expressing cells are visible (arrows), whereas in (K) (60%) a third neuron has appeared (arrows), and the cells have extended serotonin-containing processes. Note the large number of MNB progeny (open arrows in [J] and [K]) visible in (K), which is a T3 segment. The right edge of (K) is at the midline.

(L) Schematic diagram of the engrailed-positive neurons in an A3 segment of a 52% embryo. EC, IC, LE, eLNH, and eMNH are names of neurons; 1.2, 6.3, 6.4, 7.2, 7.4, and MNB refer to the NBs that generate the indicated neuronal clusters.

Bar in (A), 10 μm for (A), (I), (J), and (K); bar in (B), 10 μm for (B), (C) (E), and (F); bar in (B), 8 μm for (D) and (G).


A
a
p
B
a
p
C
a
p
D
p
a
p
a
p
E
a
p
a
p
a
p
F
a
p
G
a
p
H
a
p
I
a
p
J
a
p
K

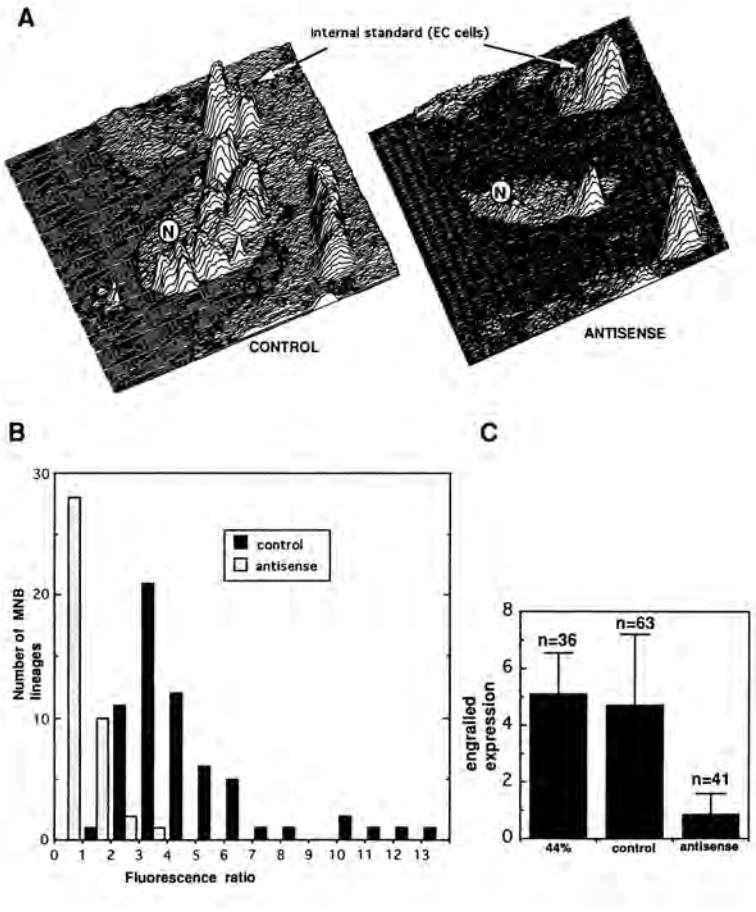


Figure 4. Quantitative Estimate of Inhibition of Engrailed Expression by Antisense *engrailed* ODN Injection

(A) Contour plot of engrailed expression in the MNB and EC clusters in a control (sense) and an antisense-injected segment. The plot was generated using the program NIH Image. The MNB is indicated by "N." Note the lower contours within the MNB cluster in the antisense segment. The EC lineages provide internal reference standards for each segment.

(B) Histogram of fluorescence ratios for 104 injected lineages. For each segment, the MNB cluster/EC cluster fluorescence ratio was determined from the contour plot and assigned to the interval between the two nearest integers.

(C) The average expression ratios obtained from the graph of (B) are plotted as single bars. The average ratio is also shown for a group of 36 segments that developed to about 44% in vivo (the histogram for these segments is not shown). The error bars indicate SD. The error bar is shorter for uncultured segments because these seldom display the highest expression ratios (>7) shown in (B).

development (Figure 6; Condron and Zinn, 1994). Antisense-injected clusters contained 24.5 ± 2.1 8B7-positive cells (n = 6). Thus, about 14 extra neurons are generated within the MNB lineage when engrailed expression is inhibited.

The absence of annulin-expressing cells at glial positions and the concomitant increase in the number of neurons in the MNB cluster suggest that glial precursors are converted to neuronal GMCs when engrailed expression is inhibited. We used double staining with MAbs against the neuronal (8B7) and glial (annulin) markers to show directly that in single antisense-injected segments midline glia are absent and midline neurons are increased in number. The segment shown in Figure 7A, in which the MNB was injected with control ODNs, displays bright green annulin staining around the MNB cluster and along the midline. In contrast, there is little annulin expression in the antisense-injected segment (Figure 7B). Many more MNB cluster neurons (red midline cells) are present in this segment than in the control segment (compare Figures 7B and 7A). The intercommissural region of the antisense-injected segment also contains a larger number of neuronal processes relative

Figure 3. Inhibition of Engrailed Expression in the MNB Lineage and Its Phenotypic Effects, as Visualized by Confocal Microscopy

The positions of the anterior and posterior commissures are indicated by a and p, respectively, in (A)–(J). Red labeling in all panels is rhodamine–dextran lineage tracer dye. Where visible, dye-labeled axonal tracts are indicated by small arrows.

(A) Tracer dye pattern. Cells at and anterior to the commissures are axonal midline glia.

(B) Control FITC-labeled ODN pattern (same segment as [A]).

(C) Antisense *engrailed* FITC-labeled ODN.

(D and E) Engrailed is visualized using FITC-conjugated secondary antibody (green). Dye-labeled cells that also express engrailed are yellow. (D) Control ODNs were injected into 3 adjacent MNBs. The red cells between the yellow neuronal clusters are glia. (E) Antisense ODNs were injected into 3 adjacent MNBs. Note the absence of labeled cells between the clusters.

(F and G) Control (F) and antisense (G) segments, stained with the 8B7 MAb (green). Yellow-labeled MNB progeny are indicated (arrow).

(H and I) Control (H) and antisense (I) segments, stained with anti-HRP (green).

(J and K) Control (J) and antisense (K) segments, stained with the 7H7 MAb against the cell surface annulin protein (green). Greener regions are glial processes (e.g., curved arrow), which contain little dye but stain brightly for annulin; redder regions are glial nuclei (e.g., arrowhead). Red dye-labeled neuronal cell bodies, which do not stain for annulin, are also indicated (large arrow). Note the lack of annulin staining in (K).

Bar in (A), 10 μm for (A), (B), (C), (F), (G), (H), (I), and (K); bar in (D), 10 μm for (D) and (E).

Figure 5. Morphology of Neurons in Antisense *engrailed*-Injected MNB Clusters

Three different cultured embryos are visualized with Nomarski optics. In each embryo, 1 MNB was injected with antisense ODNs. In (A) the MNB progeny (gray) are visualized by staining for biotin–dextran lineage tracer, using streptavidin–HRP and HRP-Ni histochemistry for visualization. The other embryos were stained with the 8B7 MAb (B) and with anti-HRP (C), using HRP immunohistochemistry (brown) for visualization. Note the round morphology of the MNB progeny (arrows) in each injected segment. In (A) and (B), the neurons appear to burst out of one side of the cluster. In all 3 embryos, there are additional neurons in these clusters that are in more dorsal focal planes and are thus not visualized here. The morphology of neurons in uninjected clusters (arrowheads) is different. These neurons have asymmetric cell bodies, and 8B7 staining within the clusters is localized primarily to the median fiber tract (small arrow in [B]). The anterior (a) and posterior (p) commissures are indicated, as is the transverse nerve (tn). Bar, 10 μm.

to the control segment, and these processes have a disorganized appearance. This indicates that the midline glia may help to confine commissural axons within tract boundaries. This phenotype could be related to the "fused commissure" phenotypes observed for mutations affecting midline glia in Drosophila (Klambt et al., 1991).

Figure 7C summarizes the effects of *engrailed* inhibition in the MNB lineage. In a normal MNB lineage, glia (green cells) and neurons (red cells) are generated from the MNB (large red cell) in distinct temporal phases. When engrailed expression is inhibited, a large number of extra neurons are produced, and no glia are generated. These phenotypes were reproducibly observed in our experiments. For example, in the experiment analyzed in Figure 4, all 63 of the control-injected MNBs developed normally and produced both neurons and glia, as evaluated by examination of the lineage tracer dye pattern (Figure 3D). In 38 of the 41 antisense-injected segments (93%), dye-labeled progeny failed to migrate anteriorly, and the MNB cluster extended laterally and dorsally beyond its normal boundaries (Figure 3E). There was little toxicity associated with ODN injection, even at the highest concentrations tested (104/114 injected lineages survived in this experiment, in which 250 μM ODN was injected).

Extra neurons were also reproducibly observed within antisense *engrailed*-injected MNB clusters, as assayed by staining with the 8B7 MAb or with anti-HRP antisera (85% of clusters in one experiment; n = 26 segments). A smaller fraction of the segments in the same experiment displayed loss of annulin-staining midline glia (55%; n = 40). The lower penetrance of the glial phenotype is due to migration of glia into antisense-injected segments (see below). None of the control-injected segments displayed extra neurons or missing glia (n = 50).

Midline Glia Can Migrate across Segment Boundaries

Although dye-labeled cells failed to migrate anteriorly from the MNB cluster in almost all antisense-injected segments, some of these segments contained annulin-expressing glia at the end of the culture period. Segments with glia were usually adjacent to segments in which the MNB was not injected. These data suggested that glia generated in uninjected segments of a cultured embryo can migrate into adjacent antisense-injected segments, in which all glial positions are vacant. To test this idea, we injected MNBs in alter-

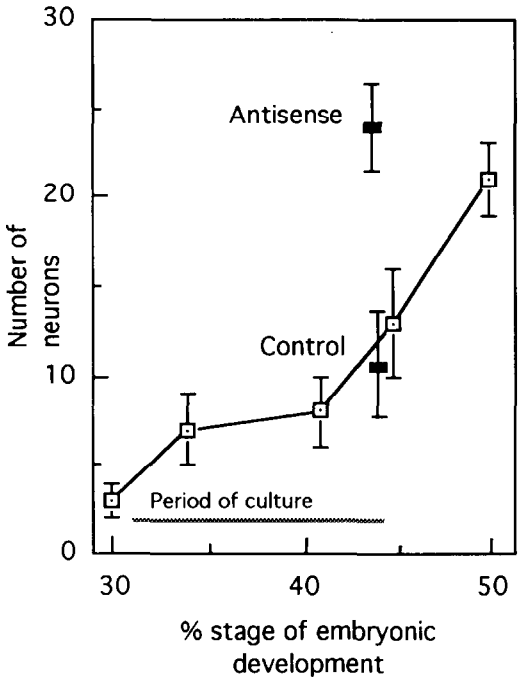


Figure 6. Time Course of Neuronal Production in Control and Antisense *engrailed*-Injected Segments

Neurons staining with 8B7 MAb within the MNB cluster were counted. The line defined by the open squares represents in vivo neuronal development in T3 segments (data from Condron and Zinn, 1994). At the end of the 72 hr culture period, the overall CNS morphology of cultured segments is similar to that of segments which have developed to 43%–45% in vivo. Control-injected segments have an average number of MNB cluster neurons (10.2 ± 2.7; n = 32) approximately equal to that observed in an uncultured 43% segment, whereas antisense-injected segments have 24.5 ± 2.1 (n = 6; this number is small because it is difficult to find antisense-injected clusters in which neurons are in a small enough range of focal planes to allow accurate counting).

nate segments with antisense and control ODNs mixed with different fluorescent lineage tracer dyes. In the antisense-injected segment shown in Figure 7D, cells labeled with fluorescein-dextran are confined to the MNB cluster. Glia labeled with rhodamine-dextran have migrated posteriorly from the control segment into the antisense-injected segment and appear to be occupying normal glial positions around the commissures. Glia can also migrate anteriorly across segment boundaries into antisense-injected segments (data not shown).

These data show that grasshopper midline glia, like vertebrate glia, are quite plastic in their phenotypes and can migrate into vacant glial positions and enwrap axons or cell bodies that they would not normally contact. It is possible that, as observed for oligodendrocytes in rat optic nerve (Barres et al., 1993), midline glial number is regulated by the number of axons (and cell bodies) that require enwrapping. Thus, midline glial precursors might undergo more rounds of division when there are vacant positions to fill. In experiments like that shown in Figure 7D, the density of glial labeling in control-injected segments did not appear to be reduced by migration, suggesting that extra glia may have been generated in response to the presence of unsheathed neuronal cell bodies and processes.

Discussion

The *engrailed* gene encodes a homeodomain transcription factor that is conserved between insects and vertebrates. In all species examined, *engrailed* is expressed in the embryonic CNS, but its functions during CNS development are unknown. The complex expression pattern of engrailed in the insect CNS (Figure 2) suggests that it may be involved in a number of different cell fate decisions.

In this paper, we show that *engrailed* controls the decision between midline glial and neuronal fates within the lineage produced by the MNB (Figure 1). This is one of the first examples of the identification of a molecular mechanism for a glial/neuronal fate decision within a multipotential cell lineage. In Drosophila, the *numb* gene controls the decision between two progenitor cell fates within the bristle sensory organ lineage, which generates both neurons and sheath (glia-like) cells. In the absence of *numb* function, the neuron and sheath cell are replaced by an extra hair and socket cell (Uemura et al., 1989). In mammals, it has recently been shown that a glial/neuronal fate decision within the lineage produced by neural crest stem cells can be influenced by glial growth factor, which is a ligand for a receptor tyrosine kinase (Shah et al., 1994).

Lineage-Specific Inhibition of Engrailed Expression

We have developed methods for inhibiting gene expression within individual grasshopper NB lineages and for assaying the resulting phenotypes. Grasshopper embryos can be maintained in culture between 28% and 44% of development, and NBs injected with tracer dye produce normal lineages in cultured embryos. To evaluate the effects of inhibiting expression of a specific gene, antisense ODNs complementary to its mRNA sequence are injected into NB nuclei in cultured embryos. At the end of the culture period, the fates of NB progeny can be assayed by examination of the lineage tracer dye pattern and by staining with specific antibodies.

In this paper we examine the effects of inhibition of engrailed expression within the MNB lineage, which produces midline glia and neurons. Injection of antisense ODNs complementary to the *engrailed* mRNA into the MNB reduced total engrailed expression within the MNB cluster by an average of 5.4-fold (Figure 4). However, this number is likely to represent an underestimate of the extent of inhibition in glial precursors. Engrailed expression is measured after 72 hr in culture, at which time most engrailed is localized to neuronal progeny that are born 8–10 divisions after

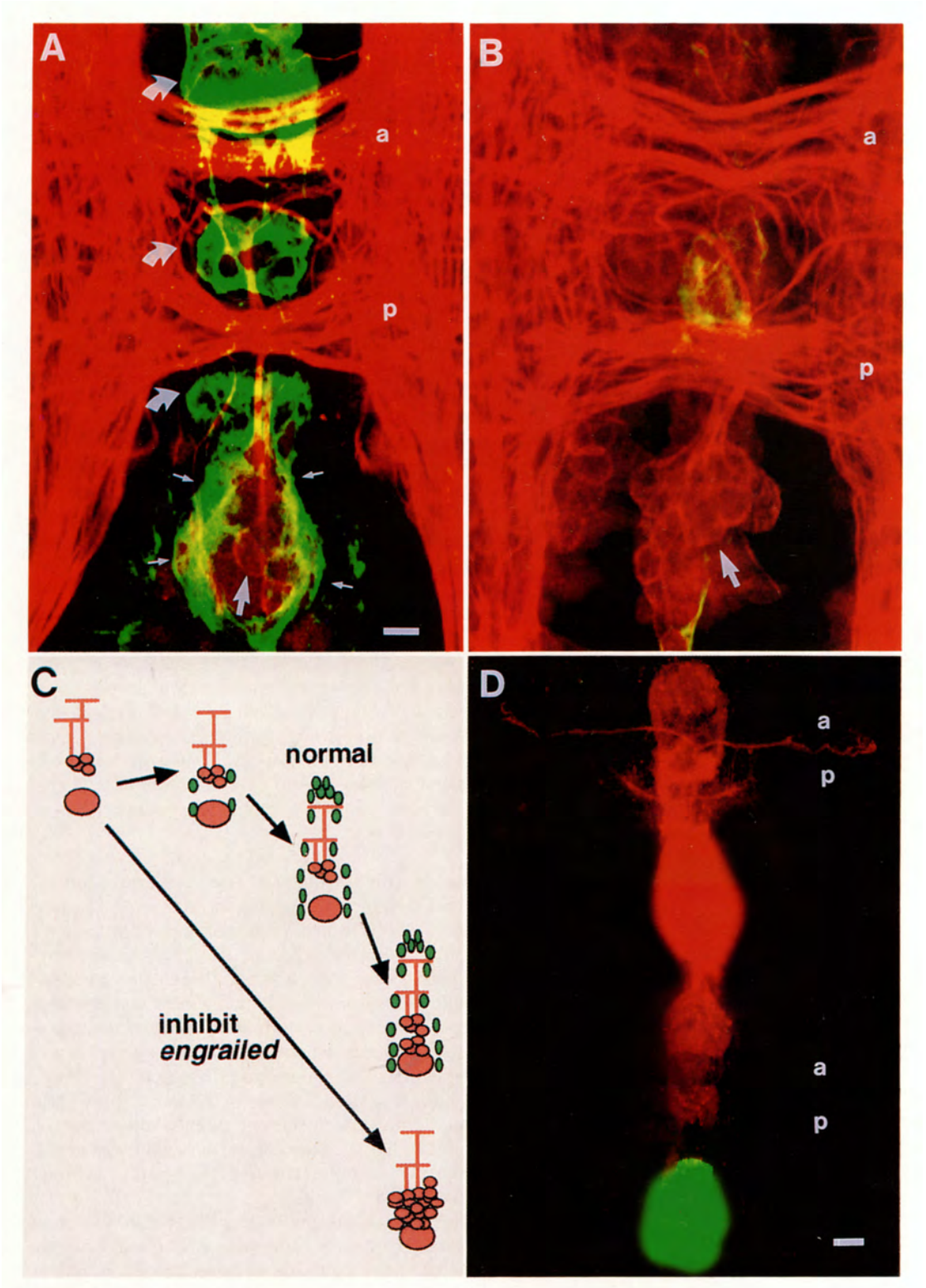


Figure 7. Visualization of Midline Neurons and Glia in Control and Antisense *engrailed*-Injected Segments

Control-injected (A) and antisense-injected (B) segments, double stained with the 8B7 (red) and 7H7 (anti-annulin; green) MAbs and visualized by confocal microscopy.

(A) The MNB cluster neurons (large arrow) are ensheathed by annulin-expressing glia (small arrows). The axonal midline glia (curved arrows) are located anterior and posterior to the commissures (a, p).

(B) In the antisense-injected segment, no annulin-expressing glia are visible. The faint green staining between the commissures is probably due to residual annulin expression in mesectodermal cells (Bastiani et al., 1992). There are more red neurons within the MNB cluster (arrow) in (B).

injection. The glial precursors are generated by the first postinjection divisions (see Figure 1), so ODN concentrations may be higher in these cells. Furthermore, a few early neurons that are generated from the MNB before the usual time of injection stably express engrailed and will thus contribute to the measured engrailed fluorescence.

engrailed Determines the Fates of MNB Progeny

There are two developmental transitions in the MNB lineage. The MNB generates neuronal GMCs during its first 3–4 divisions, then produces glial precursors for 8–10 divisions, after which it again begins making GMCs (Figure 1; Condron and Zinn, 1994). GMCs do not usually express the neuronal 8B7 antigen, but each GMC divides once to generate 2 8B7-positive neurons. An initial accumulation of these 8B7-staining neurons within the MNB cluster is observed during the first neuronal production phase, followed by a plateau during the glial phase. After this plateau, the number of neurons increases again during the second neuronal production phase (Figure 6). Like other NBs, the MNB divides regularly every 5 hr (1% of development; Shepherd and Bate, 1990). The rate of increase in neuron number during the neuronal production phases is consistent with this division rate.

Our data indicate that *engrailed* is required for the first transition in progeny cell fates, in which the MNB switches from generation of GMCs to production of glial precursors. When engrailed expression within the MNB lineage is inhibited by antisense ODN injection, no glia are produced, and the MNB continues to generate 8B7- and anti-HRP-positive cells that have the appearance of immature neurons (Figures 3, 5, and 7). The number of neurons in antisense *engrailed*-injected MNB clusters is consistent with the uninterrupted production of neuronal GMCs from the MNB during the entire culture period (Figure 6). These data suggest that MNB progeny which would have become glial precursors are converted to neuronal GMCs when engrailed expression is inhibited. It is likely that the loss of engrailed expression does not affect the division rate of the MNB or the survival of its progeny.

The molecular mechanisms involved in the glial/neuronal fate decision are unknown, but are likely to involve changes in gene expression and signal transduction in both the MNB and its progeny. One aspect of the decision is a change in the orientation of MNB cytokinesis. Glial progeny are observed to emerge laterally from the MNB and may interact with a different local environment than do GMC progeny, which emerge dorsally (see Figure 2H; Condron and Zinn, 1994). The influence of *engrailed* on the glial/neuronal fate decision is probably exerted downstream of the change in cytokinetic orientation, because engrailed is expressed in MNB progeny at the time of the decision but not by the MNB itself. Engrailed is a permissive rather than an instructive factor in the decision, since it is expressed by both glial precursors and neuronal GMCs at their births. One possibility is that surrounding cells send signals to newly born glial precursors that cause them to adopt glial fates. Engrailed might be required in the glial precursors to turn on genes that determine glial fates and/or repress genes that determine neuronal fates in response to these signals.

We do not know whether engrailed expression is also required for the acquisition of neuronal phenotypes within the MNB lineage. We observe that only a few of the extra dye-labeled neurons generated in antisense *engrailed*-injected lineages extend axons. Their axonal trajectories are indistinguishable from those of normal MNB progeny neurons, however (Figures 3G and 3I). This could indicate that engrailed is required for expression of a gene that facilitates growth cone extension by neuronal MNB progeny. Another possibility is that midline glia are necessary for axonal outgrowth by the later (non-pioneer) neurons in the MNB cluster. Midline glial precursors migrate anteriorly along the median fiber tract established by the pioneer growth cones, and a partially formed glial scaffold is present by the time the later neurons extend axons. In this model, the elimination of glia due to inhibition of engrailed expression would result in the absence of a signal required for process extension by these later neurons. The only dye-labeled neurons in antisense-injected clusters that could extend axons in the absence of this signal would be the few early (pioneer) neurons generated after injection, but before the glial production phase would normally begin (see Figure 1).

Another possible interpretation of our results is that the effect of antisense ODN injection on the MNB lineage is due to inhibition of engrailed expression within the MNB itself and that this inhibition changes the identity of the MNB into that of an NB that never expresses engrailed. In this model, the loss of midline glia might occur if the NB into which the MNB had

(C) Schematic diagram summarizing the effects of inhibition of engrailed expression in the MNB lineage. In the normal lineage, neurons (red) and glia (green) are generated in the temporal pattern shown (Condron and Zinn, 1994), whereas in antisense-injected lineages glia are absent and extra neurons are present.
(D) Posterior migration of glia into vacant glial positions in antisense-injected segments. The anterior MNB was injected with rhodamine-dextran tracer and control ODNs, whereas the posterior MNB was injected with fluorescein-dextran tracer and antisense ODNs. Bifurcating axonal pathways are visible in the anterior segment. The positions of the commissures in each segment are indicated (a, p). Note that the rhodamine-labeled glia have migrated posteriorly beyond the posterior commissure of the antisense-injected segment and are contacting the fluorescein-labeled MNB cluster. The positions of the glia relative to the commissures appear similar in the two segments.
Bar in (A), 10 μm for (A) and (B); bar in (D), 10 μm.

been transformed never produces glia as part of its normal lineage. We consider this to be unlikely, because we do not detect engrailed expression in the MNB after 28%, which is prior to the stage at which ODN injections are performed. Furthermore, the neurons generated in engrailed-inhibited segments always extend axons that have trajectories characteristic of MNB progeny, suggesting that the entire lineage has not been transformed. It was shown, however, that external sensory neurons in *cut* mutant embryos can apparently acquire the identities of chordotonal neurons without changing their CNS projection patterns to those characteristic of chordotonal neurons (Merritt et al., 1993). Thus, axonal geometry may not always be a definitive indicator of neuronal identity.

engrailed may also function within the MNB lineage to distinguish the phenotypes of two classes of neuronal MNB progeny. MNB cluster neurons that stably express engrailed (Figure 2I) have axons that bifurcate in the posterior commissure and are likely to correspond to some of the interneurons identified by Thompson and Siegler (1991). The efferent DUM motoneurons do not express engrailed, and their axons bifurcate in the anterior commissure (Goodman, 1982). Because process outgrowth is altered as a result of the absence of the midline glia, we cannot assay this potential function of engrailed in our experiments.

Midline Development in Grasshopper and Drosophila

The midline neurons and glia in grasshopper and Drosophila embryos are quite similar in shape and organization. Drosophila VUM neurons have axonal trajectories and targets like those of grasshopper DUM neurons (Sink and Whitington, 1991). The axonal midline glia in Drosophila are also arranged similarly to those in grasshopper, although fewer glia are present (Jacobs and Goodman, 1989a; Klambt and Goodman, 1991; Klambt et al., 1991; Bastiani et al., 1992; Condron and Zinn, 1994). Engrailed is expressed in a subset of the Drosophila midline neurons (Patel et al., 1989b). It is unclear whether these neurons are homologous to the grasshopper MNB progeny that stably express engrailed.

In the grasshopper embryo, all of the annulin-staining midline glia are derived from the MNB. In contrast, the Drosophila axonal midline glia are thought to arise from a separate set of glial precursors (Klambt et al., 1991). We have proposed that these Drosophila precursors could be early MNB progeny that migrate anteriorly and switch off engrailed before dividing to generate the glia (Condron and Zinn, 1994). In this model, *engrailed* could be involved in the determination of midline glial fates in the same manner as in grasshopper. Another possibility is that midline lineages are different in Drosophila and grasshopper, although the same molecules are likely to be involved in midline determination in both organisms. An example of this is provided by the role of *engrailed* in segmentation in grasshopper and fly. In both organisms engrailed is expressed in the posterior region of each segment (and probably determines the identities of cells in this region), but the mechanism by which the segmental expression pattern is generated is quite different (Patel et al., 1989a, 1992).

Evolutionary Implications of Engrailed Expression and Function in the Grasshopper CNS

Engrailed is expressed in the posterior region of each segment during the early development of arthropod embryos (Patel et al., 1989a; reviewed by Martinez-Arias, 1993). In vertebrates, however, segmental engrailed expression is not observed prior to the appearance of the metameric pattern. In chick and mouse embryos, early expression of the two *engrailed* genes (*en-1* and *en-2*) is restricted to cells at the midbrain/hindbrain junction (reviewed by Joyner and Hanks, 1991). *en-2* is strongly expressed in the internal granular layer of the cerebellum in the postnatal mouse brain, as well as in subsets of cells in the colliculi (Logan et al., 1993). In *en-2* mouse mutants, the foliated structure of the cerebellum is abnormal (Joyner et al., 1991; Millen et al., 1994). It is likely that during early vertebrate development, *engrailed* determines regional identity within the nervous system. Later in development, *engrailed* may control cell fate decisions within particular brain regions. These CNS functions could be ancestral to its segmentation function in arthropods, since the common progenitor of the arthropod and vertebrate lineages was probably unsegmented.

It is unclear whether *engrailed* genes are involved in glial/neuronal fate decisions in the vertebrate nervous system. Our analysis of the detailed pattern of engrailed expression within individual NB lineages, however, suggests that the function of *engrailed* during insect CNS development is not restricted to glial fate determination. Rather, *engrailed* is likely to control a number of different cell fate decisions during neurogenesis and could also regulate the properties of mature CNS neurons. Similarly, in vertebrates *engrailed* genes could function in the establishment or maintenance of a number of different cellular phenotypes within the brain.

Experimental Procedures

Oligonucleotides

Antisense *engrailed* ODN sequences were derived from the sequence of a portion of the single grasshopper *engrailed* gene (Patel et al., 1989a). Four ODNs were used for injection: ASEN-2 (5′-CGTGCAGTACACCCACGCGGGCCACAG); ASEN-3 (5′-CTTCAGTCGGCGCGACCGCGGACTTCT); ASEN-4 (5′-GGCCTTCTTGATCTTGGCGCGCTTGTTCTGGAA); and ASEN-5 (5′-CCAGATCTTGATCTGCGC). Control ODNs were SEN-1 (5′-CTGTGGCCCGCGTGGGTGTACTGCACG)-sense *engrailed* sequence; ASEVE-2 (5′-CAGCTGGTCGTGTCTTCCTCGGAAG); ASEVE-3 (5′-CTGGAGCCGCGCCAGCTGCTCGCGGGTGAAGGC); and ASEVE-4 (5′-GGTCCGGTAGCGCCGGAT). These four are antisense grasshopper *even-skipped* sequences (Patel et al., 1992). *even-skipped* is not expressed in the MNB lineage; SEVE-2 (5′-CAGCTGGTCGTGTCTTCCTCGGAAG)-sense *even-skipped* sequence. All ODNs were

purified by reverse phase chromatography and butanol extraction.

ODN Injections and Embryo Cultures

Microinjection and embryo culturing were done as described previously (Condron and Zinn, 1994). A total of 239 control and 354 antisense *engrailed*-injected lineages were analyzed. Each injected lineage was examined for phenotype after culturing by visualization of the coinjected rhodamine tracer dye pattern. Lineages in which all dye-labeled progeny appeared to be localized to the MNB cluster and in which the cluster was expanded dorsally and/or laterally were scored as exhibiting the engrailed phenotype. A subset of the antisense and control lineages were then analyzed further by antibody staining. ODNs were injected at a number of different concentrations. Fifty percent of lineages in which the MNB was injected with antisense ODNs at a total concentration of 20 μM or less in the injection solution displayed the phenotype (n = 41). Eighty-five percent of lineages injected with 20–50 μM antisense ODNs (n = 272) and 93% of those injected with 250 μM ODNs (n = 41) exhibited the phenotype. Control ODNs had no specific effects on development at any concentration. In the experiment of Figure 4 (250 μM control ODN mixture), 0/63 lineages displayed the engrailed phenotype; 18 of the 239 control-injected lineages were initially scored as exhibiting the phenotype. These were all from one set of embryo clutches, and these MNBs were injected with 20–50 μM ODN. When further analyzed by staining for 8B7 antigen or annulin, however, none of these 18 lineages displayed excess neurons or missing glia. These clutches may have had some developmental abnormalities, or our initial scoring based on dye morphology may have been in error. The three longer *engrailed* antisense ODNs (ASEN-2 [27 nucleotides], ASEN-3 [27 nucleotides], and ASEN-4 [33 nucleotides]) were found to be effective in generating the engrailed phenotype and in inhibiting engrailed expression when used alone at 20–50 μM, whereas the short antisense ODN (ASEN-5 [18 nucleotides]) was ineffective. In most experiments, a mixture of ASEN-2, ASEN-3, and ASEN-4 was used for injection. In the experiment of Figure 4, these three ODNs were mixed and used at a total concentration of 250 μM; the control ODNs were SEN-1 (27 nucleotides), ASEVE-2 (25 nucleotides), and ASEVE-3 (33 nucleotides), and they were mixed and used at the same total concentration. Fluorescent ODNs were made using the 5′ FAM modification (Applied Biosystems). The 33 nucleotide fluorescent ODNs used in the experiment of Figure 3 were ASEN-4 and ASEVE-3. We also made 18 nucleotide fluorescent ODNs (ASEN-5 and ASEV-4); when these were injected into the MNB, all ODN fluorescence was lost within 24 hr of culture. This suggests that the fluorescent signal in Figures 3B and 3C is actually due to ODN and not to fluorescent breakdown products, since these would probably be generated by ODNs of both lengths. In the experiment of Figures 7A and 7B, only unlysinated rhodamine–dextran 10K dye (Molecular Probes) was used in the injection mixture, so that all rhodamine dye fluoresence was lost during fixation and MAb staining.

Immunohistochemistry

Embryo staining with MAbs was performed as described (Patel et al., 1989a; Condron and Zinn, 1994), and all embryos were viewed through a Zeiss Axioplan microscope or a Bio-Rad 600 confocal microscope. The 8B7 MAb was provided by Dr. Michael Bastiani (University of Utah). Rabbit anti-HRP antiserum (Cappel) was used at a dilution of 1:1000. Double fluorescence staining of embryos with anti-annulin (7H7) and 8B7 MAbs (Figures 7A and 7B) was performed as follows: Experiments were first conducted in which the two MAbs were mixed and incubated with the same embryos. We then used different lots of FITC- and RITC-conjugated goat anti-mouse secondary antibodies for detection. We found that an RITC-conjugated secondary (Jackson ImmunoResearch 115-025-003) recognized the two MAbs equally, whereas an FITC-conjugated secondary (Jackson ImmunoResearch 111-095-003, lot #22935) preferentially recognized 7H7. For double staining, an 8B7 MAb supernatant was used at a limiting concentration (1:60), followed by an excess of the RITC-conjugated secondary (1:60). The embryos were postfixed (4% HCHO in phosphate-buffered saline, 10 min) to block further secondary binding to the 8B7 MAb. They were then incubated with 7H7 MAb supernatant at 1:6, followed by a limiting amount of the FITC-conjugated secondary (1:300).

Acknowledgments

We thank Scott Fraser for helpful discussions and for the use of injection equipment, Gilles Laurent for the use of his grasshopper colony, and David Anderson and Sarah Fashena for comments on the manuscript. This work was supported by National Institutes of Health grants #NS28182 to K. Z. and #GM47268 to N. H. P., by McKnight Scholars Awards to K. Z. and N. H. P, and by a Pew Scholars Award to K. Z. B. G. C. was supported by a Del Webb postdoctoral fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received June 28, 1994.

References

Barres, B. A., Jacobson, M. D., Schmid, R., Sendtner, M., and Raff, M. C. (1993). Does oligodendrocyte survival depend on axons? Current Biol. *3*, 489–497.

Bastiani, M. J., Couet, H. G. D., Quinn, J. M. A., Karlstrom, R. O., Kotrla, K., Goodman, C. S., and Ball, E. E. (1992). Position-specific expression of the annulin protein during grasshopper embryogenesis. Dev. Biol. *154*, 129–142.

Bate, C. M., and Grunewald, E. B. (1981). Embryogenesis of an insect nervous system. II: A second class of neuron precursor cells and the origin of the intersegmental connectives. J. Embryol. Exp. Morphol. *61*, 317–330.

Chu-LaGraff, Q., and Doe, C. Q. (1993). Neuroblast specification and formation regulated by wingless in the Drosophila CNS. Science *261*, 1594–1597.

Condron, B. G., and Zinn, K. (1994). The grasshopper median neuroblast is a multipotent progenitor cell that generates glia and neurons in distinct temporal phases. J. Neurosci., in press.

Desai, C. J., Popova, E., and Zinn, K. (1994). A Drosophila receptor tyrosine phosphatase expressed in the embryonic CNS and larval optic lobes is a member of the set of proteins bearing the 'HRP' carbohydrate epitope. J. Neurosci., in press.

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system. Development *116*, 855–863.

Doe, C. Q., Hiromi, Y., Gehring, W. J., and Goodman, C. S. (1988a). Expression and function of the segmentation gene *fushi tarazu* during Drosophila neurogenesis. Science *239*, 170–175.

Doe, C. Q., Smouse, D., and Goodman, C. S. (1988b). Control of neuronal fate by the Drosophila segmentation gene *even-skipped*. Nature *333*, 376–378.

Duffy, J. B., Kania, M. A., and Gergen, J. P. (1991). Expression and function of the Drosophila gene *runt* in early stages of neural development. Development *113*, 1223–1230.

Goodman, C. S. (1982). Embryonic development of identified neurons in the grasshopper. In Neuronal Development, N. C. Spitzer, ed. (New York and London: Plenum Publishing Corp.), pp. 000–000.

Goodman, C. S., and Doe, C. Q. (1993). Embryonic development of the Drosophila central nervous system. In The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds. (Plainview, New York: Cold Spring Harbor Laboratory Press), pp. 1131–1206.

Gutjahr, T., Patel, N. H., Li, X. L., Goodman, C. S., and Noll, M.

(1993). Analysis of the gooseberry locus in Drosophila embryos – gooseberry determines the cuticular pattern and activates gooseberry-neuro. Development *118*, 21–31.

Jacobs, J. R., and Goodman, C. S. (1989a). Embryonic development of axon pathways in the Drosophila CNS. I. A glial scaffold appears before the first growth cones. J. Neurosci. *9*, 2402–2411.

Jacobs, J. R., and Goodman, C. S. (1989b). Embryonic development of axon pathways in the Drosophila CNS. II. Behavior of pioneer growth cones. J. Neurosci. *9*, 2412–2422.

Jan, L. Y., and Jan, Y. N. (1982). Antibodies to horseradish-peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. Proc. Natl. Acad. Sci. USA *79*, 2700–2704.

Joyner, A. L., and Hanks, M. (1991). The *engrailed* genes: evolution of function. Sem. Dev. Biol. *2*, 435–445.

Joyner, A. L., Herrup, K., Auerback, A., Davis, C. A., and Rossant, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the *En-2* homeobox. Science *251*, 1239–1243.

Katz, F., Moats, W., and Jan, Y. N. (1988). A carbohydrate epitope expressed uniquely on the cell surface of Drosophila neurons is altered in the mutant *nac* (neurally altered carbohydrate). EMBO J. *11*, 3471–3477.

Klambt, C., and Goodman, C. S. (1991). The diversity and pattern of glia during axon pathway formation in the Drosophila embryo. Glia *4*, 205–213.

Klambt, C., Jacobs, J. R., and Goodman, C. S. (1991). The midline of the Drosophila central nervous system: a model for the genetic analysis of cell fate, cell migration and growth cone guidance. Cell *64*, 801–815.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G., and Wittig, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature *353*, 43–48.

Kleuss, C., Scherubl, H., Hescheler, G., Schultz, G., and Wittig, B. (1992). Different β subunits determine G-protein interaction with transmembrane receptors. Nature *358*, 424–426.

Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1993). Selectivity in signal transduction determined by γ subunits of heterotrimeric G proteins. Science *259*, 832–834.

Lawrence, P. A., and Johnston, P. (1984). On the role of the *engrailed* gene in the internal organs of Drosophila. EMBO J. *3*, 2839–2844.

Logan, C., Khoo, W. K., Cado, D. K., and Joyner, A. L. (1993). Two enhancer regions in the mouse *En-2* locus direct expression to the mid/hindbrain region and mandibular myoblasts. Development *117*, 905–916.

Martinez-Arias, A. (1993). Development and patterning of the larval epidermis of Drosophila. In The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds. (Plainview, New York: Cold Spring Harbor Laboratory Press), pp. 517–608.

Merritt, D. J., Hawken, A., and Whitington, P. M. (1993). The role of the *cut* gene in the specification of central projections by sensory axons in Drosophila. Neuron *10*, 741–752.

Millen, K. J., Wurst, W., Herrup, K., and Joyner, A. L. (1994). Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse *Engrailed-2* mutants. Development *120*, 695–706.

Myers, P. Z., and Bastiani, M. (1993). Growth cone dynamics during the migration of an identified commissural growth cone. J. Neurosci. *13*, 127–143.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., and Goodman, C. S. (1989a). Expression of engrailed proteins in arthropods, annelids and chordates. Cell *58*, 955–968.

Patel, N. H., Schafer, B., Goodman, C. S., and Holmgren, R. (1989b). The role of segment polarity genes during Drosophila neurogenesis. Genes Dev. *3*, 890–904.

Patel, N. H., Ball, E. E., and Goodman, C. S. (1992). Changing role of *even-skipped* during the evolution of insect pattern formation. Nature *357*, 339–342.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell *77*, 349–360.

Shepherd, D., and Bate, C. M. (1990). Spatial and temporal patterns of neurogenesis in the embryo of the locust (*Schistocerca gregaria*). Development *108*, 83–96.

Singer, M. A., Hortsch, M., Goodman, C. S., and Bentley, D. (1992). Annulin, a protein expressed at limb segment boundaries in the grasshopper embryo, is homologous to protein cross-linking transglutaminases. Dev. Biol. *154*, 143–159.

Sink, H., and Whitington, P. M. (1991). Pathfinding in the central nervous system and periphery by identified embryonic Drosophila motor axons. Development *112*, 307–316.

Snow, P. M., Patel, N. H., Harrelson, A. L., and Goodman, C. S. (1987). Neural-specific carbohydrate moiety shared by many surface glycoproteins in Drosophila and grasshopper embryos. J. Neurosci. *7*, 4137–4144.

Taghert, P. H., and Goodman, C. S. (1984). Cell-determination and differentiation of identified serotonin-immunoreactive neurons in the grasshopper embryo. J. Neurosci. *4*, 989–1000.

Thomas, J. B., Bastiani, M. J., Bate, M., and Goodman, C. S. (1984). From grasshopper to Drosophila: a common plan for neuronal development. Nature *310*, 203–207.

Thompson, K. J., and Siegler, M. V. S. (1991). Anatomy and physiology of spiking local and intersegmental interneurons in the median neuroblast lineage of the grasshopper. J. Comp. Neurol. *305*, 659–675.

Udolph, G., Prokop, A., Bossing, T., and Technau, G. (1993). A common precursor for glia and neurons in the embryonic CNS of Drosophila gives rise to segment-specific lineage variants. Development *118*, 765–775.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y., and Jan, Y. N. (1989). *numb*, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell *58*, 349–360.