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Engrailed Negatively Regulates the Expression of Cell Adhesion Molecules Connectin and Neuroglian in Embryonic *Drosophila* Nervous System

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Summary

Engrailed is expressed in subsets of interneurons that do not express Connectin or appreciable Neuroglian, whereas other neurons that are Engrailed negative strongly express these adhesion molecules. Connectin and Neuroglian expression are virtually eliminated in interneurons when *engrailed* expression is driven ubiquitously in neurons, and greatly increased when *engrailed* genes are lacking in mutant embryos. The data suggest that Engrailed is normally a negative regulator of *Connectin* and *neuroglian*. These are the first two "effector" genes identified in the nervous system of *Drosophila* as regulatory targets for Engrailed. We argue that differential Engrailed expression is crucial in determining the pattern of expression of cell adhesion molecules and thus constitutes an important determinant of neuronal shape and perhaps connectivity.

Introduction

engrailed-like genes are present widely across phyla. In *Drosophila*, *engrailed* and the related *invected* gene function in epidermal patterning (reviewed by Hidalgo, 1996; Lawrence and Struhl, 1996). *engrailed* is also expressed in the central nervous system (CNS) (Brower, 1984; Patel et al., 1989b) and contributes to neuroblast (NB) identity (Bhat and Schedl, 1997). *engrailed*-like genes occur in mouse (Joyner et al., 1985) and in other vertebrates (Darnell et al., 1986, J. Cell Biol., abstract; Fjose et al., 1988). Developmental expression in vertebrates is different from that in *Drosophila* and other insects, however (Patel et al., 1989a): whereas the insect pattern is segmentally reiterated, in vertebrates expression is regionalized. Engrailed expression is crucial to the development and patterning of the cerebellum and the optic tectum, for example (Joyner and Martin, 1987; Joyner et al., 1991; Wurst et al., 1994; Rétaux and Harris, 1996). This contrasts with similarities of pattern between the insect homeotic genes and vertebrate *Hox* genes (Holland, 1992), ordered in expression along anteroposterior body axes.

In grasshopper, we found a neuronal type specificity of Engrailed expression that bore an intriguing similarity to one reported in vertebrates: in mouse, *En2* is expressed in neurons intrinsic to the cerebellar cortex but not in the neurons comprising its output, the efferent Purkinje cells (Davis et al., 1988; Logan et al., 1993). In grasshopper, Engrailed is expressed in interneurons but not in efferent neurons of identified lineages (Siegler and Pankhaniya, 1997). The interneurons and the efferents have somata in a tight cluster, but their primary neurites and axons trace different pathways (Thompson and Siegler, 1991, 1993). This observation led us to consider that Engrailed genes might have a commonality of function at the cellular level, say in an aspect of pathfinding, not evident when comparing broader patterns of expression.

A notable gap in our understanding is how *engrailed*-related and homeotic/*Hox*-related genes exert their effects at the level of cell structure and function (Graba et al., 1997; Nonchev et al., 1997). In *Drosophila*, virtually all known targets of Engrailed regulation are regulatory or "selector" genes that function in segmentation and epidermal patterning (Eaton and Kornberg, 1990; Raftery et al., 1991; Tabata et al., 1992; Mann, 1994; Schwartz et al., 1995; Serrano et al., 1995). *b3 tubulin* is the first "effector" gene identified as a direct Engrailed target, but it is not expressed in the CNS (Serrano et al., 1997). In vertebrates, only two putative targets have been identified among "effector" genes. Expression of Ephrins ELF-1 and RAGS (ephrin-A5) is positively correlated with the rostrocaudal gradient of increasing Engrailed expression in the tectum (Cheng et al., 1995; Drescher et al., 1995). When ectopic *En1* expression occurs rostrally, ELF-1 and RAGS are upregulated (Logan et al., 1996).

Cell adhesion molecules in *Drosophila* are attractive potential targets of *engrailed* regulation. Within the extensive literature that describes the disparate features of Engrailed expression and misexpression and the effects of related homeotic and *Hox* genes, a commonality is evident (Siegler and Pankhaniya, 1997). *engrailed* and other homeobox genes contribute to the orderly assembly and migration of cells during morphogenesis and pattern formation, consistent with the regulation of cell affinity or cell adhesion molecules. In the nervous system, this role would be manifest in directing pathfinding in the embryo and in maintaining pathway integrity in the adult, promoting association of like cell types in neuronal tracts. Cell adhesion and other guidance molecules have been studied extensively in *Drosophila* (Goodman, 1996). Most are known to be expressed in specific but overlapping subsets of identified neurons in CNS and peripheral nervous system (PNS). How these spatial patterns are regulated is unknown. It is intriguing that homeobox genes apart from *engrailed* are also expressed in subsets of neurons. When expression is disturbed, the neurons make pathfinding errors that can be reasonably attributed to the disturbance of cell adhesion or guidance molecules (Doe et al., 1988a, 1988b; Lundgren et al., 1995; Thor and Thomas, 1997).

In the present work, we show that, as in grasshopper, in *Drosophila* the Engrailed-positive neurons are interneurons and motor neurons are Engrailed negative. When *engrailed* expression is perturbed, cell adhesion

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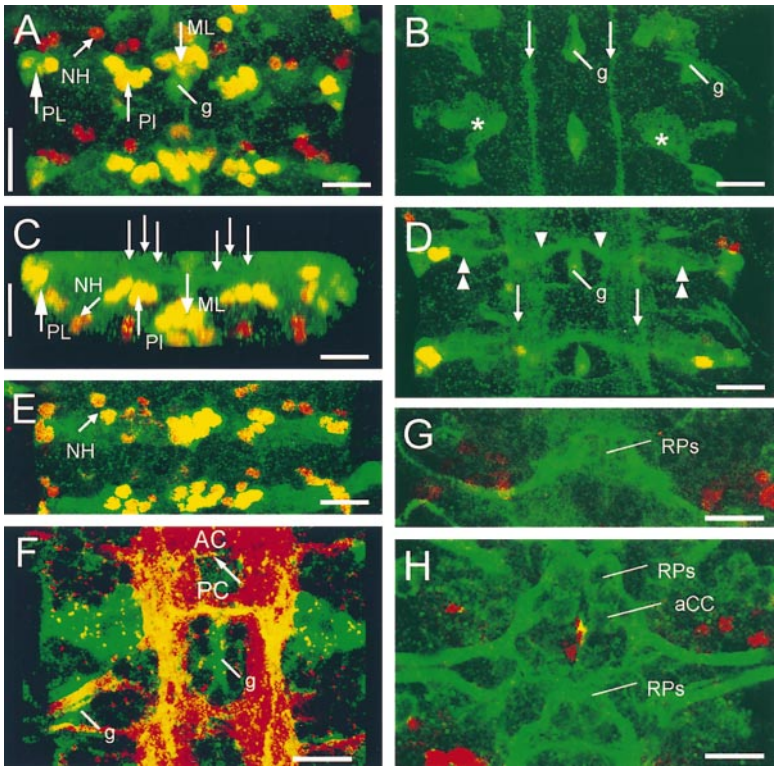

Figure 1. *engrailed* Is Expressed in Interneurons but Not Motor Neurons of CNS

Panels are confocal images from abdominal neuromeres of CNS in stage 16 embryos.

(A–D) CNS from *ryxho25* embryos immunolabeled with anti-Engrailed/Invected and anti-β-gal (*en–lacZ* expression). The region shown in (A), (B), and (D) comprises an anteroposterior distance of two neuromeres, with anterior at the top. Stripes of labeled neuronal cell bodies in (A) are at the posterior of each neuromere. Each panel is from a different level of the same Z series. (C) is a rotated view of a region of the Z series. In (A), ventrally, a single ML group and two bilateral groups of somata, the PI group and the PL group, have nuclear Engrailed/Invected (red) and cytoplasmic β-gal (green) immunolabel. The cytoplasmic label and the nuclear label only appear to overlap (yellow) in the projection. Glial cytoplasm ("g") posterior to the ML group is indicated. Three bilateral pairs of NH neurons anterior to the stripe have nuclear Engrailed/Invected immunolabel but not cytoplasmic β-gal immunolabel. One NH pair is indicated. The vertical bar at the left shows the anteroposterior distance compiled in (C). In (B), a single section from a dorsal region of the Z series shows the most dorsal of three pairs of β-gal-positive longitudinal fascicles (arrows), glial cytoplasm at ML and lateral roots ("g"), and bilateral groups of neuronal somata (asterisks) that contain faint cytoplasmic β-gal but no nuclear Engrailed/Invected immunolabel. In (C), a projected transverse view shows three longitudinal fascicles (three pairs of arrows); PI, PL, and ML β-gal-positive somata groups; and β-gal-negative NHs. The vertical bar at the left shows the dorsoventral extent of the Z series sections compiled in (A). (D) is a projection of two Z sections immediately ventral to (B) showing a thick bundle of neurites in the PC (downward single arrowheads) and neurites originating from somata of PL groups (upward double arrowheads). Two arrows indicate the course of the most lateral of the three β-gal-positive longitudinal fascicles.

(E) Abdominal neuromeres from *wxba21* embryo. NH neurons are β-gal positive, indicating *invected* expression.

(F) CNS from *ryxho25* embryo immunolabeled with anti-β-gal (green) and anti-HRP (red). A single fiber can be traced into the AC (arrow at posterior of AC). No other labeled axons are found in the ISNs or SNs. Glia ("g") are labeled at the ML and lateral roots.

(G and H) Motor neurons including RPs and aCC also immunolabeled in (G) with anti-FasIII mAb 7G10 (green) or in (H) with mAb 22C10 (green) are negative for Engrailed/Invected (red).

Scale bars, 20 μm.

molecules Connectin and Neuroglian have altered expression consistent with a negative regulation by Engrailed. In the normal CNS and PNS, patterns of expression are consistent with such a role. Engrailed-positive neurons do not express Connectin and express Neuroglian faintly, if at all; conversely, expression of Connectin and Neuroglian occurs only among neurons that are Engrailed negative. The distribution of Engrailed binding sites on polytene chromosomes is consistent with a direct regulation of *neuroglian* but is inconclusive for *Connectin*. These are the first such effector genes identified in the nervous system of *Drosophila* and the most extensively characterized to date as neural regulatory targets for Engrailed.

Results

Engrailed Is Expressed in Interneurons but Not Motor Neurons in the CNS

At stage 16, Engrailed/Invected expression occurred in two bilateral groups of neurons at the posterior of each hemineuromere, PL and PI, in midline (ML) neurons including progeny of the median neuroblast (MNB), in NH neurons, and in some glia (Figures 1A, 1B, and 1C) (Patel et al., 1989a; Cui and Doe, 1992; Mellerick et al., 1992). Engrailed and Invected, products of the *engrailed*-like genes *engrailed* and *invected*, are not distinguished by available antibodies. To distinguish between *engrailed*- and *invected*-expressing neurons, and to trace neuronal projections, the Engrailed/Invected pattern was compared with that of β-galactosidase (β-gal) expression in *ryxho25* and *wxba21* (Hama et al., 1990). In *ryxho25*, an *en–lacZ* construct is expressed in the same pattern as the endogenous *engrailed* gene, whereas in *wxba21* expression is in the *invected* pattern. The two lines further have the fortuitous property that the β-gal is cytoplasmic, and the projections of expressing neurons can be traced considerable distances.

In *ryxho25* embryos, neurons of the PL, PI, and ML groups had Engrailed/Invected-positive nuclei and β-gal-positive cytoplasm, the combination indicating the continued expression of *engrailed*. The NH neurons were Engrailed/Invected positive but did not express β-gal, indicating that Engrailed/Invected labeling came from Invected alone. Conversely, at least one cluster of neurons in each hemineuromere labeled for β-gal but not

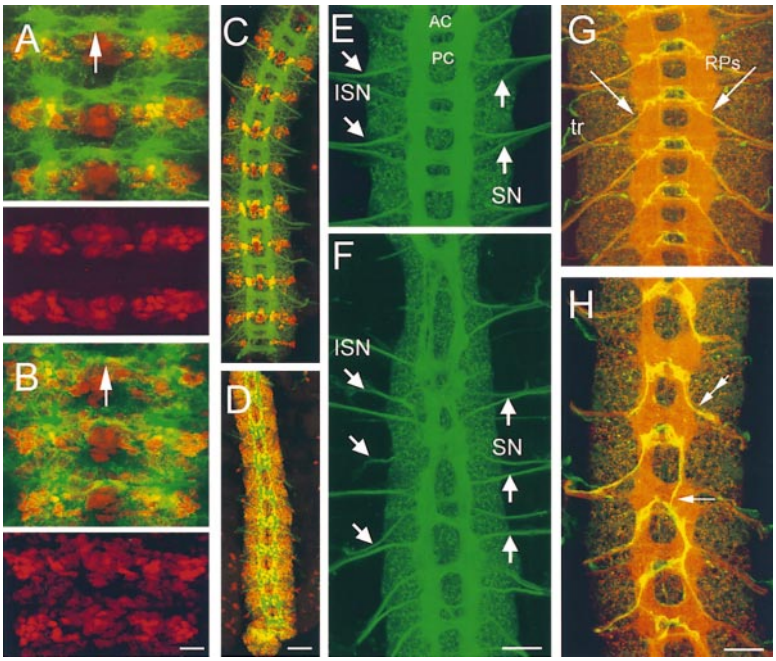

Figure 2. CNS Scaffold and Identified Motor Neurons Are Disrupted with Ubiquitous Neural Engrailed Expression

Embryos are derived from *C155–GAL4/UAS–en* crosses. (A), (C), (E), and (G) are controls and (B), (D), (F), and (H) are experimentals. Embryos were immunolabeled with anti-Engrailed/Invected (red) and anti-HRP (green) for (A) through (F). (A) and (B) show three abdominal neuromeres above (both labels) and two below (Engrailed/Invected label only) of late stage 13 embryos. The AC and PC have not separated at this stage. A commissure is indicated by an arrow in each panel. In experimentals (B), the commissures have the same relationship to the native Engrailed/Invected stripe as in controls (A) but are about half the thickness. Ectopic Engrailed expression is apparent between the native Engrailed/Invected stripes. (C) and (D) compare the abdominal portion of the CNS of stage 16 controls (C) and experimentals (D) at low magnification. (E) and (F) are stage 16 controls (E) and experimentals (F) with anti-HRP immunolabel only (green). The AC and PC, and the ISN (diagonal arrows) and SN (upward arrows), are normal in the control but are disordered or missing in the experimental. Control (G) and experimental (H) stage 16 embryos were double labeled with anti-HRP (red) and anti-FasIII (green). The RP motor neurons are among the most obvious neurons labeled with anti-FasIII (two are indicated by thin arrows). Their axons exit ISNs in each segment. Each hemisegment has a conspicuous FasIII-positive tracheole (tr). Experimental embryos (H) have aberrant RP pathways. Scale bars, 20 μm.

for Engrailed/Invected (asterisks, Figure 1B). The β-gal was weaker than in Engrailed/Invected-positive/β-gal-positive neurons, which, together with the lack of Engrailed/Invected label, suggests that *engrailed* had been expressed at an earlier stage, but downregulated, though the β-gal perdured (Hama et al., 1990). Cytoplasmic β-gal also occurred in Engrailed/Invected-positive ML glia and in lateral glia at the roots of the segmental nerve (SN) and the intersegmental nerve (ISN) ("g" in Figures 1A, 1B, 1D, and 1F). In *wxba21*, anti-Engrailed/Invected and anti-β-gal labeled all neurons and glia that were labeled in *ryxho25*, and in the same pattern (Figure 1E). In addition the NH neurons, which were β-gal negative in *ryxho25*, were β-gal positive in *wxba21*, consistent with the idea that NH neurons express *invected* only. Thus, with the exception of the NH neurons, all Engrailed/Invected-positive neurons and glia express both *engrailed* and *invected*.

The pattern of β-gal in *ryxho25* and *wxba21* indicated that the Engrailed/Invected neurons in the CNS are interneurons, extending axons in discrete bundles. In *ryxho25*, labeling occurred in a bundle of primary neurites that originate from the PL neurons (double arrowheads, Figure 1D), in at least one thick bundle of neurites that crosses within the posterior commissure (PC) (single arrowheads, Figure 1D), in the neurites of ML neurons, and in axons of three longitudinal tracts (unlabeled sets of arrows in Figures 1B, 1C, and 1D). Neurites originated from Engrailed/Invected-positive neurons and remained within the CNS. No β-gal-positive neurites were associated with Engrailed/Invected-negative somata (asterisks, Figure 1B). At stage 16, when motor neurons had extended to their target regions, or at earlier stages, no β-gal-positive neurites could be traced from the CNS (Figures 1D and 1F). In particular, we found no evidence for β-gal-positive axons in the ISNs or SNs associated with the three Engrailed-positive ventral unpaired median (VUM) neurons (see Figure 4E), and consistent with other evidence (Jia and Siegler, unpublished data) only the three Engrailed-negative VUM neurons are efferents. A single β-gal-positive axon in each hemisegment originated near the lateral pentascolopidial organ ("lch5", Figure 3E) and could be traced into the anterior commissure (AC) (diagonal arrow, Figure 1F). Anti-β-gal labeling of *wxba21* gave essentially the same pattern (data not shown).

Further evidence that motor neurons do not express *engrailed* (or *invected*) was provided by labeling wild-type embryos with antibodies that reveal identified neurons. The RP motor neurons, including RP3, are positive for Fasciclin III (FasIII), and the aCC and also the RP motor neurons label with mAb 22C10 in a well-defined pattern. None were Engrailed/Invected positive (Figures 1G and 1H). Labeling with 22C10 or with the other markers showed also that not all interneurons are Engrailed/Invected positive, including, for example, the pCC and SP1 interneurons (data not shown). Thus, the CNS neuronal expression of *engrailed* and *invected* is cell type specific, occurring only within a subset of interneurons but not in motor neurons.

Ubiquitous Neuronal Expression of *engrailed* Disrupts CNS and PNS Morphology

If differential expression of Engrailed directs the differential expression of cell adhesion molecules, then perturbing the pattern of Engrailed expression in postmitotic neurons should alter neuronal pathfinding and hence CNS and PNS morphology. As an initial test of

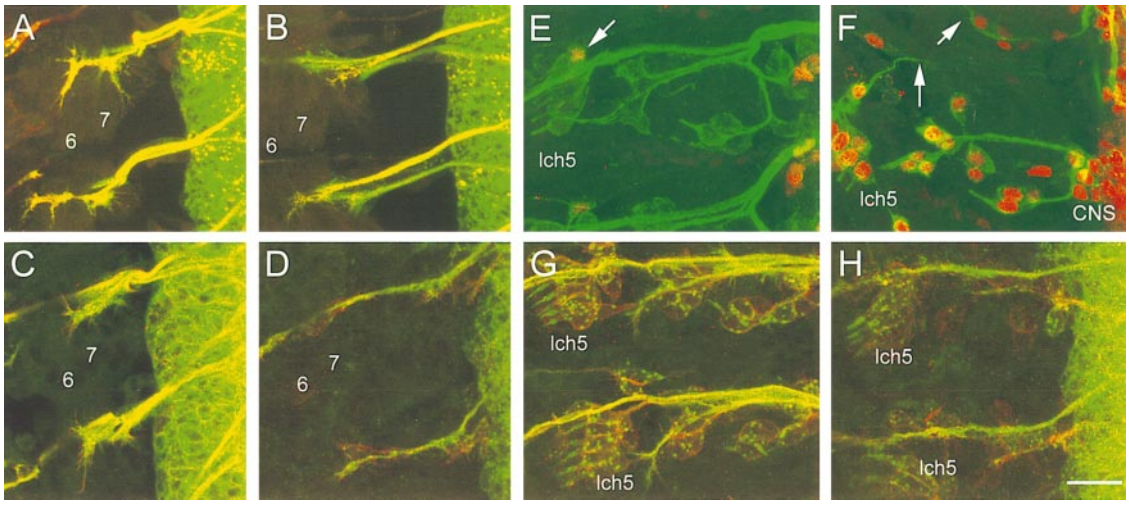

Figure 3. Motor Neurons and Sensory Neurons Develop Abnormally in the PNS

In (A) through (D), each panel shows branching of motor neurons at muscle 7 ("7") and muscle 6 ("6") for two hemisegments in Stage 16 control and experimental embryos. In controls (A) and (C), growth cones extend normally over the muscles, but in experimentals growth cones are absent or greatly reduced (B) and (D). (A) and (B) show immunolabeling with anti-FasII (red) and anti-HRP Ab (green). All motor neurons are positive for both labels (yellow). (C) and (D) show immunolabeling with BP104. In (E) through (H), panels show the lateral pentascolopidial organ ("lch5") and other sense organs and their axons in the PNS. In control embryos (E and G), sensory axons fasciculate in a regular pattern, but bundles of sensory axons are grossly reduced or absent in experimental embryos (F and H). Arrows in (F) indicate peripheral and central ends of a segmental nerve. The CNS is to the right in each panel. (E) and (F) show immunolabeling with 22C10 (green) and anti-Engrailed/Invected (red). A single sensory neuron near lch5 is Engrailed/Invected positive in the control (arrow in E), whereas all sensory neurons are Engrailed/Invected positive in the experimental (F). (G) and (H) show immunolabeling with BP104. Scale bars, 20 μm.

this idea, we used the *GAL4/UAS* system to generate ectopic neuronal expression of Engrailed (Brand and Perrimon, 1993). In the *C155–GAL4* line, the *GAL4* driver is fortuitously inserted under the control of the *embryonic lethal abnormal visual system* (*elav*) promoter (Lin and Goodman, 1994). ELAV is a neuron-specific protein, not expressed in NBs or ganglion mother cells (GMCs) (Robinow and White, 1991). The driver strain was crossed with a responder strain containing *UAS–en* (Brand and Perrimon, 1993). In controls, Engrailed/Invected labeling was confined principally to a stripe of neurons in the posterior of each neuromere (Figures 2A and 2C) and to a few sensory neurons (Figure 3E). In experimentals, Engrailed/Invected labeling occurred in all neurons of the CNS (Figures 2B and 2D) and of the PNS (Figure 3F), whereas gaps in labeling corresponded to NBs and GMCs. Ectopic expression was not observed in neurectoderm, NBs, GMCs, or glia, so all effects arise from altered neuronal expression.

Differences between control and experimental embryos were apparent by late stage 13. In controls, the incipient AC and PC were separated at the lateral extremes of the single ML commissure (Figure 2A). In experimental embryos of the same stage, there was little evidence for separation between the incipient AC and PC (Figure 2B). In mid stage 16 control embryos, the normal CNS scaffold and the SNs and ISNs were well developed (Figures 2C and 2E), but in experimentals the CNS was narrower from side to side (Figure 2D) but thicker in the dorsoventral dimension. The SNs and ISNs were missing or reduced in over half the neuromeres examined and exited the CNS at aberrant locations (Figures 2D and 2F). The intersegmental connectives were thinner than normal, and the AC and PC were partly or wholly fused.

Antibodies that detect specific tracts, commissures, or subsets of neurons revealed additional defects. FasIII is expressed on a few axon bundles and conspicuously on the RP3 motor neurons (Patel et al., 1987). In normal embryos, the RP motor neurons exited the CNS in the ISN (Figure 2G) and then joined a branch of the SN. RP motor neurons of experimentals had a range of defects (Figure 2H). In some hemisegments, the RP motor neurons projected within the CNS to more posterior hemisegments (horizontal arrow, Figure 2H). In other hemisegments, the RP neurons had grown past the edge of the CNS scaffold, but the ISN was fused to the longitudinal connective (double arrow, Figure 2H). Fasciclin II (FasII) is normally expressed in three longitudinal fascicles in the CNS and also on the axons of all motor neurons (Lin et al., 1994). In experimentals, FasII-positive longitudinal fascicles were severely disrupted. The MP1 pathway was thinner than normal, the lateral fascicle appeared only in some hemisegments, and the FN3 fascicle was missing (data not shown).

Another striking result was abnormal development of neuromuscular junctions (NMJs). In stage 16 controls, robust motor neuron growth cones occurred in virtually all abdominal segments, as seen in the region of body wall muscles 6, 7, and 13 (M6, M7, and M13) (Figures 3A and 3C). By contrast, in experimentals, motor neuron growth cones occurred in only a fraction of hemisegments examined. In embryos raised for 16 hr at 25°C, M6 and M7 were innervated in 100% of control hemisegments ($n = 36$) and 76% of experimental hemisegments

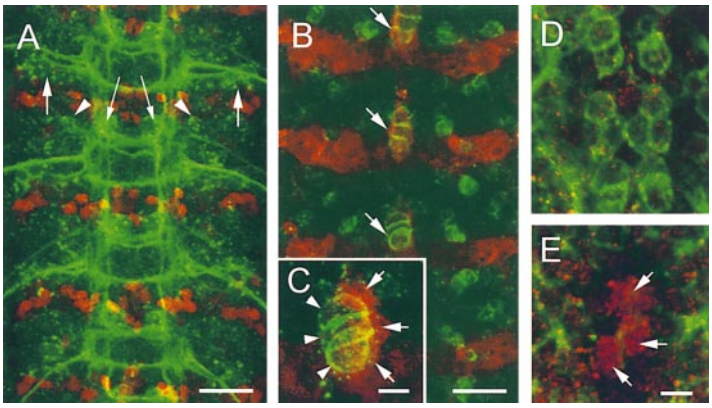

Figure 4. Connectin and Neuroglian Are Not Expressed in Engrailed-Positive Neurons

(A) Connectin-positive neurons (green) including SNa and SNc motor neurons (upward arrows) and interneurons in the CNS scaffold (downward arrows and arrowheads) are negative for Engrailed (red, anti-Engrailed/Invected mAb) in normal embryos.

(B and C) Three of the six VUM neurons per segment are Connectin positive and Engrailed/Invected negative. Anti-β-gal Ab (red) reveals *engrailed*-expressing cells in *ryxho25* embryos. In (B), VUM groups of three segments are indicated with diagonal arrows. The VUM groups are ventral in the CNS, and the underlying stripe of *engrailed*-expressing epidermal cells (red) is evident at the posterior of each segment. Underlying *engrailed* expression from this region (red) appears to coincide with the Connectin-positive VUMs (green) in one group in this projected image, but frame-by-frame analysis confirmed that labeling is in separate cells. (C) shows higher magnification of three Connectin-positive/Engrailed-negative VUMs (arrowheads) and three Connectin-negative/Engrailed-positive VUMs (arrows) in one group. In (A) through (C), Connectin-positive neurons are labeled with anti-Connectin Ab (green).

(D and E) *engrailed* and Neuroglian expression do not coincide. Anti-β-gal Ab (red) reveals *engrailed*-expressing cells in *ryxho25* embryos, and Neuroglian is detected with BP104 (green). Neuroglian-positive RP neurons at the dorsal ML do not express *engrailed* (D), whereas *engrailed*-expressing VUM neurons (arrows) do not express Neuroglian (E).

Scale bars, 20 μm (A and B); 5 μm (C–E).

(n = 34). M13 was innervated in 94% of control hemisegments (n = 35) but only 28% of experimental hemisegments (n = 32). The phenotype was more severe in embryos raised for 10 hr at 29°C. M6 and M7 were innervated in 100% of control hemisegments (n = 15) but only in 29% of experimental hemisegments (n = 14). M13 was innervated in 100% of control hemisegments (n = 15) but 0% of experimental hemisegments (n = 15). Growth cones in experimental embryos displayed a gradation of shapes. Most had sparse filopodial contact (Figures 3B and 3D) or were collapsed or club-like in shape. Growth cones in experimentals extended over an area one-third to one-half of the area of growth cones in controls. In embryos raised for 14 hr at 25°C, growth cones in experimentals were 53% the size of those in controls for M6/M7 and 47% the size of those in controls for M13. In embryos raised for 10 hr at 29°C, growth cones in experimentals were 32% the size of those in controls for M6/M7, and M13 had no growth cones. In hemisegments that lacked M6, M7, and M13 growth cones, the motor neurons were present but had not exited the CNS.

Sensory neurons were also disturbed. In controls, processes of sensory neurons traveled inward in the ISN or the SN to terminate within the CNS (Figures 3E and 3G). In experimentals, a full complement of sensory neurons was present, but the nerves and central fascicles containing their processes were disorganized. Peripheral nerves were "frayed," or the connections between afferent and efferent fibers were lacking altogether (Figures 3F and 3H). This affected the morphology of the CNS as well as the PNS, inasmuch as sensory axons contribute to certain conspicuous fascicles in the CNS.

The morphological defects that occurred when the Engrailed pattern was disturbed were consistent with an incorrect regulation of cell adhesion molecules. We therefore screened to identify potential Engrailed targets and examined en^C and en^E deficiency embryos as well as *C155–GAL4/UAS–en* embryos. Two molecules, Connectin and Neuroglian, were altered in expression in a way that suggested negative regulation by Engrailed.

Connectin Expression Is Negatively Regulated by Engrailed

Although Connectin was once considered to act via repulsive cell–cell interactions, it is now thought to function as a cell adhesion molecule (Nose et al., 1997; Raghavan and White, 1997). Connectin is expressed in a subset of lateral motor neurons and in two longitudinal and three commissural bundles that contain interneurons (Nose et al., 1992; Meadows et al., 1994). All of these neurons are Engrailed negative (Figure 4A). Connectin is also expressed in VUM neurons (Meadows et al., 1994), which have been thought to be exclusively efferent neurons. Three of the six VUM neurons per neuromere were strongly Connectin positive but were also Engrailed negative (Figures 4B and 4C). These, we suggest, are the efferent members of the VUM population. The three remaining VUMs, which are Engrailed positive, had no or negligible Connectin expression. *ryxho25* labeling shows that no Engrailed-positive neurons exit the CNS, and thus we conclude that these three VUMs are interneurons.

The neuronal pattern of expression, with Engrailed-positive/Connectin-negative neurons and Connectin-positive/Engrailed-negative neurons, was consistent with a negative regulation of Connectin by Engrailed. Results from mutant phenotypes supported this. In *C155–GAL4/UAS–en* embryos compared with controls, Connectin expression was greatly reduced or virtually lacking within the CNS scaffold and the VUM neurons (Figures 5A and 5B). The scaffold was most severely disturbed in the embryos that also had the lowest levels of Connectin. Since the defects variably included fused commissures, and thicker or thinner longitudinal connectives, we suspect that the interneurons were still

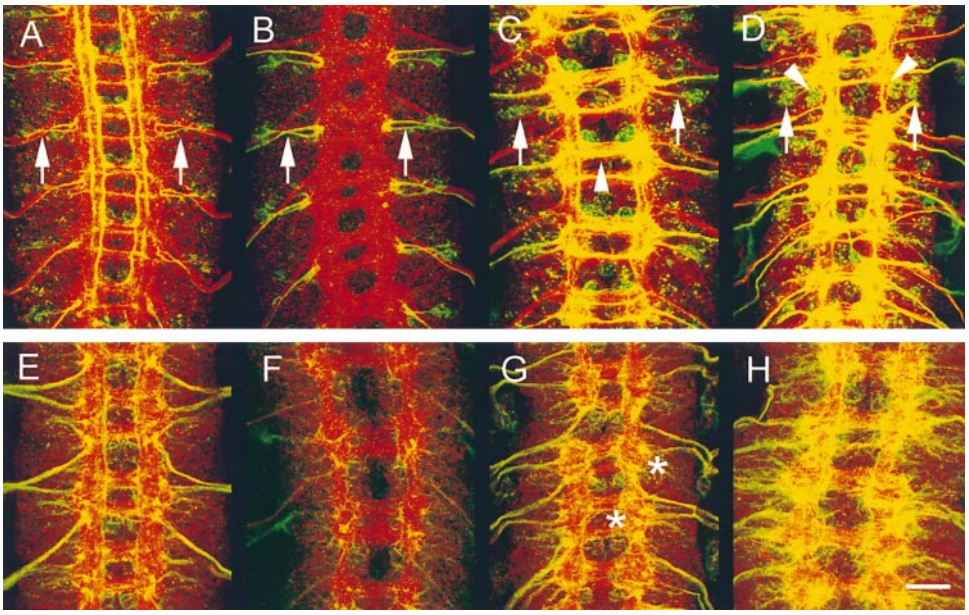

Figure 5. Connectin and Neuroglian Expression Are Altered When Engrailed Is Misexpressed

(A) through (D) show stage 16 embryos double labeled with anti-Connectin mAb (green) and anti-HRP Ab (red). (E) through (H) show stage 16 embryos double labeled with BP104 (neural form of Neuroglian) (green) and anti-HRP Ab (red).

(A–D) Panels comprise four or five posterior segments of the abdominal CNS.

(A) Control embryo shows normal pattern of Connectin expression in longitudinal fascicles, commissures, and SNa and SNc motor neurons (upward arrows in all panels).

(B) In experimental embryo, Connectin labeling is lacking in longitudinal fascicles and commissures. SNa and SNc motor neurons express Connectin at control or slightly above control levels.

(C) In en^C embryos, Connectin-positive longitudinal fascicles and commissures (an AC is indicated by an upward arrowhead) are abnormal, and Connectin labeling is conspicuously darker than normal. SNa and SNc motor neurons are also Connectin positive.

(D) en^E embryos have more extreme defects than do en^C embryos. Connectin-positive longitudinal fascicles and commissures are grossly disturbed, and Connectin labeling is yet stronger than in en^C embryos. Additional groups of cell bodies express Connectin, including interneuron groups that occur in the same position as groups that are normally Engrailed positive (diagonal arrowheads).

(E–H) Panels comprise three or four posterior segments of the abdominal CNS.

(E) Control embryo with Neuroglian immunolabel conspicuous in ISNs and SNs in each segment.

(F) In experimentals, Neuroglian immunolabel is significantly reduced in all normally Neuroglian-positive neurons.

(G) In en^C embryos, the CNS is abnormal and Neuroglian labeling is darker than normal, particularly near the roots of ISNs and SNs (asterisks).

(H) en^E embryos have more extreme defects than do en^C embryos. Increased expression of Neuroglian is again particularly evident in regions of the CNS scaffold near the roots of ISNs or SNs.

Scanning parameters were identical for (A) through (D) and for (E) through (H) and optimized for normal embryos. Scale bar, 20 μm.

present but, lacking Connectin, traced aberrant pathways. By contrast, in en^C (*engrailed* mutant) embryos, Connectin expression was greatly enhanced in the CNS scaffold, in commissural bundles in particular (arrowhead, Figure 5C). The en^E (*engrailed* and *invected* mutant) phenotype was yet stronger (Figure 5D), and in some segments clusters of Connectin-positive somata occurred ectopically in regions where clusters of Engrailed/Invected-positive interneurons are normally found (diagonal arrowheads, Figure 5D).

Unlike the effects on interneuronal expression, Connectin expression in the SNa and SNc lateral motor neurons was somewhat stronger than normal both in *C155* experimentals and in deficiency mutants (upward arrows, Figures 5A–5D). Since the change was in the same direction whether Engrailed was overexpressed or absent, it is possible that the effects were a nonspecific consequence of the failure of normal NMJ formation, as Connectin expression normally decreases in motor neurons after NMJ formation (Nose et al., 1992; Meadows et al., 1994). en^C and en^E have defects in NMJ formation but for reasons different than in *C155–GAL4/UAS–en* embryos, where muscles are apparently normal. In en^C and en^E, the muscles are grossly abnormal, probably due to earlier epidermal defects. It may also be that Engrailed failed to downregulate Connectin in SNa and SNc because the motor neurons lacked an Engrailed cofactor (Mann, 1995) or lacked some other intermediate genetic mediator.

These data from normal and mutant embryos suggest that Engrailed (and Invected) acts as a negative regulator of Connectin expression. To explore this idea further, we examined two additional experimental conditions. One concern we had was that lower levels of interneuronal Connectin expression in stage 16 experimental embryos might result indirectly from the generally disturbed growth and form of the CNS. We therefore examined hs-*en3* embryos, where *engrailed* is expressed under

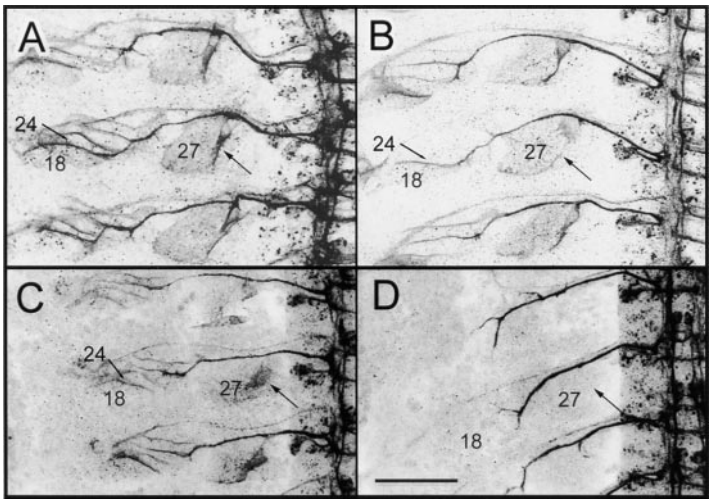

Figure 6. Connectin Expression in Muscle Is Reduced with Ectopic Engrailed Expression

Normal (A) and hs-*en3* (B) Stage 16 embryos.

(A) In controls, Connectin is expressed in a normal pattern in the CNS, and in a subset of muscles. These include M18 (18), M24 (24), and M27 (27). Expression predominates where these muscles are apposed, seen for example as a dark band between M18 and M24, and at the medial edge of M27 (diagonal arrows in all panels).

(B) In heat-shocked hs-*en3* embryos, Connectin expression is significantly reduced in the same muscles and in the PNS and CNS.

In (C) and (D), control (C) and experimental (D) embryos of a *24B–GAL4*/*UAS–en* cross are compared.

(C) In *24B* controls, Connectin is expressed in a normal pattern.

(D) In *24B* experimentals, Connectin expression is completely lacking in muscle but normal in CNS and PNS. Scale bar, 60 μm.

the control of a heat shock promoter (Poole and Kornberg, 1988). Embryos developed normally until stages 15 or 16 and then were heat shocked. Controls had a normal pattern of Engrailed expression, whereas heat-shocked embryos expressed Engrailed ubiquitously (data not shown). In heat-shocked embryos, Connectin expression was reduced in the CNS and in the subset of muscles that normally are Connectin positive (Figures 6A and 6B). Connectin expression was also reduced in SNa and SNc motor neurons, consistent with the idea that downregulation in the motor neurons fails in experimentals, and in en^C and en^E, because of failure of NMJ formation. NMJ formation will have proceeded normally in the hs-*en3* embryos prior to the heat shock experimental treatment.

We also tested whether downregulation of Connectin expression could be driven in tissue such as muscle where Engrailed is not normally expressed. Such an outcome would be consistent with CNS changes being autonomous effects of Engrailed rather than resulting from nonspecific CNS developmental disturbances. Connectin is expressed in a subset of body wall muscles innervated by the SNa and SNc motor neurons (Nose et al., 1992). Instead of the *C155–GAL4* driver used in other experiments, we crossed the *24B–GAL4* driver strain, which is expressed in all embryonic muscles (Brand and Perrimon, 1993), with *UAS–en*. In control embryos of the cross, Connectin was expressed in a normal pattern in muscle and CNS (Figure 6C). In experimental embryos of the *24B–GAL4/UAS–en* phenotype, Connectin expression was completely lacking in the normal complement of muscles but was normal in the CNS (Figure 6D). Motor axons of SNa and SNc motor neurons contacted muscle fibers near their normal locations. Labeling for Engrailed/Invected confirmed that Engrailed was expressed in all body wall muscles of the experimentals (but in none in controls), and DIC confocal microscopy confirmed that the normally Connectin-positive complement of muscles was indeed present in experimentals.

Neuroglian Expression Is Negatively Regulated by Engrailed

Neuroglian is a member of the immunoglobulin superfamily and is closely related to the mouse adhesion molecule L1 (Bieber et al., 1989). Neuroglian has two isoforms. One is expressed exclusively in the nervous system and is revealed by mAb BP104, used here. The neuron-specific isoform is expressed in all sensory neurons, and in the CNS most conspicuously in axons of motor neurons (Hortsch et al., 1990). Neurons with conspicuous Neuroglian levels, for example the RP motor neurons, did not express Engrailed (Figure 4D), whereas the Engrailed-expressing VUM neurons lacked Neuroglian expression (Figure 4E). In the region of other Engrailed/Invected interneurons at the posterior of each neuromere, Neuroglian expression was virtually absent. We could not tell whether Neuroglian expression was lacking altogether or present at a very low level in any single neuron we inspected because of the diffuse nature of Neuroglian expression (Hall and Bieber, 1997). Also, as observed for Connectin, however, the normal pattern of expression predicted a negative regulatory role for Engrailed.

In *C155* experimentals, Neuroglian expression (BP104) was greatly reduced in the PNS compared to controls (Figures 3G and 3H). This was not a result of the disorganization of the PNS, and by contrast 22C10 immunolabeling was of similar intensity in experimentals and controls (Figures 3E and 3F). Motor axons also had reduced Neuroglian expression compared with controls (Figures 3C and 3D). In experimentals, Neuroglian expression was also greatly reduced within the CNS and in the SNs and ISNs (Figures 5E and 5F). By contrast, in en^C, Neuroglian expression was stronger than in wild type in the CNS scaffold, particularly near the origins of the SNs and ISNs (Figure 5G). The en^E phenotype was yet stronger (Figure 5H). Neuroglian labeling also revealed, again, that the SNs and ISNs were grossly disturbed in en^C and en^E.

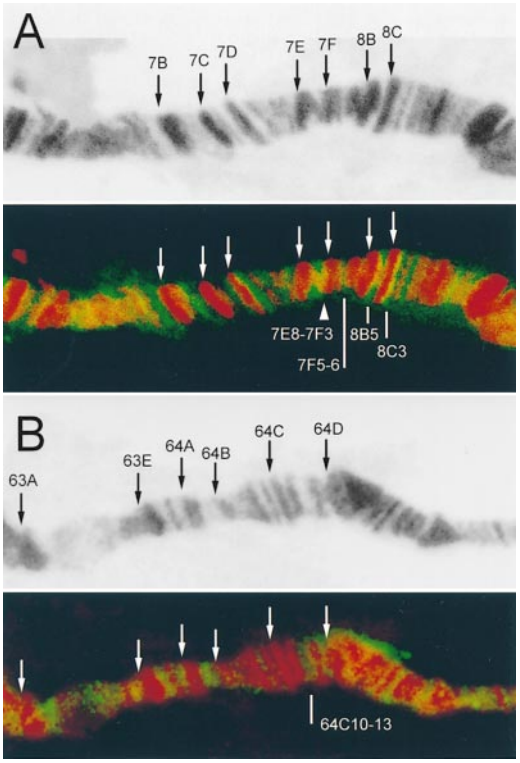

Figure 7. Engrailed Binding Sites

Salivary gland polytene chromosomes from hs-*en3* larvae. Confocal images from regions that include (A) the *neuroglian* map location (7F1) and (B) the *Connectin* map location (64C3–64C5). For each pair of images, the black and white panel shows the banding pattern revealed by propidium iodide, with several bands identified. The color panel shows the same scan, with propidium iodide in red and Engrailed immunolabel in green. Engrailed binding appears yellow at bands. The white arrows mark the reference bands from the black and white image. Regions of Engrailed binding closest to *neuroglian* and *Connectin* map locations are indicated by an arrowhead (7E8–7F3) or by vertical lines and are labeled by location.

Are *Connectin* or *neuroglian* Direct Targets for Engrailed? Evidence from Polytene Chromosome Binding Sites

To explore whether the regulatory effects of Engrailed on the *Connectin* or *neuroglian* genes might result from direct binding to these sites, we analyzed salivary gland polytene chromosomes in hs-*en3* third instar larvae. Although *engrailed* expression does not occur in normal salivary glands, numerous Engrailed binding sites of various intensities were apparent after heat shock (Figure 7). The mapping results are consistent with the possibility that *neuroglian* is a direct target of Engrailed regulation. The coding region of *neuroglian* maps to region 7F1, and a band of Engrailed labeling occurred immediately adjacent and overlapping at 7E8–7F3 (Figure 7A). A binding site at 7F has been mapped by light microscopy (Serrano et al., 1995). A weaker binding site at 7F5–7F6 and other stronger binding sites in the region seem too removed to be functional sites for *neuroglian* repression. We found no evidence for a binding site near 64C3–64C5 (Figure 7B), the map region of *Connectin*. Weak binding occurred in the regions of 63 and 64, but these Engrailed-positive sites did not map consistently in the region, save for a weak band at 64C10–64C13, and the latter seems too distant from *Connectin* to be a likely regulatory site. Such results are inconclusive, since negative data do not rule out a direct interaction.

Discussion

Differential Expression of Engrailed and Invected in Subsets of Interneurons

We show here that neuronal expression of *engrailed* is limited to a subset of interneurons in the embryonic CNS and does not occur in motor neurons. A similar division by neuronal type was found in adult grasshopper and prompted the present set of experiments (Siegler and Pankhaniya, 1997). Although *engrailed* is expressed in many cell types throughout development, including in NBs and GMCs, this clearly does not preclude a role for *engrailed* in postmitotic neuronal differentiation. Consistent with this view, when *engrailed* expression is driven ubiquitously in postmitotic neurons, the development of the embryonic nervous system is profoundly disturbed.

In wild-type embryos, all neurons that express *engrailed* also express *invected*. The converse is not true, however. The NH neurons, which lie anterior to the predominant Engrailed/Invected stripe in the CNS, express *invected* but not *engrailed*. To our knowledge, this is the best example to date of differing expression of *engrailed* and *invected* in an identified cell type. We infer that Engrailed and Invected can have additive or compensatory functions, since the ectopic expressions of Connectin and of Neuroglian are stronger in en^E than en^C.

Engrailed Downregulates Connectin in Interneurons

Our results strongly suggest that Engrailed normally functions as a negative regulator of Connectin expression within groups of interneurons of the embryonic CNS. Conversely, we infer that lack of Engrailed is permissive for expression of Connectin in other interneurons. Connectin is expressed in several sets of longitudinal and commissural interneurons (Nose et al., 1992; Meadows et al., 1994), but as we show here none of these interneurons are normally Engrailed positive. Connectin is dramatically downregulated in these interneurons, however, when Engrailed is expressed in all postmitotic neurons. Connectin is now thought to have a cell adhesive role in motor neuron pathfinding and in determination of muscle morphology (Nose et al., 1997; Raghavan and White, 1997) and likely serves the same purpose in interneurons. The most severe CNS phenotypes are observed in preparations where there is the most complete downregulation of Connectin, and we suggest that degrees of failure of Connectin expression have led to degrees of failure in axonal pathfinding. Longitudinal connectives are thinnest in the most severe of the overexpression phenotypes, for example. No null mutation of the *Connectin* gene has been isolated, however. Thus, we cannot judge whether the downregulation of Connectin in the CNS scaffold accounts for the CNS overexpression phenotype, or whether the effects we

see are more severe and thus suggest additional regulatory targets for Engrailed. The lack of Connectin expression is not simply a nonspecific effect of disturbing the CNS scaffold, however, since when embryos are allowed to develop to a stage when the scaffold is fully formed and then heat shocked, the form of the CNS is normal but expression of Connectin is nonetheless reduced. Additionally, Connectin expression is lost on muscle when Engrailed is expressed ectopically in muscle only. Although the latter manipulation does not mimic the normal distribution of Engrailed, downregulation of Connectin by Engrailed overexpression in a different tissue type lends further credence to the idea that the effects in the CNS are not simply a nonspecific effect of altered CNS morphology. The results from en^C and en^E embryos are also consistent with a specific effect on Connectin. Connectin is upregulated within axons of the CNS scaffold, and Connectin-positive clusters of neuronal cell bodies appear in regions where Engrailed/Invected-positive clusters would normally occur. These changes are the reverse of those seen in the Engrailed overexpression phenotype. In en^C and en^E, however, the lack of correct epidermal patterning and lack of glial expression of Engrailed must also contribute to the severe disruption of the CNS and PNS.

Connectin is also expressed in SNa and SNc motor neurons (Nose et al., 1992), which are Engrailed negative. When Engrailed is expressed in all neurons, Connectin is not downregulated but slightly upregulated in these motor neurons, in contrast to the effect on interneurons. Since Engrailed can act either as a repressor or as an activator (Serrano and Maschat, 1998), it is possible that ectopic Engrailed directly activated Connectin in the motor neurons. In en^C and en^E, Connectin was also expressed at normal or above normal levels in the SNa and SNc motor neurons, but because Engrailed is not normally expressed in the motor neurons anyway, this result is not informative. Some possible explanations for the inconsistent effects on motor neurons are a nonspecific result of failure of NMJ regulation, or the lack of a cofactor or a regulatory gene that is present in all interneurons but lacking in motor neurons and required directly or indirectly for normal Engrailed function. The *Connectin* locus was identified first as a direct binding site for Ultrabithorax (Gould et al., 1990). Ultrabithorax represses *Connectin* expression in the embryonic CNS, and *Ultrabithorax* is suggested from genetic interactions to be a negative target for Engrailed (Mann, 1994). Thus, if Engrailed represses *Ultrabithorax*, the net effect would be an upregulation of Connectin. More needs to be learned about the expression patterns of Ultrabithorax, or other putative intermediaries, before these possibilities can be assessed.

Analysis of salivary gland polytene chromosomes failed to reveal any consistent Engrailed binding site at or near 64C3–64C5, the cytological location of *Connectin*. This negative result does not rule out a direct effect of Engrailed on *Connectin* in embryonic interneurons, however. Again, it is possible that binding of Engrailed requires a cofactor not expressed in the salivary glands, or that a stage- or tissue-specific alteration prevents Engrailed binding to polytene chromosomes. *Connectin* is thought to be directly regulated by Ultrabithorax (Gould et al., 1990), yet heat shock overexpression of Ultrabithorax also does not reveal a binding site at the *Connectin* locus (Botas and Auwers, 1996).

Engrailed Downregulates Neuroglian in All Neurons

Our results suggest that Engrailed functions as a negative regulator of Neuroglian expression within the embryonic CNS and that lack of Engrailed permits expression of Neuroglian. In wild-type embryonic *Drosophila*, the neuronal form of the cell adhesion molecule Neuroglian is expressed at high levels in motor neurons, sensory neurons, and a small subset of interneurons (Hortsch et al., 1990; Hall and Bieber, 1997), none of which are Engrailed positive. Downregulation of Neuroglian occurred in all classes of neurons, however, when Engrailed was expressed ubiquitously in postmitotic neurons. This did not result from the loss of neurons that normally express Neuroglian: for example, the number of neurons in sense organs, assayed by markers including 22C10 and anti-HRP, appeared to be roughly normal. Conversely, Neuroglian expression was stronger than normal in en^C and en^E embryos, particularly where Engrailed-positive neurons normally occur.

As might be expected, the phenotype resulting from ectopic expression of Engrailed had aspects in common with that of extreme *neuroglian* mutants (Hall and Bieber, 1997). In *neuroglian* mutants, motor neurons do not innervate muscles properly, stalling as they reach their targets or bypassing normal routes. However, axon bundles arising from sensory organs appear to be normal. The Engrailed overexpression phenotype was more severe, however. Motor neurons additionally had collapsed growth cones in the periphery, or axons that had grown posteriorly in the CNS instead of to the periphery. Axon bundles from sensory organs were also reduced in the PNS and sometimes failed to reach the CNS. Engrailed overexpression must therefore affect the expression of target molecules in addition to Neuroglian to yield the PNS and NMJ phenotypes. Connectin misexpression cannot be responsible since, for example, it is not expressed in the motor neurons that innervate muscles 6, 7, and 13, either in wild-type or in Engrailed overexpression embryos. Nonetheless, pathfinding and growth cone elaboration were greatly disturbed in these motor neurons. Connectin misexpression also is not contributing to the sensory neuron phenotype, since it is not expressed in the sensory neurons. In *neuroglian* mutants, no significant defects are found in commissures and longitudinal pathways, whereas the Engrailed overexpression embryos have a profoundly disturbed CNS morphology. Although Neuroglian is normally expressed in a small number of interneurons, the lack of significant effects on the CNS morphology in *neuroglian* mutants leads us to conclude that the reduced Neuroglian expression makes a negligible contribution to the overexpression phenotype in the CNS.

Analysis of polytene chromosome preparations reveals an Engrailed binding site adjacent to and overlapping 7F1, the cytological location of *neuroglian*. The sequence preceding the transcription start for *neuroglian* contains numerous ATTA motifs, core recognition sites for some homeobox transcription factors (Zhao and Horscht, 1998), and our inspection of the registered

sequence reveals many sequences similar to putative "consensus" sites for Engrailed (see, e.g., Serrano et al., 1995). Molecular analysis is required to determine if, indeed, *neuroglian* is a direct target of Engrailed as our results suggest.

Neuroglian is closely related to the mouse adhesion molecule L1 (Bieber et al., 1989). In light of our evidence for repression of *neuroglian* by Engrailed, we find it intriguing that the L1 promoter has an Engrailed "consensus" binding site (Kohl et al., 1992) and that both L1 and Engrailed are expressed differentially in the cerebellum. The Purkinje cells, which express L1 (Rathjen and Schachner, 1984), are Engrailed negative (Davis et al., 1988). When *En2* is expressed ectopically in Purkinje cells late in neural development, there is a considerable loss of Purkinje cells (Baader et al., 1998). L1 has multiple roles in axon growth and guidance (Walsh and Doherty, 1997). Perhaps ectopic Engrailed downregulates the expression of L1 (just as ectopic Engrailed downregulates Neuroglian), and the Purkinje cells fail to grow normally and so die. This is a speculative leap from insect to mammal, but given the surprising degree of conservation in genetic networks between *Drosophila* and vertebrates (in the Engrailed–Wingless pathway, for example), it is not so far-fetched to suggest that interactions between regulatory genes and target genes might also be conserved.

A Neural Role for Engrailed and Other Homeobox Genes

We propose that differential Engrailed expression is crucial in determining the pattern of expression of cell adhesion molecules in the CNS and PNS and thus constitutes an important determinant of neuronal shape and, perhaps, connectivity. In the interactions we report, Engrailed acts as a negative regulator of two cell adhesion molecules, but we must assume that other cell adhesion molecules and guidance molecules, as yet unspecified, are normally expressed in the Engrailed-positive interneurons and there mediate pathfinding. We imagine that unique combinations of such molecules are selected through positive and negative regulation by Engrailed and other selector genes, in different subsets of identified interneurons and motor neurons, the combinations giving each neuronal subset its own pathfinding "signature." The present results show that Engrailed has a role in postmitotic neuronal differentiation. Engrailed expression continues throughout the adult stage in the CNS of grasshopper (Siegler and Pankhaniya, 1997) and as late as pharate adult in *Drosophila* (unpublished data), suggesting that Engrailed is involved not only in the differentiation but also in the maintenance of distinct neuronal phenotypes.

Engrailed expression was altered only in postmitotic neurons in the overexpression phenotype, and no consistent conversions of neuronal type were observed. Only occasionally did identified motor neurons assume an interneuron-like morphology, growing centrally rather than to the periphery. Thus, we do not consider that Engrailed misexpression led to a change in "cell fate" in our experiments. However, our results do not preclude the possibility that Engrailed expression can in fact determine cell fate at an earlier developmental stage. Indeed, other work points to a role for Engrailed in determination of neuroblast identity (Bhat and Schedl, 1997). Early work in *Drosophila* suggested that *fushi tarazu* and *even skipped*, homeobox genes involved in segmentation, also had a role in neuronal cell fate or pathfinding (Doe et al., 1988a, 1988b). More recently, LIM homeobox genes have been show to be involved in pathway selection among motor neurons in vertebrates (Tsuchida et al., 1994). In *Drosophila* embryos lacking the LIM homeobox gene *apterous*, interneurons that normally express Apterous fail to fasciculate with each other and do not reach correct target regions (Lundgren et al., 1995). In *islet* mutants, motor neurons that normally express Islet have substantially disturbed axonal pathfinding (Thor and Thomas, 1997). *islet* and *apterous* are expressed only in postmitotic neurons, so the effects would appear to be on differentiation rather than on determination of cell fate. By contrast, *fushi tarazu* and *even skipped* are expressed in NBs and GMCs as well as neurons, so their mode of action is unclear. As the results from Engrailed show, however, an effect on neuronal differentiation does not preclude an earlier developmental role in cell lineage and cell fate.

Connectin and Neuroglian are the first "effector" genes identified as functional neural targets of Engrailed in *Drosophila*. We suspect that other such targets will emerge, given that the *engrailed* overexpression phenotype is more severe than would be predicted from the disturbance of Neuroglian and Connectin only. Defects in the CNS are reminiscent of phenotypes described for other segment polarity or segmentation mutants (Patel et al., 1989b; Finkelstein et al., 1990). A change at two levels, one in the balance of cross-regulatory interactions between *engrailed* and other regulatory genes and another as a direct effect on genes encoding additional "effector" molecules, could explain the marked effects of perturbing *engrailed* expression. Engrailed likely has additional targets among "effector" genes, and other regulatory or "selector" genes in the genetic network will themselves have their own complement of "effector" targets. The approach we have used here may be generally useful in identifying such targets among the wealth of "effector" genes already identified in *Drosophila*. If *engrailed* and other regulatory genes are shown each to have a unique but overlapping complement of "effector" targets, then we will be some way toward understanding how genetic combinatorial codes are executed. It will be of considerable importance to discover how these interactions have been tailored to the unique demands of the nervous system.

Experimental Procedures

Fly Strains and Experimental Preparations

Virgin females of the *C155–GAL4* line were crossed to males of the *UAS–en* line, to yield a population of embryos of which half had normal *engrailed* expression and half had ubiquitous neuronal expression of *engrailed* under the control of the *elav* promoter. Embryos were raised at 25°C or 29°C. Similar crosses were done with *24B–GAL4*/*UAS–en*. Salivary glands were prepared by the methods of Serrano et al. (1995) and double labeled with propidium iodide and mAb 4D9. hs-*en3* embryos were treated by standard protocols (Poole and Kornberg, 1988).

Immunohistochemistry

Embryos were dissected in *Drosophila* saline and fixed for 20–40 min in 3.7% paraformaldehyde prior to immunohistochemistry. Earlier-stage whole embryos were processed en masse for immunohistochemistry. Tissue was incubated in the first primary (mAb 4D9) overnight at 4°C, washed, and incubated for 1.5–2 hr at 25°C with the secondary, previously preadsorbed against fixed embryos. Embryos were incubated overnight at 4°C with anti-HRP Ab, washed, and mounted in SloFade or ProLong (Molecular Probes). In preparations labeled using mAb 4D9 and another mouse mAb, cross-reactivity was minimized or eliminated by a fixation step after mAb 4D9 processing and before application of the other primary Ab. mAb 4D9 recognizes a nuclear label, but all other mAbs recognize membrane or cytoplasmic labels, so minimal cross-reactivity did not interfere with data analysis.

Primary antibodies were anti-Engrailed/Invected (mAb 4D9) and anti-FasIII (mAb 7G10) from the Developmental Studies Hybridoma Bank (DSHB), mAb 22C10 from Seymour Benzer and DSHB, anti-FasII (mAb 1D4) and anti–neuronal Neuroglian (mAb BP104) from C. S. Goodman, and anti-Connectin from Rob White and Akiro Nose. Anti-β-gal (rabbit polyclonal) was from Cappel and anti-HRP Ab and secondary Abs were from Jackson ImmunoResearch.

Data Collection

Embryos were examined using a BioRad 1024 Laser Scanning Confocal microscope. Projected images were made using BioRad software, with comparable numbers of sections projected for comparisons of control and experimental embryos. Image files were assembled using Adobe Photoshop. Growth cone data was measured from projected confocal images using NIH Image.

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