Even-skipped, acting as a repressor, regulates axonal projections in Drosophila

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Summary

Nervous system-specific eve mutants were created by removing regulatory elements from a 16 kb transgene capable of complete rescue of normal eve function. When transgenes lacking the regulatory element for either RP2+a/pCC, EL or U/CQ neurons were placed in an eve-null background, eve expression was completely eliminated in the corresponding neurons, without affecting other aspects of eve expression. Many of these transgenic flies were able to survive to fertile adulthood. In the RP2+a/pCC null background, the expression of eve was completely eliminated in the corresponding neurons, without affecting other aspects of eve expression. Many of these transgenic flies were able to survive to fertile adulthood. In the RP2+a/pCC mutant flies: (1) both RP2 and aCC showed abnormal axonal projection patterns, failing to innervate their normal target muscles; (2) the cell bodies of these neurons were positioned abnormally; and (3) in contrast to the wild type, pCC axons often crossed the midline. The Eve HD alone was able to provide a weak, partial rescue of the mutant phenotype, while both the Groucho-dependent and -independent repressor domains contributed equally to full rescue of each aspect of the mutant phenotype. Complete rescue was also obtained with a chimeric protein containing the Eve HD and the Engrailed repressor domain. Consistent with the apparent sufficiency of repressor function, a fusion protein between the Gal4 DNA-binding domain and Eve repressor domains was capable of actively repressing UAS target genes in these neurons. A key target of the repressor function of Eve was Drosophila Hb9, the derepression of which correlated with the mutant phenotype in individual eve-mutant neurons. Finally, homologues of Eve from diverse species were able to rescue the eve mutant phenotype, indicating conservation of both targeting and repression functions in the nervous system.

Key words: Axon guidance, Homeodomain, Transcriptional repressor, Evx, Hb9, Grunge, Atrophin, Groucho, Eve, Nervous system

Introduction

The homeobox-containing gene even skipped (eve) was first identified as a segmentation gene based on the cuticular pattern of hypomorphic mutants (Nüsslein-Volhard and Wieschaus, 1980). In order to function as a segmentation gene, the transcriptional repressor function of the gene product Eve is required, and also appears to be sufficient, in the context of the Eve homeodomain (HD) (Fujioka et al., 1995; Fujioka et al., 2002). The domains of Eve necessary to repress target genes were analyzed in cultured cells (Han and Manley, 1993; Jaynes and O’Farrell, 1991) and in vivo, and two distinct repressor domains were identified. One was shown to be dependent on the co-repressor Groucho (Kobayashi et al., 2001), while the other is Groucho independent (Jimenez et al., 1997), and was shown to interact functionally with the Drosophila Atrophin homologue Grunge (Erkner et al., 2002; Zhang et al., 2002). Each of these repressor domains was shown to be required for segmentation function both at the blastoderm stage and early in gastrulation, with each domain contributing roughly equally to the activity on each target gene (Fujioka et al., 2002).

Later during Drosophila development, eve is expressed in the nervous system, in the mesoderm in cells which develop into dorsal muscles and pericardial cells, and in the anal plate ring (Frasch et al., 1987). Regulatory elements sufficient to drive each of these aspects of the pattern were localized, downstream of the coding region (Fujikoa et al., 1999; Sackerson et al., 1999). In the nervous system, Eve is expressed in some ganglion mother cells (GMCs) and in their daughter neurons (Frasch et al., 1987; Patel et al., 1989): the aCC and pCC neurons (derived from GMC 1-1a), the RP2 and RP2-sibling neurons (from GMC 4-2a; eve expression in RP2-sibling is subsequently turned off), and the U/CQ neurons (which are generated by several GMCs in the neuroblast 7-1 lineage) (Bossing et al., 1996; Broadus et al., 1995). The other eve-expressing neurons, EL neurons, are derived from neuroblast 3-3 (Schmidt et al., 1997); however, the GMCs that produce them are eve negative (Skeath and Doe, 1998). The aCC, RP2 and U/CQ neurons are motoneurons, and their axons innervate the dorsal muscle field (Landgraf et al., 1997; Schmid et al., 1999; Sink and Whitington, 1991), whereas the pCC and EL
cells are interneurons. Expression of eve in the nervous system is well conserved. For example, in the grasshopper *Schistocerca americana* and in Crustaceans, Eve orthologs are expressed in identified neurons that are homologous to those expressing eve in *Drosophila* (Duman-Scheel and Patel, 1999; Patel et al., 1992; Patel et al., 1994). Studies of Eve function in adjacent V1 interneurons. When evx1 developing spinal cord to V0 interneurons and is not expressed eve dorsal muscles (Landgraf et al., 1999). In the mouse, least in some contexts) to direct motoneurons to innervate indicating that Eve function is both necessary and sufficient (at least in some contexts) to direct motoneurons to innervate dorsal muscles (Landgraf et al., 1999). In the mouse, expression of the eve homologue evx1 is restricted in the developing spinal cord to V0 interneurons and is not expressed in adjacent V1 interneurons. When evx1 function was removed, the majority of V0 interneurons failed to extend commissural axons and became similar to V1 neurons, suggesting that Evx1 is a determinant of V0 neuronal identity (Moran-Rivard et al., 2001). Consistent with the action of Eve and its homologues as repressors that use conserved co-repressors, it has been suggested that the pattern of neurogenesis in the mouse neural tube is regulated in part by the spatially controlled repression of transcriptional repressors, through a Groucho/TLE-dependent mechanism (Muhr et al., 2001), while in humans, a mutation (expansion of a polyglutamine tract) in Atrophin is associated with the neurodegenerative disease DRPLA (Koide et al., 1994; Nagafuchi et al., 1994).

Recent studies showed that several HD proteins are involved in the regulation of neuronal identity (Thor and Thomas, 2002). In *Drosophila*, the identities of ventrally projecting motoneurons appear to be specified by Islet, Lim3 and *Drosophila* Hb9 (Exex – FlyBase), while Eve regulates the identity of dorsally projecting motoneurons (Broihier and Skeath, 2002; Landgraf et al., 1999; Odden et al., 2002; Thor et al., 1999; Thor and Thomas, 1997). The expression patterns of *Drosophila* Hb9 and Eve do not overlap in the wild-type CNS (Broihier and Skeath, 2002; Odden et al., 2002), and ectopic expression of Eve represses *Drosophila* Hb9 expression, indicating that *Drosophila* Hb9 might be a direct target of Eve (Broihier and Skeath, 2002). Expression of Islet and Eve is also non-overlapping in the wild-type CNS, and ectopic expression of Eve represses islet expression in most motoneurons, although neither the absence of Islet nor its ectopic expression was found to change the eve expression pattern (Landgraf et al., 1999; Thor and Thomas, 1997). Lim3 and Eve are co-expressed in the EL neurons, but not in other Eve-positive neurons (Broihier and Skeath, 2002).

In this study, we address aspects of Eve protein function and conservation, and investigate in detail the requirements for eve function in the nervous system, by creating eve neuron-specific mutants. We accomplish this by rescuing eve-null mutants with transgenes containing the entire eve locus deleted for individual neuronal regulatory elements. This results in a complete loss of detectable Eve expression in the corresponding neurons, without affecting other aspects of the expression pattern. Combining these with reporter transgenes that specifically mark RP2 and a/pCC, we analyze the mutant phenotype. The lack of Eve causes severe alterations in axonal morphology, among other defects. We find that our constructed mutants have a more severe mutant phenotype than that caused by eveD19, and so apparently have a more complete loss of eve function. Furthermore, in situations of partially penetrant rescue, we observe a strong correlation between a mutant axonal morphology and derepression of *Drosophila* Hb9. We test which domains of Eve are required for rescue, and find that the Eve HD alone can partially rescue some aspects of the mutant phenotype. However, for full rescue, both of the Eve repressor domains are required, and this requirement can be fully supplied by a heterologous repressor domain from Engrailed. Homologues of Eve from species as diverse as the mouse are also able to rescue eve function in the developing nervous system.

**Materials and methods**

**Plasmid construction**

Wild-type eve genomic DNA from either –6.4 to +9.2 kb (EGN92) or –6.4 to +8.6 kb (EGN86) was cloned into a modified pCaSpeR vector, as described previously (Fujioka et al., 1999; Fujioka et al., 2002). Throughout this paper, we refer to the P-element transgenes *P[ eve-ΔRP2A], P[ eve-ΔRP2B] and P[ eve-ΔRP2C]* by the abbreviated names *ΔRP2A*, *ΔRP2B* and *ΔRP2C*, respectively. RP2+a/pCC neuronal mutant constructs were made by deleting the following regions from EGN92 (Fig. 1): for *ΔRP2A*, +8.2 (*Cidl*) to +9.2 kb (*EcoRI*); for *ΔRP2B*, +8.0 (*Plcl*) to +9.2 kb; and for *ΔRP2C*, +8.0 (*Plcl*) to +8.6 kb (*Nhel*). The U/CQ neuronal mutant construct *ΔCQ* was made by deleting the region +3.5 (*BglII*) to +4.0 kb (*NruI*) from EGN92, and the EL neuronal mutant construct *ΔEL* was created by deleting the region from +1.9 (*MluI*) to +2.6 kb (*EcoRI*) from EGN92.

The first generation of RP2+a/pCC-specific Gal4 drivers was constructed as previously described (Baines et al., 1999). The eve 5′ promoter region from –275 (*SfiI*) to +11 bp (*XhoI*) was fused to a fragment (flanked by *HindIII* sites) that includes the Gal4-coding region from plasmid pCEP4-Gal4 (gift from Stefan Thor) followed by the eve 5′ region from +1306 (*BsrUI*) to +1917 bp (*MluI*). The RP2+a/pCC element from +7.9 (*EcoRI*) to +9.2 kb was placed upstream of this eve 5′ promoter and Gal4-coding region. This construct was then modified as follows. A 38 bp multi-cloning

![Fig. 1. Rescue transgenes used to create neuron-specific eve mutants. Deletions were made in the context of a complete rescue transgene consisting of the eve locus from –6.4 to +9.2 kb (relative to the transcription start site, see Materials and methods for details). Three different deletions (ΔRP2A, B, C) were made in the region sufficient to drive expression in RP2, aCC and pCC neurons. Other deletions were of the minimal elements necessary to drive expression in either U/CQ neurons (ΔU/CQ) or EL neurons (ΔEL). An unbroken line indicates the region included in each construct, while a gap indicates the deleted region. The end points of each deletion are given above the line.](image-url)
sequence upstream of eve DNA from +91 to +99 nucleotides, followed by an ATG, was used to replace the yeast GAL4 translation initiation signal with that of eve. In addition, the eve 3' region from +1306 to +1521 (KpnI) was used instead of the +1917 end point. The region from +7.9 to +9.2 kb (to generate RRA-eve4), or two tandem repeats of the fragment from +7.9 to +8.6 kb (to generate RN2-eve4), was then inserted upstream of this promoter-Gal4-coding region.

A Gal4-Eve fusion protein construct has been described previously (Fujioka et al., 2002). This fusion protein coding region was placed downstream of either two tandem repeats of the RP2a/+pCC element (the eve region from +7.9 to +8.6 kb), two tandem repeats of the U/CQ element (the region from +3.5 to +4.3 kb), or the EL element (the region from +1.9 kb, MluI, to +3.0 kb). Two reporter UAS constructs were made in order to test the ability of Gal4-Eve to actively repress transcription in vivo. For testing the activity in RP2a/+pCC neurons, the mesodermal element (the region from +5.7 to +6.7 kb, Smal) and the RP2a/+pCC element (+7.9 to +8.6 kb) were cloned upstream of the eve 5' region from −275 to +99 bp, followed by the EGF coding sequence (Clontech) and the eve 3' untranslated region from +1306 to +1521 bp, followed by the EL element from +1.9 to +3.5 kb. The UAS sequence, amplified by PCR using as a template the pUAST plasmid (Brand and Perrimon, 1993), was inserted between the mesodermal element and eve 5' promoter. To test the repression activity in U/CQ neurons, the fragment from +1.9 to +4.5 kb (BamHI) was cloned upstream of the eve 5' region from −275 to +166 bp, followed by the lacZ-coding region and eve 3' sequences from +1306 to +1521 bp. The UAS sequence was inserted between the regulatory fragment and the eve 5' region.

The constructs for expressing modified versions of Eve, as well as the Eve orthologs Tc-eve and Sa-eve, from a complete rescue transgene, have been described previously (Fujioka et al., 2002). For expressing mouse Evx1, the mesodermal element from +5.7 to +6.7 kb and the RP2a/+pCC element from +7.9 to +8.6 kb were cloned upstream of the eve 5' region from −275 to +99 bp followed by the ATG of a Flag tag fused to the Evx1-coding sequence and the eve 3' untranslated region from +1306 to +1521 bp. This transgene expresses Evx1 in the mesoderm and in the RP2, aCC and pCC neurons.

### Drosophila strains

Transgenic lines were established as described previously (Fujioka et al., 2000; Rubin and Spradling, 1982). When either ∆RP2A or ∆RP2B or ∆RP2C was placed in an eve-null mutant background, expression of Eve at the blastoderm stage appeared to be normal. When either the ∆RP2A or ∆RP2B transgene insertion was homozygous, engrailed expression was regularly spaced as in the wild type, indicating that the segmentation function of eve was fully rescued (data not shown). However, at the extended germ-band stage, eve expression in the mesoderm was often weak or missing in these lines. By contrast, mesodermal expression was normal in the ∆RP2C-rescued lines (data not shown). However, in the ∆RP2C lines, the odd-numbered parasegments were narrower than normal, indicating a lower activity at the blastoderm stage. Because of this, only one out of five lines with this construct gave rescue to adulthood. When one copy of the ∆RP2C transgene was combined (in trans) with one copy of the ∆RP2A transgene, mesodermal expression was normal, segmentation was normal and these heterozygotes were efficiently rescued to adulthood. In these rescued embryos, eve expression was never observed in RP2 and a/pCC neurons, whereas eve expression was normal in EL and U/CQ neurons in all lines (see Results). Unlike some of the RP2-element deletions, deletion of the EL or U/CQ element did not cause a reduction of either mesodermal expression or segmentation function, and both were able to be maintained as stocks. Rescue transgenic lines were crossed into a Df[2R]eve mutant background unless otherwise indicated.

In order to mark clearly the axons of RP2, aCC and pCC neurons, we used the RP2 element to express Gal4 (Brand and Perrimon, 1993), and this in turn to drive UAS-lacZ. In our first such attempt using a single copy of the RP2a/+pCC element (from +7.9 to +9.2 kb) upstream of GAL4 (GAL4-RRc and RRc), only two out of 40 lines were able to reliably activate UAS reporter gene expression (Baines et al., 1999), and even in these cases, activity was rather weak. We tested whether this low activity might be due to a lack of efficient translation in the nervous system by changing the translational initiation signal (which was derived from the GAL4 gene of yeast) to that found in the eve gene (see above for details). With this modification, 11 out of 11 lines were able to drive strong UAS reporter expression (data not shown), albeit with some neurorome-to-neuromere variability. We then tested two tandem repeats of the region from +7.9 to +8.6 kb upstream of GAL4 (RN2-GAL4), and found that this drove expression that was more consistently strong in all neuromes (see Results). RN2-Gal4 on the third chromosome was recombined with either UAS-lacZ (Cullahan et al., 1995) or UAS-CD8GFP (Lee and Luo, 1999), and used in this study.

### Genetic crosses and analysis of embryos

To analyze GFP expression in the RP2 mutant (described in Results), each of the following four lines was self-crossed: (1) Df[2R]eve;RP2A/SM6a;RN2-GAL4,UAS-CD8GFP; (2) Df[2R]eve;RP2C/SM6a;RN2-GAL4,UAS-CD8GFP; (3) eve[813],RP2C/SM6a;RN2-GAL4,UAS-CD8GFP; or (4) Df[2R]eve;RP2A/eve[813],RP2C;RN2-GAL4,UAS-CD8GFP/TM3. In the case of the fourth line, the analyzed population of GFP-expressing progeny contained the following three second chromosome genotypes: Df[2R]eve;RP2A homozygotes, eve[813],RP2C homozygotes, and Df[2R]eve;RP2A/eve[813],RP2C. The subpopulation of these embryos homozygous for the RN2-GAL4,UAS-CD8GFP third chromosome (which gave a stronger GFP signal than RN2-GAL4,UAS-CD8GFP/TM3) was used for the analysis.

For all experiments using a CyO balancer chromosome (below), the progeny not carrying this chromosome (negative for wg-lacZ staining) were analyzed. For the rescue experiments with modified Eve proteins, embryos from the following crosses were analyzed. A line carrying Df[2R]eve;RP2A/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ] was crossed with lines of each of the following second and third chromosome genotypes: for the unrescued control, Df[2R]eve/Cy,O[P[wg-lacZ];∆RP2B; for wild-type Eve, Df[2R]eve,P[EGN86]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ]; for tagged wild-type Eve, Df[2R]eve,Cy,O[P[wg-lacZ];P[wg-AT]; for EveH, Df[2R]eve,P[EveH]/Cy,O[P[wg-lacZ];P[∆RP2B]; for EveNH, Df[2R]eve,P[EveNH]/Cy,O[P[wg-lacZ];P[∆RP2B]; for EveAc, Df[2R]eve,P[EveAc]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ]; for EveAR, Df[2R]eve,P[EveAR]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ]; for EveH-En, Df[2R]eve,P[EveH-En]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ]; for Tc-eve, Df[2R]eve,P[Tc-eve]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ]; for Sa-eve, Df[2R]eve,P[Sa-eve]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ]; and for Evx1, Df[2R]eve,P[Evx1]/Cy,O[P[wg-lacZ];P[∆RP2B]. More than 2 lines of each were analyzed, and the lines showing better rescue for each of the constructs were used for further analysis (however, each of the lines examined showed the same overall trends, with only small variations among them). For two-copy rescue by EveAR and EveAc, the following lines were used: Df[2R]eve,P[EveAR] (or P[EveAR]/Cy,O[P[wg-lacZ];RN2-GAL4,UAS-lacZ was crossed with Df[2R]eve,P[EveAR] (or P[EveAR]/Cy,O[P[wg-lacZ];∆RP2B. In all cases, the combination of rescue transgenes provided complete rescue of eve segmentation function (data not shown).
For analysis of the temperature-sensitive allele eve^{D19}, eve^{D19}/CyO,P[wg-lacZ];RN2-GAL4,UAS-\textit{\textgamma}lacZ was self-crossed for two copies of eve^{D19}, or for one copy, was crossed with \textit{Df(2R)eve}, AR2A/CyO,P[wg-lacZ];RN2-GAL4,UAS-\textit{\textgamma}lacZ. In each case, embryos were collected at 25°C for 1 hour, and were allowed to develop further at 18.5°C for 4 hours. Segmentation was rescued well under these conditions, as determined from analyzing cuticle preparations (Nüsslein-Volhard and Wieschaus, 1980). The embryos were then incubated at 18.5°C (permissive temperature) or 30°C (restrictive temperature), until they developed to the appropriate stages.

Antibody staining was performed as described previously using biotinylated secondary antibodies and SA-HRP (Patel, 1994). For immunofluorescent staining, FITC-conjugated anti-mouse (1:1000) and Texas Red-conjugated anti-rabbit (1:500, Jackson ImmunoResearch) secondary antibodies were used. The following primary antibodies were used: polyclonal anti-Eve at 1:10,000, a gift from M. Frasch (Frasch et al., 1987); anti-Eve monoclonal 2B8 (Patel et al., 1994) at 1:20; polyclonal anti-\textit{\textbeta}-gal at 1:200 (ICN); anti-Fas2 monoclonal 1D4 (Vactor et al., 1993) at 1:10; rabbit anti-\textit{Drosophila} HB9, a gift from J. B. Skeath (Broihier and Skeath, 2002) at 1:500; anti-Putsch monoclonal 22C10 (Fujita et al., 1982) at 1:5; and rat anti-Islet, a gift from J. B. Skeath (Broihier and Skeath, 2002) at 1:200. For the \textit{\textgamma}lacZ marker, the following modifications to the published protocol were used: secondary antibody was incubated overnight at 4°C (instead of 1-2 hours at room temperature), and the incubation time for SA-HRP was prolonged to 2 hours (from 1 hour) at room temperature.

Lucifer Yellow injections
Late stage 16 (14 hours 15 minutes±15 minutes) wild-type and RP2 mutant embryos were dissected in Sorensen phosphate buffer as described previously (Landgraf et al., 1997), with the modifications that collagenase treatment was omitted, and dissected embryos were fixed in 3.7% formaldehyde for 20 minutes. Neurons (RP2, aCC, and pCC) were identified by a combination of GFP expression (using RN2-GAL4;UAS-CD8GFP) and their position in the nerve cord. Cells
**Table 1. Summary of RP2 phenotypes**

<table>
<thead>
<tr>
<th>Rescued with</th>
<th>n</th>
<th>Wild type* (anterior extension)</th>
<th>Anterior extension, truncated</th>
<th>Posterior extension, truncated</th>
<th>Posterior extension</th>
<th>Crossed midline</th>
<th>Bipolar</th>
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<td>50</td>
<td>21</td>
<td>9</td>
<td>8</td>
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<tr>
<td>None 2</td>
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<td>48</td>
<td>23</td>
<td>7</td>
<td>6</td>
<td>17</td>
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<tr>
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<td>0</td>
<td>0</td>
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<td>2</td>
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<tr>
<td>t-wild-type Eve</td>
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<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
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<td>29</td>
<td>35</td>
<td>15</td>
<td>6</td>
<td>15</td>
<td>27</td>
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<td>29</td>
<td>43</td>
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<td>1</td>
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<td>16</td>
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<td>12</td>
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<tr>
<td>t-Evx1</td>
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<td>6</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

The first column indicates the proteins expressed in RP2 neurons (in an RP2 mutant background, see text) in order to analyze their ability to rescue the eve-mutant phenotype.

None 1, ΔRP2A/ΔRP2A; none 2, ΔRP2A/ΔRP2B; wild-type Eve, wild-type Eve protein; t-(protein name), N-terminal Flag-tagged version of the protein (other protein names are described in the legend to Fig. 6 and in the text).

The lines none 2, t-wild-type Eve, t-EveH, t-EveNH and t-Evx1 were analyzed with one copy of the t-LacZ marker, while the rest carried two copies of the marker. n, total number of axons counted. (Those neurons that showed bipolarity, extending an axon in two directions, were counted twice.)

Other columns show the percentage of neurons with the following phenotypes. Wild type* indicates a phenotype similar to that in the wild type, i.e. the axon extended anteriorly, turned laterally at the pISN and clearly extended further. However, in the unrescued lines, although these axons exited the CNS, they rarely if ever extended fully to their normal target area, the dorsal muscle field (see Fig. 5). Anterior extension, truncated indicates extended an axon posteriorly, while simultaneously removing it from the RP2, aCC and pCC neurons and their progenitors (see Materials and methods).

Rescued transgenes in the background of a null mutation at the endogenous eve locus is referred to as the RP2 mutant.

Cell-type-specific U/CQ mutant flies were created analogously, by deleting the U/CQ expression element from the full-length rescue construct and placing the resulting transgene in an eve-null background. EL mutants were similarly made using a deletion of the EL enhancer (Fig. 1). These deletions resulted in the loss of detectable expression specifically in either U/CQ or EL neurons, respectively (Fig. 2F,G,L). These data indicate that each of the neuronal regulatory elements is not only sufficient, but is also necessary for eve expression in the corresponding set of neurons. Somewhat surprisingly, each of these neuronal specific mutants survived to adulthood, and neither mutant adults nor larvae showed any obvious behavioral abnormalities.

**Results**

**Generation of neuronal cell-type-specific eve mutants**

A regulatory element capable of driving expression in neurons RP2, aCC and pCC was previously identified in the eve locus (Fujioka et al., 1999). We asked whether this region is necessary for expression in the context of a transgene capable of complete functional rescue of eve null mutants. This rescue transgene extends from –6.4 to either +8.6 or +9.2 kb, with either end point providing full function when homozygous, at most chromosomal insertion sites (Fujioka et al., 1999; Fujioka et al., 2002). We used a transgene construct with a deletion in the RP2+/a+pCC enhancer region to fully rescue segmentation function, while simultaneously removing it from the RP2, aCC and pCC neurons and their progenitors (see Materials and methods). In these rescued embryos, eve expression was never observed in RP2 and a/pCC neurons, while eve expression was normal in EL and U/CQ neurons in all lines (Fig. 2C,D, and data not shown). Throughout this paper, this combination of

were filled with Lucifer Yellow, and preparations were processed, as described previously (Zlatic et al., 2003).

**Immunocytochemistry for single-cell labeling**

We used the following primary antibodies: anti-Lucifer Yellow (at 1:1000, Molecular Probes), anti-CD8 (at 1:50, Caltag Laboratories) and Cy5-conjugated goat anti-Horseradish Peroxidase (at 1:200, Jackson ImmunoResearch); and secondary antibody Alexa488-conjugated goat anti-rabbit (at 1:500, Molecular Probes). The images shown are maximum projections of confocal z-series acquired with a Leica SP confocal microscope and processed with Adobe Photoshop software.

**Table 1. Summary of RP2 phenotypes**

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<table>
<thead>
<tr>
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<th>Posterior extension, truncated</th>
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The first column indicates the proteins expressed in RP2 neurons (in an RP2 mutant background, see text) in order to analyze their ability to rescue the eve-mutant phenotype.

None 1, ΔRP2A/ΔRP2A; none 2, ΔRP2A/ΔRP2B; wild-type Eve, wild-type Eve protein; t-(protein name), N-terminal Flag-tagged version of the protein (other protein names are described in the legend to Fig. 6 and in the text).

The lines none 2, t-wild-type Eve, t-EveH, t-EveNH and t-Evx1 were analyzed with one copy of the t-LacZ marker, while the rest carried two copies of the marker. n, total number of axons counted. (Those neurons that showed bipolarity, extending an axon in two directions, were counted twice.)

Other columns show the percentage of neurons with the following phenotypes. Wild type* indicates a phenotype similar to that in the wild type, i.e. the axon extended anteriorly, turned laterally at the pISN and clearly extended further. However, in the unrescued lines, although these axons exited the CNS, they rarely if ever extended fully to their normal target area, the dorsal muscle field (see Fig. 5). Anterior extension, truncated indicates extended an axon posteriorly, while simultaneously removing it from the RP2, aCC and pCC neurons and their progenitors (see Materials and methods).

Rescued transgenes in the background of a null mutation at the endogenous eve locus is referred to as the RP2 mutant.

Cell-type-specific U/CQ mutant flies were created analogously, by deleting the U/CQ expression element from the full-length rescue construct and placing the resulting transgene in an eve-null background. EL mutants were similarly made using a deletion of the EL enhancer (Fig. 1). These deletions resulted in the loss of detectable expression specifically in either U/CQ or EL neurons, respectively (Fig. 2F,G,L). These data indicate that each of the neuronal regulatory elements is not only sufficient, but is also necessary for eve expression in the corresponding set of neurons. Somewhat surprisingly, each of these neuronal specific mutants survived to adulthood, and neither mutant adults nor larvae showed any obvious behavioral abnormalities.

**eve expression is independent of eve function in the nervous system**

Although eve expression was eliminated in specific subsets of neurons in these transgenic lines, we did not know whether the neurons themselves were eliminated, or whether a change in neuronal cell fate had occurred that might alter the activity of the eve neuronal enhancer elements. To test whether eve regulatory element activity is affected by the loss of eve function, we crossed into these lines additional transgenes in which the regulatory elements directly drive lacZ expression (Fujioka et al., 1999). In each case, β-gal expression was able to clearly mark the eve mutant cells, showing that eve function is not required to maintain the activity of the eve neuronal enhancers, and that these neurons still exist without eve function (Fig. 2E,H,K).
As we were able to mark the mutant cells in our RP2 mutants, we could analyze the resulting morphological changes in detail. (Note that these mutants lack eve expression in RP2, aCC, and pCC neurons.) In order to mark axons more clearly, we used a Gal4 driver transgene with a multimerized enhancer region and a modified translation initiation site (see Materials and methods). We used this Gal4 driver (RN2-GAL4) in combination with either UAS-tacZ or UAS-CDSGFP to examine the mutant phenotype.

Loss of eve function causes aberrant positioning of cell bodies and abnormal axonal morphology

In combination with the modified Gal4 driver, UAS-tacZ generated strong marker expression that was consistent from
neuromere to neuromere, and clearly marked individual RP2 and a/pCC axons (Fig. 3A). When this reporter was placed in the RP2 mutant background, β-gal staining revealed several abnormalities (Fig. 3B). RP2 neurons were positioned further posterior than normal, lying almost adjacent to aCC, instead of the normal position intermediate between segmentally reiterated groups of a/pCC neurons (arrows, Fig. 3C,D). The positions of mutant RP2s were also often abnormal laterally (out of line with the a/pCCs) and dorsoventrally (data not shown). By contrast, the positions of a/pCC neurons were more normal, lying just dorsal to the positions of the most medially located U/CQ neurons (Fig. 3D), as in the wild type; however, their positions relative to each other were abnormal (see below).

We analyzed the morphologies of RP2, aCC and pCC in detail using the combination of two labeling methods. In addition to using the tLacZ marker (Fig. 3E-P), we used dye injection to anterogradely label single cells in both wild-type and mutant embryos, at late stage 16 (Fig. 4). The RP2 neurons showed the widest range of mutant phenotypes. In the wild type, RP2 axons extend anterolaterally from the cell body, through the posterior root of the intersegmental nerve (pISN; Fig. 3G,I, arrow in Fig. 4A), and out towards the muscle field (Fig. 3E). Dendritic arbors emerge from the proximal axon and extend mainly anteriorly (arrowhead in Fig. 4A) and, to a somewhat lesser extent, also posteriorly (not shown). In the RP2 mutants, about half of the RP2s extended an axon posteriorly along a lateral longitudinal fascicle, as in the wild type, but the axon failed to exit the CNS and enter the muscle field (arrowhead in Fig. 3H; Fig. 4D; Table 1 ‘anterior extension, truncated’). The second most commonly observed defect was an axon extending posteriorly and failing to enter the muscle field (arrow in Fig. 3H; Table 1 ‘posterior extension, truncated’). Some mutant RP2 axons that projected posteriorly did exit the CNS, but via the anterior root of the ISN (aISN; arrow in Fig. 3J; Table 1 ‘posterior extension’) rather than through the normal pISN (arrowhead in Fig. 3J). These posteriorly projecting RP2 axons seemed to occur more often in anterior regions of the CNS, and were not represented among the single-cell labelings. Some mutant RP2s exhibited contralateral axonal projections, mostly through the anterior commissure (Fig. 4C, Table 1 ‘crossed midline’). Consistent with an abnormal axonal searching behavior, most of the mutant RP2s appeared thicker than in the wild type, and the mutant RP2s exhibited many more filopodia (Fig. 3H,J).

In addition, about 19% of mutant RP2s appeared to have bipolar axons (Table 1 and Fig. 4C), whereas no RP2s do so in the wild type.

Overall, using the tLacZ marker, we found that only 21-23% of RP2 axons extended out of the CNS toward the muscle field (Table 1 ‘wild-type*’ and ‘posterior extension’ combined; Fig. 3F). Young first instar larvae showed a similar fraction of RP2 axons exiting the CNS (data not shown). Consistent with this, we observed mutant RP2s in embryos by single-cell labeling that had a nearly normal dendritic arbor (arrowheads in Fig. 4B) and which projected an axon in the normal direction, anteriorly into the pISN root (arrow in Fig. 4B). Out of the 23 RP2s labeled with this technique, eight were observed with these characteristics (Fig. 4B). Of these eight, two had axons that remained within the CNS, while the axons of the remaining six exited the CNS via the ISN, but did not project to their normal targets. In four out of these six cases, the mutant axon terminated within the ventral ISN (not the nerve branch ISNb, the axons of which innervate ventral muscles), while in only two cases were muscles contacted, and these were ventral muscles (ventral oblique muscles 4-6) rather than the normal dorsal muscles (data not shown). This essentially complete failure to extend to the normal target region, the dorsal muscle field, was confirmed with the CD8GFP reporter, as described below.

In the wild type, aCC neurons extend their axons posteroperipherally through the aISN and have predominantly anteriorly projecting dendrites (Fig. 3E, Fig. 4E) (Landgraf et al., 1997; Schmid et al., 1999; Sink and Whitington, 1991). In

<table>
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The first two columns are as described in Table 1. Columns 3-7 show the percentage of neurons with the following phenotypes. Wild type indicates extended out of line with the ISN and clearly extended further; however, in the unrescued lines, even the few in this category did not extend fully to the dorsal muscle field (see Fig. 5). ‘Posterior extension, truncated’ indicates extended an axon posteriorly, including short axons which contacted a nearby RP2 axon. ‘Anterior extension’ indicates extended an axon anteriorly, including short axons which contacted a nearby RP2 axon. ‘Crossed midline’ indicates axon crossed the midline (midline crossing did not occur in any of the other categories); the majority (>90%) of these crossed at the posterior commissure. ‘No axons’ indicates no visible axons. Owing to the necessity of using only one copy of the marker in some lines, the intensity of staining was reduced, especially that of aCC. In cases where pCC neurons were visible but not the corresponding aCC, they were counted as not visible, and were not included in the n (second column).
the mutant, virtually all aCC neurons showed clear abnormalities (Table 2; Fig. 3F,H,J, Fig. 4F,G). Many aCCs extended short axons anteriorly or posteriorly that usually seemed to attach to RP2 axons. Some aCC axons were also observed to cross the midline, mostly at the posterior commissure (Fig. 4F). Unlike the RP2s, one-third of aCCs had no axon (Table 2), and by stage 16, some of these cells appeared to be fragmenting, possibly as part of a cell death process (data not shown). We were able to label only two mutant aCC neurons by intracellular injection. Both had very abnormal morphologies: axons did not exit the CNS, and only one was bipolar (Fig. 4F,G).

The pCC cells are interneurons that extend axons anteriorly (arrow in Fig. 3M and Fig. 4H), and, like aCC and RP2, their axons normally do not cross the midline (Fig. 3K,M, Fig. 4H). However, in the mutant, although pCC axons extended anteriorly along a medial longitudinal fascicle as in the wild type, more than one-third of them crossed the midline at the next anterior commissure (Table 3; Fig. 3D,L,N). Single-cell fills of pCC confirmed this phenotype (Fig. 4J), although this technique revealed a smaller fraction of axons crossing the midline, probably owing to the much smaller sample size (Fig. 4; Table 3).

The aCC and pCC neurons are sibling cells that normally exhibit a consistent relative position at stage 12 (Fig. 3O). In the wild type, pCC is located either directly posterior or posterior and slightly lateral to its aCC sibling (Fig. 3O). In the mutant, the relative position of aCC and pCC appears to be random (Fig. 3P). This mislocation of relative cell body position was also seen to persist through later embryonic stages (Fig. 3B,F,L, and data not shown). At least at later stages, this seems largely to reflect an abnormal position of aCC relative to the neuropile as a whole (data not shown).

In order to test whether our cell-specific mutants have residual eve function, we compared our observed phenotypes with those of the previously studied temperature-sensitive allele eveID19 (Doe et al., 1988; Landgraf et al., 1999). Compared with our RP2 mutant, eveID19 (at the restrictive temperature) exhibited more RP2 and aCC neurons extending axons toward the muscle field in almost every segment, and fewer pCC axons crossing the midline (Fig. 3Q). We also

---

**Table 3. Summary of pCC phenotypes**

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The first two columns are as described in Table 1. Wild type indicates the percentage of neurons extending axons anteriorly, without crossing the midline. Crossed midline indicates the percentage of neurons extending axons anteriorly, which crossed the midline at the anterior commissure.
crossed these mutants to generate transheterozygotes of eve\textsuperscript{ID19} and ΔRP2A. Their phenotype was intermediate between that of our RP2 mutants and that of eve\textsuperscript{ID19} homozygotes (Fig. 3R). These data show that, even though eve\textsuperscript{ID19} exhibits cuticle (segmentation) defects very similar to those of eve nulls, it has residual function in the nervous system, while our mutant has less (if any) such residual function.

Even in wild-type embryos, we were not able to use the \(\beta\)-galactosidase (\(\beta\)-gal, \(\beta\lac\)) marker to follow the axons to the dorsal muscle field. However, we were able to do so using UAS-CD8GFP, and we were also able to clearly visualize neuromuscular junctions (Fig. 5A-D,G,H). When this marker was placed in the RP2 mutant background, although axons were strongly marked in the CNS (Fig. 5B), only a few were observed in the muscle field. About a third of embryos examined showed none (17 out of 50), while most of the remainder showed one or two axons extending outside the CNS. However, there were a few embryos (three out of 50) that had short axons outside the CNS, although these were never seen to reach the dorsal muscle field. In total, axons were observed in the muscle field in 19% of hemisegments (\(n=366\)), consistent with the data obtained using \(\beta\lac\) (21-23%, see above) and single-cell labeling (six out of 23). For those axons observed in the muscle field, the GFP intensity was weak relative to that in

---

**Fig. 5.** Without Eve function, most RP2 and aCC axons do not reach the muscle field. The combination of RN2-Gal4 and UAS-CD8GFP transgenes (two copies each) was placed in either a wild-type (left column) or a ΔRP2A/ΔRP2C mutant background (right column). Stage 16 embryos are shown, anterior towards the left, and dorsal upwards (except A and B, which are centered on the ventral midline). (A,B) Overview of the CNS. (C,D) GFP in the muscle field. Note that in the mutant, only a few axons are visible, and that they do not reach to the dorsal muscle field. The yellow arrow indicates the same lateral position in all panels (D,F,H are a more ventral view in order to show the small amount of axonal outgrowth that occurs near the edge of the CNS). (E,F) Nomarski view of C,D, respectively. (G) Merged image of C and E. (H) Merged image of D and F. (I-L) Anti-Fas2 staining. (J,L) Higher magnification of I,K, respectively. Note the attachment of some axons to DO2 muscles in both the wild type and the mutant (arrowheads; neuromuscular junctions to DA2 are also present, but not visible here), but that attachments to DO1 and DA1 (only DO1 is visible here) are barely formed in the mutant (arrows). Scale bars in B and L (equal in size): 50 μm in A-L; 20 μm in J,L.
eve+ embryos. This is also consistent with our results described above using the tlcZ marker, which showed almost no aCC axons exiting the CNS, so that we would expect only one axon to be present at each position (from RP2) rather than the wild-type combination of two in each hemisegment (from RP2 and aCC). When embryos were stained with anti-Fas2, sites of contact to DA2 (also known as muscle 2), a normal target muscle of both RP2 and U/CQ (Landgraf et al., 1997; Schmid et al., 1999; Sink and Whittington, 1991) and DO2 (a.k.a. muscle 10), a normal target muscle of U/CQ (Landgraf et al., 1997; Schmid et al., 1999) were visible in the mutants, although they were possibly less extensive than in the wild type (arrowheads in Fig. 5I-L), perhaps reflecting the lack of RP2 axons. However, contacts with DA1 muscles (also known as muscle 1), normal targets of aCC (Landgraf et al., 1997; Schmid et al., 1999; Sink and Whittington, 1991) were just barely visible in the mutant (arrows in Fig. 5K,L; compared with the wild type in Fig. 5IJ). The apparent residual innervation beyond the DA2/DO2 neuromuscular junction may be due to U/CQ neurons targeting the DO1 muscle (Schmid et al., 1999).

Eve functions in neurons to repress target genes

At the blastoderm stage, Eve uses two repressor domains to repress target genes. In addition to a generic repression activity, only the HD (with conserved flanking region) is required for Eve function during segmentation (Fujioka et al., 2002). We examined whether the function of Eve is also as a repressor in the nervous system using a series of rescue transgenes expressing modified Eve proteins. In these experiments, one copy of each modified Eve transgene was used to rescue nervous system function (in RP2, aCC and pCC), in the background of the RP2 mutant (see Materials and methods for details). Single copies of these transgenes were compared in order to allow clearer distinctions to be made among them, as we found that a single copy of the wild-type construct could provide almost complete rescue (see below). The resulting rescued axonal phenotypes are summarized in Tables 1-3. As described earlier, in the RP2 mutant alone, about 12% of RP2 axons and 2% of aCC axons exited the CNS via the normal route, while 60% of pCC axons showed a normal phenotype (Tables 1-3, Fig. 6A). When one copy of the wild-type rescue transgene was combined with the mutant, almost all (97%) RP2 axons, and 100% of aCC and pCC axons, showed a normal phenotype (Tables 1-3; Fig. 6F). When a Flag-tagged wild-type protein was used as an additional control (as modified proteins carried such a tag) the percentage of rescue was reduced slightly in RP2 and aCC (Tables 1 and 2). When the Eve HD alone was supplied to these neurons, there was some rescue of both RP2 and pCC (EveH; Tables 1-3, Fig. 6B), but not of aCC. When the N terminus of the protein was added to the HD, there was some additional rescue of pCC (EveNH; Table 3, Fig. 6C). In addition to rescuing axonal morphology, EveNH also showed some degree of rescue of the RP2 position, as well as the relative positions of the aCC and pCC cell bodies (Fig. 6C). Where either of the repressor domains alone was added to the EveNH construct, rescue of both the RP2 and aCC axonal phenotypes were substantially increased, and rescue of pCC reached 100% (EveAC, EveAR; Tables 1-3, Fig. 6D,E). Thus, both repressor domains contribute about equally to the activity of Eve in these neurons. Consistent with this, two copies of either the EveAR or the EveAC transgene were sufficient to fully rescue the phenotypes in the nervous system (data not shown). Importantly, the function of the Eve repressor domains
could be completely replaced by a heterologous repressor region from the Engrailed protein (EveH-En; Tables 1-3, Fig. 6G). Taken together, these data strongly suggest that the Eve HD (with conserved flanking region) is sufficient to recognize correct target genes in the nervous system, and that in addition, active repression function is required. Furthermore, the fact that the HD by itself is able to rescue axonal morphology to varying degrees in different neurons suggests that there may be distinct target genes or mechanisms involved in Eve function in these different neuronal cell types.

**Eve homologues are able to function in the Drosophila nervous system**

To test the extent to which these functions that are required in the nervous system have been conserved during evolution, Eve homologues from several species were tested for rescuing activity in this system. In previous studies, Eve homologues from the red flour beetle (*Tribolium castaneum* Tc-eve), grasshopper (*Schistocerca americana* Sa-eve) and mouse (*Evx1*) all showed substantial activity during *D. melanogaster* segmentation (Fujioka et al., 2002). These same proteins were analyzed in RP2 mutant embryos in the same way as were the modified Eve proteins described above. All three were able to rescue the mutant phenotype essentially completely in both RP2 and pCC (Tables 1, 3; Fig. 6H,I,J). In aCC, both Tc-eve and Sa-eve were able to rescue as well as did the *Drosophila* protein, while the activity of *Evx1*, although detectably weaker, was nonetheless very substantial (Table 2; Fig. 6H,I,J). As such rescue requires both the HD and a strong repressor activity, these data suggest that both targeting to and repression of specific target genes have been conserved in the function of these proteins during nervous system development.

**Eve can act as a direct repressor in RP2 and a/pCC**

Whether Eve acts as a transcriptional repressor of direct endogenous target genes in these neurons could not be addressed extensively, as the target genes in this tissue are only beginning to be identified. Therefore, we tested whether a chimeric protein of the Gal4 DNA-binding domain fused with the Eve repressor domains (Fujioka et al., 2002) could repress an activated UAS-containing target gene in these cells. Expression of such a transgene in RP2 and a/pCC, as well as in EL neurons to provide an internal control (Fig. 7A), was monitored in the presence or absence of Gal4-EveRC, expressed from a transgene specifically in RP2 and a/pCC (but not ELs). We found that the reporter GFP expression was clearly reduced in both RP2 and a/pCC neurons in the presence of Gal4-EveRC, indicating that Eve can, indeed, act as a direct transcriptional repressor in these cells (Fig. 7B,C). Using a similar strategy, we found that Eve can also act as a direct repressor in U/CQ neurons (Fig. 7D,E). However, we were not able to detect repressor activity clearly in EL neurons using a similar assay (data not shown).

**Derepression of Drosophila Hb9 correlates with eve mutant phenotypes in RP2 and aCC**

The expression of the *Drosophila* Hb9 has previously been shown to be non-overlapping with that of Eve in the CNS (Broihier and Skeath, 2002; Odden et al., 2002). Furthermore, ectopic expression of Eve repressed *Drosophila* Hb9 gene expression (also known as extra-extra), and expression of *Drosophila* Hb9 was found to be derepressed in RP2 and in either aCC or pCC (which could not be reliably distinguished) in *eveΔID19* and in EveAC embryos (Broihier and Skeath, 2002). In that study, segmentation was only partially rescued, as Eve
activity during segmentation was also reduced, owing to the lack of the Groucho interaction domain in the rescuing EveΔC protein (Kobayashi et al., 2001). Therefore, we examined *Drosophila* Hb9 expression in our neuron-specific mutants. We found that in RP2 mutants rescued by a wild-type transgene, Hb9 was absent from neurons that showed a normal phenotype (e.g., green arrow, Fig. 8A), which represented the great majority, as described above. However, in the minority of RP2 neurons that exhibited an abnormal axonal morphology, such as those extending axons posteriorly (black arrows, Fig. 8A), Hb9 was often detectably derepressed. In the RP2 mutant itself, Hb9 was largely derepressed both in RP2 (black arrows, Fig. 8B) and in aCC (yellow arrow, Fig. 8B). However, derepression of Hb9 in pCC neurons was never observed.

When the EveH transgene was used to rescue the RP2 mutant, expression of Hb9 was often absent or reduced in RP2 neurons relative to the mutant (green arrow, Fig. 8C), although not always (black arrow, Fig. 8C), consistent with the partial rescue of the RP2 mutant phenotype by this protein. In aCC, Hb9 was almost always strongly derepressed (yellow arrow, Fig. 8C), consistent with the inability of this protein to rescue the phenotype of aCC (Table 2). When the RP2 mutant was partially rescued using EveAC, the degree to which Hb9 was repressed in aCC and RP2 neurons similarly correlated with the degree of their phenotypic rescue: the expression of Hb9 was usually repressed only in those aCC and RP2 neurons with a normal axonal morphology (aCC – yellow arrows, RP2 – black arrows in Fig. 8D). We also obtained very similar results for EveΔR-rescued embryos (data not shown). Thus, overall in these rescued lines, there is a strong correlation between abnormal axonal phenotypes and the derepression of Hb9. However, on a cell-by-cell basis, the correlation is not 100%. In particular, although we observed that neurons with abnormal axons almost always showed derepression of Hb9, a few of those with normal axons also had detectable derepression, although it was never very strong.

The monoclonal antibody 22C10 (Fujita et al., 1982) recognizes a subset of neurons, including RP2 (green arrows, Fig. 8E-H) and aCC (yellow arrow, Fig. 8E,F), but not pCC. This antibody was recently shown to recognize the *futsch* gene product, which is homologous to vertebrate MAP1B, a microtubule-associated protein (Hummel et al., 2000). We used this antibody to examine expression of the antigen in our mutants. We found that although it was still detectable in the
RP2 mutant, its expression was weaker than in the wild type, especially in aCC neurons (23 out of 24 neurons showed decreased intensity; yellow arrow, Fig. 8G,H), indicating that it is a downstream target of Eve regulation. We also examined expression of the transcription factor Islet, which is known to be involved in axonal guidance (Thor and Thomas, 1997). However, we did not observe clear derepression in the RP2 mutant (data not shown).

Discussion
Testing the cell-specific requirements for eve function in the nervous system
In this study, we created transgenic embryos that lack detectable eve expression in specific sets of neurons by deleting individual neuronal regulatory elements from a complete rescue transgene (Fig. 1). These deleted rescue transgenes were placed in the background of an eve deficiency mutant that is both a protein and transcript null. In such embryos, eve segmentation function can be completely rescued, providing a true neuron-specific eve mutant (Fig. 5). Importantly, we found in our analysis that eveD19, a temperature-sensitive allele that has been used previously to examine eve function at later developmental stages, has residual activity in the nervous system (see below), so that the phenotype of our mutant is closer to that of an eve null.

The mutants that we constructed lack all detectable Eve expression either in the combination of RP2 and a/pCC neurons, or in the U/CQ neurons, or in the EL neurons (Fig. 2), and here we analyzed in detail the defects in the first of these. In this case, despite significant defects in axonal architecture (discussed below), individuals were able to survive to fertile adulthood. In a preliminary analysis, we did not observe any behavioral abnormalities in either larvae or adult flies (M.F. and J.B.J., unpublished). Eve is normally also expressed in the parental GMCs of RP2, aCC, pCC and the U/CQ neurons, and this expression is also eliminated in our mutants. The removal of Eve from these neuronal lineages did not cause a loss of neurons (Fig. 2), showing that eve function in the GMCs is not required for their normal cell division to occur.

In order to identify the mutant neurons and to analyze the axonal phenotypes, the eve neuronal elements were used to drive marker gene expression. Expression in RP2+a/pCC was enhanced using the Gal4-UAS system (Brand and Perrimon, 1993). In order to be effective, we found that Gal4 activity needed to be increased by replacing the yeast translational initiation signal in the Gal4 driver transgene with that from eve, and by multimerizing the enhancer (see Materials and methods). With these modifications, in combination with a UAS-\(\text{lacZ}\) transgene (Callahan et al., 1995), the axons of RP2, aCC and pCC were clearly marked (Figs 3, 6 and 8). Combining the same Gal4 driver with UAS-C\(\text{D8GFP}\) (Lee and Luo, 1999) allowed us to mark axons with membrane-localized GFP. This allowed us to examine how far mutant axons grew towards the muscle field, and to visualize connections to specific target muscles (Fig. 5).

The regulation of eve neuronal elements
The locations of individual neuronal regulatory elements sufficient for eve expression in subsets of neurons were identified previously (Fujio et al., 1999). Because deleting each of these regulatory elements in the context of the rescue transgene eliminates eve expression in the corresponding neurons, the elements are also necessary in the context of the entire gene (Fig. 2), as was found for the eve mesodermal enhancer (Han et al., 2002).

In the absence of eve function, all neuronal enhancer elements remain active. This suggests that the cells do not completely change their identities in the absence of Eve, but continue to express the combination of factors that normally initiate and maintain eve expression, and which presumably act in concert with Eve to specify the phenotype.

Requirements for Eve in RP2 motoneurons
Using a combination of the constructed cell-specific marker and the mutant transgenes described above, we analyzed the morphology of RP2 mutant neurons. The mutant RP2s exhibit a variety of defects in axonal morphology (Figs 3 and 4, summarized in Table 1), and in addition they are defective in their ability to migrate to their normal position within the CNS (Fig. 2E, Fig. 3). Although the abnormal position of mutant RP2s might affect their ability to extend axons normally, it does not seem to be a primary determinant of whether they extend to the muscle field, because the position defect is often rescued by the Eve protein without its repressor domains (EveNH, supplied by an additional rescue transgene expressing this protein; Fig. 6C), and yet in this case, axons still fail to extend properly. In addition, there are a few abnormally located RP2s in wild-type embryos, and they still extend their axons normally (data not shown). The mutant RP2s seem to retain some axonal guidance capability, as their axons often recognize their normal point of exit from the CNS along the ISN, once they ‘happen’ onto it, although many of these fail to extend further (Figs 3-5, Table 1). The RP2 mutant axons were never observed to extend as far as their normal target muscles, and indeed, many of them do not exit the CNS (Fig. 5). Of those that do exit the CNS, most appear to be unable to defasciculate from the ISN onto muscle targets. It is unlikely that the failure of mutant RP2 axons to exit the CNS by stage 16 is due solely to a delay in either axon outgrowth or recognition of the nervous system, because we find similar percentages of peripherally projecting RP2 neurons in both stage 16 embryos and young first instar larvae.

The inability of the mutant axons to reach the muscle field was partially rescued by the Eve HD without its repressor domains (Fig. 6B,C). However, in addition to the HD, the two distinct repressor domains of Eve contribute strongly to rescue of the mutant phenotype (Fig. 6D,E). Interestingly, complete rescue can also be provided by the Eve HD fused with a heterologous repressor domain from Engrailed (Fig. 6G). Therefore, in addition to the functions provided by the DNA-binding HD, a generic repressor function of sufficient strength is required for normal function in these neurons. The fact that the HD alone can provide a detectable degree of rescue, which contrasts with the lack of its ability to rescue segmentation function (Fujio et al., 2002), suggests that perhaps competition for binding sites with activators of downstream target genes plays a more prominent role in Eve function in the nervous system. Consistent with the requirement for its repressor domains, an Eve-Gal4 fusion protein was able to
actively repress a UAS-containing target gene in RP2 neurons (Fig. 7B,C).

Requirements in aCC motoneurons
The requirement for Eve in axonal guidance is somewhat more stringent in aCC than in RP2 neurons. Although a significant fraction of mutant RP2s initially extend axons in the same direction as wild-type RP2s, essentially none of mutant aCCs do so (Table 2). In addition, unlike for RP2s, the aCC phenotype was not significantly rescued by either the HD alone or the HD with the N terminus (which provides no detectable repression activity, but might stabilize the protein). In aCC, as in RP2, the phenotype was partially rescued by including either repressor domain, and the Engrailed repressor domain was able to provide full activity (Fig. 6). Furthermore, Eve repressor domains were able to actively repress a UAS target gene in aCC neurons (Fig. 7). These data indicate that the primary function of eve in aCC is to actively repress target genes. The more stringent requirements in aCC versus RP2 suggest that there may be different target genes in these two motoneurons, although Drosophila Hb9 is a common target (discussed below).

Requirements in the interneuron pCC
In mutant pCC neurons, in contrast to the wild type, 40% of axons crossed the ventral midline to the contralateral side (Table 3). This phenotype was rescued quite effectively by the HD alone, suggesting that the target gene(s) involved may be passively repressed through a competition for activator binding sites (although more complex possibilities cannot be ruled out). Recent studies have shown that midline crossing is regulated by a complex interplay of responses to attractive and repellant signals secreted by midline cells (reviewed by Dickson, 2002). One possibility is that the midline crossing phenotype of mutant pCCs might be caused by derepression of the DCC/Frazzled receptor (Kolodziej et al., 1996) for the midline attractant Netrins (Harris et al., 1996; Mitchell et al., 1996).

In addition to the defects in axonal morphology, the cell body position of pCC relative to that of its sibling aCC is apparently randomized in the mutant (Fig. 3P). We do not know whether this is due to the lack of Eve in aCC, in pCC, or in both. As other neurons in the CNS are wild type, it is unlikely to be an effect involving surrounding neurons. This defect is rescued effectively only when the Eve HD is accompanied by at least one repressor domain. It is unclear whether the normally tight control of this characteristic has a role in the subsequent morphogenesis of the neurons.

Null and hypomorphic neuronal phenotypes
Previous studies using the eve temperature-sensitive allele eveD19 showed that eve is required in eve-positive motoneurons for proper axonal morphology, including the ability to reach the dorsal muscle field (Doe et al., 1988; Landgraf et al., 1999). The eveD19 allele contains a point mutation in the HD (Frasch et al., 1988), and shows a near-null segmentation phenotype at the restrictive temperature (Nüsslein-Volhard et al., 1985). However, our data indicate that eveD19 is not a true null in the nervous system (Fig. 3). This might explain the differences between the morphological phenotypes of mutant RP2, aCC and pCC neurons that we observe in our mutant as compared with those seen in eveD19 (Doe et al., 1988).

A significant variation in phenotype with a small change in the level of function is consistent with an interpretation wherein loss of Eve leads to the absence of a particular subset of neuronal properties. Interestingly, even in our mutant, which has no detectable Eve expression in these lineages, some RP2s as well as some pCCs show several of the characteristics of their wild-type counterparts. If we assume that our mutant represents the complete null phenotype, this indicates that, to a limited extent, Eve acts in parallel with other factors in the specification of these cell types, rather than being an overall determinant of the cell fate. This notion is also consistent with the fact that the eve regulatory elements, which are specific markers for these cell types, continue to be active in the absence of eve function.

Evolutionary conservation of function
A single copy of a wild-type transgene was sufficient to almost completely rescue the mutant phenotypes in the nervous system, in contrast to the requirement for two copies to rescue segmentation (Fujikawa et al., 1999). This relative lack of eve dosage sensitivity in the nervous system might be related to the apparently ancestral nature and greater conservation of the nervous system function of eve.

We tested the extent to which Eve homologues from Tribolium, grasshopper, and mouse could rescue the mutant neuronal phenotypes. We found that a single copy of either of the insect orthologs could provide essentially complete function in Drosophila, while mouse Evx1 also showed quite strong rescuing capability. Coupled with the fact that active repression function is required for this degree of rescue, these results indicate that each of these homologues has retained both the ability to be targeted to and to repress the key direct target genes of Eve in the nervous system.

Target genes in the nervous system
It has previously been shown that Drosophila Hb9 and Eve are expressed in a non-overlapping pattern in the wild-type CNS (Broihier and Skeath, 2002; Odden et al., 2002), and that ectopic Eve expression represses Hb9, indicating that Hb9 is a target gene of Eve (Broihier and Skeath, 2002). We found that Hb9 is derepressed in the RP2 mutant in both RP2 and aCC (Fig. 8), but not in pCC neurons (the RP2 mutant lacks Eve in all three cell types), showing that there are significant differences in target gene regulation in different neurons, even in those derived from the same GMC (in the case of aCC and pCC).

When the Eve HD alone is used to rescue the RP2 mutant, Hb9 is repressed in many of the RP2 neurons, and this seemingly stochastic repression correlates with a more normal axonal morphology. However, effective repression, particularly in aCC, requires active repression domains, with either of the repressor domains of Eve alone providing partial activity (in the context of the Eve HD). Although there is a strong correlation in situations of partial rescue between the axonal phenotypes of individual neurons and derepression of Hb9, this correlation is not 100%. This suggests that there may be other key target genes that mediate Eve neuronal function in addition to Hb9. We also found that the level of expression of the antigen (Futsch) of the monoclonal antibody 22C10 (Hummel
et al., 2000) is reduced in RP2 and aCC in the absence of Eve (Fig. 8). However, the gene encoding this antigen is likely to be an indirect target of Eve, because its expression is activated rather than repressed by Eve.

Either of the repressor domains of Eve is sufficient to give a similar degree of partial rescue of each of the phenotypes we have studied in the nervous system, including the repression of Hb9, showing that these repressor domains provide a similar function. In fact, two copies of a transgene expressing either EveC or EveAR are able to rescue to a similar degree as that of one copy of the wild-type transgene (data not shown). Thus, we see that the recruitment of either of two apparently distinct co-repressors, Groucho or Atrophin, produces the same net result. The two are used in these neurons in an additive fashion to generate the appropriate level of Eve repressor activity, with no apparent target gene specificity.

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References


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