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The *Caenorhabditis elegans* ems class homeobox gene *ceh-2* is required for M3 pharynx motoneuron function

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SUMMARY

Several homeobox genes, for example those of the ems class, play important roles in animal head development. We report on the expression pattern and function of *ceh-2*, the *Caenorhabditis elegans ems*/*Emx* ortholog. CEH-2 protein is restricted to the nuclei of one type of small muscle cell, one type of epithelial cell, and three types of neurons in the anterior pharynx in the head. We have generated a deletion allele of *ceh-2* that removes the homeobox. Animals homozygous for this deletion are viable and fertile, but grow slightly slower and lay fewer eggs than wild type. We assayed the function of two types of pharynx neurons that express *ceh-2*, the pairs M3 and NSM. M3 activity is substantially reduced in electropharyngeograms of *ceh-2* deletion mutants; this defect can account for the observed retardation in larval development, as M3 activity is known to be necessary for effective feeding. NSM function and metabolism are normal based on the assays used. All cells that express *ceh-2* in wild type are present in the *ceh-2* mutant and have normal morphologies. Therefore, unlike other *ems/Emx* genes, *ceh-2* seems to be important for a late differentiation step and not for neuron specification or regional patterning. Because the CEH-2 homeodomain is well conserved, we tested whether *ceh-2* can rescue *ems*$^-$ brain defects in *Drosophila*, despite the apparent differences in biological roles. We found that the *C. elegans ems* ortholog is able to substitute for fly *ems* in brain development, indicating that sequence conservation rather than conservation of biological function is important.

Key words: *Caenorhabditis elegans*, Homeobox, Pharynx, *ceh-2*, *empty spiracles*, Evolution, Cross-species rescue

INTRODUCTION

Bilaterally symmetric animals possess a head with sensory organs, central nervous system, and mouth, usually facing the direction of locomotion. Several transcription factors, which exist in highly conserved forms in animals of diverse phyla, play a role in embryonic head development. Some factors even act in organs of similar function like brain or eyes. Such similarities suggest a common evolutionary origin of the corresponding organs and the head itself. The actual function of these genes can be very different in diverse animals, reflecting divergent evolution from the proposed common ancestor to extant species.

Homeobox genes of the *ems/Emx* class have been characterized from fruit fly, amphioxus, several vertebrates and a hydrozoan. Fly *ems* mutant embryos lack two brain segments and other head structures that normally develop from the *ems* expression domain (Dalton et al., 1989; Hartmann et al., 2000; Hirth et al., 1995; Jürgens et al., 1984; Walldorf and Gehring, 1992). Comparable with the expression of *Drosophila ems*, orthologs from several vertebrates are expressed in the developing cortex (Kastury et al., 1994; Morita et al., 1995; Pannese et al., 1998; Simeone et al., 1992). Mouse *Emx1* and *Emx2* define the identity of their cortical expression domains and are necessary for formation and migration of specific neurons (Bishop et al., 2000; Mallamaci et al., 2000; Tole et al., 2000; Shinozaki et al., 2002). Expression of a hydrozoan *ems* homolog in endodermal cells around the mouth opening suggests that *ems* genes have played a role in head development since very early in metazoan evolution (Mokady et al., 1998; Meinhardt, 2002). Other *ems/Emx* functions are apparently unrelated to head development. An amphioxus *ems* ortholog is expressed only in trunk and tail epidermis (Oda and Saiga, 2001). *ems* and *Emx2* later act in the development of fly tracheae (Dalton et al., 1989; Walldorf and Gehring, 1992) and formation of the mouse urogenital system (Miyamoto et al., 1997), respectively. Thus, it is of interest to understand the molecular role of *ems* genes at the cellular level in different phyla.

In the nematode *Caenorhabditis elegans*, we have the

opportunity to study gene function at the level of single cells. Furthermore, head development of this nematode is still poorly understood. Nematodes have recently been proposed to be a sister group to arthropods based on molecular data (Aguinaldo et al., 1997), but they have a simple body plan with fewer cell types. The main head structures of nematodes are the head ganglia, with sensory organs and interneurons, and the pharynx, a muscular feeding organ, around which the ganglia are arranged. We have now identified and studied the *C. elegans ems/Emx* ortholog to understand its role in nematode head development. Additional sequencing of the previously identified homeobox gene *ceh-2* (Bürglin et al., 1989) confirmed that this is an *ems*/*Emx* ortholog. We find that *ceh-2* is expressed in a few cells of the pharynx. This organ rhythmically pumps suspended bacteria, grinds them and passes them on to the intestine. Pharynx muscle activity is modulated by a small independent nervous system that consists of 20 neuron types (Avery and Horvitz, 1989). We characterized defects in some of the *ceh-2*-expressing neurons in a *ceh-2* mutation that we generated. Unlike in other animals, the *C. elegans ems* ortholog is not required for the formation or morphological differentiation of the cells expressing it. Given the apparent discrepancy in the biological roles of *ceh-2* and *ems* between nematodes and flies, we were interested to see if cross-species rescue would be possible. Thus, we examined the degree of functional conservation between the *Drosophila* and *C. elegans ems* orthologs by rescuing the fly *ems* mutant brain phenotype with *ceh-2*.

MATERIALS AND METHODS

Worm strains, maintenance and mutagenesis

Strains [N2 (wild type), *unc-40(e271) bli-4(e937)*, *dpy-5(e61) unc-13(e51)* and *pha-4(q490)*] were obtained from the *Caenorhabditis* Genetics Center and maintained according to standard techniques. N2 worms were mutagenized with EMS (ethyl methanesulfonate, Sigma) and screened by nested PCR for a deletion within 2.7 kb (Jansen et al., 1997) using primers c2ko2 (acttaaatgtcaaataaatc-ccagctag)/c2ko1 (cgatgaatgttatggcactaaaatgtg) (outer set) and c2ko4 (gaatgagccactttcacagacactctc)/c2ko5 (cgtcgtttgacatcggagcgttcgg) (inner set). One allele, *ceh-2(ch4)*, was obtained and outcrossed ten times against N2. PCR of crude single worm lysates (50 mM KCl, 10 mM Tris pH 8.2, 0.045% Tween-20, 0.01% gelatine, 200 μg/ml proteinase K, 1 hour at 65°C, 10 minutes at 95°C) with primers c2ko4/c2ko5 identified the deletion; absence of a PCR product with c2ko4/c2ko6 (tgttcctcttgatcatctggccgag, within the deletion) identified homozygous mutants. A linked lethal mutation that was not rescued by cosmid C27A12 (The *C. elegans* Sequencing Consortium, 1998; Janke et al., 1997) was separated from *ceh-2(ch4)* by recombining *bli-4(e937)* on and off. Two independently amplified PCR products from mutant worm lysate were sequenced to determine the deletion breakpoints as (exon 2)...gaaacacag to gtaaaagttt...(intron 4). We identified transcripts of the deletion allele by sequencing several RT-PCR clones from homozygous mutant total RNA.

Cloning *ceh-2* cDNA, reporter and rescue constructs

A *C. elegans* embryonic stage cDNA library (a gift from Peter Okkema) (Okkema and Fire, 1994) was hybridized according to standard techniques with a labeled 5′ fragment of the *ceh-2* cDNA that had been obtained by PCR from the same library. We isolated two different clones with identical open reading frames. Accession Numbers are AY246428 and AY246429. *ceh-2* reporter constructs were cloned in vectors pPD95.79 (*gfp*) or pPD95.07 (*lacZ*) (kind gifts from A. Fire, S. Xu, J. Ahnn and G. Seydoux). For pTRB201, genomic DNA was amplified with primers PCR2-10 (tgcttctcttgtcgacaaaactg-gcatg)/PCR2-7(cgcggatccccttctcactatccaccagtcgttccaccg), cloned *Sal*I-*Bam*HI into pPD95.79, and shortened to 1.6 kb by *Hind*III digestion and religation. pTRB202 is a PCR subclone amplified with primers PCR2-10/PCR2-9 (cgcggatccgattttgaaaccaaacttttacctga) and cloned *Sal*I-*Bam*HI into pPD95.79.

For rescue, we subcloned an *Sph*I-*Sal*I fragment from cosmid C27A12 (AF003137) (The *C. elegans* Sequencing Consortium, 1998) and excised the *Eco*RV fragment, which leaves only the *ceh-2* gene with surrounding sequences up to the next upstream and downstream open reading frames (pTRB203; Fig. 1B). pTRB204, a *Sac*I deletion subclone of pTRB203 lacking most of *ceh-2* served as negative control and showed no rescuing activity in transgenic animals. Constructs were injected into the gonads of wild-type or mutant animals to obtain transgenic lines and the dominant co-injection marker *rol-6* was used (Mello and Fire, 1995).

C. elegans antibody staining and microscopy

A synthetic peptide C-terminal to the homeodomain of CEH-2 (LEGSDPNAPMSNDEDDE) was synthesized and coupled to BSA with glutaraldehyde. Coupled antigen was injected into rabbits using RIBI adjuvants, and antisera were affinity purified against peptide-coupled BSA coupled to CNBr-activated sepharose (Pharmacia). Mouse monoclonal antibody 3NB12 (Miller et al., 1983) was used to detect a myosin specific for pharyngeal muscle cells. Worms were fixed and stained as described (Finney and Ruvkun, 1990). Microscopic analyses were performed on a Nikon Microphot FXA equipped with DIC (Nomarski) optics, fluorescence and a Sony 3CCD color video camera, or on a Leica TCS SP laser confocal microscope.

Electrophysiology and behavioral assays

Electropharyngeograms were recorded from isolated heads as described (Avery et al., 1995). The tip of the head was sealed by suction into fire-polished borosilicate glass electrodes of about 3 μm outer diameter. Bath and electrode were filled with Dent's saline (140 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH ad 7.4) containing about 1 μM serotonin hydrochloride (Sigma) to stimulate moderate pumping. EPGs were recorded with an AxoClamp microelectrode amplifier (Axon) connected to a Gould RS3200 pen recorder.

Locomotion rate assays were performed according to Sawin et al. (Sawin et al., 2000). Staged populations of young adult worms were deprived of food for 30 minutes on standard agar plates with or without 75 μg/ml fluoxetine hydrochloride (Sigma) and transferred to assay plates covered with a thin lawn of *E. coli* OP50 or with no *E. coli*. Worms were transferred between plates in a drop of buffer, washed before each transfer, and assayed five minutes after transfer. Control and experimental worms as well as plates were prepared in parallel and treated exactly the same to ensure constant conditions. At least 30 animals were assayed under each condition.

Ectopic expression of *ceh-2* in *Drosophila*

The *ceh-2*-coding sequence was amplified by PCR from *ceh-2* cDNA clones, verified by sequencing and cloned into plasmid pCaSpeR-hs (Thummel and Pirrotta, 1992). Transgenic flies were prepared by P element transformation in a *white*$^{-}$ mutant background. Induction of *ceh-2* transcription by heat-shock was verified by RT-PCR of total RNA isolated from heat-shocked hs-*ceh-2*/hs-*ceh-2* embryos. No RT-PCR band was obtained from embryos that were not heat-shocked. One hs-*ceh-2* and one *hs-ems* line (the latter a gift from Uwe Walldorf) were marked on chromosome III with TM3 Sb *Ubx-lacZ*/TM6 *hm* and crossed into *ems*^{9H83}/TM3

sb *Ubx-lacZ* (a gift from Markus Affolter) to produce fly strains of the genotype *hs-ceh-2*/*hs-ceh-2* [II]; *ems*^{9H83}/TM3 *sb Ubx-lacZ*[III] and *hs-ems*/*hs-ems*[II]; *ems*^{9H83}/TM3 *sb Ubx-lacZ*[III]. Stage 11 embryos from these lines were heat-shocked for 45 minutes at 37°C, raised to stage 15, fixed and stained. FITC-conjugated goat-anti-horseradish-peroxidase antibody (Jackson) was used to stain the central nervous system. Absence of anti-β-galactosidase staining (mouse monoclonal, anti-mouse TRITC; Promega) identified homozygous *ems* mutant animals.

RESULTS

ceh-2 is the *C. elegans* ortholog of *Drosophila* ems and vertebrate Emx genes

ceh-2 (*C. elegans* homeobox) was originally identified in a screen for *C. elegans* genes containing homeobox motifs (Bürglin et al., 1989). We isolated two independent full-length *ceh-2* cDNA clones from an embryonic stage cDNA library

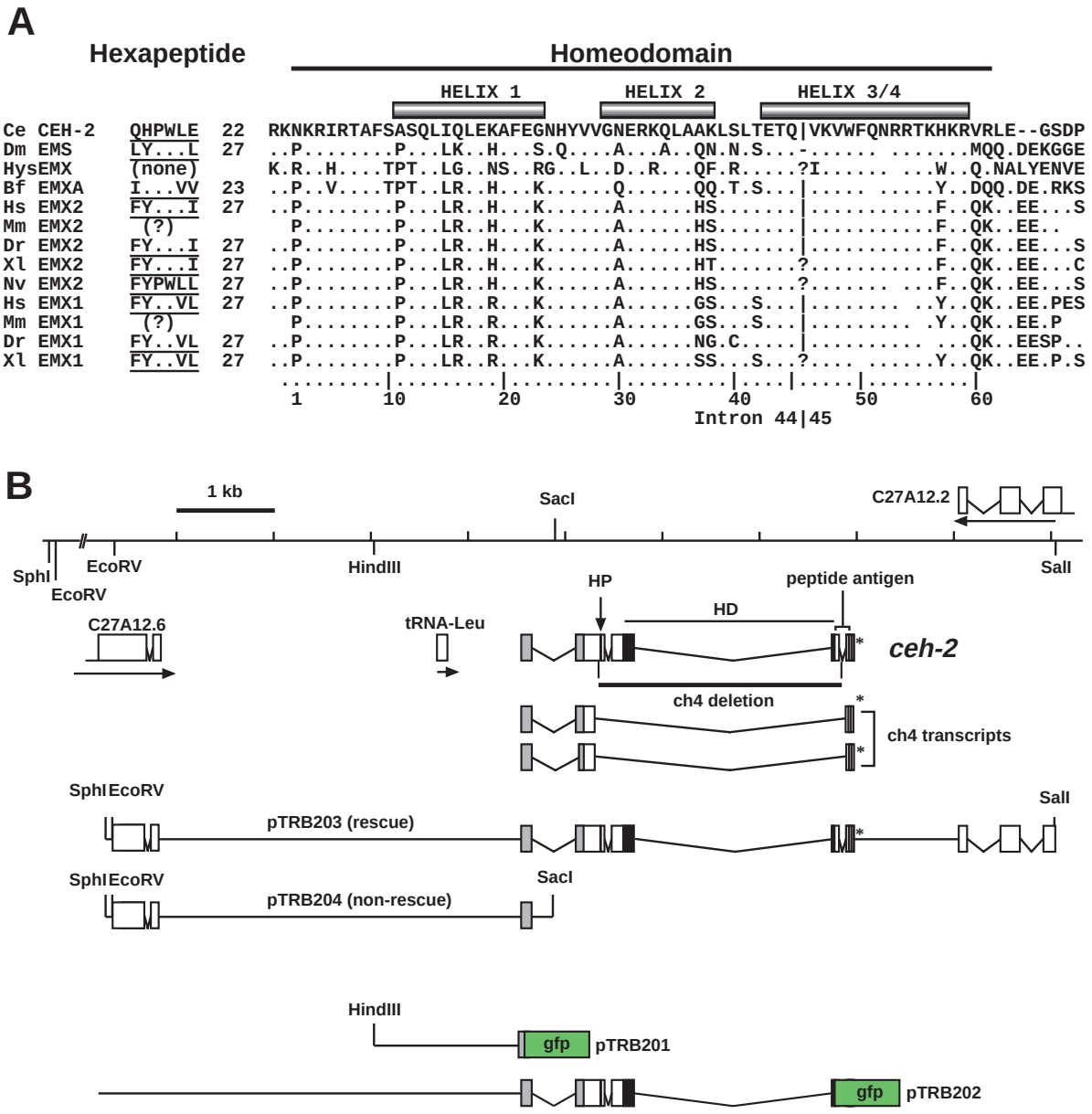

Fig. 1. Genomic structure of *ceh-2* and sequence comparisons. (A) Alignment of homeodomains and hexapeptides of ems class genes from various species. Dots designate identical amino acids, vertical lines indicate intron positions and dashes denote artificial gaps that are introduced for alignment purposes. Abbreviations and sources of sequence data: *Bf*, *Branchiostoma floridiae* (amphioxus) (AF261146) (Williams and Holland, 2000); *Dm*, *Drosophila melanogaster* (X51653, X66270) (Dalton et al., 1989; Walldorf and Gehring, 1992); *Dr*, zebrafish (D32214, D32215) (Morita et al., 1995); *Hs*, human (X68879, X68880) (Simeone et al., 1992); *Hys*, *Hydractinia symbiolongicarpus* (cnidarian) (Mokady et al., 1998); *Nv*, *Notophthalmus viridescens* (newt) (Beauchemin et al., 1998); *Mm*, mouse (X68881, X68882) (Simeone et al., 1992); *Xl*, *Xenopus laevis* (Pannese et al., 1998). (B) Genomic structure of the *ceh-2* gene and flanking ORFs on cosmid C17A12. The extent of the *ch4* deletion is indicated underneath the gene. Rescue subclones (pTRB203, pTRB204) and reporter constructs (pTRB201, pTRB202), as well as the homeodomain (HD), hexapeptide (HP) and the position of the peptide antigen are shown. Grey boxes indicate acidic regions.

(Okkema and Fire, 1994) that encode the same 209 amino acid long homeodomain protein (Fig. 1B; Materials and Methods). The full sequence for the 5 kb genomic *ceh-2* locus was subsequently obtained by the Genome Sequencing Project (The *C. elegans* Sequencing Consortium, 1998).

ceh-2 is most closely related to *ems* class homeobox genes and shares all their typical features (Bürglin, 1994). Within and immediately adjacent to the homeodomain, 79-82% amino acids are identical between the CEH-2, fly *EMS* and vertebrate EMX predicted protein products (Fig. 1A). Like many Hox cluster genes, the *ems* class genes, including *ceh-2*, encode a hexapeptide motif upstream of the homeodomain, and an intron disrupts the homeobox between codons 44 and 45 (Bürglin, 1994; Bürglin, 1995). The hexapeptide motif of Hox genes interacts with PBC class homeodomain proteins, which contributes to cooperative and specific DNA binding (reviewed by Mann and Affolter, 1998). Whether the hexapeptide in *ems* class genes is essential or serves a similar function is not known, though *C. elegans* has two conserved PBC class homeobox genes [*ceh-20* and *ceh-40* (F17A2.5) (Bürglin and Ruvkun, 1992; Bürglin, 1997; Liu and Fire, 2000; Van Auken et al., 2002)]. A short region around the hexapeptide of CEH-2 is rich in proline and other amino acids and may therefore be used to activate or repress transcription (Lee and Young, 2000). N and C termini contain 30% acidic residues and may serve similar purposes. Acidic and proline-rich stretches are present in other *ems/Emx* genes as well as in other homeobox genes (Bürglin, 1994).

ceh-2 is expressed in five cell types of the anterior pharynx

We determined the *ceh-2* expression pattern using an antiserum raised against a peptide located C terminally to the homeodomain, as well as *gfp* and *lacZ* reporter fusions (Fig. 1B). Antibody staining is nuclear as expected for a transcription factor (Fig. 2). We find *ceh-2* expression restricted to eleven cells (fourteen nuclei) of five types in the anterior pharynx (corpus) of larvae and adults: the I3 neuron that lies embedded in the dorsal sector of the pharynx muscle; the pairs of NSM and M3 motoneurons in the left and right subventral sectors; the three m2 muscle cells, each possessing two nuclei resulting from cell fusion during development; and the three e2 epithelial cells with the anterior-most pharynx nuclei (Fig. 2A).

We see earliest expression with the antibody in a small cluster in late-gastrulation embryos (Fig. 2C). From their position, these cells may be the precursors of the pharyngeal cells that express GFP in the L1 larva (Schnabel et al., 1997). Reporter gene expression under the control of the *ceh-2* promoter is detected later, shortly after formation of the pharynx primordium (Fig. 2D), and is strongest in elongated embryos and early larvae. 1.6 kb sequence upstream from the start codon drives expression only in the neurons (pTRB201, Fig. 2E); a fourth exon *gfp* fusion that includes 4.5 kb of upstream sequence and the large intron within the homeodomain is expressed also in e2 and m2 cells (pTRB202; data not shown). A 10 kb genomic promoter sequence is also expressed in vulval cells, which may not reflect the natural expression pattern of the *ceh-2* gene, as that promoter extends into the next gene upstream (Inoue et al., 2002).

The transcription factor PHA-4 specifies organ identity during pharynx development; embryos lacking *pha-4* activity produce no pharyngeal cells (Azzaria et al., 1996; Horner et al., 1998; Mango et al., 1994). Thus, *pha-4* is expected to be upstream of *ceh-2*, and we find that *ceh-2* reporter constructs are not expressed in *pha-4* mutants (data not shown).

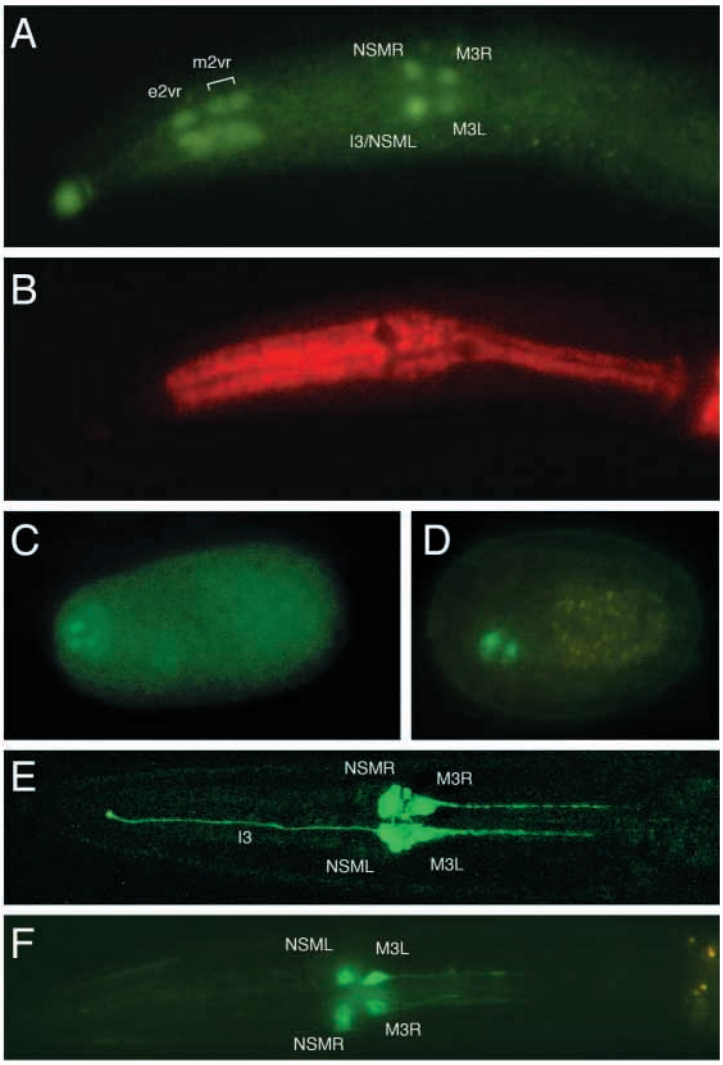

Fig. 2. Expression pattern of *ceh-2* in the anterior pharynx, determined by anti-peptide antibody staining (A,C) and *gfp* reporter constructs (D-F). Animals are oriented with anterior towards the left; cells are labeled. (A,B,E) Right subdorsal views to show all cell types; (D) dorsal view; (F) ventral view showing both pairs of M3 and NSM neurons. (A,C) An antibody against a CEH-2 peptide stains 14 nuclei in the anterior pharynx of larvae (A) and five or six cells in gastrulating embryos (C). (B) Pharynx muscle corresponding to A, stained with 3NB12 mouse monoclonal antibody (Miller et al., 1983) against a pharyngeal myosin. (D-F) *ceh-2::gfp* under the control of 1.6 kb promoter sequence (pTRB 201) in wild-type (D,E) and *ceh-2(ch4)* animals (F) is expressed in the embryonic pharynx primordium (D) and neurons of the pharynx (E,F). *ceh-2::gfp* expression levels and cell morphology in *ceh-2(ch4)* mutants are unchanged (F).

A *ceh-2* deletion mutation causes starvation and larval growth retardation

To investigate the function of *ceh-2* we isolated a deletion allele, *ceh-2(ch4)*, from an EMS mutant screen (see Materials and Methods). The 2.5 kb deletion in *ceh-2(ch4)* joins exon 2 to intron 4, thus eliminating exonic sequences that encode the complete hexapeptide and homeodomain (Fig. 1B). We sequenced several RT-PCR clones from *ceh-2(ch4)* animals and found two almost identical splice variants stemming from the remaining exons of *ceh-2* (Fig. 1B); in both cases, the 5′ end of exon 5 is spliced to a cryptic splice donor site within exon 2, which creates a functional open reading frame. Thus the *ceh-2(ch4)* allele encodes at least two peptides of about 100 amino acids that consist essentially of the *ceh-2* regions located N- and C-terminally to the hexapeptide and homeodomain. With 27% glutamate and aspartate residues these peptides are very acidic. As *ceh-2(ch4)* lacks both the potential DNA binding and protein binding domains, it is presumably a null allele; no deficiency is available to confirm this assumption. The M3 neuron phenotype described below is recessive, supporting the notion that *ceh-2(ch4)* is a loss-of-function allele.

Homozygous *ceh-2(ch4)* mutant worms are viable and fertile. About 20% of them are obviously starved: they are shorter and thinner at all stages and pale, and severely affected adults bear only a few eggs. Homozygous mutants proliferate slightly more slowly than wild type. Single mutant animals and their offspring take a day longer to exhaust food on a standard agar plate, even when the parent animal does not appear starved. We found that slow proliferation is due to both retarded larval development (Fig. 3) and reduced brood size (not shown). Worms that took longest to develop into adulthood also laid fewest eggs and appeared most starved. The mean larval development time in *ceh-2(ch4)* mutants was 54±7 hours (n=57) compared with 48±2 hours in wild type; single worms took up to 75 hours to develop into adults. A few homozygous *ch4* mutants arrested as L1 larvae (<5%). Timing of embryonic development was not affected, which is consistent with a feeding defect. However, we did not observe reliable differences in feeding behavior or pharynx pumping between mutant and wild-type animals under the light microscope.

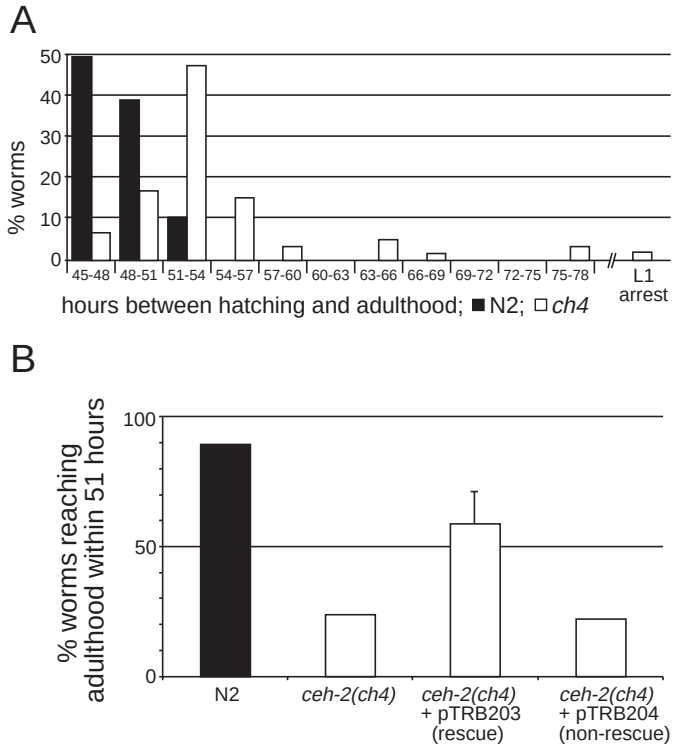

Fig. 3. Homozygous *ceh-2(ch4)* mutants are retarded in larval development. (A) Duration of larval development in wild type (N2) and *ceh-2(ch4)*. (B) Rescue of the slow growth defect by a *ceh-2* transgene. Most *ceh-2(ch4)* mutants take more than 51 hours to develop into adults. Worms carrying the genomic rescue transgene pTRB203 develop faster. The error bar represents the s.d. of four independent transgenic strains; less than wild-type rescue of *ceh-2(ch4)* is probably due to a slight growth disadvantage of the *rol-6*D co-injection marker used.

ceh-2 deletion mutants have defective M3 neuronal transmission but wild-type NSM functions

Cells normally expressing *ceh-2* also express *ceh-2::gfp* in *ceh-2(ch4)* and have normal morphology as judged by *ceh-2::gfp* expression (Fig. 2F). We therefore expected to find defects, if at all, in the function of the cells expressing *ceh-2*. Laser ablation and mutant analysis have revealed functions for two of the *ceh-2*-expressing pharynx neurons, the pairs of motoneurons M3 (Avery, 1993; Avery and Horvitz, 1989; Raizen and Avery, 1994) and NSM (Sawin et al., 2000).

The two glutamatergic M3 motoneurons are essential for effective feeding (Avery, 1993). Raizen and Avery (Raizen and Avery, 1994) have developed an external recording technique that allows visualization of M3 action. During each pharynx pump, changes in the membrane potential of pharynx muscle are recorded as stereotyped pattern of spikes called an electropharyngeogram (EPG). An EPG, formally the time derivative of a pharynx muscle action potential, consists of large spikes in the depolarizing and repolarizing direction at the start and the end of a contraction, respectively (Raizen and Avery, 1994) (Fig. 4A). In the plateau phase between the large depolarization and repolarization spikes, several smaller spikes in the inhibitory direction exist. M3 neurons are necessary and sufficient to generate these plateau phase spikes (Raizen and Avery, 1994).

We recorded 10-20 EPGs from each of 24 homozygous *ceh-2(ch4)* mutants. Seventy-five percent of the mutants exclusively produced EPGs without M3 transients (Fig. 4B). In recordings from the remaining 25%, EPGs with and without transients were interspersed. If present, most M3 mutant transients were considerably smaller than wild type, typically 10% of the repolarizing spike. A 13 kb genomic clone that contains only the *ceh-2* gene (pTRB203, Fig. 1B), as well as *ceh-2* cDNA driven by the 1.6 kb pTRB201 neural promoter sequence, restores M3 transients at least in some EPGs of all transgenic animals tested (Fig. 4C), showing that the partial loss of M3 function is due to reduction or lack of *ceh-2* function. Because M3-ablated animals are retarded in development (Avery, 1993), the severe reduction of M3 function in *ceh-2(ch4)* mutants presumably causes the growth retardation phenotype described above.

M3 activity shortens pump duration and speeds up relaxation of the contracted pharynx (Avery, 1993; Raizen and

Avery, 1994). This is reflected in the duration of electropharyngeograms, which is considerably longer in *ceh-2(ch4)* than in wild-type EPGs. We measured lengths of EPGs from the peak of the depolarization spike to the peak of the repolarization spike in the first four or five EPGs recorded from each of six to eight animals. In *ceh-2(ch4)*, pump duration was extended to 210±20 ms compared with 130±10 ms in wild type. A rescue transgene shortened the average pump duration of *ceh-2(ch4)* to 160±30 ms, compared with 220±20 ms for the non-rescue control. Errors are standard errors of the mean.

We assayed the *ceh-2(ch4)* mutants also for possible defects of NSM neuron function, but found only minor abnormalities that may not be related to NSM. The two NSM motoneurons contain serotonin (5-hydroxytryptamine, 5-HT) (Horvitz et al., 1982) and are able to take up exogenous serotonin via a serotonin reuptake transporter (Ranganathan et al., 2001). *ceh-2(ch4)* mutants both possess serotonin and are the same as wild type in terms of serotonin uptake. This was determined by antibody staining against serotonin in a wild-type or serotonin-deficient (*tph-1*) (Sze et al., 2000) background (data not shown). We tested for NSM function using NSM-dependent behavioral assays developed by Sawin et al. (Sawin et al., 2000): *C. elegans* moves less actively in the presence of food; worms that have been starved for half an hour will slow down even more when they encounter food again. This 'enhanced slowing response' is potentiated by the serotonin reuptake inhibitor fluoxetine. The enhanced slowing response depends in part on the presence of NSM neurons; its potentiation by fluoxetine is completely abolished upon NSM ablation (Sawin et al., 2000). *ceh-2(ch4)* homozygous mutants showed both responses (Fig. 5), which indicates that the NSM neurons are still functional.

We did observe a difference in the locomotion rate of *ceh-2(ch4)* compared with wild type in the absence of food. *ceh-2(ch4)* animals move more actively than wild-type animals when satiated, but less when starved (Fig. 5). However, this effect does not depend

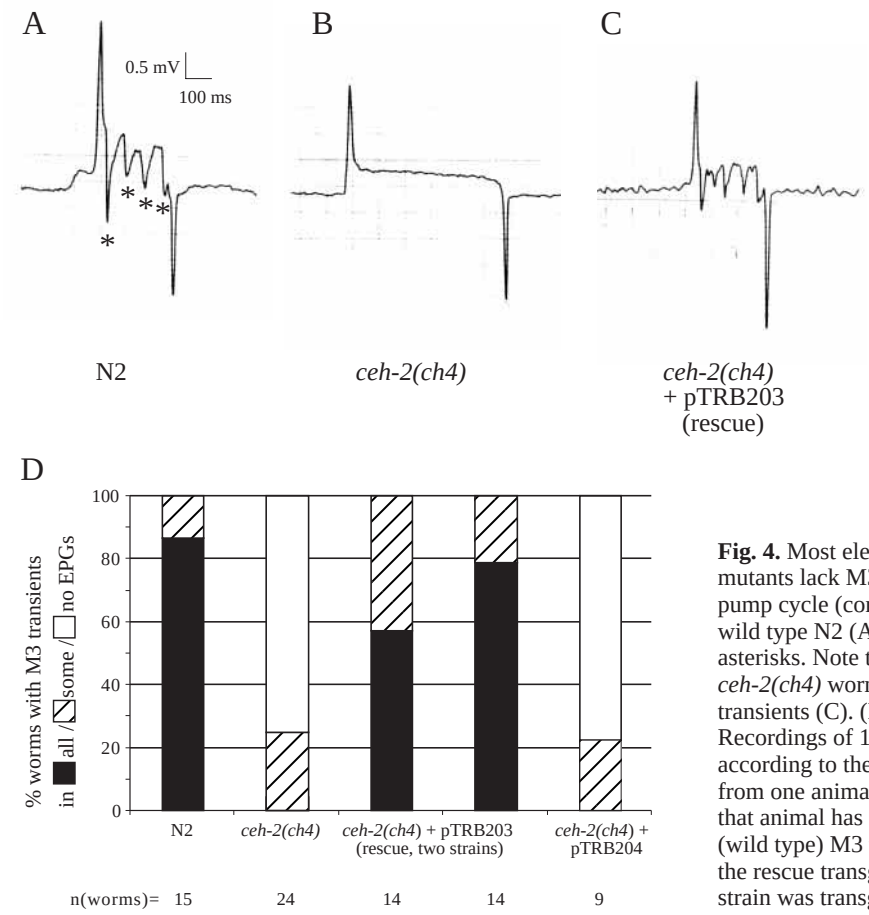

Fig. 4. Most electropharyngeograms (EPGs) from *ceh-2(ch4)* mutants lack M3 transients. Each EPG corresponds to one pharynx pump cycle (contraction and relaxation). (A-C) Typical EPGs from wild type N2 (A) and *ceh-2(ch4)* (B). M3 transients are marked with asterisks. Note the longer pump duration in mutants (B). EPGs of *ceh-2(ch4)* worms carrying a rescue transgene have normal M3 transients (C). (D) Percentage of animals with wild-type EPGs. Recordings of 10-20 EPGs from each of 9-24 animals were classified according to the presence of M3 transients in the EPGs: all EPGs from one animal lack M3 transients (white), at least one EPG from that animal has M3 transients (hatched), or all EPGs have normal (wild type) M3 transients (black). Two independent strains carrying the rescue transgene pTRB203 show rescue. The non-rescue control strain was transgenic with pTRB204.

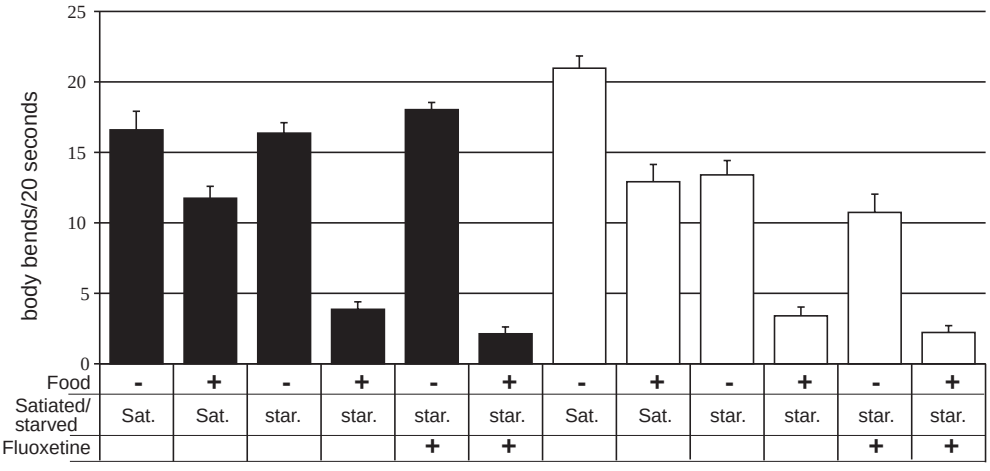

Fig. 5. *ceh-2(ch4)* animals display locomotion behaviors that require functional NSM neurons. Locomotion rates on agar plates of wild-type (black columns) or *ceh-2(ch4)* animals (white columns) were recorded in the presence (+) or absence (–) of food, with or without prior food deprivation for 30 minutes (starved/satiated) on plates containing either no fluoxetine or 75 μM fluoxetine (+). Wild type, black; *ceh-2(ch4)*, white. Error bars indicate s.e.m.

on NSM neuron function (Sawin et al., 2000). We think that the locomotion rate differences between *ceh-2(ch4)* and wild type may be due to constant malnourishment or starvation, induced by the reduction of M3 function.

ceh-2 can substitute for ems in *Drosophila* ems mutants

Although similarities in orthologous protein sequences are often restricted to domains, many orthologous genes are functionally exchangeable between organisms. For example, the mouse *Pax6* gene can substitute for the *eyeless* gene in *Drosophila* eye development (Halder et al., 1995), and defects in the brain of *ems* mutant *Drosophila* embryos can be rescued by expression of the human *EMX2* gene (Hartmann et al., 2000). We were interested to see whether *ceh-2* could function in place of the fly *ems* gene in a similar manner.

All homozygous *ems* mutant fly embryos lack neuromeres b2 and b3, this is visible as a distinct gap between the anteriormost neuromere b1 and the ventral nerve cord in antibody stainings for endogenous horseradish peroxidase crossreactivity (Hirth et al., 1995) (Fig. 6A). In addition, the frontal connectives that usually emerge laterally from the frontal ganglion and project into the b3 neuromere on both sides (Nassif et al., 1998) ectopically project into neuromere b1 in *ems* mutants (Hartmann et al., 2000) (Fig. 6B). The fly *ems* gene is able to restore neuromeres b2 and b3 as well as the frontal connective projections when expressed ubiquitously at embryonic stage 11 under the control of a heatshock promoter. The mouse *Emx2* gene is able to restore the missing neuromeres but not the frontal connective projections in the brain (Hartmann et al., 2000).

We repeated the rescue experiments described by Hartmann et al. (Hartmann et al., 2000). We expressed fly *ems* and worm *ceh-2*, respectively, using a heat-shock promoter in *ems* mutant flies and found rescue of the brain gap defect with *ceh-2* in 9% of *ems* mutant embryos (29 of 349 embryos). In 18 of the rescued embryos, one or both frontal connectives also projected correctly (Fig. 6C). We observed the same extent of rescue of the brain gap (9% or 7 of 80 embryos) and frontal connectives (4/80) using the fly *hs-ems* construct (not shown). Heat-shocked *ems* mutants without the transgene showed no rescue. From these data, we conclude that *ceh-2* is able to rescue the two main morphological brain defects of *ems* mutants, brain gap and frontal connective projection, albeit the latter only partially.

DISCUSSION

ceh-2 function in M3 pharynx motoneurons

We have shown that the homeodomain protein and putative transcription factor CEH-2 is expressed in M3 and NSM motoneurons. M3 function is greatly reduced in *ceh-2(ch4)*, as determined from electrophysiological recordings on the postsynaptic target muscle. However, M3 function seems not to be completely abolished. This may be either because *ceh-2(ch4)* is a reduction of function allele, or because loss of *ceh-2* function does not completely abolish M3 function. Because the M3 phenotype is recessive (not shown), and because *ceh-2(ch4)* lacks both the putative DNA-binding domain (homeodomain) and a putative protein-protein interaction site (hexapeptide), we assume that it is a null allele. We think that other transcription factors together with *ceh-2* are involved in expression of the downstream gene or genes that convey M3 function.

eat-4 is the only other gene known to be expressed in M3 and required for its function (Lee et al., 1999). *eat-4* is necessary for the function of several neurons considered glutamatergic, and may therefore be a general glutamatergic factor (Lee et al., 1999). Its vertebrate ortholog, a vesicular glutamate transporter, is sufficient to induce glutamate-mediated synaptic transmission in GABAergic hippocampal neurons (Bellocchio et al., 2000; Takamori et al., 2000). It is therefore possible that the glutamatergic phenotype of a neuron is largely defined by the uptake of this abundant amino acid into synaptic vesicles. *eat-4* may thus be the major factor needed for M3 function, perhaps apart from enhanced glutamate synthesis activity. Like *ceh-2*, *eat-4* is expressed in NSM as well as M3 but is not required for NSM function (Lee et al., 1999). *ceh-2* may thus positively regulate *eat-4* expression in M3 and NSM neurons, and may be deficient in glutamate uptake into presynaptic vesicles. We have examined the expression of an *eat-4::lacZ* reporter in *ceh-2(ch4)* mutants, but found no obvious regulation of *eat-4* by *ceh-2* (G.A. and

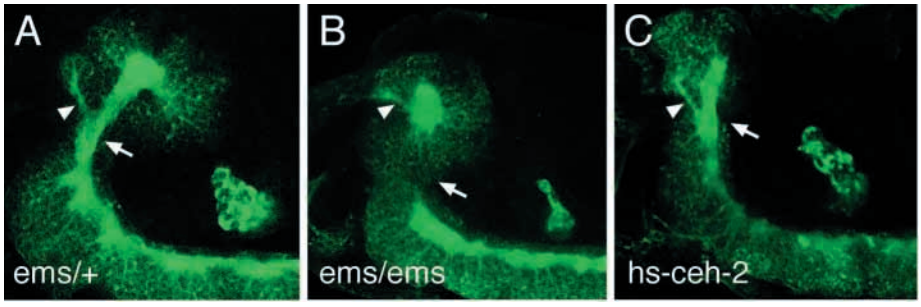

Fig. 6. *hs-ceh-2* is able to partially rescue the fly *ems* phenotype in the brain. Laser confocal images of stage 15 embryonic *Drosophila* brains, lateral views. Anterior is towards the left, dorsal is upwards. *ceh-2* cDNA was expressed under the control of a heat-shock promoter at embryonic stage 11 in offspring of ems^{9H83}/TM3 *sb Ubx-lacZ* heterozygotes. Embryos were fixed at stage 15 and stained with anti-horseradish peroxidase (green) and anti-β-galactosidase antibodies (not shown). Homozygous *ems* mutant embryos were recognized by the absence of anti-β-galactosidase staining. (A) *ems*/+ heterozygous mutants have wild-type brain morphology. Head involution movements cause a backward bending of the brain towards the ventral nerve cord. Arrow in A indicates connectives at the level of neuromere b3. (B) *ems/ems* mutant embryos have no neurons or connectives between neuromere b1 and the ventral nerve cord (arrow) (Hirth et al., 1995). The frontal connectives that usually project to b3 (arrowhead in A) project ectopically into b1(arrowhead in B). (C) *hs-ceh-2* is able to restore the missing neuromeres (arrow) and correct the projection of the frontal nerve (arrowhead) in homozygous *ems* mutants. Head involution movements do not occur in *ems* homozygous mutants (Jürgens et al., 1984) and are not restored by *hs-ceh-2* expression (nor by *hs-ems*; data not shown). Therefore the angle between brain and ventral nerve cord is larger in rescued animals (C) compared with heterozygotes (A).

T.R.B., unpublished); nevertheless, a regulation that is more difficult to detect may exist. Alternatively, *ceh-2* may regulate other, as yet unidentified components necessary for M3 function.

ceh-2(ch4) mutants seem to have normal NSM function as far as we can tell from presently available tests, and the only defect we see in these assays may be due to starvation, a consequence of the M3 failure. The role of *ceh-2* in I3 could not yet be determined, as no function has been described for this neuron. Furthermore, we did not observe any obvious morphologically defects in the m2 muscle and e2 epithelial cells. Thus, any function of *ceh-2* in these cells appears to be very subtle.

Apart from being located in the anterior pharynx, the cells expressing *ceh-2* are unrelated by cell type or lineage. *ceh-2* expressing cells have muscular, neuronal and epithelial identities and descend from all pharynx lineages, the ABara, ABalp and MS blastomeres. Most closely related by lineage are the M3 and NSM neurons, granddaughter and great-granddaughter of ABaraap(a/p)pp, respectively (Sulston et al., 1983). Thus, the *ceh-2* expression/function domain is most probably not regulated at the level of lineage, but rather positional/regional cues trigger expression of *ceh-2* during embryogenesis, when the cells are closely clustered.

ceh-2 in *ems/Emx* evolution

ceh-2 is the only clear *ems/Emx* ortholog in the sequenced *C. elegans* genome. It shares the features of ems class genes from *Drosophila*, amphioxus, a hydrozoan, and several vertebrate species; the degree of sequence conservation between the vertebrate and invertebrate ems genes is similar. Besides the homeodomain (with a conserved intron position) and the hexapeptide, there is little detectable sequence similarity between *ems* class genes from different phyla, except for general proline-rich and acidic regions.

We showed by cross-species rescue experiments that *ceh-2* can functionally replace the fly *ems* gene in the brain to a comparable extent to the fly *ems* gene. Thus, CEH-2 protein can presumably associate with the same co-factors as fly Ems and bind to the same enhancer elements. The identities of these co-factors and enhancer elements are not known, but because this function has been conserved, it is likely that similar interactions are used in *C. elegans* to regulate target genes. The human *EMX2* gene, for example, is able to rescue the missing *Drosophila* prosomeres but never the axonal pathfinding defect of the frontal nerve (Hartmann et al., 2000), and therefore might have lost some functions needed in the fly.

The evolutionary origin of the *C. elegans* pharynx nervous system is not clear. The posterior part of the pharynx itself has physiological and molecular similarities to vertebrate and fly hearts and is derived mainly from the MS blast cell (Haun et al., 1998; Okkema and Fire, 1994). Most of the anterior part of the pharynx is derived from the anterior AB.a blast cell from which many ring ganglia neurons are also derived, thus two rather separate cell lineages contribute to the pharynx. However, pharynx identity is conferred by a single gene, the fork head/winged-helix class transcription factor *pha-4* (Azzaria et al., 1996; Horner et al., 1998; Mango et al., 1994). *pha-4* mutants possess cells of the pharynx lineages, but do not develop a pharynx. *ceh-2* reporter constructs are not expressed in *pha-4* mutants so that *ceh-2* is downstream of *pha-4*. The *Drosophila* ortholog of *pha-4*, the gene *fork head*, is expressed in the terminal regions of the embryo and functions in anterior gut development and has been shown to play a role in brain patterning through inductive events (Page, 2002). Hence, both *ceh-2* and *pha-4* may have an evolutionary origin in the development of anterior structures.

The common theme between *ceh-2* and its hydrozoan, fly and chordate orthologs is that they are expressed at the anterior. As a sister phylum of arthropods and other molting animals (ecdysozoa) (Aguinaldo et al., 1997), one could expect nematode genes to be similar to fly genes and thus have a CNS function, in particular given that both fly *ems* and vertebrate *Emx* genes function in the brain (Reichert and Simeone, 1999). However, an ascidian *ems/Emx* ortholog is expressed in the anterior trunk and lateral tail epidermis and, like *ceh-2*, not in the CNS. This observation lead to the proposal that *ems/Emx* gene functions in the CNS may have been acquired independently in *Drosophila* and vertebrates (Oda and Saiga, 2001). Likewise in Hydractinia, an *ems* homolog is expressed in the anterior digestive tract and not in neurons (Mokady et al., 1998). Thus, although the orientation of the anterior/posterior axis in cnidarians is being discussed (Meinhardt, 2002), the more basic role of *ems* class genes may have been in anterior patterning, and the notion that they always play a role in the CNS may have to be revised. A possible hypothesis is that in some animal phyla, cells contributing to the CNS are derived from the *ems/Emx* expression domain, but in others not, depending on how the *ems/Emx* domain overlaps with the fate map in embryos of different phyla.

A second conclusion we can draw from our cross-species experiments is that an orthologous gene does not necessarily have the same function, even if it can substitute for its ortholog in a cross-species rescue experiment. For example, the fact that mouse *Pax6* can substitute for its fly counterpart and generate ectopic eyes has been taken as an indicator that these two genes have the same function in development (for a review, see Gehring and Ikeo, 1999). By contrast, *ceh-2* can rescue fly *ems* brain defects, even though it plays no role in the development of the *C. elegans* ring ganglia: the 'brain'. *ceh-2* does have a function in pharynx neurons, which may have a common function with neurons in the CNS. We suggest that the primary reason for cross-species rescue lies in appropriate sequence conservation, and is not necessarily related to particular functions in the ontogeny of an animal.

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