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Analysis of molecular marker expression reveals neuronal homology in distantly related arthropods

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SUMMARY

Morphological studies suggest that insects and crustaceans of the Class Malacostraca (such as crayfish) share a set of homologous neurons. However, expression of molecular markers in these neurons has not been investigated, and the homology of insect and malacostracan neuroblasts, the neural stem cells that produce these neurons, has been questioned. Furthermore, it is not known whether crustaceans of the Class Branchiopoda (such as brine shrimp) or arthropods of the Order Collembola (springtails) possess neurons that are homologous to those of other arthropods. Assaying expression of molecular markers in the developing nervous systems of various arthropods could resolve some of these issues. Here, we examine expression of Even-skipped and Engrailed, two transcription factors that serve as insect embryonic CNS markers, across a number of arthropod species. This molecular analysis allows us to verify the homology of previously identified malacostracan neurons and to identify additional homologous neurons in malacostracans, collembolans and branchiopods. Engrailed expression in the neural stem cells of a number of crustaceans was also found to be conserved. We conclude that despite their distant phylogenetic relationships and divergent mechanisms of neurogenesis, insects, malacostracans, branchiopods and collembolans share many common CNS components.

Key words: Neurogenesis, Neuron, Neuroblast, Homology, Arthropod, Even-skipped, Engrailed

INTRODUCTION

Comparative analysis of arthropod CNS development will improve our understanding of the principles of neural development, the phylogeny of arthropods and the ways in which changes in development produce neuronal diversity. The first step in such a comparative analysis is to determine if different arthropod species have homologous neural cells. According to Whitington (1996), neuronal homology can be determined through the examination of a number of morphological criteria, such as a neuron's cell body location and axonal morphology. Based on such morphological criteria, neurons resembling a number of insect embryonic neurons, including the Even-skipped (Eve)-expressing neurons (Fig. 1) known as U, RP2, aCC (motor neurons) and pCC (an interneuron), were previously identified in three malacostracan crustaceans (Thomas et al., 1984; Whitington et al., 1993). Although comparison of neuronal morphology is a powerful way of ascertaining whether or not two neurons are homologous, a number of researchers have demanded additional non-morphological tests for the determination of neuronal homology.

The discovery of a number of neural molecular markers in *Drosophila* and grasshoppers (Patel et al., 1989a, 1992; Condron et al., 1994; Broadus and Doe, 1995) has provided us with such a test. It is now possible to examine molecular marker expression in putative neuronal homologues. The use of cross-reactive antibodies that recognize the same antigens in a variety of arthropods provides us with a convenient means of collecting data from a number of species. Cross-reactive antibodies that recognize Eve and Engrailed (En), two transcription factors that serve as insect molecular neuronal markers, have been described (Patel et al., 1992, 1994a, 1989b). These two proteins have distinct functions during CNS development that are separate from their roles during insect segmentation. For example, Doe et al. (1988) showed that following its role in segmentation, Eve is responsible for generating the proper axonal projection of the RP2 neuron. In the grasshopper, when *en* expression in the median neuroblast (MNB) is disrupted by injection of antisense oligonucleotides, extra neurons develop at the expense of midline glial cells (Condron et al., 1994). Here, we examine Eve and En neuronal expression in a variety of arthropods, including insects, malacostracan crustaceans, branchiopod crustaceans and collembolans. This study enables us to verify the homology of previously identified neurons and to discover novel putative homologues.

The issue of neuroblast (NB) homology has also prompted

debates in recent years (Scholtz, 1992; reviewed by Whitington, 1996). Insect and malacostracan crustacean NBs have a number of similarities. For example, in both insects and malacostracan crustaceans, NBs function as neural stem cells. Insect and crustacean NBs divide asymmetrically perpendicular to the surface and produce ganglion mother cells (GMCs). Apically budded insect and malacostracan GMCs then divide symmetrically to produce neurons (reviewed by Campos-Ortega, 1993; Dohle and Scholtz, 1988; Whitington, 1996). Insect and malacostracan NBs also differ in a number of ways. In insects, a process of lateral inhibition results in neuroectodermal cells becoming either NBs or epidermoblasts (reviewed by Campos-Ortega, 1993). However, in the malacostracan ventral neuroectoderm, NB formation is lineage invariant, and a lateral inhibition process does not appear to be used to specify NB cell fate (Dohle, 1970; Dohle and Scholtz, 1988). Furthermore, once insect cells are specified as NBs, they delaminate from the ventral neuroectoderm before dividing to produce GMCs; crustacean NBs do not delaminate, but remain in the ventral-most cell layer. Crustacean NBs differ from insect NBs in another interesting way. In crustaceans, most ventral neuroectodermal cells are initially NBs (N. H. P., unpublished). After their first division, some NBs then switch and become epidermoblasts, but they can later switch back to producing GMCs (Dohle, 1970; Dohle and Scholtz, 1988). This switching phenomenon has never been observed in insects. For these reasons, crustacean NBs are not generally thought to be homologous to those of insects (Dohle and Scholtz, 1988; Scholtz, 1992).

The NB homology debate becomes even more complicated in non-malacostracan crustaceans. NBs that remain on the surface, dividing unequally to produce GMCs, have been observed in only one member of the Class Branchiopoda (Gerberding, 1997). In other branchiopods, neural stem cells produce neurons through a general inward proliferation of cells (Weygoldt, 1960; Benesch, 1969). Despite arguments suggesting that insect and crustacean NBs may not be homologous, our studies indicate that arthropods possess a conserved set of Eve- and En-expressing neurons. This observation led us to hypothesize that despite their differences, insect and crustacean NBs may still express the same molecular markers.

In order to test this hypothesis, we have examined En NB expression in a number of arthropods. En is expressed in a subset of insect NBs (Condron et al., 1994; Broadus and Doe, 1995; Broadus et al., 1995). En has specific CNS functions, and maintenance of En expression in insect NBs is not simply a remnant of its earlier expression in the ventral neuroectoderm (Patel et al., 1989a). En expression was found to be conserved in the neural stem cells of a variety of crustacean species, despite the fact that these cells are formed and behave somewhat differently from typical insect NBs. Although early neurogenic events may not be well conserved among arthropods, the remarkable conservation of Eve and En expression in the neurons of various arthropods, in conjunction with previously obtained morphological data (Thomas et al., 1984; Whitington, 1993) leaves little doubt that hexapods and crustaceans share a set of homologous neurons. We conclude that the common ancestor of hexapods and crustaceans possessed a set of Eve- and En-expressing neurons and neural stem cells that expressed En.

MATERIALS AND METHODS

Animal sources and culturing conditions

Thermobia domestica were obtained from T. Kaufman and were maintained as described by Rogers et al. (1997). *Schistocerca americana* were cultured as described by Bentley et al. (1979). *Folsomia candida* (obtained from J. Burns) were kept at room temperature in plastic containers which held a hardened mixture of 4 parts plaster of Paris: 1 part activated charcoal; once a week, the containers were lightly sprayed with water and sprinkled with baker's yeast. *Porcellio laevis* were obtained from Carolina Biologicals and maintained at room temperature in plastic containers which held dirt, corrugated cardboard and a piece of chalk (calcium source). Weekly, the dirt was sprayed lightly with water, and the animals were fed small pieces of raw vegetables (usually carrots). *Procambarus clarki* adults were obtained from Lemberger Co., Inc. during their breeding season (May); embryos were dissected and fixed upon arrival. Adult *Mysidium columbiae* were collected in their native habitat in Belize and were fixed immediately. *Triops longicauditus* and *Artemia franciscana* cysts were obtained from Carolina Biologicals and were hatched and maintained according the instructions provided.

Immunohistochemistry

Anti-Eve monoclonal antibodies 2B8 and 7H5 are described by Patel et al. (1992, 1994a). Anti-En monoclonal antibodies 4D9 and 4F11 have also been described (Patel et al., 1989b). The 2B8 antibody was used to stain *Drosophila melanogaster, Schistocerca americana,* and *Mysidium columbiae. Procambarus clarki* and *Triops longicauditus* were stained with the 7H5 antibody. Both 7H5 and 2B8 were used successfully in *Folsomia candida* and *Porcellio laevis*. The 4F11 antibody was used to examine En expression in *Folsomia candida*, *Triops longicauditus* and *Artemia franciscana,* and 4D9 was used to stain all other arthropod species described.

In general, staining was completed according to the procedures discussed by Patel et al. (1989b,c) and Patel (1994b). *Drosophila melanogaster* and *Folsomia candida* were fixed according to the standard fly protocol (Patel, 1994b). Crustaceans and *Thermobia domestica* were fixed for 15-20 minutes in 4% formaldehyde in PEM (a heptane interface was not necessary). Vitelline membranes were removed by hand dissection during or following fixation, as needed. After their fixation, *Mysidium columbiae, Triops longicauditus* and *Artemia franciscana* were sonicated for 1-3 seconds in a Branson 250 sonicator set at its lowest power setting. Following dissection or sonication, both of which allowed for better penetration of antibodies, animals were rinsed briefly in PBS+0.2% Triton-X, blocked in PBS+0.2% Trition X+5% NGS, and stained with the appropriate antibodies. Primary and secondary antibody incubations were completed at 4°C overnight.

RESULTS

Criteria for identifying neurons

After thorough examinination of the Eve and En neuronal expression patterns in *Drosophila melanogaster* (fruit fly) and *Schistocerca americana* (grasshopper), we began to look at the expression of these proteins in other arthropods, including an apterygote insect, a collembolan, three malacostacan crustaceans and a brachiopod crustacean (species' names, sources and culturing conditions are listed in the Materials and Methods section). Cell body position was the most important criterion for determining neuronal identity. We paid close attention to each Eve- or En-expressing neuron's anterior/posterior and dorsal/ventral location, as well as its

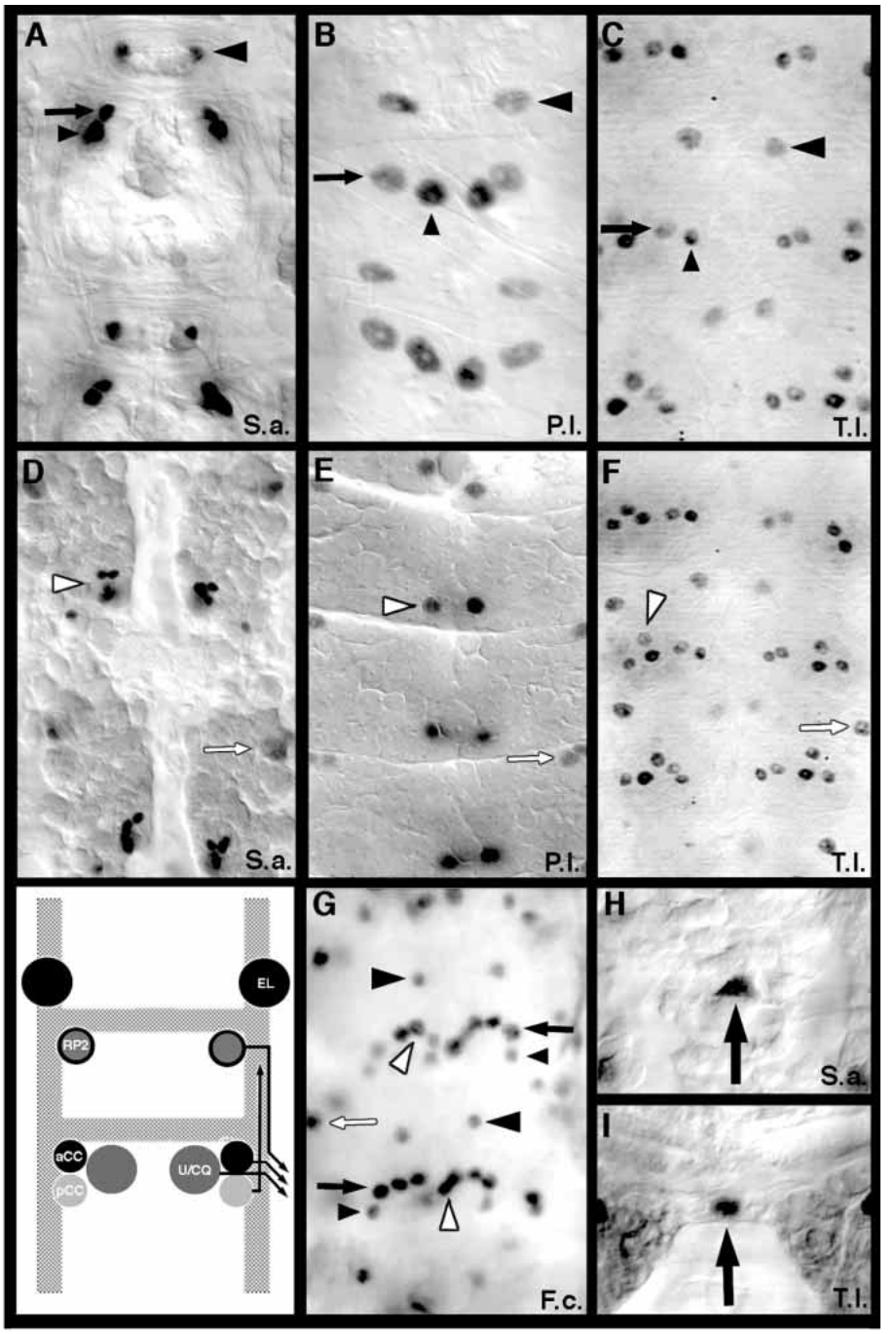

Fig. 1. Neural expression of Eve is conserved among arthropods. In all figures, anterior is up. The Latin names of each organism are abbreviated in the lower right hand corner of each figure as follows: *Schistocerca americana* = S.a., *Porcellio laevis* = P.l., *Triops longicauditus* = T.l. and *Folsomia candida* = F.c. Eve-positive neurons, anterior and posterior commissures and longitudinals in one bilaterally symmetrical segment are illustrated in the schematic at the bottom-left corner of the figure. In this schematic, single circles marked U/CQ and EL actually represent the clusters of U/CQ (three U neurons and two CQ neurons/hemisegment) and EL neurons (eight to ten cells/hemisegment) found in insect abdominal segments. Axon projectories (reviewed by Whitington, 1996) of the RP2, aCC, pCC and U neurons are shown. The arrow drawn from the U/CQ circle represents the projectory of a typcial U (not CQ) neuron. Eve expression is conserved among insects (represented by the grasshopper *Schistocerca americana* in A, D and H), malacostracan crustaceans (represented by the sow bug *Porcellio laevis* in B and E), branchiopod crustaceans (represented by the tadpole shrimp *Triops longicauditus* in C, F and I) and collembola (represented by the springtail *Folsomia candida* in G). In dorsal planes of focus (A-C), the RP2 (large black arrowhead), aCC (black arrow) and pCC (small black arrowhead) neurons can be seen. The EL (white arrow) and U/CQ (white arrowhead) neuron clusters are found more ventrally (D-F). Dorsal and ventral Eve-expressing neurons appear to be in the same plane in G because the embryo has been flattened so that it could be photographed more easily. Although all five cells of the U/CQ cluster are not shown in E and F, additional U/CQ neurons (typically four to five/hemisegment) are found in slightly more ventral planes of focus and at different stages of development. Similarly, although only a small number of EL neurons are visible in E and F, we expect that additional neurons would be detected if we were to analyze older embryos. The AUN (large black arrow) is found in the mandibular segment of *Schistocerca americana* (H) and *Triops longicauditus* (I). This Eve-expressing neuron is found in all arthropods that were examined in this study.

location in reference to the commissures and other neurons. Whenever possible, we compared cell body positions of Eve-expressing neurons to the cell body positions of neurons filled by Whitington et al. (1993). The timing of Eve or En expression was also noted; different neurons express various molecular markers at different times during development, and this factor helped us to identify neurons. We also paid close attention to levels of protein expression; in some cases, neurons could be identified by either their relatively high or low levels of Eve or En expression.

Analysis of Eve neuronal expression

Eve is an excellent neural marker because a relatively small number of neurons express this protein. In most cases, these cells can be identified based solely on their cell body location. Expression of Eve in the insect CNS has been described (Patel et al., 1989a; Patel et al., 1992) and is schematized in Fig. 1. In a dorsal plane of focus in *Schistocerca americana*, the RP2, aCC and pCC neurons found in each hemisegment express Eve (Fig. 1A; Patel et al., 1992). The Eve-expressing U/CQ and EL neuron clusters are found in more ventral positions in each

hemisegment (Fig. 1D; Patel et al., 1992). In the midline of the mandibular segment, an anterior unpaired neuron (AUN) is observed (Fig. 1H, Patel et al., 1992). This Eve expression pattern is conserved in all insects examined to date (18 species spanning six Orders, N. H. P., unpublished).

We looked for Eve CNS expression in a number of crustaceans. Eve-expressing cells resembling the aCC, pCC and RP2 neurons are found dorsally in a number of malacostracans, including the sow bug *Porcellio laevis* (Fig. 1B), the crayfish *Procambarus clarki* and the opossum shrimp *Mysidium columbiae* (not shown). The positions of these Eve-stained cell bodies agree precisely with the cell body positions of crustacean neurons whose axon morphologies resemble those of the insect aCC, pCC and RP2 neurons (see schematic in Fig. 1; Whitington et al., 1993).

In comparison to *Schistocerca americana* (Fig. 1A), the position of aCC and pCC shifts slightly in *Porcellio laevis* (Fig. 1B). Though pCC is found just posterior of the aCC neuron (as depicted in the schematic in Fig. 1) in *Schistocerca americana* and *Drosophila melanogaster* (not shown), in some arthropods, including the insect *Tribolium* (not shown; N. H. P., unpublished observation), pCC is found just medially to aCC. In cases where this shift has occurred, pCC is easily distinguished from aCC because its level of Eve expression is relatively higher (Fig. 1B). This shift in the relative position of pCC in *Porcellio* was predicted by Whitington's morphological study (1993).

We also searched for cells resembling the more ventral insect U/CQ and EL neurons. Ventral Eve-expressing cells corresponding to the U/CQ and EL neurons (Fig. 1E) are found in all three malacostracan crustacean species studied. The position of the Eve-stained U neurons that we observe matches the cell body locations of the neurons whose axonal morphologies resemble the insect U neurons (Whitington et al., 1993). Furthermore, a cell resembling the *Schistocerca americana* AUN is found in all malacostracans examined (not shown).

Eve expression was also analyzed in a member of the crustacean Class Branchiopoda and a member of the arthropod Order Collembola. Extensive CNS morphological data has not been collected from these organisms in the past. We therefore hoped that gathering data from these two species would be informative from an evolutionary standpoint, particularly in the case of the collembolans, who may represent the hexapod sister taxon of the insects (Gillott, 1980). Homologues of all Eve-expressing neurons could be identified in the branchiopod *Triops longicauditus* (tadpole shrimp; Fig. 1C,F,I). As in *Porcellio laevis*, pCC is found more medially to aCC in this organism (Fig. 1C). A conserved set of Eve-positive neurons was also found in the collembolan *Folsomia candida* (springtails; Fig. 1G). We conclude that the Eve nervous system expression pattern is conserved among insects, malacostracans, brachiopods and collembolans. These data are summarized in Fig. 4.

En neuronal expression

In general, En neuronal expression is more complex than Eve neuronal expression; En is expressed in a larger number of neurons, and its expression is rather dynamic. For these reasons, we limited our analysis of En expression to a small number of neurons that have very distinct locations within each segment. These neurons are also easier to identify because most of them express En relatively early and maintain En expression through later stages of development. The cells chosen for analysis are schematized in Fig. 2 and have been described in the grasshopper (Condron et al., 1994). They include (from ventral to dorsal and posterior to anterior): the progeny of NB 1-2, the MNB progeny (Fig. 2C), the ECp,l,a (Fig. 2F), LE (Fig. 2A) and IC (Fig. 2A) neurons.

Although the En-positive neurons listed above express En rather early, they initiate En expression at slightly different times; this factor assisted us in identifying particular neurons. For example, the MNB progeny and EC neurons express En first, and the LE and IC neurons initiate En expression slightly later. The IC neurons could also be identified by the fact that the En expression level is relatively lower in these neurons. We find En-positive cells resembling the NB 1-2 progeny, MNB progeny, ECa,p,l, LE and IC neurons in a number of insects, including *Drosophila melanogaster* (with the exception of IC neurons) and the primitive apterygote insect *Thermobia domestica* (firebrat; data not shown, NB 1-2 progeny not analyzed). With the exception of the ECl neuron, all other En-positive neurons, with conserved anterior/posterior and dorsal/ventral positions, timing and levels of En expression are found in malacostracans, including *Porcellio laevis*, *Procambarus clarki* and *Mysidium columbiae* (NB 1-2 progeny not analyzed in *Porcellio*). For example, the dorsally located LE and IC neurons (Fig. 2B), the ventrally located MNB progeny (Fig. 2D) and the ventral ECa and ECp neurons (Fig. 2H) of *Porcellio laevis* are shown. In *Procambarus clarki* (not shown), a large number of En-positive MNB progeny are visible only at late stages. We see a comparatively smaller number of cells that resemble MNB progeny in *Porcellio laevis* and *Mysidium columbiae*, but embryos of a similar age to the late-staged *Procambarus clarki* were not analyzed. In summary, the En CNS expression pattern is conserved among winged and non-winged insects and malacostracan crustaceans.

We also analyzed En expression in collembolans and branchiopods. *Folsomia candida* (Collembola) have a conserved set of En-expressing neurons (NB 1-2 progeny not analyzed), including all three EC neurons (Fig. 2G). Since we find three EC neurons in insects, but only two in malacostracan crustaceans, these data are consistent with grouping collembolans as hexapods. A number of En-positive neurons are also found in the branchiopod crustacean *Triops longicauditus*. A large unpaired cell at the midline resembles an MNB or one of its progeny (Fig. 2E). However, the other En-positive cells (not shown) did not resemble any of the neurons assayed in this study, and we are uncertain of their relationship to insect neurons. Though this result is surprising, as mentioned above, due to the complexity of the En neuronal expression pattern, we have only attempted to identify a relatively small number of En-positive neurons. It remains possible that En-positive neurons that were not examined in this investigation are conserved in *Triops longicauditus*. With the exception of the *Triops longicauditus* En pattern, we find, overall, a remarkable degree of conservation of the arthropod Eve and En neural expression patterns among crustaceans and hexapods. Our results are summarized in Fig. 4.

Expression of En in neural stem cells

NB expression of En has been described in insects. In

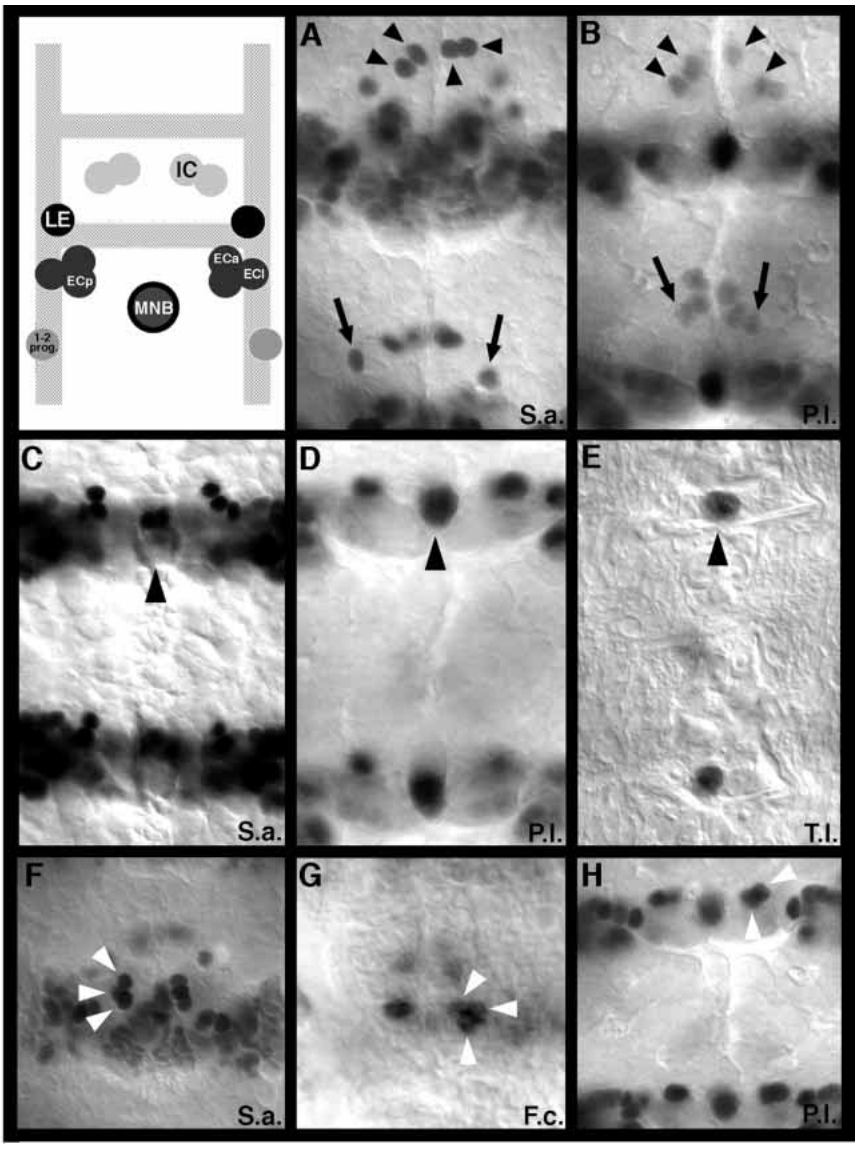

Fig. 2. A conserved set of En-positive neurons. In all figures, anterior is up. The Latin names of the organism are abbreviated in the lower right hand corner of each figure as follows: *Schistocerca americana* = S.a., *Porcellio laevis* = P.l., *Triops longicauditus* = T.l. and *Folsomia candida* = F.c. The En-positive cells analyzed in this study, anterior and posterior commissures and longitudinals of one bilaterally symmetrical segment are schematized at the top-left corner of the figure. In dorsal planes of focus in insects (represented by the grasshopper *Schistocerca americana* in A) and malacostracans (represented by the sow bug *Porcellio laevis* in B), the LE (black arrow) and IC (black arrowheads) neurons are found. These cells are also found in collembola (not shown). In insects (*Schistocerca americana* shown in C), an unpaired MNB (large En-positive cell marked by black arrowhead) and several of its En-positive progeny (cells just anterior to the MNB) are found at the midline in more ventral planes of focus. Large cells resembling MNB progeny are observed in malacostracans (*Porcellio laevis* shown in D), branchiopods (represented by the tadpole shrimp *Triops longicauditus* in E) and collembola (not shown). In ventral planes, the EC neurons are found in insects (represented by *Schistocerca americana* in F), collembola (*Folsomia candida* in G) and malacostracan crustaceans (*Porcellio laevis* in H). In insects and collembola, three EC neurons, ECa, ECl and ECp, are found (top, middle and bottom white arrowheads in F and G, respectively). In malacostracan crustaceans (H), only two EC neurons, ECa (top white arrowhead) and ECp (bottom white arrowhead), are found.

Schistocerca americana (Fig. 3A) and *Drosophila melanogaster*, En is expressed in a subset of NBs, including row six and seven NBs and NB 1-2 (Fig. 3A; Condron et al., 1994; Broadus and Doe, 1995; Broadus et al., 1995). Although expression of En initiates in the ventral neuroectoderm prior to NB specification, En continues to be expressed in NBs once these cells have delaminated. Genetic evidence indicating that En has specific CNS functions has shown that maintained expression of En in NBs is not simply a remnant of its earlier expression pattern in the neuroectoderm (Patel et al., 1989a).

We completed a detailed analysis of En NB expression in the malacostracan *Procambarus clarki*. Although crustacean NBs do not delaminate, NBs are easily distinguished from epidermal cells because they are much larger in size (this is true for insect NBs, as well). We found conservation of the En NB expression pattern (Fig. 3B), including expression in row six and seven NBs and a single row one NB. In addition to the En-positive rows of NBs, five additional rows of NBs that do not express En are found in *Procambarus clarki*, just as they are in *Schistocerca americana* (Condron et al., 1994) and *Drosophila melanogaster* (Broadus et al., 1995). In summary, expression of En and the overall arrangement of NBs is conserved between insects and *Procambarus clarki*.

We also looked for En NB expression in a number of other crustaceans. Though we did not examine these arthropods in as much detail as we examined *Procambarus clarki*, we find comparable En NB expression patterns in the malacostracans *Porcellio laevis* and *Mysidium columbiae* (not shown), as well as the branchiopods *Triops longicauditus* (not shown) and the brine shrimp *Artemia franciscana* (Fig. 3C). The *Artemia* result is somewhat surprising because the CNS of these branchiopods was previously reported to form by a general inward proliferation of cells (Benesch, 1969). However, within the *Artemia* ventral neuroectoderm, we can identify two rows of large-sized En-expressing cells, as well as five rows of large unstained cells. We speculate that these large cells are NBs. In summary, NB expression of En is conserved in all arthropod species examined. Although insect and crustacean NBs differ in a number of ways, these differences do not appear to affect the expression of at least one molecular NB marker and appear to be, in the end, of little consequence to the generation of specific neural progeny.

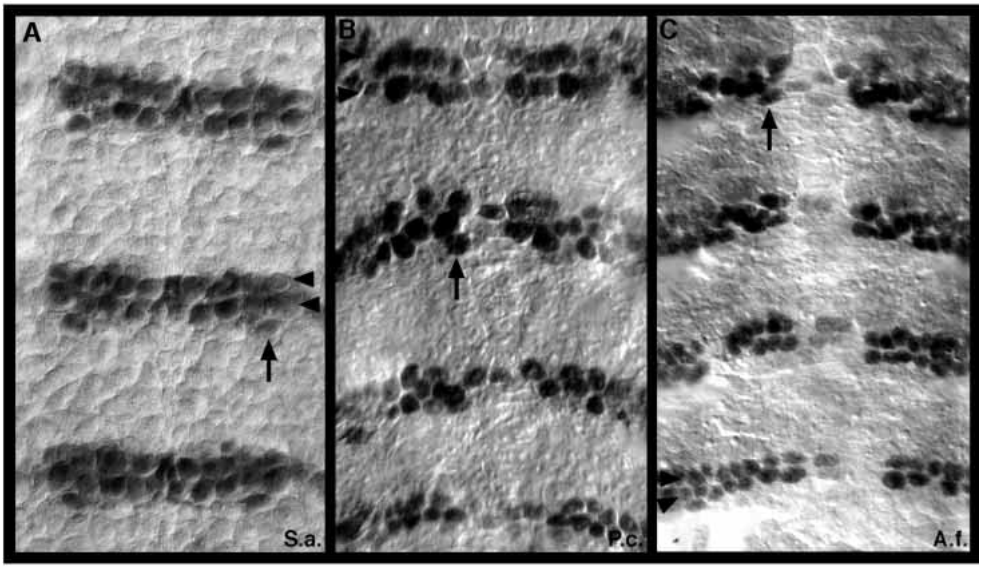

Fig. 3. Conservation of En expression in arthropod neural stem cells. Anterior is up in each photo. The Latin names of each organism are abbreviated in the lower right hand corner of each figure as follows: *Schistocerca americana* = S.a., *Procambarus clarki* = P.c. and *Artemia franciscana* = A.f. En expression in NBs (large En-positive cells) can be visualized in insects (represented by the grasshopper *Schistocerca americana* in A), malacostracans (represented by the crayfish *Procambarus clarki* in B) and branchiopods (represented by the brine shrimp *Artemia franciscana* in C). En is expressed in row six (upper arrowhead in each panel) and seven (lower arrowhead in each panel) NBs and in NB 1-2 (arrow; Condron et al., 1994; Broadus and Doe, 1995). En-expressing neural stem cells are found in comparable positions in all other arthropods examined.

DISCUSSION

CNS expression of Eve and En in the common ancestor of insects and crustaceans

In this investigation, we have examined the neural expression of Eve and the neural and NB expression of En in a number of arthropod species. Our data is summarized in Fig. 4. These data have allowed us to verify the homology of four malacostracan crustacean neurons whose axonal morphologies had led researchers to suspect that they were crustacean homologues of the Eve-expressing insect aCC, pCC, RP2 and U neurons (Thomas et al., 1984; Whitington et al., 1993). We find these neurons in a number of arthropods, including the malacostracan crustaceans *Procambarus clarki*, *Porcellio laevis* and *Mysidium columbiae*, the branchiopod crustacean *Triops longicauditus* and the collembolan *Folsomia candida*. We have also discovered a number of neurons not previously identified in these arthropods, including cells resembling the Eve-expressing CQ, EL and AUN neurons and the En-expressing NB 1-2 progeny, MNB progeny, EC, LE and IC neurons. A set of En-expressing neurons was also found in the primitive apterygote insect *Thermobia domestica*. The fact that we could find a conserved set of Eve and En neurons in a wide variety of crustaceans led us to speculate that the neural stem cells responsible for the production of these neurons might express the same molecular markers, despite the fact that they are formed and behave somewhat differently than typical insect NBs. En expression was found to be conserved in the neural stem cells of all arthropod species examined. These data have allowed us to conclude that the common ancestor of hexapods and crustaceans possessed neurons resembling the Eve-expressing aCC, pCC, RP2, U/CQ, EL and AUN neurons and the En-expressing NB 1-2 progeny, MNB progeny, EC, LE and IC neurons, as well as NBs that expressed En.

Criteria for detecting neuronal homology

We have utilized a new criterion for detecting neuronal homology: the examination of molecular neuronal and NB markers. In most previous studies, researchers have relied on the location of a neuron's cell body and its axonal projection in order to demonstrate its homology. Adding the criterion of molecular marker expression allows us to test neuronal homology more stringently. For example, in the case of the aCC, pCC, RP2 and U neurons, we now possess agreeable morphological and molecular evidence indicating that these four neurons are conserved among hexapods and crustaceans. In the case of these neurons, three criteria for judging neuronal homology were used: (1) similar cell body positions, (2) similar axonal projections and (3) similar molecular marker expression. We feel that these three criteria can serve as a guideline for judging neuronal homology; when two neurons have similar cell body positions, axonal projections and marker expression, they can be referred to as homologues. We do realize, however, that no method for determining neuronal homology is completely foolproof. Indeed, we expect that during evolution, homologous neurons will diverge in axonal morphology, for example, and that convergent patterns will occassionally emerge. Still, the combination of both morphological and molecular criteria provides us with an extremely powerful method of detecting neuronal homology.

Our molecular investigation has allowed us to identify additional neurons with similar cell body positions that express Eve or En. Axonal morphology data is not available for any of these neurons in insects or crustaceans. Since we currently lack axonal morphology data, we refer to these neurons as 'putative homologues.' Future studies will determine if they should be called homologues, as defined by the three criteria listed above.

Neural stem cell homology

The question remains, are insect and crustacean NBs homologous? Insect and crustacean NBs have similar segmental positions, express the same markers and produce homologous neurons. For these reasons, we believe that these cells are homologous. Although arthropod neural stem cells are

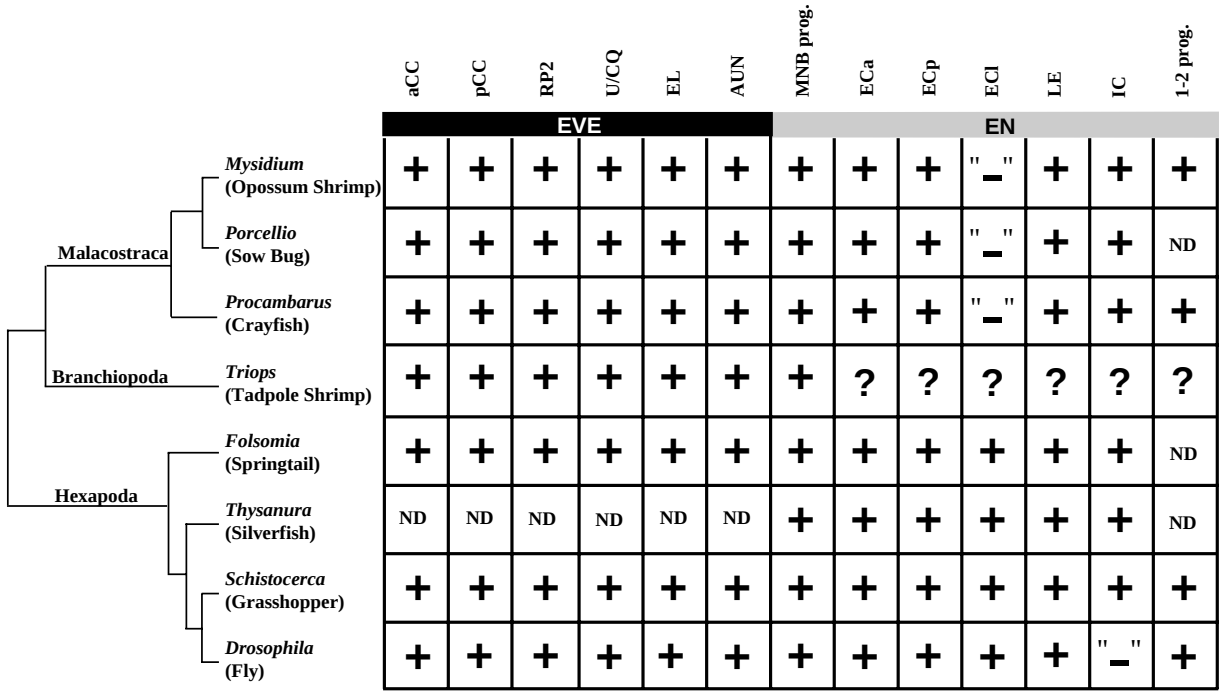

Fig. 4. Phylogenetic distribution of Eve- and En-positive neurons among arthropods. For each arthropod species examined, we indicate the presence or absence of Eve- and En-expressing homologous neurons. The crustacean branch of the phylogenetic tree is drawn according to Brusca and Brusca (1990). The hexapod portion of the tree is drawn according to Gillott (1980) because our data support the grouping of insects and collembola within the hexapods as sister taxa. A high degree of similarity is observed in the central nervous systems of members of the Class Hexapoda and species belonging to the crustacean Classes Malacostraca and Branchiopoda. We conclude that the common ancestor of hexapods and crustaceans possessed the Eve- and En-positive neurons surveyed in this study. Eve expression was not analyzed in *Thysanura* (marked ND) due to technical problems encountered with staining this species. En expression in NB 1-2 progeny was not analyzed in a number of arthropods (marked ND) because these cells are most easily identified in late-staged-embryos which were not examined in this investigation. "?" denotes the uncertain relationship between *Triops* and insect En-positive neurons (see text). A minus sign surrounded by quotation marks ("–") signifies instances where particular En-expressing neurons could not be identified. We cannot rule out the possibility that these neurons are present in these organisms, but that they fail to express En. Alternatively, En expression in a particular neuron may have shifted to a different developmental time point which was not assayed.

homologous, the processes of NB formation (see Introduction) have most likely diverged among arthropods. The alternative explanation, convergent evolution, seems less likely due to the degree of NB and neuronal similarities that we have found in this investigation. Thus, the most parsimonious explanation of our current data is that arthropod neural stem cells are homologous.

There are other examples in which equivalent cells/tissues have been produced despite the fact that earlier developmental processes have diverged. For example, in some crustaceans, a grid-like pattern of cells in post-naupliar segments is generated by the proliferation of ectoteloblasts. However, in regions that will become the first and second maxillary segments and the anterior part of the first thoracic segment, cells that are not of ectoteloblastic origin are capable of generating the same grid-like pattern of cells produced by ectoteloblasts (Dohle and Scholtz, 1988). In other words, homologous cells are produced in different ways even within the same embryo. The development of Meckel's cartilage serves as a second example. Although Meckel's cartilage is induced by different tissues in amphibians, birds and mammals, the Meckel's cartilage that is eventually produced is homologous in all of these animals (Hall, 1984). Once again, although earlier developmental processes have diverged, homologous structures are produced.

Evolutionary considerations

We had initially hoped that our data would allow us to determine if insect nervous systems more closely resemble those of malacostracan or branchiopod crustaceans. However, because we see mainly similarities among all arthropods analyzed, our data do not allow us to say much about arthropod evolution, with one exception. It is interesting that we only find the ECl neurons in insects and in a collembolan, but not in any crustaceans. These data are consistent with grouping Collembolans, which have a debated phylogenetic status, as the hexapod sister taxon of the insects, as suggested by Gillott (1980). Although this study does not have great ramifications for our current understanding of arthropod evolution, future studies with additional neuronal markers in these arthropods, as well as the chelicerate and myriapod arthropod outgroups, may be useful.

As studies with additional markers are completed, it will be interesting to examine situations where marker expression in different species of arthropods varies. Identifying such variation will eventually help us to understand how changes in development create neuronal diversity. For example, in this investigation, we found the ECl neuron in hexapods, but not in crustaceans. Future studies may address how this difference is generated.

A neural marker toolbox

One achievement of this study is the identification of molecular markers for particular neurons in a variety of arthropods. The identification of neural markers was the first step toward making *Drosophila* a powerful system in which to study neural development, and we must continue to gather neural marker data in a variety of arthropods. Knowledge of the distinct molecular markers expressed by various arthropods' neurons will be particularly important as we enter the next, more challenging phase of experimentation: the manipulation of gene expression in non-model organisms. Acquiring a neural marker toolbox in a variety of organisms will help us to proceed toward more detailed investigations of arthropod neural development in the future.

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