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COMPARATIVE MORPHOLOGY OF CENTRAL NEUROPILS IN THE BRAIN OF ARTHROPODS AND ITS EVOLUTIONARY AND FUNCTIONAL IMPLICATIONS*

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Most insects and decapod crustaceans possess an assemblage of midline neuropils, the central complex. Recent phylogenetic studies show a sister-group relationship between hexapods and decapods, suggesting that central complexes in both groups are homologous structures derived from a basal ancestral neuropil [22]. This ancestral archetype of the central complex (lacking the protocerebral bridge) might be represented in the chilopods. Until recently, diplopods were regarded as closely related to chilopods and united within the taxon Myriapoda . The entire lack of a midline neuropil in diplopods, however, renders the monophyletic origin of the class Myriapoda unlikely [15]. In this study we used a palette of immunocytochemical and neuroanatomical methods to investigate mid-line neuropils in hitherto poorly examined arthropod groups. Of special interest for resolving arthropod phylogeny are onychophorans, who are believed to be an evolutionary ancient group that resembles the ancestors of modern arthropods. Striking similarities in central brain neuroarchitecture of the onychophoran *Euperipatoides rowellii* and of a chelicerate species, however, suggest a close phylogenetic relationship between these two groups. Our findings imply that onychophorans either represent the oldest form of the chelicerates or that extant onychophorans have developed from chelicerate-like ancestors by neoteny.

Keywords: Central complex immunocytochemistry neuroanatomy Onychophora locomotor control

INTRODUCTION

Arthropods are the most abundant and structurally varied metazoan group [4] and their behavioral diversification is enormous [3]. Yet, within any group of arthropods, at least to the level of order, the organization of their cerebral neuropils seems quite conserved [11, 19]. This suggests that the arthropods are an ideal taxon from which to infer phylogenetic relationships based on discrete cerebral characters [22]. In both crustaceans and insects, the protocerebrum provides many structural characters that are useful for phylogenetic reconstructions. Amongst these characters is a system of

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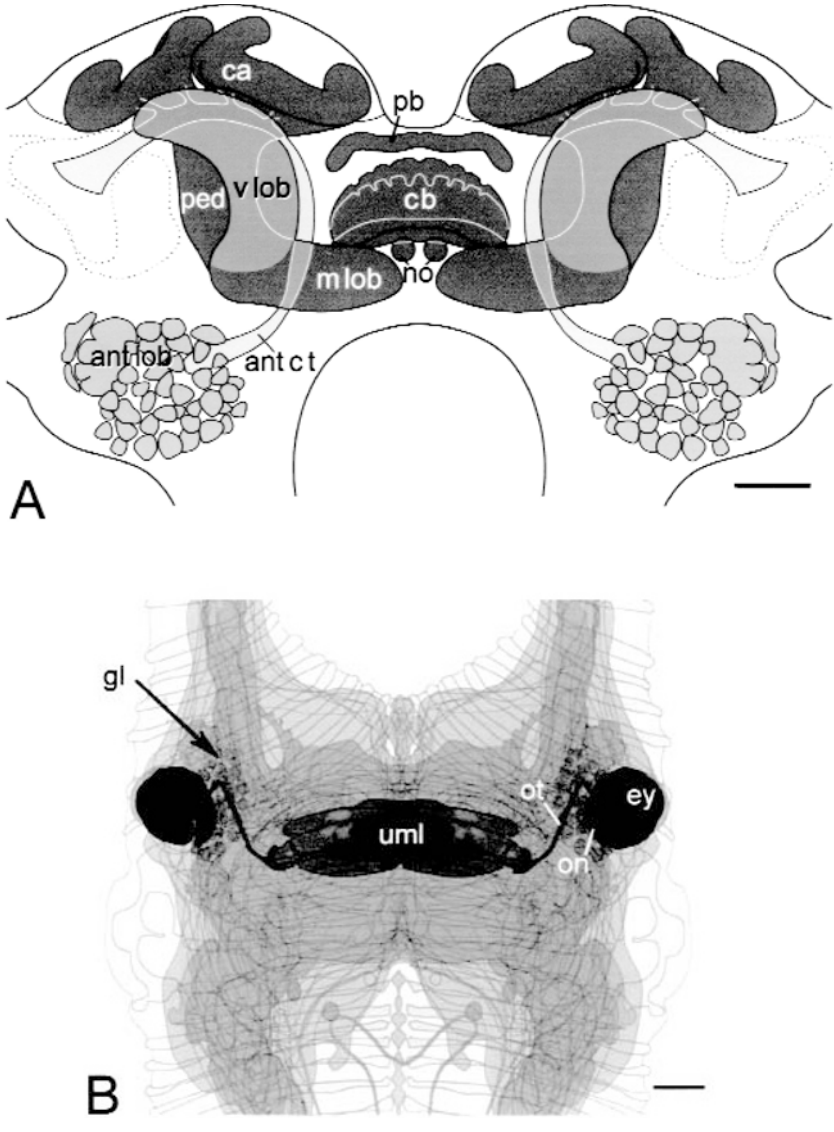

Fig. 1. Schematic drawings showing the position of unpaired midline neuropils in insects (in A) and in onychophorans (in B). A: Frontal view of the brain of the cockroach *Periplaneta americana.* The central complex consisting of the central body *(cb),* the protocerebral bridge *(pb)* and the paired noduli *(no)* is positioned at the midline of the brain between the mushroom bodies. Medial lobe *(m lob),* vertical lobe *(v lob),* pedunculus *(ped),* and calyx *(ca)* of the mushroom body. *ant lob,* antennal lobe; *ant c t,* antennocerebral tract. B: See-through drawing of the head of *Euperipatoides rowellii* as seen from dorsal. Photoreceptor fibers from the eye *(ey)* supply input to the optic neuropil *(on).* From there, an optic tract connects to the unique midline neuropil *(uml),* usually termed crescent-shaped body in the central brain. *gl,* glomeruli. Scale bars = 200 m (Drawings kindly provided by Dr. Nicholas J. Strausfeld)

unique (unpaired) midline neuropils and their immediately connected satellite neuropils. The most prominent of these midline neuropils in hexapods was termed central body by Fl gel in 1878 [8].

In insects literature on the neuroarchitecture of the central body and its satellite neuropils (together usually termed central complex) is abundant. In summary, the central body consists of several more or less horizontally arranged layers, some of which comprise similar vertical modules. The modules are essentially columnar, spread out like the staves of a fan. Neurons of these columns (columnar neurons) link the central body to the paired noduli and via a complicated arrangement of chiasmata to the protocerebral bridge. These features seem to be common across neopteran insects and have been described from *Schistocerca gregaria* [33], *Musca domestica,* and *Drosophila melanogaster* [10, 18, 21], the cockroach *Periplaneta americana*, and the paper wasp *Polistes canadensis* [23]. Figure 1A provides a schematic drawing showing the position of the central complex relative to other major neuropils in the brain of *P. americana*.

Intracellular recordings from individual neurons of the central complex in locusts and bees demonstrate its sensitivity to multimodal stimuli [12, 16] and to the e-vector of polarized light [30]. The fly central body incorporates H^3-2-deoxyglucose during visual stimulation with moving gratings [1]. However, it seems unlikely that visual information processing is the main function of the central complex because large central complexes exist in blind insects such as the eyeless workers of the ant *Mystrium* sp. (W. Gronenberg, personal communication).

Functions ascribed to the central complex come mainly from comparative studies and studies of behavioral mutants of *D. melanogaster*, where specific defects in limb coordination relate to structural defects in central complex mutants [26, 27, 28]. The notion that the central complex relates to limb coordination is further suggested by the correlation between complexity and precision of modular organization and the animal s ability to perform complex limb movements. The cellular organization of the modules of the central body and protocerebral bridge are elaborate in cell-building social insects as well as in species, including *D. melanogaster*, that can perform complex and often heterolaterally independent limb movements. In nocturnal Lepidoptera, which mainly use their legs for grasping but not for walking, the protocerebral bridge and the central body modules are reduced [23]. In aquatic Hemiptera, which mainly perform bilateral swimming strokes, central complex modules are greatly reduced [8] and the protocerebral bridge is split [23] as it is in the uncoordinated *no-bridge D. melanogaster* mutant [26].

Recently Utting et al. [29] published the first detailed description of the neuroarchitecture of the central complex in the brain of the decapod crustacean *Cherax destructor* (crayfish). Similarities between crayfish and insects extend to the number and position of the neuropils that comprise the central complex, the numbers of tracts that link some of the constituent neuropils together, and the form and immunoreactivity of many of the individual neuron classes. These similarities were taken as evidence to support a possible homology between the decapod central complex and that of insects.

In a broad taxonomic comparison, Loesel et al. [15] used a palette of immunocytological and neuroanatomical staining methods to investigate the morphology of unpaired midline neuropils in several classes of arthropods and ascribe various components of the central complex to a phylogenetic tree. While this study supports the findings of Utting et al. [29] it also demonstrated that several anatomical features of the central body in crustaceans, namely the number and sequence of immunoreactive layers to certain antisera, differed in different species, even between representatives of the same order. In chilopods, a midline neuropil with layers and columnar fibers, reminiscent of the insect central body, but lacking the protocerebral bridge and noduli, was found. Several staining techniques failed to identify a central body in diplopods, suggesting that the taxon Myriapoda (including diplopods and chilopods) is an artificial group. No unpaired central neuropil was found in the brain of the lophotrochozoan outgroup, the Annelida.

The present study is a continuation of this initial paper by Loesel et al. [15]. It investigates the correlation between central complex structure and limb coordination by comparing female and male specimens of a crustacean species whose limb movement repertoire shows a pronounced sexual dimorphism. Additional data on central brain morphology of non-decapod crustaceans, an aranean, and an onychophoran representative are included.

MATERIAL AND METHODS

Taxa

Immunostaining and Bodian silver staining was used to reveal midline neuropils in representatives of several groups of arthropods and in onychophorans. Crustaceans used for this study were the branchiopod *Triops longicaudatis* (raised in the laboratory from eggs) the decapod *Uca spec*. (fiddler crab, obtained from a local pet shop), and the isopod *Ligia occidentalis* (collected at sites on the Pacific Coast, south of Los Angeles). The arachnid *Lycosa coloradensis* (wolf spider, obtained through Hatari Invertebrates, Portal, Arizona) represented the Chelicerata. The onychophoran (velvet worm) *Euperipatoides rowellii* was collected at designated sites in New South Wales, Australia.

Histology

The polyclonal antiserum to tachykinin-related peptide (TRP) was raised at Stockholm University using rabbits inoculated with locust tachykinin-related peptide, LemTRP-1, conjugated to bovine serum albumin [34]. This antiserum is known to recognize the C-terminus of known insect and crustacean TRPs. Antiserum was used at a dilution of 1:3000. Allatostatin-like (AS) immunoreactivity was demonstrated using a monoclonal antiserum raised in mice to *Diploptera punctata* allato-

statin I (courtesy of Dr. Barbara Stay, University of Iowa) at a dilution of 1 : 100 [20]. Serotonin-like (5HT) immunoreactivity was demonstrated using a rabbit serotonin antiserum (IncStar, DiaSorin, Stillwater, Minnesota, USA) at a dilution of 1 : 2000. A detailed description of the immunostaining protocol and the imaging procedure can be found in Loesel et al. [15]. For Bodian silver staining, serial 10 m sections were stained according to Bodian s original method [2].

RESULTS

Antisera have been used to reveal architectural features across a variety of arthropods. The use of these substances is not intended to suggest functional attributes of the central complex. It is also recognized that the antisera used, single out only a small number of the total elements that must be present in these structures. For example, reduced silver stains of the isopod s central body suggest elements in addition to those revealed by the present antisera (compare Fig. 2G, H). Therefore reduced silver methods are also useful in their own right for identifying neural characters for cladistic analysis. However, where these methods are difficult to use, as on many marine organisms, antisera prove to be a practical and simple diagnostic tool.

Differences in central body architecture between female and male specimens of Uca spec.

Double immunostaining revealed obvious dissimilarities in the neuroarchitecture between the central bodies of female and male specimens of the fiddler crab *Uca spec*. In the female, the lateral tips of the spindle-shaped central body are curved towards dorsal (Fig. 2E), whereas in the male, the dorsal border of the central body follows approximately a horizontal line (Fig. 2F). Further dissimilarities occur in the level of immunoreactivity of individual layers of the central body. This is especially obvious in the pattern of TRP-immunoreactivity (TRP-ir). In the female, only one dorsal layer is weakly immunopositive, dorsal aspects of the central body contain no detectable TRP-ir (Fig. 2A). In the male, however, the dorsal layer exhibits strong TRP-ir and a ventral layer is TRP immunopositive, too, but to a lesser degree than in the dorsal layer (Fig. 2B). In other decapod crustaceans, whose legs do not show sexual dimorphism, differences in central body neuroarchitecture have not been found (data published by Loesel et al. [15]).

The isopod s central body consists of layers and of columnar neurons

In the isopod *Ligia occidentalis* Bodian silver staining (Fig. 2G) revealed an elongated central body that contains fibers that are arranged in a more or less columnar manner within the neuropil. As these fibers reach the dorsal surface of the central

body they form a criss-crossing pattern and extend further towards the protocerebral bridge. The bridge in *L. occidentalis* is split, i.e. it consists of two separate halves. The split architecture of the protocerebral bridge is also visible in the double immunolabeling (Fig. 2H). Here, the two halves exhibit TRP-ir, but no AS-ir. Double immunostaining also shows that the central body consist of at least two horizontal layers, a dorsal AS-ir, and a ventral TRP-ir one. Small patches of TRP-ir in the dorsal layer do not colocalize with AS-ir but are situated in areas that lack AS-ir.

The neuroarchitecture of the central body of a branchiopod

Double immunostaining reveals an unpaired midline neuropil in the central brain of the basal crustacean branchiopod *Triops longicaudatis* (Fig. 2I-K). This central body is approximately bean-shaped and exhibits TRP-ir (Fig. 2I) as well as AS-ir (Fig. 2J). TRP-ir cell bodies situated in two clusters at the dorsal border of the brain innervate the central body with a dense meshwork of fibers. In principal the same is true for AS-ir neurons with the exception that AS immunostaining in cell bodies is faint and that fibers that innervate the central body are of somewhat smaller diameter as compared to TRP-ir neurons. No parallel columnar elements or distinct horizontal layers were identified. A protocerebral bridge is absent.

Similarities in the neuroarchitecture of the arcuate body of an onychophoran and of a spider

Serotonin immunostaining in the brain of the onychophoran species *Euperipatoides rowellii* reveals a crescent-shaped unpaired midline neuropil. Combined anatomical studies (data not shown) demonstrate that in *E. rowellii* each optic nerve supplies a lenticular optic neuropil in the brain that is connected by a second nerve to the flank

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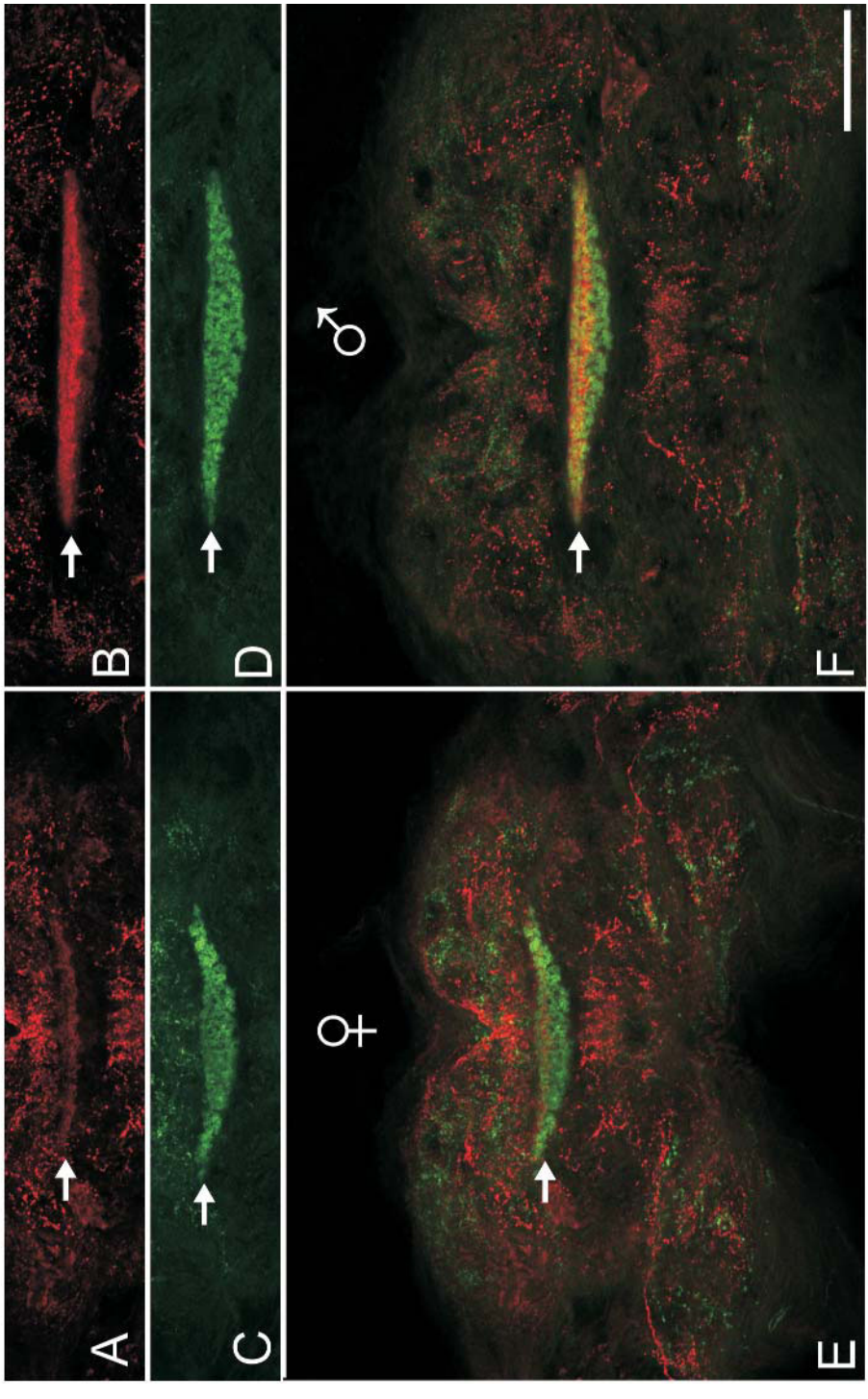
Fig. 2. The central body in the brains of a decapod (A F), an isopod (G, H), and a branchiopod crustacean species as seen from frontal. A F: Differences in the shape of the central body (arrows) between females (A, C, E) and males (B, D, F) of the highly sexual dimorphic decapod genus *Uca* as revealed by TRP- (coded red in A and B) and AS-immunostaining (coded green in C and D). Double immunolabelings for the female shown in (E) and for the male in (F). Differences in the shape of the central body and in the level of immunoreactivity in its horizontal layers (especially for TRP-ir) are apparent. E, F: The central complex of the isopod *Ligia occidentalis.* Bodian silver impregnation (E) reveals columnar fibers in the central body *(arrow).* While these fibers run parallel throughout most of the central body s extension, they form a criss-crossing pattern on its dorsal surface *(asterisk). Arrowheads* denote the position of the two halves of the split protocerebral bridge. Double immunostaining (F) shows a TRP-ir ventral layer (coded red) and a AS-ir dorsal layer (coded green) in the central body *(arrow),* the split protocerebral bridge *(arrowheads)* exhibits TRP-ir, but no AS-ir. I K: The central body *(arrow)* of *Triops longicaudatis* as revealed by double immunolabeling (K). Images I and F show TRP immunostaining and AS immunostaining individually. Scale bars = 200 m in (A F), 100 m in (G) and (H), and 50 m in (I K)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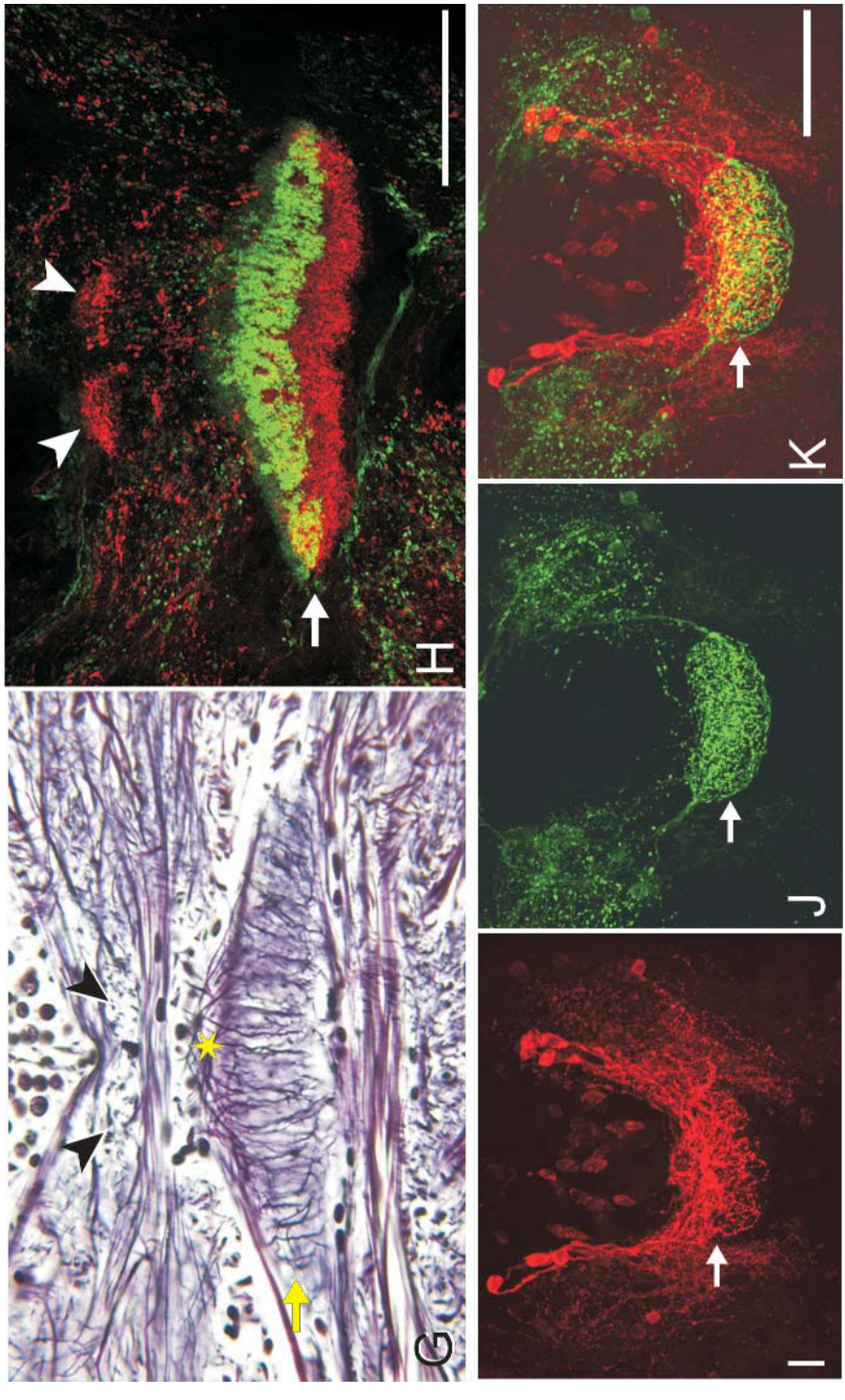
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of the crescent-shaped body (Fig. 1B), as is the case with the arcuate body in chelicerates. A comparison between immunolabeling (Fig. 3A, B) in *E. rowellii* and Bodian silver staining in the wolf spider *Lycosa coloradensis* (Fig. 3B, D) show that the neuronal assemblage in the crescent-shaped body of *E. rowellii* has the same cellular architecture as seen in the arcuate body of the chelicerate and the same nested sequence of looped axons, columns and tangential strata. A separate neuropil comparable to the protocerebral bridge in insects and malacostracan crustaceans is absent in both, onychophorans and araneans.

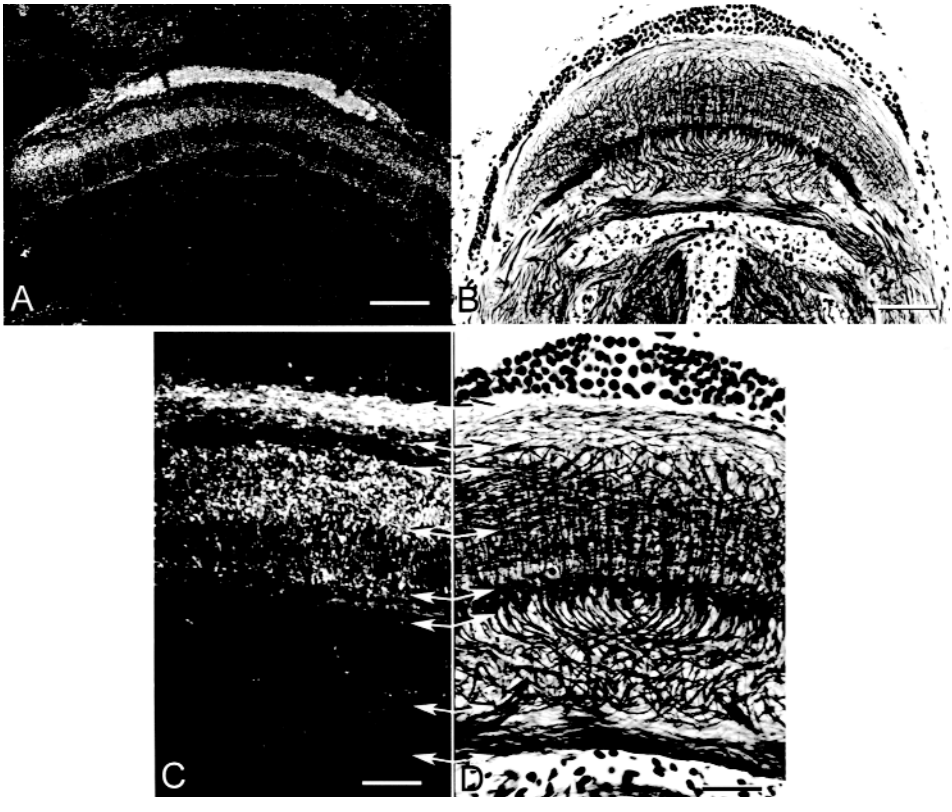

Fig. 3. Comparison of the neuronal design of the unique midline neuropil of the onychophoran *Euperipatus rowellii* and the arcuate body of the wolf spider *Lycosa coloradensis.* A: Overview of the unique midline neuropil of *E. rowellii* as revealed by serotonin immunostaining. B: Bodian silver staining of the arcuate body of *L. coloradensis.* At higher magnification, borders of homologous layers in the neuroarchitecture of the unique midline neuropil (in C) and the arcuate body (in D) are denoted by arrows. Scale bars = 200 m in (A) and (B), 100 m in (C) and (D)

DISCUSSION

Neuroarchitecture of the crustacean central complex

While Utting et al. [29] have demonstrated that the basic neuroarchitecture of the crayfish s central complex is virtually identical to that of the locust, Loesel et al. [15] demonstrated that certain anatomical characteristics differ in different decapod species. The latter finding complicates the task of relating individual features of the central complex to certain locomotor abilities. To address the question whether the limb movement repertoire of a given species is indeed reflected by central complex morphology as has been suggested [15, 23, 27], the central brains of female and male specimens of a crustacean species whose locomotor repertoire shows a pronounced sexual dimorphism were compared. In fiddler crabs of the genus *Uca* the chelipeds on either side of the body of females are of equal size. In the male, however, one cheliped is vastly enlarged to attract females while the other cheliped that is used for feeding is of approximately the same size as in females. This difference in cheliped use and morphology is coincident with a difference in the shape of the central body between females and males. From all decapod species investigated, *Uca* was the only genus to show that kind of central body dimorphism. While there might be other explanations for this finding it certainly is consistent with the idea that the central complex is a higher control center of locomotor behavior.

In the representative of another malacostracan order, the isopod *Ligia occidentalis*, the neuroarchitecture of the central complex is similar to that in decapod crustaceans. The central body comprises distinct layers and columnar fibers that form a criss-crossing pattern on its dorsal surface. The TRP-ir protocerebral bridge is split, paired noduli have not been identified. In contrast, double-immunolabeling the central body of a basal crustacean, the branchiopod *Triops longicaudatis* revealed no internal substructures comparable to the neuroanatomical features in malacostracan crustaceans, insects or chilopods. This might be due to shortcomings of the staining technique or it might imply that the hexapods (and chilopods) originated within the crustaceans and are closely related to the Malacostraca instead of being the sister group of the Crustacea as a whole.

Is the celicherate arcuate body homologous to the central complex?

Despite similarities between the arcuate bodies of chelicerates and the insect central complex (including their position within the brain and their principal neuroarchitecture that comprises layers and columnar neurons which form chiasms), an argument against arcuate bodies being homologous to the central body was their relationship with the visual system. In insects, central complex neuropils receive numerous axons from other midbrain regions including centers that relay information about the e-vector of polarized light [30]. None of these afferents arise directly from peripheral optic neuropils, however [13, 21]. This contrasts with some araneans (and onychopho-

rans), where the lateral margins of the arcuate body are directly supplied by afferents from the principal eye medullae [24, 25]. For this reason, the arcuate body of chelicerates was optimistically compared to the insect lobula and it might be argued that the Acari (mites), with their vestigial eyes, support this analogy because their arcuate bodies are so small relative to the rest of the protocerebrum [14].

These objections are undermined by the following observations. In spiders, the arcuate body also receives many projections from the midbrain and is certainly not a visual neuropil alone. Indeed, many other chelicerates, such as solpugids, amblypygids, and uropygids, which have minute eyelets, have prominent arcuate bodies, the largest being in the highly mobile solpugids (Strausfeld, unpublished data). Likewise ctenid and salticid spiders, both of which have exquisite motor control, possess huge multilayered arcuate bodies although in each the elaboration of their principal eyes differs greatly [32]. The chelicerate arcuate body is likely to receive multimodal inputs and, like the insect central complex, is unlikely to serve as a sensory analyzer alone.

The phylogentic postion of onychophorans

Neural staining and immunocytology reveal that the unpaired midline assemblage in *E. rowellii* has the same cellular architecture as in the arcuate bodies of *L. coloradensis*, and the same nested sequence of looped axons, columns and tangential strata. This arrangement is found in no arthropods other than the chelicerates. The present study on central brain morphology along with numerous other neuroanatomical features (Strausfeld et al., in preparation) suggests the taxonomic position of the Onychophora as integrated within the chelicerate clade. While the classical view of chelicerates does not include a vermiform adult equipped with at least 15 pairs of legs, limb buds on the opithsomata of chelicerate embryos [5, 6, 7], and the common segmental expression of engrailed class genes [6, 31] admits this possibility within chelicerate ontogeny and, possibly, phylogeny. The expression of the gene *Distal-less* also indicates possible affinities of chelicerates and onychophorans in that the mouthparts of both groups are whole limb type as opposed to gnathobasic [9, 17]. The present results support the view that the Onychophora possess brain architectures that are diagnostic of the chelicerates and that the chelicerates are therefore likely to be the most ancient group of arthropods. We propose that onychophorans are basal chelicerates or that extant onychophorans have developed from chelicerate-like ancestors by neoteny. The assemblage Chelicerata + Onychophora might therefore deserve the annotation Chelicericerebraliformes.

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