LETTERS

# Neuroanatomy of sea spiders implies an appendicular origin of the protocerebral segment

Amy Maxmen[1], William E. Browne[3], Mark Q. Martindale[3] & Gonzalo Giribet[2]

**Independent specialization of arthropod body segments has led to more than a century of debate on the homology of morphologically diverse segments[1,2], each defined by a lateral appendage and a ganglion of the central nervous system. The plesiomorphic composition of the arthropod head remains enigmatic because variation in segments and corresponding appendages is extreme. Within extant arthropod classes (Chelicerata, Myriapoda, Crustacea and Hexapoda—including the insects), correspondences between the appendage-bearing second (deutocerebral) and third (tritocerebral) cephalic neuromeres have been recently resolved on the basis of immunohistochemistry[1] and Hox gene expression patterns[3,4]. However, no appendage targets the first ganglion, the protocerebrum, and the corresponding segmental identity of this anterior region remains unclear[5]. Reconstructions of stem-group arthropods indicate that the anteriormost region originally might have borne an ocular apparatus and a frontal appendage innervated by the protocerebrum[6]. However, no study of the central nervous system in extant arthropods has been able to corroborate this idea directly, although recent analyses of cephalic gene expression patterns in insects suggest a segmental status for the protocerebral region[7–10]. Here we investigate the developmental neuroanatomy of a putative basal arthropod[11], the pycnogonid sea spider, with immunohistochemical techniques. We show that the first pair of appendages, the chelifores, are innervated at an anterior position on the protocerebrum. This is the first true appendage shown to be innervated by the protocerebrum, and thus pycnogonid chelifores are not positionally homologous to appendages of extant arthropods but might, in fact, be homologous to the 'great appendages' of certain Cambrian stem-group arthropods.**

The traditional view of the arthropod head has been that the anteriormost region, containing the eyes and protocerebrum, belongs to a presegmental anterior cap, the 'acron'[6,12]. The term acron was developed in the context of the Articulata hypothesis in which annelids and arthropods share a recent common ancestor, with the acron being homologous with the annelid presegmental prostomium[2]. Although the Articulata hypothesis has fallen out of favour, the acron is often cited as part of the arthropod ground plan[12]. Evidence against the acron concept, drawn from onychophoran neuroanatomy[13] and insect development, demonstrates that the arthropod protocerebral neuromere may be truly segmental[7,9] and involved with the coordination of motor/sensory behaviour associated with appendages[14].

Pycnogonids are most commonly placed as a sister taxon to the chelicerates[15,16] or as a separate class, basal to all remaining extant arthropods[11] (Fig. 1). The fossil record indicates that pycnogonids branched off early during the emergence of stem-group arthropods with earliest larvae identified from the Upper Cambrian[17] (about 490 Myr ago) and a crown-group (or near-crown-group) adult from the Silurian period[18] (about 425 Myr ago). The antiquity of the lineage is congruent with the observation that pycnogonids share traits with other ecdysozoans (tardigrades, nematodes, priapulans and their kind), including a terminal mouth[19] and a triradiate pharynx[20], that could be found in the arthropod ground pattern.

Because of the critical position of pycnogonids as putative basal arthropods, the structure and development of the nervous system of a Hawaiian species belonging to the genus *Anoplodactylus* were examined by immunohistochemistry in conjunction with confocal laser-scanning microscopy. The scaffolding and segmental nature of the larval central nervous system (CNS) can be clearly identified before the fusion of cephalic ganglia. The *Anoplodactylus* sp. protonymphon larva (Fig. 2a) is microscopic (about 0.05 mm) when it hatches from eggs carried exclusively by the male. The protonymphon bears a pair of functional chelifores (claw-like appendages) followed by two postcheliforal appendages[21]. The digestive tract is initially incomplete and consists of a short proboscis with a terminal mouth and a highly innervated, muscularized oesophagus. After the second protonymphon stage, the *Anoplodactylus* sp. larva encysts within a hydroid until it emerges as a juvenile. Protonymphon development involves a series of transformations during which the larval chelifores transform into adult chelifores, and the second and third larval appendages are transformed into palps and ovigers, respectively[21]. The more posterior walking legs are added in a succession of moults, beginning with limb buds forming the fourth

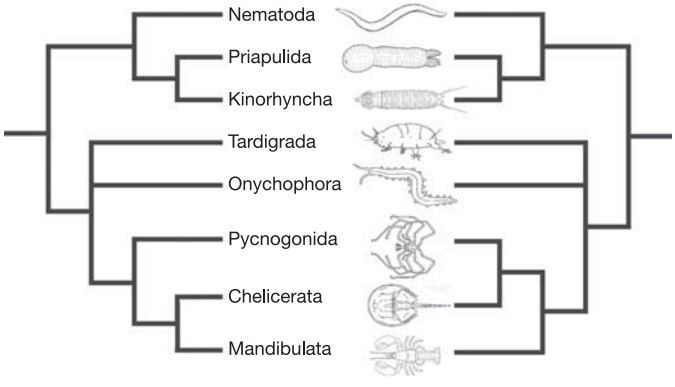


**Figure 1 | Proposed relationships of pycnogonids among other ecdysozoans.** The tree to the left reflects the Cormogonida hypothesis, with Pycnogonida as sister group to remaining extant arthropods. The tree to the right reflects the classical hypothesis of pycnogonids as sister group to chelicerates.

[1]Department of Organismic & Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 26 Oxford Street, [2]Department of Organismic & Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138, USA. [3]Kewalo Marine Lab, Pacific Biosciences Research Center, University of Hawaii, 41 Ahui Street, Honolulu, Hawaii 96813, USA.

larval appendage pair[22]. Of particular relevance with regard to the interpretation of CNS organization is that the body of the protonymphon effectively becomes the head of the adult[22] and the protonymphon appendages correspond to the adult cephalic appendages (the chelifores, palps and ovigers; Fig. 2b)[22,23].

Immunostaining against acetylated α-tubulin revealed the axonal architecture of the developing protonymphon CNS, which is organized in a ring around the oesophagus (Fig. 3). Four bilateral pairs of distinct ganglia are connected by commissures across the oesophagus along the protonymphon anterior–posterior axis (Fig. 3a). The anteriormost supraoesophageal neuromere, the protocerebrum, consists of axon tracks that extend medially across the midline and receive the bifurcating nerves of the future optical apparatus dorsally, and of the cheliforal nerves anterolaterally (Fig. 3b, and Supplementary Movie 1). The anterior portion of each cheliforal ganglion innervates a chelifore by means of two nerve tracks (Fig. 3d, e), one innervating the fixed finger of the pincer, and the other the movable finger. In addition to the cheliforal ganglia at the lateral edges of the protocerebrum, the protonymphon CNS includes three posterior pairs of ganglia (Fig. 3a). The second (A2G) and third (A3G) pair are in close proximity and innervate the second and third appendages, respectively (Fig. 3c). The fibrous commissures between A2G appear to extend both above and below the oesophagus, whereas those of A3G extend only below. The terminal fourth pair of ganglia is positioned ventrolaterally at the base of the oesophagus (digestive tract is incomplete), at the posterior end of the protonymphon (Fig. 3d, f). Neuronal staining with anti-Elav (embryonic lethal abnormal visual system) was robust, and confirmed the above structural organization, including the ocular nerves at the dorsal surface of the protocerebrum (Supplementary Fig. 2).

Double immunostaining against acetylated α-tubulin and serotonin reveals a distinct subset of serotonergic neurons in the cheliforal ganglia (Fig. 3b), along two parallel tracks through the longitudinal connectives, and into the appendicular and posteriormost ganglia of the protonymphon CNS (Fig. 3f). In other arthropods examined, serotonergic neurons are identified most anteriorly in the median and lateral edges of the protocerebrum[24]. Thus, anteriormost staining of serotonin in the cheliforal ganglia supports the association of these ganglia with the first neuromere of the arthropod CNS, the protocerebrum. Absence of reactivity in the medial region of the protocerebrum may be attributable to the delayed formation of the medial optical apparatus in the early protonymphon, in which the ocular nerves have only begun to develop above the protocerebral commissure.

Given the correspondence between the early protonymphon and the adult pycnogonid head[22], and the resemblance in shape of the protonymphon CNS to the larval brain ('neuropil ring') observed in other arthropods, we conclude that the three anterior pairs of ganglia in the early protonymphon CNS correspond to the tripartite, circumoral arthropod brain: the protocerebral commissure (presumably including the central/arcuate body), early ocular nerves and lateral cheliforal neuropils comprise the protocerebrum; the deutocerebrum, A2G; and the tritocerebrum, A3G. Because of the terminal position of the stomodeum, the longitudinal connectives encircling the oesophagus are oblique to the body axis (Supplementary Movies 1 and 2b, c) rather than bent dorsally in relation to the ventrally directed oesophagus as in other arthropods, such that the third poststomodeal commissure of the neuropil ring is suboesophageal rather than supraoesophageal (Fig. 3a, d). The proportionate size of the protonymphon neuromeres to one another is similar to those of other immature arthropods; the protocerebrum is significantly larger than both the deutocerebrum and the tritocerebrum[8]. The presence of strong reactivity to serotonin in the pair of suboesophageal posterior ganglia (Fig. 3f) indicates that the posteriormost protonymphon ganglia might correspond to the fourth cephalic ganglia, the mandibular ganglia of other arthropods[24].

The hypothesis that the pycnogonid protocerebrum consists of a single neuromere targeting the chelifores is the simplest interpretation of the data, requiring the fewest assumptions. However, owing to the minute size of the protonymphon, we are unable to eliminate alternative models unequivocally. Alternatives assume a hypothetical fusion of neuropils in the anteriormost region, such that either the protocerebrum, consisting only of the protocerebral commissure and ocular nerves, is fused to the cheliforal ganglia, which migrated from a deutocerebral position, or the protocerebral region includes two fused segmental neuromeres anterior to the deutocerebrum. In the first case, the assumptions are that the commissure between the ganglia targeting functional chelifores has not formed or is indistinguishable from the protocerebral commissure. Additionally, A2G must be interpreted as the tritocerebrum and thus the tritocerebral commissure as partly preoral, a condition not typical of arthropods. Conversely, if our initial interpretation is correct (A2G corresponds to the second neuromere) the commissure between A2G concurs with the proposal based on recent neuroembryological studies in Chelicerata[1] and Hexapoda[25] showing that the deutocerebral ganglia are transversely connected by preoral commissures as well as postoral fibres. The second alternative, in which the protocerebrum consists of two neuromeres, would support a controversial[9] hypothesis based on the expression of segment polarity and dorsoventral patterning genes in insects claiming a bisegmental protocerebrum (labral and ocular neuromere)[8]. However, the arthropod protocerebrum generally includes a collection of neuropils that vary between taxa[14] (for

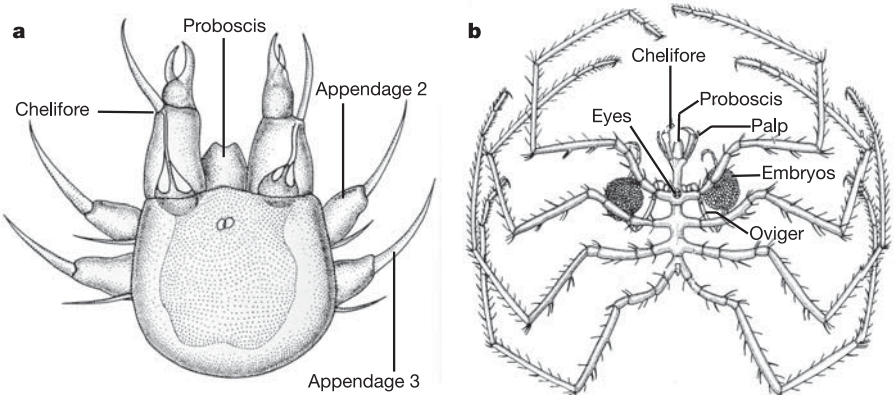


**Figure 2 | The unique adult and larval morphology of pycnogonids. a**, The three appendages of the protonymphon larva (shown) correspond to the cephalic appendages of the adult pycnogonid. (Modified from ref. 26.) **b**, The adult male cares for embryos until hatching (*Nymphon rubrum*; modified from ref. 32).

example 'ellipsoid body', 'paired noduli'), and there is no indication here that the ocular nerves and cheliforal ganglia belong to separate neuromeres.

Pycnogonids are often placed with chelicerates on the basis of the morphological similarities of their uniramous chelate (claw-like) appendages[15,18], although separate terms—pycnogonid chelifores versus chelicerate chelicerae—were explicitly chosen to reflect uncertain homology[23,26]. Positional support for homology has been based on classical descriptions of tritocerebral innervation of both chelifores and chelicerae in pycnogonids and arachnids[15,27,28]. Recently, the chelicerae–tritocerebrum correspondence was disputed after the identification of deutocerebral ganglia targeting the chelicerae of horseshoe crabs[1]. Evidence for tritocerebral innervation of the pycnogonid chelifores was presented in a descriptive model

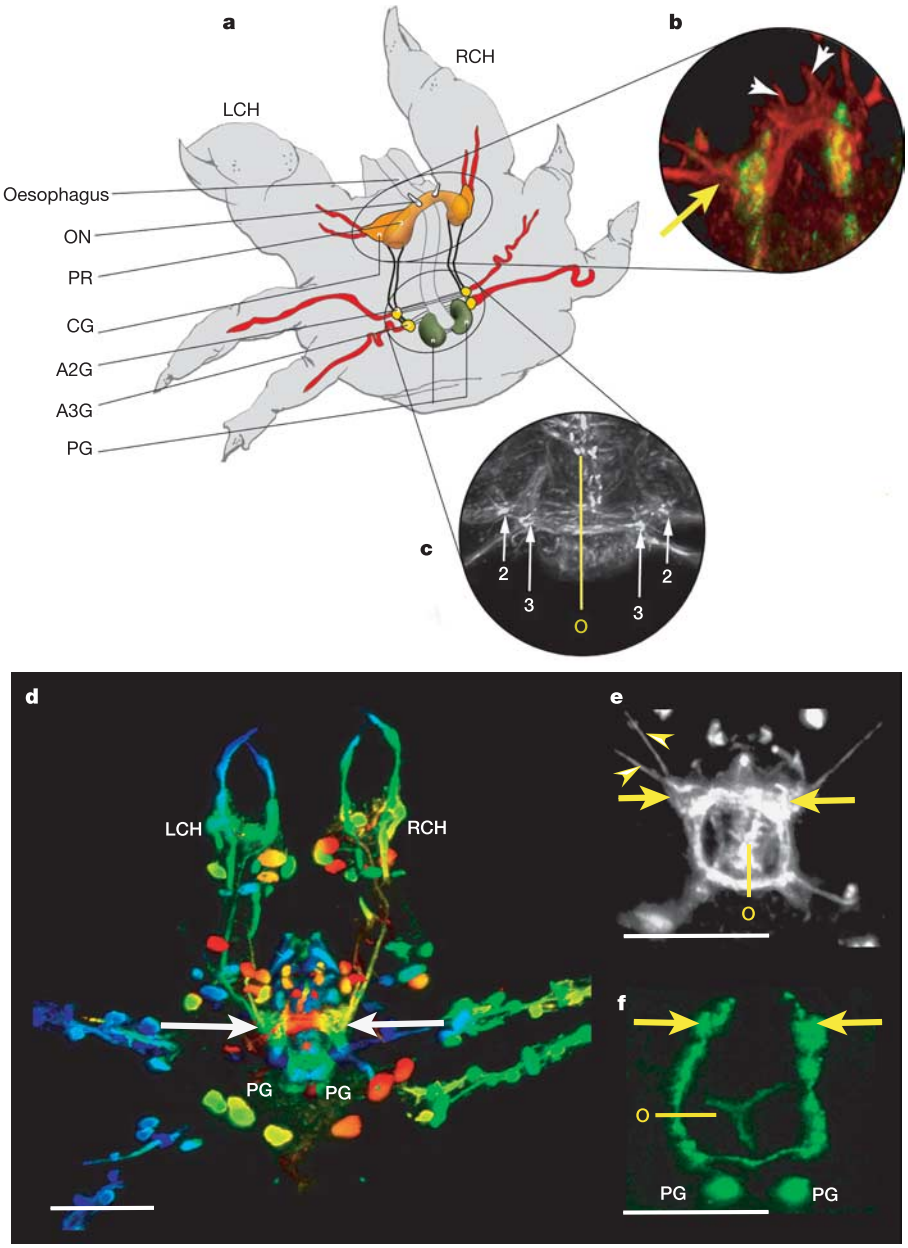


**Figure 3 | CNS of the *Anoplodactylus* sp. larva (protonymphon).** **a**, Diagram of the protonymphon seen from an oblique posteriolateral view based on reconstructions from confocal stacks (**b**–**f**). The CNS consists of four pairs of ganglia connected by commissures across the midline. The oesophagus runs through the proboscis between the left (LCH) and right (RCH) chelifore, and ends incompletely at the posterior ganglia (PG). The first neuromere is the protocerebrum, consisting of anteriolateral cheliforal ganglia (CG) connected by a prominent supraoesophageal protocerebral commissure (PR), and ocular nerves (ON). **b**, High magnification of the protocerebrum stained for tubulin (red) and serotonin (green) showing the cheliforal ganglion (arrows) and bifurcating ocular nerves (**b**, arrowheads). Circumoesophageal connectives run posteriorly from the PR, leading to the second (A2G) and third (A3G) ganglia that innervate the second and third appendages (**c**). **c**, High magnification (α-tubulin, grey scale) of A2G and A3G and the fibrous appendicular commissures conecting the ganglia across the innervated oesophagus (O). **d**, Depth-coded image (α-tubulin): colours range from warm (red) indicating dorsal, to cooler (blue) indicating ventral. **e**, Transverse optical section (α-tubulin, grey scale) showing the circumoral shape of the protonymphon 'neuropil ring' in relation to the oesophagus (O); the anterior bifurcating cheliforal nerves (arrowheads) target the cheliforal ganglia at the top of the ring. **f**, Same view of the CNS as in **e**, stained for serotonin (green). Immunoreactivity is visible in the cheliforal ganglia (arrows), along the circumoesophageal connectives, the suboesophageal appendicular commissure, and separately in the posterior ganglia (PG). Note background staining in the tripartite luminal surface of the oesophagus (O). Scale bars, 25 μm.

by Wiren[28], and later affirmed by histology[27], in which a subpharyngeal commissure connecting the larval cheliforal ganglia (chelicerenneuromere) was illustrated as support for a postoral origin of the anterior chelifore ganglia. However, in his dissertation on pycnogonid development, T. H. Morgan[29] noted a discrepancy between innervation of chelifores and chelicerae: as embryos, arachnid chelicerae were innervated by postoral ganglia whereas pycnogonid chelifores arose heterolateral to the stomodeum and were not innervated by postoral ganglia. Our results confirm the observation that the commissure between the cheliforal ganglia is not postoral. The first set of midline commissures extending below the oesophagus are located between the second pair of ganglia, which innervate the second larval appendages. The chelifores clearly arise on segmental precursors anterior to the second larval appendages, and correspondingly to the first appendages of other extant arthropods, which are innervated by the second neuromere (chelicerae of chelicerates and (first) antennae of mandibulates)[1]. In terms of structural similarity, chelate appendages have evolved convergently in many other arthropod groups, such as crustaceans, and appear on a variety of other segments, such as scorpion pedipalps[12].

This is direct neuroanatomical evidence of an appendage-bearing protocerebrum in an extant arthropod and corroborates previous data from onychophoran innervation[19] and analyses of neuronal elements in *Drosophila* head gap gene mutants, used to reject the term 'acron' in favour of 'ocular segment'[7,9]. The prostomium of annelids is derived from nonsegmental first-quartet micromere derivatives[12]; our data indicating a segmental origin of the anteriormost region in arthropods therefore further weaken the Articulata hypothesis.

In certain Palaeozoic stem-group arthropods, a pair of robust raptorial frontal appendages emerge from the anteriormost ocular region, theoretically innervated by the protocerebrum[6]. These frontal 'great appendages', are controversially thought to have been transformed into the labrum, a lip-like structure derived from the anterior portion of the arthropod head[6]. An argument has been revived for an association of the labrum with the protocerebral region based on the expression pattern of segment polarity genes during embryonic *Drosophila* development[8]. Pycnogonids do not possess a labrum, yet the homology of the labrum with pycnogonid chelifores is currently difficult to assess because of clashing views on labrum-neuromere innervation and the appendicular nature of the labrum. Further investigation with segment-specific candidate gene expression data might help to explain the potential homology of the labrum in pycnogonids. Apart from the labrum, the only possible segmental homologue of the chelifore is the frontal appendage found in stem-group arthropods such as *Kerygmachela*, *Leanchoilia* and basal anomalocaridids.

Innervation of the first appendages from the anteriormost part of the brain in onychophorans[13] (sister group to arthropods), possibly in stem-group arthropods[6], and in pycnogonids will affect future phylogenetic analyses by increasing the support for an independent clade of pycnogonids at the base of the arthropod tree, making the presence of a protocerebral appendage a plesiomorphic state. If pycnogonids are found to be sister group to chelicerates, and the protocerebral innervation of frontal appendages an ancestral feature, at least two independent transformations (if chelifores correspond to the labrum) or losses of the protocerebral appendages must be inferred within arthropod phylogeny. Alternatively, the possibility remains that chelifores are a pycnogonid apomorphy, and are thus a derived condition in arthropods that still supports previous suggestions that the protocerebrum maintains the potential to act as an appendage-bearing neuromere[10,14].

By investigating the CNS of a basal arthropod we have gained insight into the evolution of arthropod head segmentation and appendage correspondences. Pycnogonid chelifores and chelicerate chelicerae are convergent structures, innervated from different segmental neuromeres. Our finding supports previous analyses predicting that arthropods once had 'great appendages' innervated by the protocerebrum, which have been lost or unrecognizably transformed in all extant arthropods except pycnogonids. This study further closes the gap in diagnosing positional homology between stem and crown-group arthropod head segments. Still, the defining criterion for homology, common ancestry, cannot be certain without a well-resolved phylogeny. If evidence is found sufficient to deem pycnogonids the most basal group of extant arthropods, our results support previous models of head evolution that predict that the original arthropod bore an acronless[6,7,9,13] four-segmented head[30], encapsulating a tripartite circumoral brain rotated in an axial position, reminiscent of that found in onychoporans, nematodes and other cycloneuralians[13,19].

## METHODS

**Specimen collection.** Protonymphae of *Anoplodactylus* sp. were reared from embryos collected from males living in Kewalo Basin, Oahu, Hawaii. Detailed examination of adults indicates that our specimens might belong to a new, as yet undescribed species (C. P. Arango, personal communication).

**Immunohistochemistry of CNS.** Neuronal subsets were labelled as described previously[31], with the following modifications. Before labelling, specimens were treated with a proteinase K solution ($10\,\mu g\,ml^{-1}$) for 5 min and bath-sonicated for 5 s at a low setting. Staining of microtubules, such as the axonema of neurons, was obtained with a monoclonal anti-acetylated α-tubulin antibody and, independently, with Elav (for each, mouse IgG; Developmental Studies Hybridoma Bank) at dilutions of 1:5 and 1:200, respectively, in block solution (PBS containing 0.1% Triton X-100 and 5% normal goat serum) overnight at 4 °C. Serotonergic neurons were labelled with monoclonal anti-serotonin-specific antibodies (rabbit IgG; Immunostar) diluted 1:100 in block solution, overnight at 4 °C. Specimens were incubated for 5 h at 27 °C in secondary antibodies, Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories) and Alexa-488-conjugated donkey anti-rabbit (Molecular Probes), at a dilution of 1:250. Omission of the primary antibody abolished staining. Images were taken with a Zeiss LSM510 META confocal laser-scanning microscope.

**Received 10 February; accepted 30 June 2005.**


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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

**Acknowledgements** We thank W. Morrisey for diagram preparation; E. C. Seaver for assistance with immunohistochemistry; and J. Hanken, G. Das, G. Edgecombe and A. Hejnol for advice and discussion. We thank the Developmental Studies Hybridoma Bank for the anti-tubulin and Elav antibodies. This material is based on work supported by the National Science Foundation AToL program to G.G. and M.Q.M.

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