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SHORT COMMUNICATION

The fate of the onychophoran antenna

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Abstract Recent gene expression data suggest that the region on which the onychophoran antenna is situated corresponds to the anteriormost, apparently appendage-less region of the arthropod head. The fate of the onychophoran antenna (or any appendage-like precursor), also called the primary antenna, has been discussed intensively, and there are conflicting suggestions that this anteriormost non-segmental appendage gave rise either to the arthropod labrum or, alternatively, to the so-called frontal filaments found in certain crustaceans. Our data on early axogenesis in anostracan crustaceans show that even in the earliest embryos, before the antennula and antennal nerves are developed, the circumoral anlagen of the brain display very prominent nerves which run into the frontal filament organ (also known as the cavity receptor organ). This situation resembles the development of the antennal nerves in onychophorans, which leads us to conclude that the frontal filaments are indeed homologous to the primary antenna. Frontal filaments also appear to be more common in crustaceans than previously thought, removing the need for a complicated scenario of transformation from a primary antenna into the labrum.

Keywords Labrum · Primary antenna · Secondary antenna · Frontal filament · Organ of bellonci

Introduction

The evolution of the arthropod head is an ongoing enigma in metazoan evolution. Good starting points for any understanding of the composition of the head are the two potential sister groups Onychophora and Tardigrada, which together with the arthropods form the Panarthropoda (Dunn et al. 2008).

The homology of the deutocerebral (i.e. cheliceral, cheliforal, antennular) and tritocerebral (antennal, intercalary) segments in arthropods is now well established (Scholtz and Edgecombe 2006). Their alignment with the onychophoran head also appears to be settled now, the jaw segment corresponding to the deutocerebral segment and the slime papilla segment corresponding to the tritocerebral segment (Eriksson et al. 2010), although this does not necessarily mean that a brain encompassing a tritocerebrum was already present in the last common ancestor of onychophorans and arthropods (Mayer et al. 2010). These findings support the suggestion that the onychophoran antenna belongs to a head region anterior to the deutocerebral segment and therefore does not correspond to the mandibulate antennules (see also Eriksson and Budd 2000; Eriksson et al. 2003). Scholtz and Edgecombe (2006) suggested calling the onychophoran antenna 'primary' antenna to distinguish them from the arthropod 'secondary' antenna (i.e. the antennule). One structure of the arthropod head, which remains enigmatic, is the labrum, a relatively small upper lip that lies in front of the mouth. According to Maas et al. (2003), a fleshy labrum evolved only in the taxon Labrophora; other arthropods, particularly trilobites and chelicerates, are assumed to have a structure called a hypostome. The development of the 'upper lip', however, is strikingly similar in chelicerates and mandibulates (Kimm and Prpic 2006): in many cases, the 'labrum' anlagen appears as a paired structure at the front of the embryo which later moves backwards and fuses into a single organ (e.g. Ungerer and Wolff 2005; Mittmann and Wolff 2012). We consider this as a strong support for the notion that at least the anlage of the upper lip, which is generally termed labrum, is homologous throughout arthropods (a comparable anlage is absent in Pycnogonida;

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Brenneis et al. 2011). The segmental affinities of the labrum have been debated intensively (see Scholtz and Edgecombe 2006 for a detailed discussion of labrum homology and segmental affinities). Recently, Posnien et al. (2009) showed that the labrum is formed by an appendage-regulatory gene network and concluded as a result that the labrum is an appendage-like structure. Steinmetz et al. (2010) found evidence of *six3* expression anterior to *otx* expression in the anteriormost region of the developing brain in both arthropods (the area where the labrum originates) and onychophorans (the area where the antenna originates). It is interesting to note that this comes close to the test Scholtz and Edgecombe (2006) suggested as a means of obtaining direct support for the homology of the onychophoran antenna and the labrum. On the basis of these findings and the alignment of the onychophoran jaw segment with the mandibulate antennule segment suggested by *labial*, *proboscipedia*, *Hox3* and *Deformed* expression, Eriksson et al. (2010) proposed that the onychophoran antenna is indeed homologous to the labrum. This view was supported by Strausfeld (2012), who hypothesized a complex scenario for the evolutionary transformation from the location of the frontal appendage to the more posterior position of the labrum.

Although we are not able to solve the labrum problem, we do have new evidence to support the alternative hypothesis for the fate of the onychophoran primary antenna (or any appendage-like precursor) put forward by Scholtz and Edgecombe (2006), i.e. that the frontal filaments on the anterior part of the head of Remipedia and cirripedian nauplius larvae are the remnants of the primary antenna. In branchiopods, Fritsch et al. (2013) distinguish between the filamentous external 'frontal filament' and an internal region beneath the frontal filaments which they term the 'frontal filament organ'. Although the two structures undoubtedly form one functional unit, we support this terminological distinction, which reflects the history of discovery of the two structures (see Fritsch et al. 2013). A pair of frontal filaments is present in Notostraca, Laevicaudata, Spinicaudata and Cyclestherida. Internally, the situation in Anostraca corresponds to that in the remaining Branchiopoda: a frontal filament organ is present which Elofsson and Lake (1971) described as the 'cavity receptor organ'. In *Eubranchipus* at least, an externally observable pair of cavities is present (Møller et al. 2004, Fig. 3B), but frontal filaments are not.

Our evidence is based on nervous system development in the anostracan *Artemia franciscana* Kellogg 1906.

Materials and methods

Cysts of *A. franciscana* were bought at a specialist aquarium shop (Dohse Aquaristik). Species identity was checked by sequencing the 16S rRNA and conducting a BLAST search. Samples were found to be 100 % identical to sequences of *A. franciscana* in GenBank. To make it possible to study the embryos, the chorion was removed following the protocol established by Sorgeloos et al. (1977): cysts were rehydrated for 90 min in water under strong air supply. After rehydration, cysts were incubated for 10 min in 2.5 % of hypochlorite bleach (Eau de Javel, Floreal Haagen GmbH), which dissolved the chorion, changing the colour of the cysts to orange. The bleach was removed by rinsing the cysts in water. Some of the cysts were fixed directly after rinsing (0 h), and the remainder was transferred into saline water (32 PSU) from which batches were fixed every 4 h (at 4, 8, 12,…, 44 h). It should be mentioned, however, that even cysts fixed at the same time varied with regard to the state of advancement of their development. Fixation followed the protocol set out by Patel (1994) for *Drosophila* embryos using *n*-heptane (AppliChem) later replaced by methanol. Shaking for 1 min led to the disruption of the egg membrane and the release of the embryos. Embryos were rinsed in methanol and stored at 4 °C.

Antibody staining

Immunohistochemical labelling was performed as described in Fritsch and Richter (2010). Before antibody staining, embryos were exposed to several short pulses in a bath ultrasonicator (Elmasonic One) to facilitate permeation. Specimens were then washed several times in 0.1 M PBT (with 0.3 % Triton X-100, 1.5 % DMSO, 0.5 % BSA) and pre-incubated in PBT containing normal goat serum. The primary antibodies, monoclonal mouse anti-acetylated α-tubulin (clone 6–11 B-1, Sigma T6793, dilution 1:100) and polyclonal rabbit anti-serotonin (Sigma S5545, dilution 1:100), all in PBT + NGS, were applied overnight. Subsequently, specimens were rinsed in PBT and incubated with secondary fluorochrome-conjugated antibodies (goat anti-mouse Cy3, Jackson Immunoresearch 155-165-003, dilution 1:200; goat anti-rabbit Alexa488, Molecular Probes A-11008, dilution 1:33) in PBT + NGS overnight. Once antibody staining was complete, the specimens were incubated in SYTOX Green (Molecular Probes, S-7020, dilution 1:600 in 0.1 M PBS) for 30 min to stain the cell nuclei. Finally, the embryos were washed several times in PBT and mounted in glycerin (70 %).

Microscopy

Labelled specimens were analysed using a Leica DMI6000 CFS microscope equipped with a Leica TCS SP5 II confocal laser scanning unit. Image stacks of optical sections were recorded at a step size of 0.5–1 μm. The following images were processed using the software IMARIS 6.40 (Bitplane,

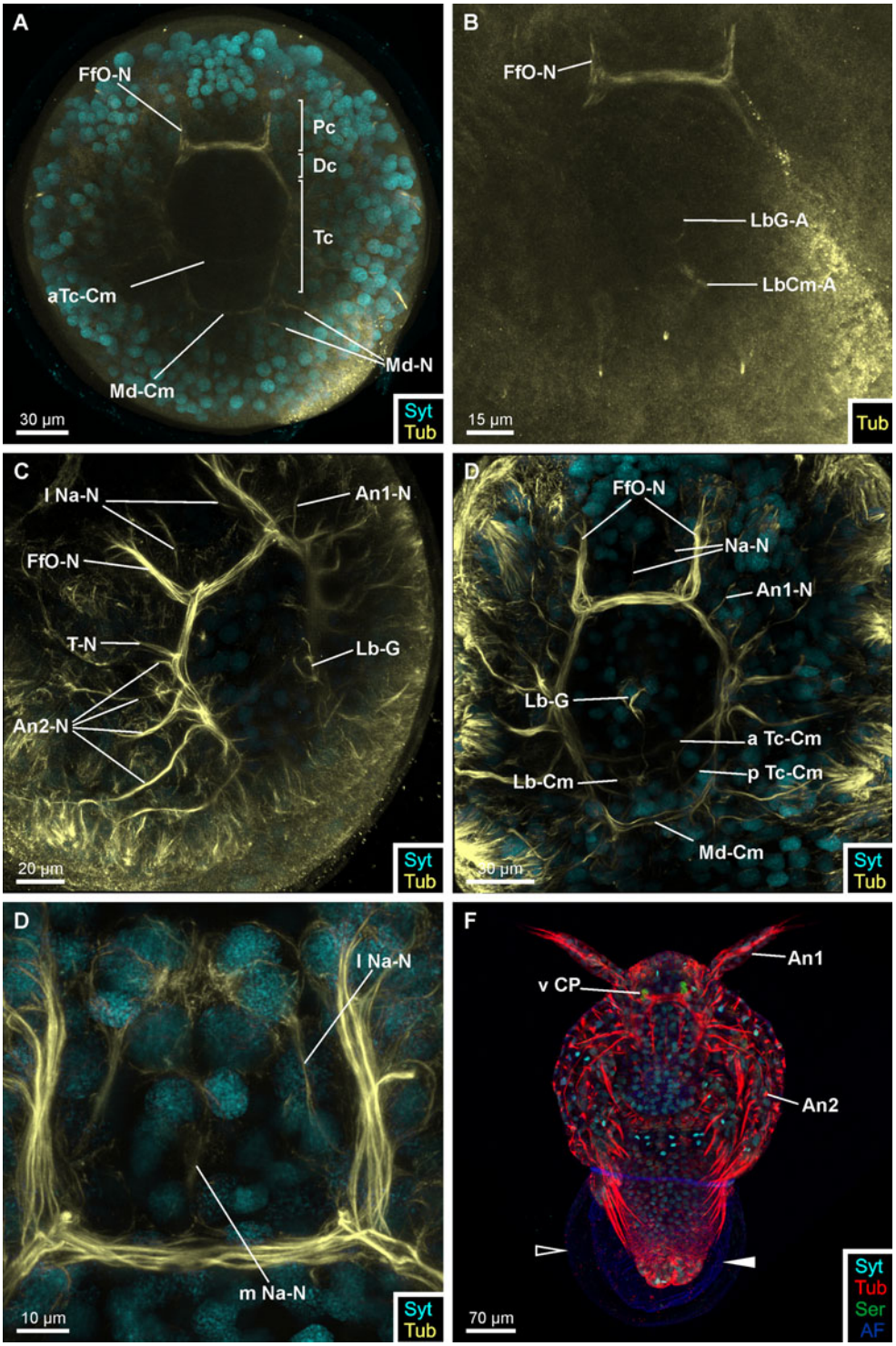

Fig. 1 Development of the embryonic nervous system in *A. franciscana*. **a** A 0-h embryo. Ventral view showing all parts of the syncerebrum (protocerebrum *Pc*, deutocerebrum *Dc*, tritocerebrum *Tc*) including anterior tritocerebral commissure (*a Tc-Cm*), posterior tritocerebral commissure (not shown) and mandibular commissure (*Md-Cm*) as early anlage with the frontal filament organ nerves (*FfO-N*) most prominent. **b** Same embryo showing establishment of labral ganglion (*LbG-A*) and labral commissure (*LbCm-A*). **c** An 8-h embryo. Anterolateral view showing *FfO-N* together with tegumental nerve (*T-N*), antennula nerve (*An1-N*) and the four antennal nerves (*An2-N*). **d** A 16-h embryo. Ventral view, nervous scaffold slightly more advanced than in **c**. **e** A 16-h embryo. Anlage of the nauplius eye with lateral nerves (*l Na-N*) originating close to the *FfO-N* at the lateral protocerebral commissure, nerve of median nauplius eye cup (*m Na-N*) originating medially. **f** Hatching nauplius (44 h) shedding egg membrane (*open arrow*) and embryonic cuticle (*white arrow*) showing first signs of serotonin expression in protocerebral ventral cell pairs (*v CP*). *An1* antennula, *An2* antenna, *Lb-Cm* labral commissure, *LbCm-A* labral commissure anlage, *Lb-G* labral ganglion, *LbG-A* labral ganglion anlage, *l Na-N* lateral nauplius eye nerves, *Md-N* mandibular nerve, *m Na-N* median nauplius eye nerve, *Pc* protocerebrum, *p Tc-Cm* posterior tritocerebral commissure

Switzerland) and edited in Microsoft CorelDRAW version 13 (Corel).

Results and discussion

Even the earliest embryos (0 h) show signs of a developing nervous system, with the neurites of the protocerebral commissure and of the developing frontal filament organ nerves clearly visible. Other embryos of the same 0-h stage additionally exhibit a complete circumoral neurite ring encompassing the anterior and posterior tritocerebral commissure (only the anterior one labelled, aTc-Cm) and the mandibular commissure (Md-Cm), plus some condensation of the protocerebrum and the frontal filament organ nerves (Fig. 1a, b). The labral ganglion anlage is present anteriorly

of the labral commissure (Fig. 1b). In an 8-h-stage embryo (Fig. 1c), the frontal filament organ nerves are still very prominent, equally developed are the four antennal nerves (An2-N, Fig. 1c), while the antennule nerve is less developed (An1-N). The paired nerves running into the lateral cups of the nauplius eye (l Na-N) originate together with the frontal filament organ nerves (FfO-N) from the lateral portion of the protocerebral commissure. In 16-h embryos (Fig. 1d, e), the nauplius eye is developed further and a median nauplius eye nerve (m Na-N) progresses from the central part of the protocerebrum into the median nauplius eye cup. The labral commissure (Lb-Cm) is only now connected to the tritocerebrum. From anterior, the labral ganglion (Lb-G) is medially connected via neurite bundles to the labral commissure (Lb-Km). The embryo in Fig. 1f (44 h) is close to hatching. During the hatching process, the egg membrane (open arrow head) and the embryonic cuticle (white arrow head) become disrupted. Only now is serotonin-like immunoreactivity present in the protocerebrum, possibly indicating some functionality. Two pairs of strongly labelled ventral cells (sv-Cp) ventrally of the frontal filament organ nerves send their neurites into the region of the first developing neuropil, which in branchiopod larva is the median protocerebral neuropil (see Fritsch and Richter 2010). In the larval stages (not shown), the compound eyes develop dorsally of the frontal filament organs. As the protocerebrum and the compound eyes grow, they cover the frontal filament organs and their nerves, which lose their prominence.

Benesch (1969) and Raineri and Falugi (1983) mistook the embryonic frontal filament organ nerves for optic nerves, but the optic nerves clearly only develop in later larval stages, and more dorsally.

The correspondences between the nerves of the frontal filament organs and those of the onychophoran antennae are remarkable (see particularly Eriksson et al. 2003, Figs. 56, 57 and 58). In both cases, they originate in the anterolateral area of the protocerebrum (Eriksson and Budd 2000; Eriksson et al. 2003; Mayer et al. 2010), appear early in the development at the same time as the protocerebrum and are connected to the protocerebrum early in the development via substantial neurite bundles (see Mayer et al. 2010). The situation in Anostraca therefore resembles the situation in onychophorans more closely than that in cirripeds, where only a few neurites connect the frontal filaments to the protocerebrum (Scholtz and Edgecombe 2006; Semmler et al. 2008). We suggest that anostracan embryos recapitulate a situation in which the original targets of the nerves were much more prominent structures than they are now. There is also a functional correspondence between onychophoran antennae and frontal filaments/frontal filament organs in that both are apparently chemosensory organs (Elofsson and Lake 1971; Storch and Ruhberg 1977).

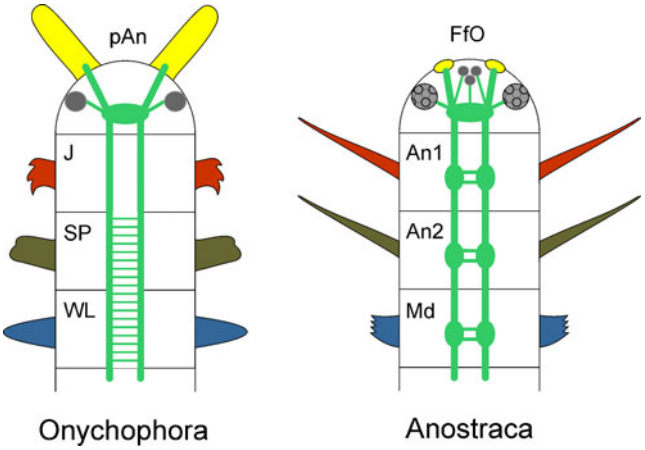

Fig. 2 Schematic drawing showing an onychophoran nervous system compared to that in Anostraca. Homologous appendages are indicated by colour correspondences (jaw—antennula in *red*, slime papilla—antenna in *green*, walking leg—mandible in *blue*). The protocerebral frontal filament organ is interpreted as partly homologous to onychophoran (primary) antenna in *yellow*. Anostracan compound eyes and nauplius eye are in *grey*, but the exact homology to onychophoran eye is uncertain. *An1* antennula, *An2* antenna, *FfO* frontal filament organ, *J* jaw, *Md* mandible, *pAn* primary antenna, *SP* slime papilla, *WL* walking leg

Because the frontal filaments also originate from the anteriormost region of the head, our suggestions do not conflict with the findings reported by Steinmetz et al. (2010). Unfortunately, no data are available so far on whether/which appendage genes are expressed in frontal filaments. Although we are aware that *Distal-less* is expressed in other outgrowths, including the labrum, it is interesting to note that it is also expressed in the external region of the frontal filament organs in *Artemia* (Panganiban et al. 1995; Popadíc et al. 1998).

Frontal filaments might also be more common than previously thought. In addition to being found in cirripeds and remipeds, they are present in various branchiopods (Fritsch et al. 2013) and also found in certain copepods (Elofsson 1971) and certain ostracodes (Andersson 1977). In the light of the suggested paraphyly of crustaceans (Regier et al. 2010), the presence of frontal filaments in various crustacean species indicates that they were already present in the stem species of Tetraconata. They are apparently absent in chelicerates, but *Cambropycnogon* (probably a representative of the stem lineage of Pycnogonida) possesses structures very similar to those of Notostraca (see Waloszek and Dunlop 2002)—their presence in the arthropod ground pattern, therefore, appears plausible. We think that the homology of the onychophoran antenna and the (crustacean) frontal filaments (Fig. 2) is still a valid hypothesis and not inferior to the hypothesis that the primary antenna and the labrum are homologues.

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