Brain organization in Collembola (springtails)

Martin Kollmann, Wolf Huetteroth¹, Joachim Schachtner*

Department of Biology – Animal Physiology, Philipps-University Marburg, Karl-von-Frisch-Str. 8, D-35032 Marburg, Germany

ARTICLE INFO

Article history:

Received 13 September 2010

Received in revised form

5 January 2011

Accepted 17 February 2011

Keywords:

Neuropil

Evolution

Central body

Mushroom body

Antennal lobe

ABSTRACT

Arthropoda is comprised of four major taxa: Hexapoda, Crustacea, Myriapoda and Chelicerata. Although this classification is widely accepted, there is still some debate about the internal relationships of these groups. In particular, the phylogenetic position of Collembola remains enigmatic. Some molecular studies place Collembola into a close relationship to Protura and Diplura within the monophyletic Hexapoda, but this placement is not universally accepted, as Collembola is also regarded as either the sister group to Branchiopoda (a crustacean taxon) or to Pancrustacea (crustaceans + hexapods). To contribute to the current debate on the phylogenetic position of Collembola, we examined the brains in three collembolan species: *Folsomia candida*, *Protaphorura armata* and *Tetradontophora bielensis*, using antennal backfills, series of semi-thin sections, and immunostaining technique with several antisera, in conjunction with confocal laser scanning microscopy and three-dimensional reconstructions. We identified several neuroanatomical structures in the collembolan brain, including a fan-shaped central body showing a columnar organization, a protocerebral bridge, one pair of antennal lobes with 20–30 spheroidal glomeruli each, and a structure, which we interpret as a simply organized mushroom body. The results of our neuroanatomical study are consistent with the phylogenetic position of Collembola within the Hexapoda and do not contradict the hypothesis of a close relationship of Collembola, Protura and Diplura.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

The ground pattern of brain architecture and neurochemistry of the neopteran insects has been described in a variety of species and seems to be highly conserved, but considerable morphological and functional modifications are readily observed (e.g., Pflugfelder, 1937; Strausfeld, 1976, 2005; Homberg, 1994; Burrows, 1996; Nässel, 2002; Schachtner et al., 2005; Homberg, 2008; Strausfeld et al., 2009; Nässel and Winther, 2010). In contrast, comparable data from the remaining hexapod groups, such as the Palaeoptera (Odonata and Ephemeroptera), the apterygote insects (Zygentoma and Archeognatha) and the Entognatha (Diplura, Protura and Collembola), are rare or even missing.

Traditional phylogenies based on morphological data considered Hexapoda as the sister group to Myriapoda, forming the clade Tracheata (also termed Atelocerata or Monoantennata (discussed in Osorio et al., 1995; Dohle, 1997a,b; Wheeler, 1997; Mallatt et al., 2004)). However, recent studies favor the clade Pancrustacea

(Hexapoda + Crustacea), also termed the Tetraconata due to the unique composition of the compound eyes with four ommatidial cone cells (Friedrich and Tautz, 1995; Zrzavý and Stys, 1997; Dohle, 2001; Richter, 2002; Mallatt et al., 2004). According to the Pancrustacea/Tetraconata concept, Hexapoda is regarded as either the sister group or an in-group of Crustacea.

Analysis of 18S rRNA sequence dataplaced Collembola as a sister group to a monophyletic clade comprising the crustacean taxa Branchiopoda, Anostraca and Notostraca, while Archaeognatha sister groups with Pterygota (Spears and Abele, 1998). Other studies based on mitochondrial gene sequences (Nardi et al., 2001, 2003) instead support a position of Collembola as the sister group to Pancrustacea. However, more recent analyses of the 28S rRNA and 18S rRNA genes and numerous nuclear protein-coding genes suggest the monophyly of a clade comprising Collembola, Diplura and Protura, which is the sister group to the remaining hexapods (Mallatt and Giribet, 2006; Misof et al., 2007; Regier et al., 2010), thus supporting the traditional placement of Collembola close to the base of the hexapod tree (Bitsch and Bitsch, 2000).

In summary, there is still no consensus about the definitive position of Collembola within the arthropods and additional data are required. In particular, an analysis of neuroanatomy in representatives of Collembola seems promising as it might help clarify the phylogenetic position of this group. Furthermore, such an

* Corresponding author.

E-mail address: schachtj@staff.uni-marburg.de (J. Schachtner).

¹ Present address: Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA.

analysis would provide important insights into the evolution of the hexapod brain.

So far, only a few studies have dealt with the collembolan brain architecture (e.g., Kühnle, 1913; Hanström, 1940; Tysiewicz, 1981; Korr, 1968), but some additional information is available from studies dealing with the collembolan endocrine system (e.g., Cassagnau and Juberthie, 1966, 1967a,b; Cassagnau et al., 1968; Lauga-Reyrel, 1984a,b). These classical studies provide an important source of information about the basic composition of the collembolan brain. However, the techniques used allowed only limited insights into its neuropilar organization. Currently, only one detailed study of at least one major neuropil in the collembolan brain, the central body, is available (Strausfeld et al., 2006).

To contribute to a better knowledge of the collembolan brain, we applied different neuroanatomical techniques, including immunohistochemistry in conjunction with confocal laser scanning microscopy and three-dimensional reconstructions, and analyzed the brains in three distantly related collembolan species. The transparent cuticle of the two smaller species (*Folsomia candida* and *Protaphorura armata*, with a body length of 1.5–3 mm) allowed for *in situ* scanning of the brain through the intact head capsule whereas the third species (*Tetradontophora bielanensis* or “giant” springtail, with a body length of 5–10 mm) was large enough to dissect out the brains for our analyses.

In the current study, we use wholemount immunostainings of Collembola brains using antibodies against synapsin to resolve the entire neuropilar brain anatomy in combination with antibodies against several neuromediators (including the biogenic amine serotonin and several neuropeptides: allatotropin, allatostatin-A, orcokinin, tachykinin, myoinhibitory peptide, periviscerokinin, and FMRFamides) which have in several insect species been shown to depict several distinct neuropils (for reviews see Nässel, 2002; Homberg, 2002; Nässel and Homberg, 2006). An α -tubulin antibody was used to identify fiber tracts and commissures (Kononenko and Pflüger, 2007), and an antibody against the catalytic subunit of protein kinase A (DCO) to visualize the mushroom bodies (MB) (Farris and Strausfeld, 2003). To resolve the projection areas of the sensory and chemosensory neurons of the antennae, we performed antennal backfills for *F. candida* and *P. armata*. To further analyze brain structures we produced methylene blue-stained semi-thin sections of whole *F. candida* heads.

Our findings revealed more similarities in brain composition between collembolans and hexapods as compared to crustaceans. These findings contradict the phylogenetic placement of Collembola as either the sister group to Branchiopoda or to Pancrustacea (Spears and Abele, 1998; Nardi et al., 2001, 2003). They instead suggest an inclusion of Collembola in Hexapoda, which is in line with the hypothesis of a close relationship of collembolans, proturans and diplurans (Mallatt and Giribet, 2006; Misof et al., 2007; Regier et al., 2010).

2. Materials and methods

2.1. Animals

Three different collembolan species were used: (1) *F. candida* (Willem, 1902), obtained from b.t.b.e. Insektenzucht GmbH (Schnürpfingen, Germany), (2) *P. armata* (Tullberg, 1869), kindly provided by Dr. Mark Maraun (TU Darmstadt, Germany), and (3) *T. bielanensis* (Waga, 1842), kindly provided by Dr. David Russell (Public Museum of Natural History, Görlitz, Germany). Specimens of *F. candida* and *P. armata* were bred in small plastic boxes in darkness by room temperature and supplied with yeast as food source. Specimens of *T. bielanensis* were caught in the area near Görlitz, Germany.

2.2. Primary antisera

Details on all used primary antisera, including their dilution, animal source and corresponding references, are listed in Table 1. All used neuropeptide antisera were raised against neuropeptide sequences, which are highly conserved among insects (for reviews, see Nässel, 2002; Homberg, 2002; Nässel and Homberg, 2006).

The monoclonal primary antibody from mouse against a fusion protein consisting of a glutathione-S-transferase and the first amino acids of the presynaptic vesicle protein synapsin I coded by its 5'-end (SYNORF1; 3C11, #151101) was used to selectively label neuropilar areas. It was used in combination with one additional primary antibody raised in rabbit. The synapsin antibody was kindly provided by Dr. Erich Buchner (University of Würzburg, Germany) and was first described by Klagges et al. (1996). The antibody was used at a dilution of 1:50. The polyclonal antiserum against serotonin (5HT) was raised in rabbit (DiaSorin, Kansas City, MO, USA). Its specificity for the insect nervous system was shown in several studies (e.g., Dacks et al., 2006). The antiserum against *Manduca sexta* allatotropin (Mas-AT, pGFKNVEMMTARGFamide) was raised in rabbit. It was kindly provided by Dr. J. Veenstra (University of Bordeaux, Talence, France) and first described by Veenstra and Hagedorn (1993). The antiserum against *Diptera punctata* allatostatin 7 (Dip-AST 7, APSGAQRLYGFGFLamide) was raised in rabbit. It was kindly provided by Dr. H. Agricola (University of Jena, Germany) and was first described by Vitzthum et al. (1996). The antiserum against Asn¹³-orcokinin (Asn¹³-OK, NFDEIDRSFGFN-OH) was raised in rabbit. It was kindly provided by Dr. H. Dirksen (University of Stockholm, Sweden) and was first characterized by Bungart et al. (1994). The antiserum against *Locusta migratoria* tachykinin II (Lom-TK II, APLSGFYGVRFamide) was raised in rabbit. It was kindly provided by Dr. H. Agricola (University of Jena, Germany) and first described by Veenstra et al. (1995). The antiserum against *Periplaneta americana* myoinhibitory peptide (Pea-MIP, GWQDLQGGWamide) was raised in rabbit. It was

Table 1

List of antisera used, including dilution, source, donor, and reference for each antiserum.

Name	Shortcut	Dilution	Source	Donor (Reference)
Anti-Synapsin I (SYNORF1)	Anti-synapsin	1:50	Mouse	Dr. E. Buchner (Klagges et al., 1996)
Anti-Serotonin	Anti-5HT	1:15000	Rabbit	DiaSorin, Kansas City, MO, USA
Anti- <i>Manduca sexta</i> Allatotropin-A	Anti-Mas-AT	1:4000	Rabbit	Dr. J. Veenstra (Veenstra and Hagedorn, 1993)
Anti- <i>Diptera punctata</i> Allatostatin 7	Anti-Dip-AST 7	1:15000	Rabbit	Dr. H. Agricola (Vitzthum et al., 1996)
Anti-Asn 13-Orcokinin	Anti-Asn ¹³ -OK	1:1000	Rabbit	Dr. H. Dirksen (Bungart et al., 1994)
Anti- <i>Locusta migratoria</i> Tachykinin II	Anti-Lom-TK II	1:3000	Rabbit	Dr. H. Agricola (Veenstra et al., 1995)
Anti- <i>Periplaneta americana</i> Myoinhibitory Peptide	Anti-Pea-MIP	1:5000	Rabbit	Dr. H. Agricola (Predel et al., 2001)
Anti- <i>Periplaneta americana</i> Periviscerokinin 2	Anti-Pea-PVK 2	1:4000	Rabbit	Dr. M. Eckert (Eckert et al., 2002)
Anti-FMRFamide	Anti-FMRFamide	1:4000	Rabbit	Dr. E. Marder (Marder et al., 1987)
Anti-DCO	Anti-DCO	1:1000	Rabbit	Dr. D. Kalderon (Lane and Kalderon, 1993)
Anti- α -tubulin	Anti- α -tubulin	1:200	Rabbit	Abcam, Cambridge, UK

kindly provided by Dr. H. Agricola (University of Jena, Germany) and described by Predel et al. (2001). The antiserum against *P. americana* periviscerokinin 2 (Pea-PVK 2, GSSGLISMPRVamide) was raised in rabbit. It was kindly provided by Dr. M. Eckert (University of Jena, Germany) and first described by Eckert et al. (2002). The antiserum against FMRamide was raised in rabbit. It was kindly provided by Dr. Eve Marder (#671, Brandeis University, Waltham, MA, USA) and first described by Marder et al. (1987). The antiserum recognizes FMRamide and FLRFamide peptides (Marder et al., 1987; Kingan et al., 1990), including the three FaRPs identified in *M. sexta* (Kingan et al., 1990, 1996; Miao et al., 1998). The DC0 antiserum is against the catalytic subunit of the protein kinase A (PKAc) of *Drosophila melanogaster*. It was raised in rabbit and was kindly provided by Dr. D. Kalderon (Columbia University, NY, USA). It was first described by Lane und Kalderon (1993). The α -tubulin antiserum raised in rabbit is against the α -tubulin of microtubules and supplied by Abcam (Cambridge, UK). The ability of α -tubulin to label tracts and commissures in insect brains was described by Kononenko and Pflüger (2007).

2.3. Secondary antibodies

Goat anti-rabbit antibodies conjugated to Cy3 (GAR-Cy3) and to Cy2 (GAR-Cy2) and goat anti-mouse antibodies conjugated to Cy5 (GAM-Cy5) were used as secondary antibodies (each 1:300; Jackson ImmunoResearch, Westgrove, PA, USA).

2.4. Further tissue markers

Alexa Fluor 488-coupled phalloidin (phalloidin; Molecular Probes, Eugene, OR, USA) at a dilution of 1:200 was used to visualize f-actin in the head musculature. To counterstain semi-thin sections we used methylene blue (Löffler's methylene blue solution, Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

2.5. Whole mount double immunostainings

Heads were cut off the animals. To prevent heads from floating on top of the surface of the 0.1 M phosphate-buffered saline (PBS) it was necessary to diminish the surface tension by adding 0.3% Triton-X 100 (PBT) (Sigma–Aldrich, Steinheim, Germany). To ensure that the heads sunk into the PBS, the cup was carefully vortexed followed by a short centrifuge spin. For the bigger *T. bialensis*, whole brains were dissected out of the head capsule in cold PBS. Both heads and dissected brains were fixed in PBS containing 4% formaldehyde for 2 h at room temperature (RT) or overnight at 4 °C. Then the heads/brains were rinsed (6 × 10 min) with PBT at RT followed by pre-incubation for two or three days in PBT with 5% normal goat serum (NGS) (Jackson ImmunoResearch, Westgrove, PA, USA) at 4 °C. Primary antibodies (Table 1) were applied in PBT with 5% NGS and the heads/brains were incubated for four days at 4 °C. After rinsing (6 × 10 min) with PBT at RT, heads/brains were incubated in secondary antibodies (GAR-Cy2/GAR-Cy3 and GAM-Cy5; 1/300) in PBT with 1% NGS and 0.5% Alexa Fluor 488 Phalloidin at 4 °C for three days in the dark. After rinsing (6 × 10 min with PBT at RT and washing in distilled H₂O for 10 min), heads/brains were dehydrated in an ascending alcohol series (30%, 50%, 70%, 90%, 95%, 2 × 100% ethanol, 5 min each). Following clearing the tissue in methyl salicylate (10 min; Merck, Darmstadt, Germany) the heads/brains were finally mounted in resin (Permount, Fisher Scientific, Pittsburgh, PA, USA).

2.6. Antennal backfills

Antennal backfills were performed in *F. candida* and *P. armata*. After cooling for 5 min in the refrigerator, the specimens were placed in PBT, the PBT with the animals was then vortexed and centrifuged (see above), to overcome the surface tension. Thereafter, the antenna was cut off at the third segment, since in *F. candida* the olfactory sensilla are housed exclusively in segments three and four of the four antennal segments (Slifer and Sekhon, 1978). The animals were rinsed for 5 min in distilled H₂O to widen the axons in the antenna. The animals were then placed in a 2% dextran-coupled biotin dilution for 20 min (Molecular Probes, Eugene, OR, USA) followed by synapsin immunostaining as described before. To visualize the backfill we used Cy3-coupled streptavidin (Jackson ImmunoResearch, Westgrove, PA, USA at a dilution of 1:1000).

2.7. Semi-thin sections

Heads of *F. candida* were fixed overnight at 4 °C in 4% PFA and 0.25% glutaraldehyde (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in 0.2 M sodium cacodylate buffer (SCB) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The heads were then rinsed (4 × 10 min) in PBT at RT, followed by pre-fixation with 1% osmium tetroxide (OsO₄) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in SCB at 4 °C for 2 h and subsequent rinsing in PBT (3 × 10 min). The heads were then dehydrated in an ascending alcohol series (distilled H₂O for 10 min, 30%, 50%, 70%, 90% and 95% ethanol for 5 min, 2 × 100% ethanol and 2 × acetone for 10 min) followed by replacement of the acetone in an ascending Durcupan resin series (Durcupan Resin-Kit, Fluka Chemika-BioChemika, Buchs, Switzerland) (4/1, 1/1, 1/4 for 1 h each), and in pure Durcupan overnight. Resin cured for 24 h at 40 °C and for 48 h at 60 °C. Resin blocks were cut in 2 µm sections with a glass knife on a 11800 Pyramitome (LKB-BROMMA, Stockholm, Sweden), stained for 5 min with Löffler's methylene blue solution (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and rinsed shortly in distilled water. Semi-thin sections were embedded in Durcupan and cured for 24 h at 40 °C and for 48 h at 60 °C.

2.8. Data processing

Fluorescence was analyzed with a confocal laser scanning microscope (Leica TCS SP2), with the object lenses HC PL APO 20×/0.70 Imm Corr CS, HCX PL APO 40×/1.25–0.75 Oil CS, und HCX PL APO 63×/1.32–0.60, Oil Ph3 CS. We scanned with a resolution of 1024 × 1024 pixels, a line average of 2–4, speed of 200 Hz, and z-steps varying from 0.4 to 1.0 µm. Semi-thin sections were analyzed with a Zeiss-Axioplan 2 imaging system (Carl Zeiss MicroImaging GmbH, Jena, Germany) using a Plan-Neofluar 63×/1.25 Oil DIC objective lens.

2.9. Image segmentation, reconstruction, and visualization

The gross anatomy of collembolan brains was labeled by using the module LabelVoxel of AMIRA 4.1 (Visage Imaging, Fürth, Germany). For the segmentation and reconstruction of the brain structures we principally refer to Kurylas et al. (2008), el Jundi et al. (2009), and Dreyer et al. (2010). For the visualization of the brain including subesophageal ganglion we also used the volume rendering module Voltex of AMIRA. Snapshots were taken in AMIRA and subsequently processed in Corel Draw 13 (Corel Corporation, Ottawa, Ontario, CA; Figs. 1–4), diagrams generated with Excel XP (Microsoft Corporation, Redmond, WA, USA; Fig. 1B).

Fig. 1. Position, size, and shape of the collembolan brain and SEG. A) Horizontal view on a voltex projection of an *F. candida* head. Synapsin-ir represents neuronal tissue (including nerves, brain, and SEG), phalloidin staining labels filamentous actin (mainly muscle tissue) (colored voltex presentation can be seen in Suppl. 1). B) Comparison of the size of the brain of three different collembolan species (length: from the most anterior glomerulus to the posterior end of the brain; width: from the most left lateral side of the brain to the most right lateral side; depth: from the most dorsal part of the esophagus hole to the most dorsal part of the brain). C–C''') Horizontal, lateral, frontal, and oblique view of the brain of the three species. Orientation bars (relative to body axis): P = posterior, A = anterior, D = dorsal, and V = ventral. Scale bars: 50 μm .

Fig. 2. Central body (CB) of the collembolan brain. A) Overlay of synapsin-ir (green) and Pea-MIP-ir (magenta) of an *F. candida* CB showing the columnar area (arrowhead) and the non-columnar area (arrow). A') Optical section 0.6 μm dorsal to the optical section presented in A. It shows the projections sent from the Y- and Z-columns to the dorsal cell group (Fig. 2B, asterisk). A'') Optical section 1.2 μm dorsal to the optical section presented in A. It shows the projections sent from the W-columns to the dorsal cell group (Fig. 2B, arrowhead). B) Schematic drawing of the CB of *F. candida*, showing in light gray the dorsal part of the columns and in dark gray the ventral part of the columns. Each column typically receives one fiber (arrowhead, asterisk) stemming from a group of somata situated in a posterior area, similar to the pars intercerebralis of insects. The X-, Y-, W-columns each send one fiber, the fused part of the Z-columns send four fibers anteriorly. For further information see text. C) Asn¹³-OK-ir in an arc-shaped structure (asterisk) in the *F. candida* brain. Optical section is 2 μm ventral to the CB (arrowhead). D) Asn¹³-OK-ir of an arc-shaped structure (asterisk) in *T. bielanensis*, anterior to the CB. Note the bright Asn¹³-OK-ir in the columnar posterior part (arrowhead) and less Asn¹³-OK-ir in the non-columnar anterior part (arrow). E–E''') Protocerebral bridge (PB) of *T. bielanensis* from frontal (E) and horizontal (E'–E'''). F) Asn¹³-OK-ir of two spherical noduli-like structures (arrows) ventrally to the CB (arrowhead) and the Asn¹³-OK-ir arc-shaped structure (asterisk; see also Fig. 2C, asterisk). Orientation bars (relative to body axis): P = posterior, A = anterior, D = dorsal, V = ventral. Scale bars: 10 μm ; insets (A' and A'') 5 μm .

were imported and revised in Corel Draw 13 without any further modification.

2.10. Axis determination

Since we scanned the heads of two of the three species *in situ*, the terms anterior, posterior, dorsal, and ventral refer to the body axis throughout the paper (not to the neuraxis), as demonstrated in Fig. 1A. For a better comparison, this nomenclature is also adapted to

the dissected *T. bielanensis* brains. To transform from the body axis to the neuraxis, replace the terms “anterior”/“posterior” (body axis) by “ventral”/“dorsal” (neuraxis) and the terms “ventral”/“dorsal” (body axis) by “anterior”/“posterior” (neuraxis), respectively.

3. Results

In the present study, we describe the gross anatomy of the collembolan brain, characterize several neuropils of the brain

Fig. 3. Mushroom body (MB) of the collembolan brain. A) Voltex projection of a DCO staining (yellow) within the brain outline of *F. candida*, based on synapsin-ir (transparent). The DCO antibody stained two cap-shaped structures at the dorsal border and about 7.5 μm from the posterior border (arrows). The structures are innervated by a DCO-ir cell group of about 3–4 cells (arrowheads); an additional, paired cell group without traceable projections lies at the posterior border (asterisks). B) $\text{Asn}^{13}\text{-OK-ir}$ of a cap-like structure at the posterior border of the *F. candida* brain (arrow). C) Mas-AT staining of a cap-like structure at the posterior border of the *F. candida* brain (arrow). D) α -Tubulin staining of the *F. candida* brain revealed pedunculus-like structures (arrows). The CB (arrowhead) lies 2 μm dorsal to the presented optical section. E) Methylene blue staining of a Durcupan resin semi-thin section. Because of the oblique section it is only possible to see the right pedunculus-like structure (arrow) including a structure resembling a medial lobe (arrowhead). Orientation bars (relative to body axis): P = posterior, A = anterior. Scale bars: 10 μm .

(central complex, a structure, that we interpret as simple mushroom bodies, and antennal lobes, including the antennal glomeruli), and discuss their specific characteristics in comparison to other hexapods and crustaceans. Except for their size, the brains of the three collembolan species show no obvious differences in their principal neuroarchitecture. Interestingly, some of the used anti-peptide antisera differ in their ability to label the central body (anti-Dip-AST 7, anti- $\text{ASN}^{13}\text{-OK}$) and the antennal lobes (anti- $\text{ASN}^{13}\text{-OK}$, anti-FMRamide) in the three investigated collembolan species (Tables 2, 3).

3.1. Position, size, and gross anatomy of the brain and the subesophageal ganglion

The small size and the shiny cuticle of the heads of *F. candida* and *P. armata* allowed us to analyze the gross anatomy of their brains *in situ*, by scanning the entire heads (Fig. 1A). By performing double-labelings of the heads using phalloidin and an antiserum against synapsin, it was possible to visualize the musculature and the neural tissue, including the brain and the subesophageal ganglion (SEG), within the head capsule. In both species, the brain fills a small portion of the head capsule and is located dorsally

(movie of rotating head of *F. candida*, suppl. 1). Our three-dimensional (3D) reconstructions of head preparations in *F. candida* and *P. armata* demonstrate the shape of the brain and the SEG without artificial deformation (Fig. 1C–C’). In contrast, the brain and the SEG of *T. bielanensis* had to be dissected out of the head capsule, which has led to a tilted orientation of the brain 90° down toward the SEG (Fig. 1C’').

Supplementary video related to this article can be found at doi: 10.1016/j.asd.2011.02.003.

For the brain size measurements (Fig. 1B), the length is considered as the distance from the anterior-most antennal glomerulus to the posterior border of the brain. The width is considered as the distance from the outermost left border to the outermost right border of the brain. The depth is considered as the distance from the dorsal border of the esophageal foramen to the dorsal border of the brain. Our data show that the brain size in *F. candida* and *P. armata* is similar between the two species and might mirror the similar body lengths (1.5–3 mm) in these two species (Fig. 1B). In contrast, the 2–3 times larger species *T. bielanensis* (5–10 mm) exhibits a brain, which is almost twice the size of the brains in *F. candida* and *P. armata* (Fig. 1B).

Fig. 4. Antennal lobe (AL) of the collembolan brain. **A**) Maximal projection of an antennal backfill (magenta) and anti-synapsin staining (green) of an *F. candida* brain. **A')** A maximal projection shows a soma of a neuron (large arrow) located in the maxillary ganglion, its axon (small arrow) projecting into the AL/DL (not clearly identifiable). From AL/DL, two processes (asterisk) project to an area located in the mandibular ganglion (arrowhead; also arrowhead in 4 A), and continue further down, until weak staining prohibits further tracking. **A'')** A single optical section (dorsal deutocerebrum) of the AL showing the antennal nerve (AN) projecting towards clearly identifiable glomeruli (G). **A''')** A single optical section (ventral deutocerebrum) of the DL showing an agglomerular structure. **B–B''')** Reconstruction of an AL with 27 glomeruli of *F. candida* based on a backfill; horizontal (**B'**), frontal (**B''**), and lateral (**B'''**) view. In red are 6 large median glomeruli and in green 21 smaller glomeruli. **C–E**) Anti-5HT staining of an AL of *F. candida* (**C**), *P. armata* (**D**), and *T. bielanensis* (**E**). The outlines represent the shape of the AL based on anti-synapsin staining. In both *F. candida* and *P. armata* only one glomerulus in each AL exhibits 5HT immunoreactivity. In *T. bielanensis*, additionally three neighboring glomeruli are weakly innervated, two anteroventrally (v), one posterodorsally (d). The source of the innervation of the glomeruli is a large fiber (arrowheads) originating in the SEG. **F**) Anti-Pea-MIP staining of an AL of *T. bielanensis*. The outline represents the shape of the AL based on synapsin-ir. The anti-Pea-MIP staining varies in intensity in the stained glomeruli (* intensive, ** medium, and *** weak). Orientation bars (relative to body axis): P = posterior, A = anterior, D = dorsal, V = ventral, L = lateral, and M = median. Scale bars: A 25 µm, all others 10 µm.

Table 2

Staining results in the central bodies of the investigated collembolan species by using different antibodies.

Antibody	<i>F. candida</i>	<i>P. armata</i>	<i>T. bielanensis</i>
Anti-5HT	×	×	n.a.
Anti-Mas-AT	—	—	—
Anti-Dip-AST 7	—	×	×
Anti-Asn ¹³ -OK	— ^a	×	×
Anti-Lom-TK II	×	n.a.	n.a.
Anti-Pea-MIP	×	×	n.a.
Anti-Pea-PVK 2	—	—	—
Anti-FMRFamide	—	—	—

“×”: staining observed; “—”: no staining observed; “n.a.”: immunolabeling not performed.

^a Observed in the arc-shaped structure, discussed as part of the central body.

3.2. The central body

Presence and shape of the central body: Within the collembolan brain, we found a fan-shaped, unpaired midline neuropil that we regard as a central body (CB). The CB of the three analyzed collembolan species shows the fan-shaped design of a typical insect CB (reviewed by Homberg, 2008). The collembolan CB lies in the center of the protocerebrum and the flat side of the fan-shape points toward the anterior. With the anti-synapsin immunostaining alone, it is difficult to identify the substructures of the CB, but in combination with additional antisera against serotonin and three peptides (Asn¹³-OK, Pea-MIP, and Lom-TK II), it was possible to analyze the CB in more detail.

Columns in the central body: The CB exhibits eight clearly distinct columns in its posterior portion. These columns can be best identified with anti-Pea-MIP (anti-Pea-MIP staining, Fig. 2A) and anti-5HT immunostainings (Suppl. 2). According to Williams (1975) these eight columns are named W, X, Y and Z (from lateral to median). In the Pea-MIP immunostaining, a separation into a dorsal (Fig. 2B, light-gray) and a ventral part of the CB is found (Fig. 2B, dark-gray). The dorsal parts of both Z-columns are fused to a single structure. The ventral parts of the W, X, Y, Z-columns exhibit one fiber each (Fig. 2B, arrowheads, asterisks), which lead to a group of Pea-MIP-ir somata in the posterior brain portion, likely part of the pars intercerebralis, as described for insects (Panov, 1959; Younossi-Hartenstein et al., 2003). The fibers of the Y- and Z-columns in each brain hemisphere (Fig. 2B, asterisks) either run closely in parallel to each other, or both columns are innervated by one fiber. In addition, the dorsal parts of the columns exhibit one fiber each, which cross the midline of the CB, thus forming a chiasma. The two fused dorsal Z-columns send four fibers to the center of the CB (Fig. 2B, small arrows), which fuse to one bundle on each side. Before they reach the center of the CB, they converge to one fiber bundle. From the inside of the fan-shaped CB, one fiber in

each brain hemisphere (Fig. 2B, large arrow) leads to the Pea-MIP-ir area in the anterior portion of the brain.

Layers in the CB: Some of the immunostainings against various neuromediators (5HT, Asn¹³-OK, Pea-Pea-MIP, and Lom-TK II) suggest two different layers in the CB, which cannot be discerned by anti-synapsin staining alone. The posterior part of the CB, which exhibits a clear columnar structure and a very bright staining against several neuromediators, represents the first layer (Fig. 2A, D and Suppl. 2, arrowheads). The inner, non-columnar and less brightly stained part represents the second layer of the CB (Fig. 2A, D and Suppl. 2 arrows).

Subdivision of the CB in an upper and a lower unit: By using an antiserum against Asn¹³-OK it was possible to identify in *F. candida* and *T. bielanensis* an arc-shaped structure associated with the CB (Fig. 2C and D, asterisks). Depending on the species, this structure is located more or less antero-ventral to the non-columnar layer of the CB. This additional arc-shaped structure could be either a third layer of the CB or, alternatively, the lower unit of the CB. Correspondingly, the columnar and non-columnar layers of the central body would then be substructures of the upper unit of the CB.

3.3. The protocerebral bridge

A structure resembling a protocerebral bridge (PB) can only be visualized in *T. bielanensis* whereas the brains of *F. candida* and *P. armata* might be too small to identify a PB. In *T. bielanensis*, this elongated structure is located posteroventrally to the CB, with its tips pointing ventrally (Fig. 2E–E’). Both arms of the PB join medially together to a single structure. At the dorsal border, in the middle of the arc, the PB exhibits two protrusions (Fig. 2E’). The lateral ends of the PB seem to be connected to neuropil of the protocerebrum. In contrast to the CB, the PB features no significant immunoreactivity against any of the used neuromediator antibodies. It is not possible to identify a clear columnar structure in the PB.

3.4. The noduli

Only with the anti-Asn¹³-OK antibody and only in *F. candida* it was possible to stain two small spherical structures (about 4 µm in diameter) situated ventral to the CB (Fig. 2F). By only analyzing the anti-synapsin staining, it is not possible to identify these spherical structures. The anti-Asn¹³-OK staining is the only indication for the existence of a nodule-like structure in the collembolan brain.

3.5. The mushroom bodies

Some of the antibodies used in this study labeled structures in the collembolan brains, which resemble the insect mushroom bodies (MBs). The best evidence for these “mushroom body-like structures” is provided by the DC0 immunolabeling in *F. candida*. This immunolabeling revealed a paired structure in the posterior part of the protocerebrum (Fig. 3A, arrows). The cap-shaped design of the labeled structure resembles the insect MB calyces. This calyx-like structure is innervated by a small cell cluster of a few (about 3–4) DC0-ir cells at each posterior lateral side of the brain (Fig. 3A, arrowheads). In addition, a few cells in the posterior brain region were stained (Fig. 3A, asterisks), but no projections of these cells could be found. In *F. candida* an elongated cap-shaped area posterior to the DC0-ir structure and at the posterior border of the protocerebrum was labeled with the Asn¹³-OK antibody (Fig. 3B, arrow). In *P. armata*, this calyx-like structure is subdivided in two parts, which lie close together (data not shown). A comparable

Table 3

Number of stained glomeruli in the antennal lobes of the investigated collembolan species by using different antibodies.

Antibody	<i>F. candida</i>	<i>P. armata</i>	<i>T. bielanensis</i>
Anti-5HT	1	1	1(+3) ^a
Anti-Mas-AT	1	1	0 ^b
Anti-Dip-AST 7	3–4	1–2	2
Anti-Asn ¹³ -OK	0	3–5	7
Anti-Lom-TK II	0	n.a.	n.a.
Anti-Pea-MIP	4–5	4–5	17
Anti-Pea-PVK 2	0	0	0
Anti-FMRFamide	0	4	1

^a Three weakly innervated neighbor glomeruli.

^b We could not find Mas-AT-ir glomeruli, but we found three Mas-AT-ir somata in the periphery of the antennal lobes.

immunostaining pattern was found in *F. candida* with anti-Mas-AT staining (Fig. 3C, arrow).

The immunostainings with the α -tubulin antibody exhibit a pedunculus-like structure, possibly homologous to the pedunculi of insects (Fig. 3D, arrows). This structure extends from the posterior dorsal border of the brain to the ventral side of the lateral ends of the CB. In semi-thin sections stained with methylene blue, a pedunculus-like structure is also seen, which resembles in position and shape the α -tubulin staining pattern (Fig. 3E, arrow). A short medial extension of this structure resembles the median lobes typically found in dicondylic insects (Fig. 3E, arrowhead). A vertical lobe, however, is missing.

3.6. The antennal lobes

In all three collembolan species, the first integration center of the antennal afferents exhibits a glomerular pattern, which is therefore regarded as the antennal lobes (ALs). The deutocerebral paired ALs represent the anterior-most neuropils in the collembolan brain. Because of the small brain size, it was difficult to analyze details of the ALs in *F. candida* and *P. armata* by using anti-synapsin immunohistochemistry alone. However, our backfills of the antennal nerve with dextran-coupled biotin revealed distinct glomerular structures and dorsal lobes. Based on our backfill data, we could reconstruct 27 glomeruli in one AL of *F. candida* (Fig. 4B⁺–B⁺). However, due to the small size and the resulting difficulty to distinguish glomerular borders, this number gives only an estimate. This seems to be also a good estimate for the number of the glomeruli in the ALs of the two other investigated collembolan species. We identified 4–6 median glomeruli in *F. candida* (Fig. 4B⁺–B⁺, labeled in red) and 3–4 median glomeruli in *P. armata*, which are obviously larger than the other glomeruli of the ALs. This glomerular size dichotomy between the large median and the smaller lateral glomeruli was not found in *T. bielanensis*.

By backfilling the antennal nerve, the dorsal lobes (DLs) were also filled (Fig. 4A⁺). The DLs are attached ventrally to the ALs. The backfills in *F. candida* and *P. armata* revealed fibers passing through the ALs/DLs region to the SEG (Fig. 4A⁺, small arrow and asterisks). The lateral fiber (Fig. 4A⁺, small arrow) seems to belong to an efferent projection neuron with its soma in the maxillary ganglion (Fig. 4A⁺, arrow). The median fibers (Fig. 4A⁺, asterisk) project in A area (Fig. 4A and A⁺, arrowheads) in the mandibular ganglion and further upward to the maxillary ganglion. Further backtracing was prohibited by a fading backfill quality.

Stainings with an antibody against the biogenic amine 5HT showed a similar pattern in the ALs in all investigated species: one centrifugal neuron innervating one anterior-lateral glomerulus, plus three weakly innervated neighboring glomeruli in *T. bielanensis* (Fig. 4C–E). The soma is most likely located in the mandibular region of the SEG. All antibodies against different neuromediators used in this study labeled, depending on the species, either a subset or no glomeruli within the ALs but never all glomeruli (Table 3). In different preparations with the same antibody in the same species, we always stained a similar subset of glomeruli. This subset varied, however, from species to species. Analyzing the neuropeptide immunostainings in more detail, we found varying staining intensities between different glomeruli in the same AL (Fig. 4F, asterisks).

4. Discussion

4.1. Comparative anatomy of the central body

The fan-shaped CB of the investigated collembolan species compares in its principal anatomy to a typical CB of insects

(reviewed by Homberg, 2008), but differs from the flattened, spinal-shaped CB in representatives of the crustacean taxa Malacostraca and Remipedia (Sandeman, 1992; Fanenbruck et al., 2004), which are assumed to be close relatives of hexapods (Garcia-Machado et al., 1999; Wilson et al., 2000). The posterior border of the CB in the collembolans *F. candida* and *P. armata* exhibits a columnar structure, especially well defined in anti-peptide (Asn¹³-OK, Pea-MIP and Lom-TK II) and anti-5HT stainings. The columnar structure, consisting of 8 columns (W-, X-, Y-, and Z-columns according to Williams, 1975), is restricted to the posterior part of the CB. Fibers extending from these columns cross the midline of the CB and form a chiasma. A columnar arrangement of 8 or 16 columns has also been reported from many insects (Williams, 1975; Hanesch et al., 1989; Müller et al., 1997). Such an internal modular organization of the CB composed of layers and columns with chiasmal fiber crossings might be an apomorphy of Panarthropoda (Arthropoda, Onychophora and Tardigrada) (reviewed by Homberg, 2008).

Only with the Asn¹³-OK antibody, we stained in *F. candida* and *T. bielanensis* an arc-shaped structure in a close antero-ventral neighborhood to the non-columnar layer of the CB. Owing to its close proximity, this structure could be a third layer of the collembolan CB. This interpretation corresponds with descriptions of a multi-layered upper unit of the CB in numerous insect species (e.g., Williams, 1975; Hanesch et al., 1989). Alternatively, the arc-shaped structure could also be homologous to the lower unit of the central body of insects (also termed lower division or ellipsoid body in Diptera, see Hanesch et al., 1989). Kühnle (1913) identified a large CB in the collembolan *Tomocerus flavescens* (a close relative of *F. candida*, see D'Haese, 2002), which he did not distinguish as an upper and a lower unit or as different layers. However, a columnar organization of the fan-shaped CB is evident from his illustrations (Kühnle, 1913; Fig. 30). Strausfeld et al. (2006) also described an unpaired midline neuropil within the brain of the collembolan *Neanura* sp., which they termed a “fan-shaped body”, but the authors did not subdivide it into an upper and a lower unit. Tyszwicz (1981) analyzed the brains of *T. bielanensis* and described an oval CB, which he named “corpus centrale”. He characterized the CB as a structure composed of ramifying nerves originating in other parts of the brain and forming separate entanglements that divide the CB into parts, but he did not describe these parts as columns or layers. However, Tyszwicz (1981) recognized a kidney-shaped upper part and an oval lower part in the CB lying next to each other, which might be homologous to the upper and lower units of the CB in Dicondylia (Zygentoma + Pterygota) (reviewed by Homberg, 2008).

A CB not divided in an upper and a lower unit in the collembolan brain would be congruent with the theory that all hexapods basal to Dicondylia possess undivided CBs, but would oppose Tyszwicz (1981). However, the absence of a lower unit of the CB could reflect the close relation of the phylogenetic basal Hexapoda to the Decapoda, which also lack the lower unit of the CB.

4.2. Organization of the protocerebral bridge and associated structures

The PB of *T. bielanensis* is an elongated, arc-shaped structure, with its open side pointing ventrally. The PB compartments of both hemispheres are fused to a single structure. This compares well, e.g., to the PB of the orthopteran *Schistocerca gregaria* (see Kurylas et al., 2008), the hymenopteran *Apis mellifera* (see Brandt et al., 2005) and the coleopteran *Tribolium castaneum* (see Dreyer et al., 2010), but contrasts with the findings in the lepidopteran *M. sexta* (el Jundi et al., 2009), where the PB neuropil is divided along the brain midline. We were unable to identify columns in the PB of the collembolan *T. bielanensis*, which have been described from other

insects (e.g., Williams, 1975; Hanesch et al., 1989; Wegerhoff et al., 1996; Müller et al., 1997). This is in accordance with a previous study of the same species. Tyszwicz (1981) characterized a PB in *T. bielaniensis* (he termed it “pons protocerebralis”) close to the pars intercerebralis, but he did not describe any additional anatomical details in this structure. Kühnle (1913) also identified in the collembolan *T. flavescens* a well-defined PB and described it as a conspicuous arc-shaped structure, but he did not report any further details on its organization either.

Only in *F. candida* and only with anti-Asn¹³-OK immunostaining, we were able to describe structures located ventrally to the CB resembling the noduli. Thus far, such noduli have been reported only from pterygote insects as they are obviously lacking in apterygote insects and crustaceans (reviewed by Homberg, 2008). Their functional organization is largely unknown. Because noduli appear only in Pterygota, they might be associated with the evolution of wings. However, the loss of flight does not necessarily lead to a lack of noduli (Kühnle, 1913; Goll, 1967). The presence of noduli in Collembola would suggest a close relationship of this group to the pterygote insects and a reduction of wings in this taxon. However, this assumption contradicts the results of phylogenetic analyses of molecular and morphological data, which place Collembola into a close relationship to other monocondylic hexapod taxa (e.g., Mallatt and Giribet, 2006; Misof et al., 2007; Regier et al., 2010; Bitsch and Bitsch, 2000), or even outside of Hexapoda (Spears and Abele, 1998; Nardi et al., 2001, 2003). We therefore suggest that the noduli might be a ground pattern feature of Hexapoda, which supports the inclusion of Collembola in this taxon. Future analyses of brain architecture in diplurans and proturans will show whether noduli-like structures occur in their brains.

4.3. Homology of the mushroom body-like structures in collembolans and dicondylic insects

MBs are composed of neurites from globuli cells (termed Kenyon cells in Hexapoda) that form parallel arranged bundles, the so-called peduncles, and usually terminate in medial and vertical lobes. MBs with a calyx are a character of dicondylic insects and are missing in Archaeognatha and Crustacea (reviewed in Strausfeld et al., 2009).

The anti-DCO antiserum reliably labels all Kenyon cell subpopulations in the MBs of the neopteran insects (Farris and Sinakevitch, 2003; Farris and Strausfeld, 2003; Farris et al., 2004; Farris, 2005a, b).

Using the same anti-DCO antibody, we stained a cap-like structure in each hemisphere of the collembolan brain. These cap-like structures resemble the calyx structures of the MBs in the terrestrial insects possessing olfactory glomeruli (Farris, 2005b; Strausfeld et al., 2009). An insect-like arrangement of Kenyon cells (Farris, 2005b; Strausfeld et al., 2009) could not be observed in the investigated collembolan species, but we found a small posterolateral DCO-immunoreactive cell group on each side of the brain associated with each cap-like structure. These cell groups might be homologous to the Kenyon cells of the dicondylic insects. However, the number of putative Kenyon cells labeled with the anti-DCO antiserum is much lower in collembolans as compared to the higher numbers of Kenyon cells in the neopteran insects ranging from about 2000 Kenyon cells in the dipteran *D. melanogaster* to 170,000–180,000 in the hymenopteran *A. mellifera* and 175,000 in the blattodean *P. americana* (see Neder, 1959; Hinke, 1961; Witthöft, 1967; Technau, 1984; Aso et al., 2009). Also in the wingless Zygentoma, representatives of which are assumed to show the most ancestral architecture of the MBs among the hexapods (Strausfeld et al., 2009), higher numbers of Kenyon cells have been described (Farris, 2005a). If the cap-like structures of collembolans are homologous to those of the dicondylic insects, it remains unclear whether the low number of putative Kenyon cells was

reduced in this group or whether it represents an ancestral feature of Hexapoda.

Further evidence suggesting homology of the MB-like structures in Collembola with the insect MBs is provided by immunostainings against Asn¹³-OK-ir, α -tubulin and by our analyses of semi-thin sections stained with methylene blue. The MBs of the cockroach *Leucophaea maderae*, the locust *S. gregaria*, and the silverfish *Lepisma saccharina* also show an Asn¹³-OK-ir (Hofer et al., 2005). Like in these insect species, the cap-like structure in the collembolan species *F. candida* and *P. armata* exhibits Asn¹³-OK immunoreactivity. This correspondence suggests a possible homology of the collembolan cap-like structures with the typical caps of the neopteran MBs.

Apart from the cap-like structures, the MBs of collembolans possess pedunculus-like structures. These structures are evident as conspicuous bundles of nerves, visualized by anti- α -tubulin immunohistochemistry, which reliably shows the pedunculi in the insect MBs (e.g., Kononenko and Pflüger, 2007). Furthermore, in semi-thin sections, stained with methylene blue, we identified a pedunculus-like structure resembling the α -tubulin staining pattern in position and shape. In addition, we identified a short median structure in the anterior portion of the pedunculus-like structure, which might be homologous to the medial lobes of the MBs found in dicondylic insects. Kühnle (1913) also reported similar structures from the brain of the collembolan *T. flavescens*. He described the MB-like structures as “Pilze”, the calyx as “Pilzhut”, the Kenyon cells as “Pilzzellen” or “Pilzzellenhaube” and the pedunculus as “Pilzstiel”. Furthermore, he reported the lack of the fibrous mass and described the pedunculi as accumulations of fiber bundles and the calyx as a branch of these bundles at the posterior border of the brain, which is in accordance with our findings.

Based on the results of our antibody labelings against DCO, Asn¹³-OK and α -tubulin and analyses of the methylene blue-stained semi-thin sections, which correlate with Kühnle's (1913) observation, we therefore interpret the MB-like structures in the collembolan brain as the homologs of the MBs in dicondylic insects. The structure of the collembolan MBs, with missing vertical lobes and with only a few Kenyon cells, seems to be simpler than that in various dicondylic insects (Strausfeld et al., 2009). However, the architecture of the MBs is also simple at least in some dicondylic insects. For example, the MBs of mayflies lack a calyx and a vertical lobe and possess only small clusters of Kenyon cells (Strausfeld et al., 2009). This variation in structural composition of the MBs might be due to functional constraints and does not necessarily contradict the homology hypothesis of these structures among the hexapods.

Interestingly, the brain of representatives of Archaeognatha, the sister group of Dicondylia (Zygentoma + Pterygota), does not show any MB-like structures (Strausfeld et al., 2009). In contrast, anatomical structures very similar to the MBs of the terrestrial Pterygota have been demonstrated in the zygentoman *Thermobia domestica* (see Farris, 2005a). In the light of our new findings, thus, the MBs were either reduced secondarily in Archaeognatha or, alternatively, the MB-like structures might have evolved independently in Collembola and Dicondylia. Future detailed analyses of the brain in Protura and Diplura, the putative sister groups of Collembola (see Mallatt and Giribet, 2006; Misof et al., 2007; Regier et al., 2010), will shed light on the homology and evolution of the MB-like structures in hexapods.

4.4. Insights into the antennal lobes and their function

The ALs and their glomeruli, which located anteriorly in the deutocerebrum are, together with the CB, the most conspicuous neuropils in the collembolan brain. The collembolan ALs are

remarkably similar to those in Pterygota (Schachtner et al., 2005). Kühnle (1913) also identified ALs including glomeruli (he termed them “Riechlappen” with “Riechbällchen”) in the collembolan brain. However, he did not provide any numbers of glomeruli but described them as less numerous. Tysiewicz (1981) characterized the ALs (“lobi olfactorii”) in the collembolan *T. bielanensis*, which display a small number of about nine antennal glomeruli in each AL.

Based on the antennal backfills in *F. candida* and *P. armata*, we estimate the number of glomeruli in the ALs of these species between 20 and 30. In contrast, the anti-synapsin immunostainings of the large collembolan species *T. bielanensis* revealed only 10–15 glomeruli in each AL. However, double immunostainings against synapsin and several neuromediators suggest a higher number of glomeruli, similar to the numbers estimated for the two other collembolan species studied. Thus, in either case we found more glomeruli than described in a previous study of the *T. bielanensis* brain (Tysiewicz, 1981).

The glomerular organization of the olfactory brain centers differs substantially across the Tetraconata. Within the crustaceans, the olfactory lobe of Decapoda consists of wedge- or barrel-shaped glomeruli ranging in number between 150 and 1300 and which establish a peripheral cortex; a columnar organization into several vertical columns (or sub-lobes) has been described in Cephalocarida (see also Stegner and Richter, 2011); spherical glomeruli can be found in Stomatopoda and Remipedia, and slightly elongated glomeruli of irregular shape in Leptostraca (reviewed by Schachtner et al., 2005). Archaeognatha possess a small number of elongated glomeruli (Mißbach et al., 2011). Within most dicondylic insects, typically heteromorphic spheroidal glomeruli ranging from about 40 to 60 glomeruli, e.g., in the ensiferan Orthoptera and in Diptera, to about 500 glomeruli in ants have been described (Ignell et al., 2005; Schachtner et al., 2005; Ghaninia et al., 2007; Mysore et al., 2009). In contrast, the ALs of the caeliferan Orthoptera contain about 2000–3000 so-called microglomeruli (Ignell et al., 2001; Schachtner et al., 2005) and several taxa, including Palaeoptera (Odonata and Ephemeroptera), certain Coleoptera and Hemiptera, lack olfactory glomeruli, which might be due to a secondary loss of these structures in these taxa (reviewed by Schachtner et al., 2005). In summary, the neuroarchitecture of the collembolan AL resembles that in most insects rather than that in crustaceans.

In both *F. candida* and *P. armata*, we found large median glomeruli (4–6 in *F. candida* and 3–4 in *P. armata*) located near the entrance of the antennal nerve. These glomeruli exhibit higher immunoreactivity to several neuromediator antibodies (Dip-AST 7, Asn¹³-OK, and Pea-MIP) than the remaining smaller glomeruli. It is known from some insect species, that morphologically diverse glomeruli are involved in special functions, e.g., in the detection of pheromones or carbon dioxide. In male cockroaches (Boeckh et al., 1970; Rospars, 1988; Hösl, 1990) and male moths (Hansson, 1997; Anton and Homberg, 1999), morphologically distinct larger glomeruli are innervated by pheromone-sensitive olfactory receptor neurons. In female sphinx moths, corresponding large female glomeruli are specialized for identifying host plant-specific odors. (King et al., 2000). These pheromone-specific glomeruli are typically clustered near the entrance of the antennal nerve into the AL. According to our data, a similar position of large glomeruli is evident in the ALs of Collembola.

The labial pit organ glomerulus (LPOG) is a single glomerulus in the ALs of Lepidoptera, innervated exclusively by carbon dioxide-sensitive olfactory receptor neurons of the labial palps (Kent et al., 1986; Guerenstein et al., 2004). We observed the larger, median glomeruli only in the collembolans *F. candida* and *P. armata*, but not in *T. bielanensis*. Notably, *F. candida* and *P. armata* have a parthenogenetic mode of reproduction (Hopkin, 1997; Wiles and Krogh,

1998). Thus, pheromone sensing seems less important in these species. However, such prominent glomeruli could serve a variety of other functions, including search for the optimal substrate for egg laying.

Typical for insect glomeruli, the antibodies against neuromediators label all glomeruli in a similar pattern (Schachtner et al., 2005). Only recently, exceptions for some neuropeptides were described in two dipteran insects. In the fly *D. melanogaster*, 13 glomeruli express short neuropeptide F, which stems from innervating olfactory receptor neurons (Carlsson et al., 2010). In the nematoceran *Aedes aegypti*, antisera against allatostatin-A and FMRFamide strongly label subsets of a few glomeruli, while other glomeruli show only a weak or no immunostaining (R. Ignell and J. Schachtner, unpublished data). A striking feature of the collembolan ALs is the fact that none of the used neurotransmitter antisera labels all glomeruli. Instead, only individual glomeruli or subsets of glomeruli are stained.

In all investigated collembolan species, one glomerulus per AL was intensely stained by the anti-5HT antiserum. Only in *T. bielanensis* three additional neighboring glomeruli showed a weak staining. The innervation arises from a centrifugal neuron with its soma most likely located in the mandibular ganglion of the SEG. The 5HT-ir fibers stemming from a single or a few (centrifugal) neurons with multi-glomerular arborizations have been suggested as a synapomorphic character of the primary olfactory centers of crustaceans and hexapods (reviewed by Schachtner et al., 2005).

In summary, the described pattern of neuromediator immunostaining in collembolans is unique compared to insects and crustaceans. It could well represent a basic innervation pattern provided by neurons, which regulate the function of a defined glomerulus or a set of glomeruli responsible for a certain olfactory context. Assuming that collembolans have a relatively simple olfactory system, with only a few olfactory receptors and receptor neurons, such an organization could indeed represent a simple way of coordinating the olfactory signaling in the ALs. Thus, more complex mechanisms of regulating the AL activity might be reflected by the multi-glomerular innervation, which is found for most neuromediators in the neopteran insects.

5. Conclusions

Paired antennal lobes with spheroidal glomeruli, a fan-shaped, columnar-structured central body divided in upper and lower units, a protocerebral bridge and mushroom bodies are typical characters of the brain of the dicondylic insects. Our analysis of the neuroarchitecture of the collembolan brain revealed paired antennal lobes with 20–30 spheroidal glomeruli, a fan-shaped, columnar-structured central body, a protocerebral bridge-like structure and simply organized mushroom bodies. However, the subdivision of the central body into an upper and a lower unit remains uncertain. A structure, associated with the central body, can either be interpreted as a lower unit or as a third layer of the central body. The mushroom bodies show a simple organization as they contain only a few Kenyon cells and the vertical lobes are missing. Furthermore, immunostaining with several antisera against neuropeptides and serotonin revealed individual glomeruli or small groups of glomeruli in the collembolan antennal lobes, even though their low number is uncommon for insects.

In summary, the results of our analysis of the collembolan brain correspond well with the composition of a typical hexapod brain. The similar organization of the central body and the protocerebral bridge, the occurrence of noduli, the specific composition of the mushroom bodies and antennal lobes in collembolans and dicondylic insects suggest a close relationship of Collembola to one of the hexapod taxa (Bitsch and Bitsch, 2000; Mallatt and Giribet, 2006; Misof et al., 2007;

Regier et al., 2010) rather than to one of the subgroups of Crustacea or to the entire Pancrustacea (Spears and Abele, 1998; Nardi et al., 2001, 2003). However, the precise phylogenetic position of Collembola within Hexapoda remains unclear. The simple architecture of the mushroom bodies and the apparent absence of the lower unit of the central body might be either ancestral features of Hexapoda or derived characters of Collembola. To polarize these characters, detailed analyses of the architecture of the brain are required in Diplura and Protura. These analyses will help clarify the phylogenetic relationships of different hexapod subgroups.

Acknowledgments

The authors thank Dr. H. Agricola (University Jena, Germany; anti-Dip-AST 7, anti-Lom-TK II, and anti-Pea-MIP), Dr. E. Buchner (University Würzburg, Germany; anti-synapsin), Dr. H. Dirksen (University of Stockholm, Sweden; anti-Asn¹³-OK), Dr. M. Eckert (University of Jena, Germany; anti-Pea-PVK 2), Dr. E. Marder (Brandeis University, MA, USA; anti-FMRFamide), Dr. D. Kalderon (Columbia University, NY, USA; anti-DC0), and Dr. J. Veenstra (University of Bordeaux, France; anti-Mas-AT) for providing antibodies. Animals were kindly provided by Dr. M. Maraun (TU Darmstadt, Germany; *P. armata*) and Dr. D. Russell (Public Museum of Natural History, Görlitz, Germany; *T. bielanensis*). The authors are also grateful to Dr. U. Homberg and Dr. C. Heuer (both University of Marburg, Germany) and Dr. S. Heinze (University of Massachusetts Medical School, Worcester, MA, USA) for many fruitful discussions and M. Kern and H. Kisselbach-Heckmann (University of Marburg, Germany) for expert technical assistance. We are indebted to two anonymous reviewers and the guest editors whose valuable comments greatly improved the manuscript.

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.asd.2011.02.003.

References

- Anton, S., Homberg, U., 1999. Antennal lobe structure. *Insect Olfaction* 1, 98–122.
- Aso, Y., Grübel, K., Busch, S., Friedrich, A.B., Siwanowicz, I., Tanimoto, H., 2009. The mushroom body of adult *Drosophila* characterized by GAL4 drivers. *Journal of Neurogenetics* 23, 156–172.
- Bitsch, C., Bitsch, J., 2000. The phylogenetic interrelationships of the higher taxa of apterygote hexapods. *Zoologica Scripta* 29, 131–156.
- Boeckh, J., Sandri, C., Akert, K., 1970. Sensory inputs and synaptic connections in the insect CNS. Experimental degeneration in the antennal afferent pathway in the suprasophageal ganglia of flies and cockroaches. *Zeitschrift für Zellforschung und mikroskopische Anatomie* 103, 429–446.
- Brandt, R., Rohlfing, T., Rybak, J., Kroczyk, S., Maye, A., Westerhoff, M., Hege, H.C., Menzel, R., 2005. Three-dimensional average-shape atlas of the honeybee brain and its applications. *The Journal of Comparative Neurology* 492, 1–19.
- Bungart, D., Dirksen, H., Keller, R., 1994. Quantitative determination and distribution of the myotropic neuropeptide orokinin in the nervous system of astacidean crustaceans. *Peptides* 15, 393–400.
- Burrows, M., 1996. *The Neurobiology of an Insect Brain*. Oxford University Press, Oxford, 682 p.
- Carlsson, M.A., Diesner, M., Schachtner, J., Nässel, D.R., 2010. Multiple neuropeptides in the *Drosophila* antennal lobe suggest complex modulatory circuits. *The Journal of Comparative Neurology* 518, 3359–3380.
- Cassagnau, P., Juberthie, C., 1966. Neurosécrétion et organes endocrines chez *Tomocerus minor* (Collembolus). *Comptes Rendus de l'Académie des sciences* 267, 793–796.
- Cassagnau, P., Juberthie, C., 1967a. Structures nerveuses, neurosécrétion et organes endocrines chez les Collembolus: I. Poduromorphes. *Bulletin de la Société d'histoire naturelle de Toulouse* 103, 178–222.
- Cassagnau, P., Juberthie, C., 1967b. Structures nerveuses, neurosécrétion et organes endocrines chez les Collembolus: II. Le complexe cérébral des Entomobryomorphes. *General and Comparative Endocrinology* 8, 489–502.
- Cassagnau, P., Juberthie, C., Raynal, G., 1968. Structures nerveuses, neurosécrétion et organes endocrines chez les Collembolus: III. Le complexe cérébral des Symphyléones 10, 61–69.
- Dacks, A.M., Christensen, T.A., Hildebrand, J.G., 2006. Phylogeny of a serotonin-immunoreactive neuron in the primary olfactory center of the insect brain. *The Journal of Comparative Neurology* 498, 727–746.
- D'Haese, C.D., 2002. Were the first springtails semi-aquatic? A phylogenetic approach by means of 28S rDNA and optimization alignment. *Proceedings of the Royal Society* 269, 1143–1151.
- Dohle, W., 1997a. Myriapod–insect relationships as opposed to an insect–crustacean sister group relationship. In: Fortey, R.A., Thomas, R.H. (Eds.), *Arthropod Relationships*. Chapman and Hall, London, pp. 305–316.
- Dohle, W., 1997b. Are the insects more closely related to the crustaceans than to the myriapods? *Entomologica Scandinavica* 51 (Suppl.), 7–16.
- Dohle, W., 2001. Are the insects terrestrial crustaceans? A discussion of some new facts and arguments and the proposal of the proper name 'Tetraconata' for the monophyletic unit Crustacea + Hexapoda. *Annales de la Société entomologique de France* 37, 85–103.
- Dreyer, D., Vitt, H., Dippel, F., Goetz, B., el Jundi, B., Kollmann, M., Huetteroth, W., Schachtner, J., 2010. 3D standard brain of the red flour beetle *Tribolium castaneum*: a tool to study metamorphic development and adult plasticity. *Frontiers in Systems Neuroscience* 4, 3. doi:10.3389/fnro.0003.2010.
- Eckert, M., Herbert, Z., Pollak, E., Molnar, L., Predel, R., 2002. Identical cellular distribution of all abundant neuropeptides in the major abdominal neurohemal system of an insect (*Periplaneta americana*). *The Journal of Comparative Neurology* 452, 264–275.
- Fanzenbruck, M., Harzsch, S., Wägele, J.W., 2004. The brain of the Remipedia (Crustacea) and an alternative hypothesis on their phylogenetic relationships. *Proceedings of the National Academy of Sciences United States of America* 101, 3868–3873.
- Farris, S.M., 2005a. Developmental organization of the mushroom bodies of *Thermobia domestica* (Zygentoma, Lepismatidae): insights into mushroom body evolution from a basal insect. *Evolution and Development* 7, 150–159.
- Farris, S.M., 2005b. Evolution of insect mushroom bodies: old clues, new insights. *Arthropod Structure and Development* 34, 211–234.
- Farris, S.M., Sinakevitch, I., 2003. Development and evolution of the insect mushroom bodies: towards the understanding of conserved developmental mechanisms in a higher brain center. *Arthropod Structure and Development* 32, 79–101.
- Farris, S.M., Strausfeld, N.J., 2003. A unique mushroom body substructure common to both basal cockroaches and to termites. *The Journal of Comparative Neurology* 456, 305–320.
- Farris, S.M., Abrams, A.I., Strausfeld, N.J., 2004. Development and morphology of Class II Kenyon cells in the mushroom bodies of the honey bee, *Apis mellifera*. *The Journal of Comparative Neurology* 474, 325–339.
- Friedrich, M., Tautz, D., 1995. Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. *Nature* 376, 165–167.
- Garcia-Machado, E., Pempera, M., Dennebouy, N., Olivia-Suarez, M., Mounolou, J.-C., Monnerot, M., 1999. Mitochondrial genes collectively suggest the paraphyly of crustacea with respect to insecta. *Journal of Molecular Evolution* 49, 142–149.
- Ghaninia, M., Hansson, B.S., Ignell, R., 2007. The antennal lobe of the African malaria mosquito, *Anopheles gambiae* – innervation and three-dimensional reconstruction. *Arthropod Structure and Development* 36, 23–39.
- Goll, W., 1967. Strukturuntersuchungen am Gehirn von *Formica*. *Zeitschrift für Morphologie und Ökologie der Tiere* 59, 143–210.
- Guerenstein, P.G., Christensen, T.A., Hildebrand, J.G., 2004. Sensory processing of ambient CO₂ information in the brain of the moth *Manduca sexta*. *Journal of Comparative Physiology A* 190, 707–725.
- Hösl, M., 1990. Pheromone-sensitive neurons in the deutocerebrum of *Periplaneta americana*: receptive fields on the antenna. *Journal of Comparative Physiology A* 167, 321–327.
- Hanesch, U., Fischbach, K.F., Heisenberg, M., 1989. Neuronal architecture of the central complex in *Drosophila melanogaster*. *Cell and Tissue Research* 257, 343–366.
- Hansson, B.S., 1997. Antennal lobe projection patterns of pheromone specific olfactory receptor neurons in moths. In: Carde, R.T., Minks, A.K. (Eds.), *Insect Pheromone Research: New Directions*. Chapman and Hall, New York, pp. 164–183.
- Hanström, B., 1940. Inkretorische Organe, Sinnesorgane und Nervensystem des Kopfes einiger niederer Insektenordnungen. *Kungl Svenska Vetenskaps Akademiens Handlingar* 18, 1–266.
- Hinke, W., 1961. Das relative postembryonale Wachstum der Hirnteile von *Culex pipiens*, *Drosophila melanogaster* und *Drosophila* mutanten. *Zeitschrift für Morphologie und Ökologie der Tiere* 50, 81–118.
- Hofer, S., Dirksen, H., Tollback, P., Homberg, U., 2005. Novel insect orokinin: characterization and neuronal distribution in the brains of selected dicondylid insects. *The Journal of Comparative Neurology* 490, 57–71.
- Homberg, U., 1994. *Distribution of Neurotransmitters in the Insect Brain*. Gustav Fisher, Stuttgart, Jena, New York.
- Homberg, U., 2002. Neurotransmitters and neuropeptides in the brain of the locust. *Microscopy Research and Technique* 56, 189–208.
- Homberg, U., 2008. Evolution of the central complex in the arthropod brain with respect to the visual system. *Arthropod Structure and Development* 37, 347–362.
- Hopkin, S.P., 1997. *Biology of the Springtails (Insecta: Collembola)*. Oxford University Press, Oxford, UK.
- Ignell, R., Anton, S., Hansson, B.S., 2001. The antennal lobe of Orthoptera anatomy and evolution. *Brain, Behavior and Evolution* 57, 1–17.
- Ignell, R., Dekker, T., Ghaninia, M., Hansson, B.S., 2005. Neuronal architecture of the mosquito deutocerebrum. *Journal of Comparative Neurology* 493, 207–240.

- el Jundi, B., Huetteroth, W., Kurylas, A.E., Schachtner, J., 2009. Anisometric brain dimorphism revisited: implementation of a volumetric 3D standard brain in *Manduca sexta*. The Journal of Comparative Neurology 517, 210–225.
- Kühnle, K.F., 1913. Vergleichende Untersuchungen über das Gehirn, die Kopfnerven und die Kopfdrüsen des gemeinen Ohrwurms (*Forficula auricularia* L.) mit Bemerkungen über die Gehirne und Kopfdrüsen eines Springschwanzes (*Tomocerus flavescens* Tullb.), einer Termitenarbeiterin (*Eutermes peruanus* f. *aequatorianus* Holmgr.) und der indischen Stabheuschrecke (*Dixippus morosus*). Jenaische Zeitschrift für Naturwissenschaften 50, 147–276.
- Kent, K.S., Harrow, I.D., Quartararo, P., Hildebrand, J.G., 1986. An accessory olfactory pathway in Lepidoptera: the labial pit organ and its central projections in *Manduca sexta* and certain other sphinx moths and silk moths. Cell and Tissue Research 245, 237–245.
- King, J.R., Christensen, T.A., Hildebrand, J.G., 2000. Response characteristics of an identified, sexually dimorphic olfactory glomerulus. Journal of Neuroscience 20, 2391–2399.
- Kingan, T.G., Teplow, D.B., Phillips, J.M., Riehm, J.P., Rao, K.R., Hildebrand, J.G., Homberg, U., Kammer, A.E., Jardine, I., Griffin, P.R., Hunt, F., 1990. A new peptide in the FMRFamide family isolated from the CNS of the hawkmoth, *Manduca sexta*. Peptides 11, 849–856.
- Kingan, T.G., Shabanowitz, J., Hunt, D.F., Witten, J.L., 1996. Characterisation of two myotropic neuropeptides in the FMRFamide family from segmental ganglia of the moth *Manduca sexta*: candidate neurohormones and neuromodulators. Journal of Experimental Biology 199, 1095–1104.
- Klages, B.R.E., Heimbeck, G., Godenschwege, T.A., Hofbauer, A., Pflugfelder, G.O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S., Buchner, E., 1996. Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. Journal of Neuroscience 16, 3154–3165.
- Kononenko, N.L., Pflüger, H.J., 2007. Dendritic projections of different types of octopaminergic unpaired median neurons in the locust metathoracic ganglion. Cell and Tissue Research 330, 179–195.
- Korr, H., 1968. Das postembryonale Wachstum verschiedener Hirnbereiche bei *Orchesella villosa* L. (Ins., Collembola). Zoomorphologie 62, 389–422.
- Kurylas, A.E., Rohlfing, T., Kroficzki, S., Jenett, A., Homberg, U., 2008. Standardized atlas of the brain of the desert locust, *Schistocerca gregaria*. Cell and Tissue Research 333, 125–145.
- Lane, M.E., Kalderon, D., 1993. Genetic investigation of cAMP-dependent protein kinase function in *Drosophila* development. Genes and Development 7, 1229–1243.
- Lauga-Reyrel, F., 1984a. Modifications ultrastructurales des corps allates au cours du développement et de l'écomorphose chez *Hypogastrura tullbergi* (Tullberg) (Collembola: Hypogastruridae). International Journal of Insect Morphology and Embryology 13, 411–424.
- Lauga-Reyrel, F., 1984b. Etude anatomique et ultrastructurale des organes neuro-hémaux et des corps allates d'*Hypogastrura tullbergi* (Tullberg) (Collembola: Hypogastruridae). International Journal of Insect Morphology and Embryology 13, 399–410.
- Mallatt, J.M., Giribet, G., 2006. Further use of nearly complete 28S and 18S rRNA genes to classify Ecdysozoa: 37 more arthropods and a kinorhynch. Molecular Phylogenetics and Evolution 40, 772–794.
- Mallatt, J.M., Garey, J.R., Shultz, J.W., 2004. Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin. Molecular Phylogenetics and Evolution 31, 178–191.
- Marder, E., Calabrese, R.L., Nusbaum, M.P., Trimmer, B., 1987. Distribution and partial characterization of RFamide-like peptides in the stomatogastric nervous system of the rock crab, *Cancer borealis*, and the spiny lobster *Panulirus interruptus*. The Journal of Comparative Neurology 259, 150–163.
- Miao, Y., Waters, E.M., Witten, J.L., 1998. Developmental and regional-specific expression of FLRFamide peptides in the tobacco hornworm, *Manduca sexta*, suggests functions at ecdysis. Journal of Neurobiology 37, 469–485.
- Miñobach, C., Harzsch, S., Hansson, B.S., 2011. New insights into an ancient insect nose: The olfactory pathway of *Lepismachilis y-signata* (Archaeognatha: Machilidae). Arthropod Structure & Development 40, 317–333.
- Misof, B., Niehuis, O., Bischoff, I., Rickert, A., Erpenbeck, D., Staniczek, A., 2007. Towards an 18S phylogeny of hexapods: accounting for group-specific character covariance in optimized mixed nucleotide/doublet models. Zoology 110, 409–429.
- Müller, M., Homberg, U., Kühn, A., 1997. Neuroarchitecture of the lower division of the central body in the brain of the locust (*Schistocerca gregaria*). Cell and Tissue Research 288, 159–176.
- Mysore, K., Subramanian, K.A., Sarasij, R.C., Suresh, A., Shyamala, B.V., VijayRaghavan, K., Rodrigues, V., 2009. Caste and sex specific olfactory glomerular organization and brain architecture in two sympatric ant species, *Camponotus sericeus* and *Camponotus compressus* (Fabricius, 1798). Arthropod Structure and Development 38, 485–497.
- Nässel, D.R., 2002. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Progress in Neurobiology 68, 1–84.
- Nässel, D.R., Homberg, U., 2006. Neuropeptides in interneurons of the insect brain. Cell and Tissue Research 326, 1–24.
- Nässel, D.R., Winther, A.M., 2010. *Drosophila* neuropeptides in regulation of physiology and behavior. Progress in Neurobiology 92, 42–104.
- Nardi, F., Carapelli, A., Fanciulli, P.P., Dallai, R., Frati, F., 2001. The complete mitochondrial DNA sequence of the basal hexapod *Tetradontophora bielensis*: evidence for Heteroplasmy and tRNA Translocations. Molecular Biology and Evolution 18, 1293–1304.
- Nardi, F., Spinsanti, G., Boore, J.L., Carapelli, A., Dallai, R., Frati, F., 2003. Hexapod origins: monophyletic or paraphyletic? Science 299, 1887–1889.
- Neder, R., 1959. Allometrisches Wachstum von Hirnteilen bei drei verschieden großen Schabenarten. Zoologische Jahrbücher Anatomie 77, 411–464.
- Osorio, D.C., Averof, M., Bacon, J.P., 1995. Arthropod evolution: great brains, beautiful bodies. Trends in Ecology and Evolution 10, 449–454.
- Panov, A.A., 1959. Structure of the insect brain at successive stages of postembryonic development. II. The central body. Entomological Review 38, 276–284.
- Pflugfelder, O., 1937. Vergleichend-anatomische, experimentelle und embryologische Untersuchungen über das Nervensystem und die Sinnesorgane der Rhynchoten. Zoologica 93, 1–102.
- Predel, R., Rapus, J., Eckert, M., 2001. Myoinhibitory neuropeptides in the American cockroach. Peptides 22, 199–208.
- Regier, J.C., Shultz, J.W., Zwick, A., Hussey, A., Ball, B., Wetzer, R., Martin, J.W., Cunningham, C.W., 2010. Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature 463, 1079–1083.
- Richter, S., 2002. The Tetraconata concept: hexapod–crustacean relationships and the phylogeny of Crustacea. Organisms Diversity and Evolution 2, 217–237.
- Rospars, J.P., 1988. Structure and development of the insect antennodeutocerebral system. International Journal of Insect Morphology and Embryology 17, 243–294.
- Sandeman, D., 1992. Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. The Biological Bulletin 183, 304–326.
- Schachtner, J., Schmidt, M., Homberg, U., 2005. Organization and evolutionary trends of primary olfactory brain centers in Tetraconata (Crustacea + Hexapoda). Arthropod Structure and Development 34, 257–299.
- Slifer, E.H., Sekhon, S.S., 1978. Sense organs on the antennae of two species of Collembola (insecta). Journal of Morphology 157, 1–19.
- Spears, T., Abele, L.G., 1998. Crustacean phylogeny inferred from 18S rDNA. In: Fortey, R.A., Thomas, R.H. (Eds.), Arthropod Relationships. Chapman and Hall, London, pp. 169–187.
- Stegner, M.E.J., Richter, S., 2011. Morphology of the brain in *Hutchinsoniella macracantha* (Cephalocarida, Crustacea). Arthropod Structure & Development 40, 221–243.
- Strausfeld, N.J., 1976. Atlas of an Insect Brain. Springer, Heidelberg.
- Strausfeld, N.J., 2005. The evolution of crustacean and insect optic lobes and the origins of chiasmata. Arthropod Structure and Development 34, 235–256.
- Strausfeld, N.J., Strausfeld, C.M., Loesel, R., Rowell, D., Stowe, S., 2006. Arthropod phylogeny: onychophoran brain organization suggests an archaic relationship with a chelicerate stem lineage. Proceedings of the Royal Society B: Biological Sciences 273, 1857–1866.
- Strausfeld, N.J., Sinkevitch, I., Brown, S.M., Farris, S.M., 2009. Ground plan of the insect mushroom body: functional and evolutionary implications. The Journal of Comparative Neurology 513, 265–291.
- Technau, G.M., 1984. Fiber number in the mushroom bodies of adult *Drosophila melanogaster* depends on age, sex, and experience. Journal of Neurogenetics 1, 113–126.
- Tyszwiecki, K., 1981. Anatomy and histology of brain of *Tetradontophora bielensis* (Waga) (Collembola). Acta Biologica Cracoviensia. Zoologia 23, 177–184.
- Veenstra, J.A., Hagedorn, H.H., 1993. Sensitive enzyme immunoassay for Manduca allatotropin and the existence of an allatotropin–immunoreactive peptide in *Periplaneta americana*. Archives of Insect Biochemistry and Physiology 23, 99–109.
- Veenstra, J.A., Lau, G.W., Agricola, H.J., Petzel, D.H., 1995. Immunohistochemical localization of regulatory peptides in the midgut of the female mosquito *Aedes aegypti*. Histochem. Cell Biology 104, 337–347.
- Vitzthum, H., Homberg, U., Agricola, H., 1996. Distribution of Dipallatostatin I-like immunoreactivity in the brain of the locust *Schistocerca gregaria* with detailed analysis of immunostaining in the central complex. Journal of Comparative Neurology 369, 419–437.
- Wegerhoff, R., Breidbach, O., Lobemeier, M., 1996. Development of locustatachykinin immunopositive neurons in the central complex of the beetle *Tenebrio molitor*. The Journal of Comparative Neurology 375, 157–166.
- Wheeler, W.C., 1997. Sampling, groundplans, total evidence and the systematics of arthropods. In: Fortey, R.A., Thomas, R.H. (Eds.), Arthropod Relationships. Chapman and Hall, London, pp. 87–96.
- Wiles, J.A., Krogh, P.A., 1998. Tests with the collembolans *Isotoma viridis*, *Folsomia candida* and *Folsomia fimetaria*. In: Løkke, H., Van Gestel, C.A.M. (Eds.), Handbook of Soil Invertebrate Toxicity Tests. John Wiley, Chichester, pp. 131–156.
- Williams, J.L.D., 1975. Anatomical studies of the insect central nervous system: a ground–plan of the midbrain and an introduction to the central complex in the locust, *Schistocerca gregaria* (Orthoptera). Journal of Zoology, London 176, 67–86.
- Wilson, K., Cahill, V., Ballment, E., Benzie, J., 2000. The complete sequence of the mitochondrial genome of the crustacean *Pennaeus monodon*: are malacostracan crustaceans more closely related to insects than to branchiopods? Journal of Molecular Evolution 17, 863–874.
- Witthöft, W., 1967. Absolute Anzahl und Verteilung der Zellen im Hirn der Honigbiene. Zeitschrift für Morphologie der Tiere 61, 160–164.
- Younossi-Hartenstein, A., Salvaterra, P.M., Hartenstein, V., 2003. Early development of the *Drosophila* brain: IV. Larval neuropile compartments defined by glial septa. Journal of Comparative Neurology 455, 435–450.
- Zrzavý, J., Štys, P., 1997. The basic body plan of arthropods: insights from evolutionary morphology and developmental biology. Journal of Evolutionary Biology 10, 353–367.