SYMPOSIUM

A Hypothesis for the Composition of the Tardigrade Brain and its Implications for Panarthropod Brain Evolution

Frank W. Smith,[1,*] Paul J. Bartels[†] and Bob Goldstein[*]

*Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA; [†]Department of Biology, Warren Wilson College, PO Box 28815, Asheville, NC 9000, USA

[1]E-mail: smithfw@live.unc.edu

Synopsis Incredibly disparate brain types are found in Metazoa, which raises the question of how this disparity evolved. Ecdysozoa includes representatives that exhibit ring-like brains—the Cycloneuralia—and representatives that exhibit ganglionic brains—the Panarthropoda (Euarthropoda, Onychophora, and Tardigrada). The evolutionary steps leading to these distinct brain types are unclear. Phylogenomic analyses suggest that the enigmatic Tardigrada is a closely related outgroup of a Euarthropoda + Onychophora clade; as such, the brains of tardigrades may provide insight into the evolution of ecdysozoan brains. Recently, evolutionarily salient questions have arisen regarding the composition of the tardigrade brain. To address these questions, we investigated brain anatomy in four tardigrade species—*Hypsibius dujardini*, *Milnesium* n. sp., *Echiniscus* n. sp., and *Batillipes* n. sp.—that together span Tardigrada. Our results suggest that general brain morphology is conserved across Tardigrada. Based on our results we present a hypothesis that proposes direct parallels between the tardigrade brain and the segmental trunk ganglia of the tardigrade ventral nervous system. In this hypothesis, brain neuropil nearly circumscribes the tardigrade foregut. We suggest that the tardigrade brain retains aspects of an ancestral cycloneuralian brain, while exhibiting ganglionic structure characteristic of euarthropods and onychophorans.

Introduction

The evolutionary steps that connect disparate metazoan brains remain unclear (Schmidt-Rhaesa 2007; Hejnol and Lowe 2015). Ecdysozoa includes lineages characterized by ring-like brains—the Cycloneuralia (Nematoda, Nematomorpha, Priapulida, Kinorhyncha, and Loricifera)—and lineages characterized by ganglionic brains—the Panarthropoda (Euarthropoda, Onychophora, and Tardigrada). The cycloneuralian brain typically consists of anterior and posterior clusters of neuronal somata with a ring of circumesophageal neuropil positioned between them (Schmidt-Rhaesa 1997/1998; Richter et al. 2010; Rothe and Schmidt-Rhaesa 2010; Martín-Durán et al. 2016; Henne et al. 2017b; reviewed in Hejnol and Lowe 2015; Schafer 2016). The brains characteristic of Nematomorpha have diverged from this architecture (Schmidt-Rhaesa 1996), but retain evidence of

cycloneuralian ancestry (Henne et al. 2017a). Although often referred to as ganglia, the brain clusters of cycloneuralians are not true ganglia, because they do not contain neuropil (Richter et al. 2010; Schafer 2016). In contrast to cycloneuralian brains, panarthropods exhibit ganglionic brains (Scholtz and Edgecombe 2006; Mayer 2016; Mayer et al. 2013a). Intriguingly, Panarthropoda appears to be nested within Cycloneuralia (Campbell et al. 2011; Rota-Stabelli et al. 2013; Borner et al. 2014), suggesting that the ganglionic brains of panarthropods evolved from a ring-like cycloneuralian brain (Hejnol and Lowe 2015).

The relatively simple tardigrade brain may hold important clues regarding the transition from a cycloneuralian brain to a ganglionic brain in the panarthropod stem lineage. Unlike the brains of euarthropods and onychophorans, which are composed of multiple segmental ganglia (Scholtz and Edgecombe 2006; Strausfeld et al. 2006a;

Mayer et al. 2010; Whitington and Mayer 2011; Martin and Mayer 2015), the tardigrade brain is composed of a single segmental ganglion (Hejnol and Schnabel 2005; Gabriel et al. 2007; Mayer et al. 2013a; Gross and Mayer 2015; Smith et al. 2016; Gross et al. 2017; see Zantke et al. 2008 and Persson et al. 2012 for alternative views)—a condition that is thought to be plesiomorphic for Panarthropoda (reviewed in Strausfeld 2012; Smith and Goldstein 2017; Ortega-Hernández et al. 2017). Furthermore, Tardigrada is most likely the sister group of a Euarthropoda + Onychophora clade (Campbell et al. 2011; Rota-Stabelli et al. 2013; Dunn et al. 2014; Giribet 2016; for an alternative view, see Borner et al. 2014 and Laumer et al. 2015; reviewed in Edgecombe and Giribet, this volume). Under this hypothesis, the most recent common ancestor of Panarthropoda exhibited a brain composed of a single segmental ganglion, referred to as the protocerebrum; tardigrades retain a protocerebral brain, while more posterior segmental ganglia were incorporated into the brains of euarthropods and onychophorans after the euarthropod + onychophoran lineage diverged from Tardigrada (Martin and Mayer 2015; reviewed in Strausfeld 2012; Hejnol and Lowe 2015; Scholtz 2016; Strausfeld et al. 2016; Ortega-Hernández et al. 2017; Smith and Goldstein 2017). Additionally, in terms of its position along the body axis, the entire brain of tardigrades is most likely homologous to the entire brain of non-panarthropod invertebrate animals (Steinmetz et al. 2010; Smith et al. 2016).

Unlike the circumesophageal brains of cycloneuralians, the protocerebrum of euarthropods and onychophorans is restricted to a supraesophageal position (Eriksson and Budd 2000; Strausfeld et al. 2006a, 2006b; Martin and Mayer 2014; Ortega-Hernández et al. 2017), which raises the question of when during panarthropod evolution the protocerebrum became restricted to a supraesophageal position. The position of the tardigrade brain relative to the foregut clearly has important implications for addressing this question, but recent investigations of tardigrade brain anatomy have led to several opposing views regarding the composition and position of the tardigrade brain (Fig. 1A; Zantke et al. 2008; Persson et al. 2012; Mayer et al. 2013a, 2013b; Schulze and Schmidt-Rhaesa 2013; Persson et al. 2014; Schulze et al. 2014; Smith and Jockusch 2014). In one model, tardigrades exhibit a circumesophageal brain (Zantke et al. 2008); the circumesophageal component of the tardigrade brain—the dorsal commissure (dco) + the circumbuccal connectives (cco) + the post-oral commissure (poc)—is suggested to be directly homologous to the ring of neuropil characteristic of cycloneuralian brains

(Fig. 1B, top panel). Tardigrades also exhibit a circumesophageal brain in a second model (Fig. 1B, middle panel), but in this model the tardigrade brain is composed of three paired brain lobes—the outer lobes (ol), inner lobes (il), and ventrolateral lobes (vll)—that are not clearly relatable to cycloneuralian brains (Persson et al. 2012). In a third model, tardigrades exhibit a supraesophageal brain (Mayer et al. 2013a). This model includes a circumesophageal nervous system component—the nerve ring (nr), but the nerve ring is predicted to be part of the stomodeal nervous system, rather than part of the brain (Fig. 1B, bottom panel).

Distinguishing between different models of tardigrade brain anatomy is difficult. Different tardigrade species were investigated in different studies (Fig. 1A), raising the possibility that reported differences represent true taxonomic variation. Furthermore, different immunohistochemical and imaging approaches were used in different studies. Both of these factors may have contributed to different results, and ultimately, different conclusions. Here, we investigate brain morphology in four tardigrade species, using identical immunohistochemical and imaging approaches. This includes the first study of a member of the tardigrade lineage Apochela using these techniques. Our results suggest that brain neuropil wraps around the tardigrade foregut. Based on our results, we present a hypothesis for the evolution of the ganglionic brains of panarthropods from an ancestrally cycloneuralian state.

Materials and methods

Specimen collection

Hypsibius dujardini (Eutardigrada; Parachela) specimens were collected from a laboratory culture (Gabriel et al. 2007). We collected three wild tardigrade species from habitats in North Carolina. Based on taxonomic keys, these appear to be three new species. We refer to them as *Milnesium* n. sp., *Echiniscus* n. sp., and *Batillipes* n. sp. We collected *Milnesium* n. sp. specimens (Eutardigrada; Apochela) from lichen samples originating from Durham County, North Carolina (location: 35°59′47.5″N, 78°52′34.1″W). *Echiniscus* n. sp. (Heterotardigrada; Echiniscoidea) specimens were collected from lichen originating in Orange County, North Carolina (location: 35°54′46.4″N, 79°03′21.2″W). *Batillipes* n. sp. (Heterotardigrada; Echiniscoidea) specimens were collected from intertidal sand in Brunswick County, North Carolina (location: 33°51′58.8″N, 78°30′20.1″W). When we refer to hatchlings, we are referring to specimens that were collected as embryos and fixed within 24 h after they hatched.
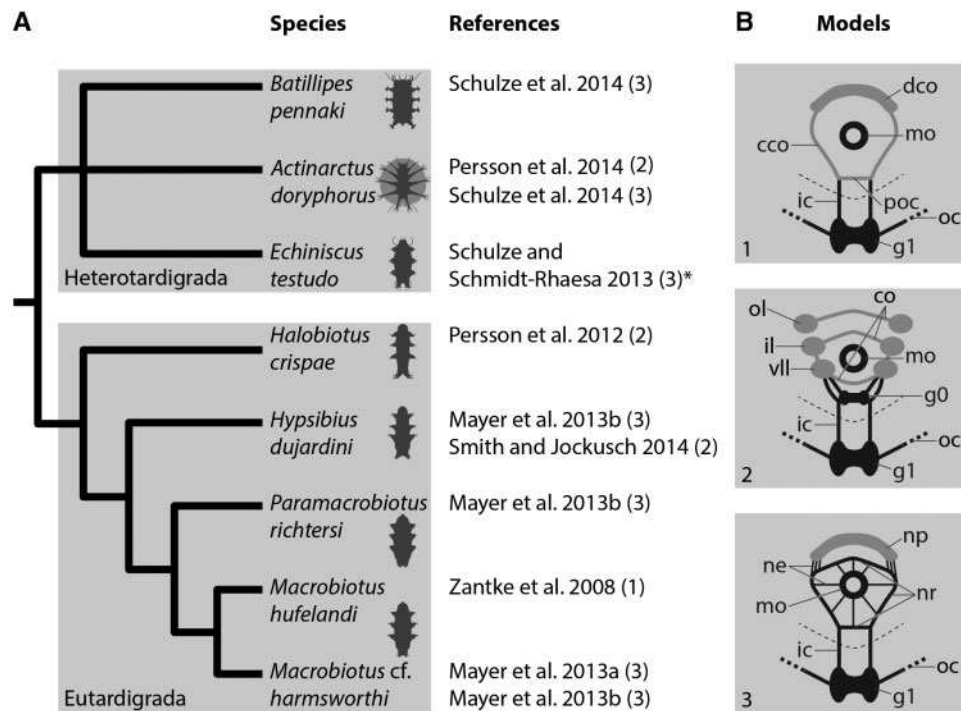
Fig. 1 Summary of recent studies that investigated tardigrade brain morphology. (**A**) Diversity of tardigrade species investigated with a combination of immunohistochemistry and laser scanning confocal microscopy. The numbers in parentheses refer to the models in (**B**) that the results of the studies most closely match, in terms of neuronal connectivity and the regions in the head that were considered to be part of the brain. *The model for *Echiniscus testudo* nervous system anatomy was shown in Schulze et al. (2014), based on data from Schulze and Schmidt-Rhaesa (2013). The phylogeny is based on Jørgensen et al. (2010), Guil and Giribet (2012), and Bertolani et al. (2014). (B) Models of tardigrade brain anatomy, based on Zantke et al. (2008; top panel), Persson et al. (2012; middle panel), and Mayer et al. (2013a; bottom panel). Gray structures are considered to be part of the brain; black structures are not considered to be part of the brain. The dashed line demarcates the boundary between the head and the trunk. Heads are modeled in cross section; trunks are modeled in frontal view. For simplicity, the outer region of the brain that connects to the first trunk ganglion via the outer connectives—the outer lobe in the middle panel—is not diagrammed in the top and bottom panels. Abbreviations: cco, circumbuccal connective; co, commissure; dco, dorsal commissure; g0, subesophageal ganglion; g1, first trunk ganglion; ic, inner connective; il, inner lobe; mo, mouth; ne, neurites; np, neuropil; oc, outer connective; ol, outer lobe; nr, nerve ring; poc, post-oral commissure; vll, ventrolateral lobe.

Specimen preparation

We prepared specimens to visualize neurites, nuclei, and muscles. Specimens were stretched in carbonated water. They were then transferred to 4% formaldehyde in $0.5\times$ PB-Triton ($0.5\times$ phosphate-buffered saline, 0.1% Triton X-100, pH 7.4). They were stored in this solution at $4\,^{\circ}C$ for between 1 day and 1 week. Specimens were then washed five times with $0.5\times$ PB-Triton. To permeabilize specimens, we bisected them transversely with a 25-gauge needle. Specimens were then washed two times for 1 h in 0.2% bovine serum albumin in 0.5X PB-Triton, followed by a 1.5 h wash in 5% normal goal serum (NGS in $0.5\times$ PB-Triton). Specimens were incubated overnight in a 1:100 dilution of a β-tubulin antibody (E7, Developmental Studies Hybridoma Bank), which stains the tardigrade nervous system (Smith and Jockusch 2014). They were then washed three times

for 5 min and two times for 1 h in $0.5\times$ PB-Triton. This was followed by two $1/2$ h washes in NGS. Specimens were then incubated overnight at $4\,^{\circ}C$ in a 1:200 dilution of a goat anti-mouse Cy3-conjugated secondary antibody (Jackson ImmunoResearch) in NGS. Next, specimens were washed two times for 1 h and three times quickly in $0.5\times$ PB-Triton. They were then incubated in a 1:40 dilution of Oregon Green 488 phalloidin (Molecular Probes), which stains tardigrade muscles (Smith and Jockusch 2014), for at least 16 h at $4\,^{\circ}C$. We then washed the specimens three times quickly in $0.5\times$ PB-Triton. We mounted specimens on slides in DAPI Fluoromount-G (SouthernBiotech) to visualize nuclei.

Imaging and analysis

Z-series of specimens were collected on a Zeiss 710 laser scanning confocal microscope. To help visualize

brain morphology, we show the Cy3 excitation channel (neurons) using the Glow look up table (LUT) and the DAPI excitation channel (nuclei) using the Cyan Hot LUT available in ImageJ. Maximum projections were produced using the Zprojection tool in ImageJ. Virtual slices were produced using the Volume Viewer plugin in ImageJ. Levels were adjusted in either ImageJ or Adobe Photoshop CS4.

Results

We investigated brain morphology in a suite of species that span tardigrade phylogeny (Jørgensen et al. 2010; Guil and Giribet 2012; Bertolani et al. 2014). *Milnesium* n. sp. is similar to undescribed *Milnesium* specimens from the Great Smoky Mountains National Park with a [3-3]–[3-3] claw structure, smooth cuticle, and cylindrical buccal tube. *Echiniscus* n. sp. is most similar to members of the *Echiniscus bigranulatus* species group, but differs from these species by lacking basal spurs on claws. *Batillipes* n. sp. is most similar to *Batillipes mirus*, but has smaller body size and lacks lateral processes between legs III and IV. We follow the guidelines set by Richter et al. (2010) for labeling nervous system morphology.

Hypsibius dujardini brain morphology

We started this project by focusing on *H. dujardini* hatchlings because hatchlings are small and do not contain autofluorescent algae that *H. dujardini* eat in culture; both traits facilitate investigations with laser scanning confocal microscopy. In *H. dujardini*, inner connectives (ic) extended between the first trunk ganglion and the inner head (Figs. 2A and 3C). At a ventral position in the head, each inner connective met a neurite cluster (nc; Figs. 2A and 3B). Neurite clusters were in regions of low nucleus density (lower panels of Figs. 2A and 3B). A post-oral commissure (poc) extended between the neurite clusters at this position (Fig. 2A). From this position, the neurite clusters traveled dorsally along the internal head cavity around the foregut; we identified the foregut based on the positions of neurites extending posteriorly from the mouth (mo), that is, the stomodeal nervous system (Dewel et al. 1999), stylet muscles (st)—which lie directly above, below, and along the buccal tube—and the muscular pharynx (ph) (top panels of Fig. 2B–D). Nuclei were still absent within the neurite clusters in this region (lower panels of Figs. 2B,C and 3B). Near the top of the mouth, a preoral commissure (prc) extended between the neurite clusters (Fig. 2C). Immediately dorsal to this position, a thick band of neurites extended across the dorsal mid-line of the brain (Figs. 2D and 3C); this band of neurites was located in a region that lacked cell nuclei (lower panels of Figs. 2D and 3C).

Therefore, we refer to this structure as the dorsal neuropil (dnp). In virtual cross sections made using the Volume Viewer plugin in ImageJ, part of the dorsal neuropil appeared to be a continuation of the neurite clusters (Fig. 2E). Additional neurites extending from the outer brain region (ob)—the region that connects to the first trunk ganglion via the outer connective (oc)—contributed to the dorsal neuropil (Figs. 2E and 3A,B).

Brain morphology in other tardigrade species

Next, we analyzed brain morphology in the other species of this study (Figs. 4–6). Given the large size of the *Milnesium* n. sp. specimens that we collected, we opted to analyze morphology of hatchlings that came from embryos that adult *Milnesium* n. sp. laid after we collected them. As in *H. dujardini*, the inner connectives met neurite clusters in the ventral head of *Milnesium* n. sp. specimens (Fig. 4A,B), *Echiniscus* n. sp. (Fig. 5A,B), and *Batillipes* n. sp. (Fig. 6B). A post-oral commissure extended between the neurite clusters at this position in these species (Figs. 4A, 5A, and 6B). The neurite clusters extended dorsally around either side of the internal head cavity in regions of low nucleus density (lower panels of Figs. 4B,C, 5B,C, and 6B,C). A preoral commissure extended between the neurite clusters above the foregut (Figs. 4C, 5C, and 6B). From here, the neurite clusters continued to extend dorsally, ultimately reaching the dorsal neuropil (Figs. 4D,E, 5D,E, and 6C, E). As with *H. dujardini*, neurites emanating from the outer brain region also contributed to the dorsal neuropil in the other species of this study (Figs. 4E, 5D,E, and 6D,E). We identified anterior (ac) and posterior (pc) neurite clusters in *Milnesium* n. sp. in positions similar to those seen in other eutardigrades (Supplementary Fig. S1).

Trunk ganglion neuropil

We were surprised by how ganglion-like the ventral part of the neurite clusters appeared in *H. dujardini* hatchlings. To test for serial homology between the ventral neurite clusters and trunk ganglia, we compared the structure of these regions across species (Fig. 7). In both *H. dujardini* and *Milnesium* n. sp., ganglionic neuropil and ventral neurite clusters were composed of densely distributed thin neuronal fibers (Fig. 7A,B). In *Echiniscus* n. sp., ganglionic neuropil and ventral neurite clusters were composed of thicker and less densely distributed neuronal fibers, relative to *H. dujardini* and *Milnesium* n. sp. (Fig. 7C). Both thick and thin neuronal fibers were present in ganglionic neuropil and ventral neurite clusters of *Batillipes* n. sp. (Fig. 7D).
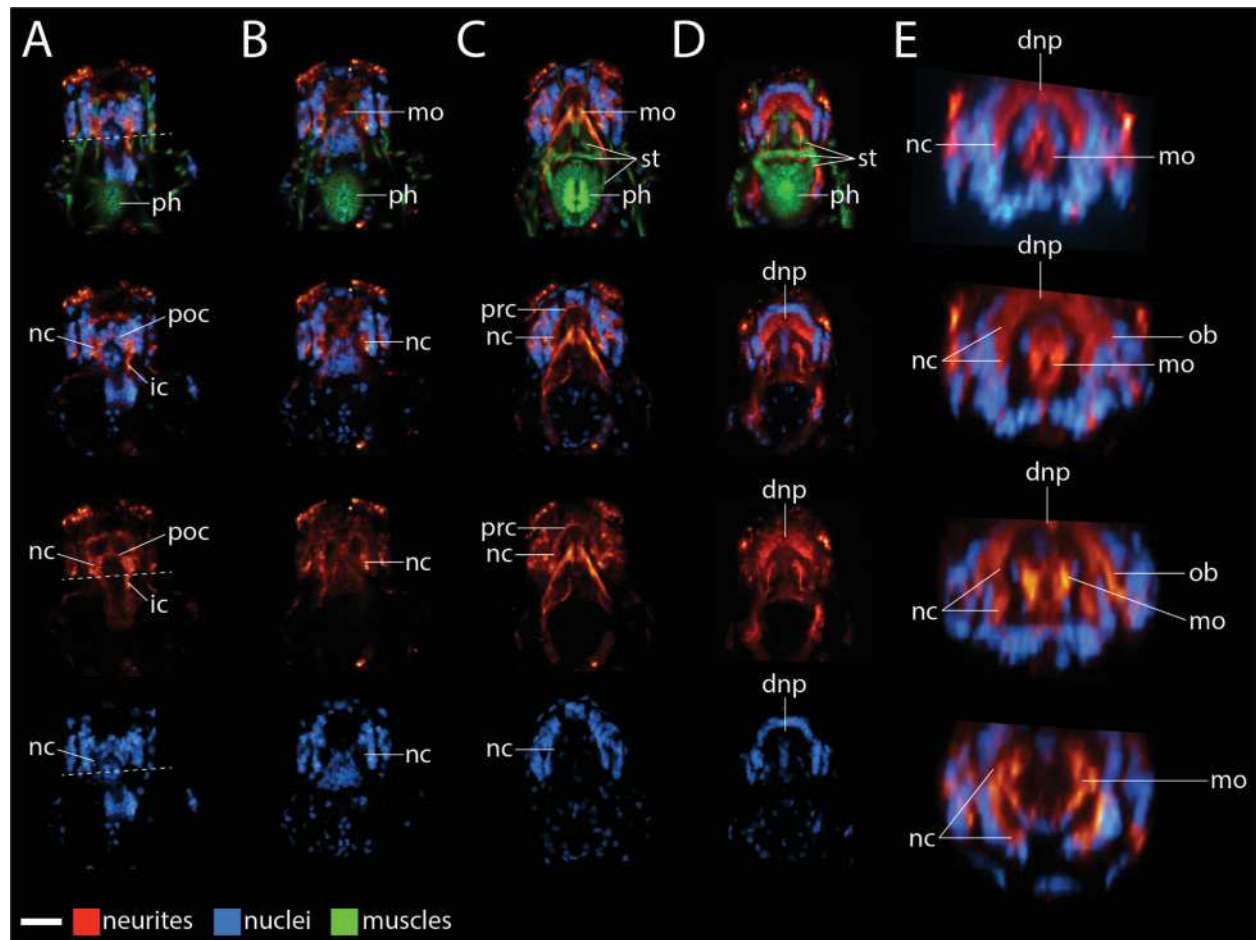
Fig. 2 Frontal views of *Hypsibius dujardini* brain morphology. (**A–D**) Confocal *Z* series slices from ventral (left) to dorsal (right). Anterior is to the top. Neurites are stained with a β-tubulin antibody (red), muscles are stained with phalloidin (green), and nuclei are stained with DAPI (blue). The top panels show neurites, nuclei, and muscles. Lower panels show a subset of these structures. We use the top panels to label structures associated with the mouth and foregut. The scale bar in the lower left hand corner equals 10 μm. (A) Dashed line represents the boundary between the head and the trunk. (**E**) Virtual cross-sections through the brain of the specimen shown in A–D, from anterior (top) to posterior (bottom). Neurons are rendered red and nuclei are rendered blue. Images are not to scale with each other in E. Abbreviations: dnp, dorsal neuropil; ic, inner connective; mo, mouth, that is, the stomodeal nervous system; nc, neurite clusters; ob; outer brain; ph, pharynx; poc, post-oral commissure; prc, preoral commissure; st, stylet muscles.

Discussion

General brain anatomy is conserved across Tardigrada

We have investigated anterior nervous system morphology in members of Parachela, Apochela, Echiniscoidea, and Arthrotardigrada. These lineages span Tardigrada, allowing us to make inferences about anterior nervous system anatomy in the most recent common ancestor of Tardigrada. Across species, the inner connectives meet the head at ventral neurite clusters. From this position, the neurite clusters wrap around the foregut. Pre- and post-oral commissures connect the neurite clusters. Part of the dorsal brain neuropil appears to be a continuation of the neurite clusters in confocal *Z*-series that we analyzed and in virtual cross sections

that we produced; the remaining part of the dorsal neuropil appears to emanate from the outer brain region (Figs. 2–6)—the region that connects to the anteriormost trunk ganglion via the outer connective (Zantke et al. 2008; Persson et al. 2012; Mayer et al. 2013a, 2013b; Schulze and Schmidt-Rhaesa 2013; Persson et al. 2014; Schulze et al. 2014; Smith and Jockusch 2014). Based on our comparisons, we predict that this general nervous system architecture was present in the most recent common ancestor of Tardigrada (Fig. 8A).

A new hypothesis for the composition of the tardigrade brain

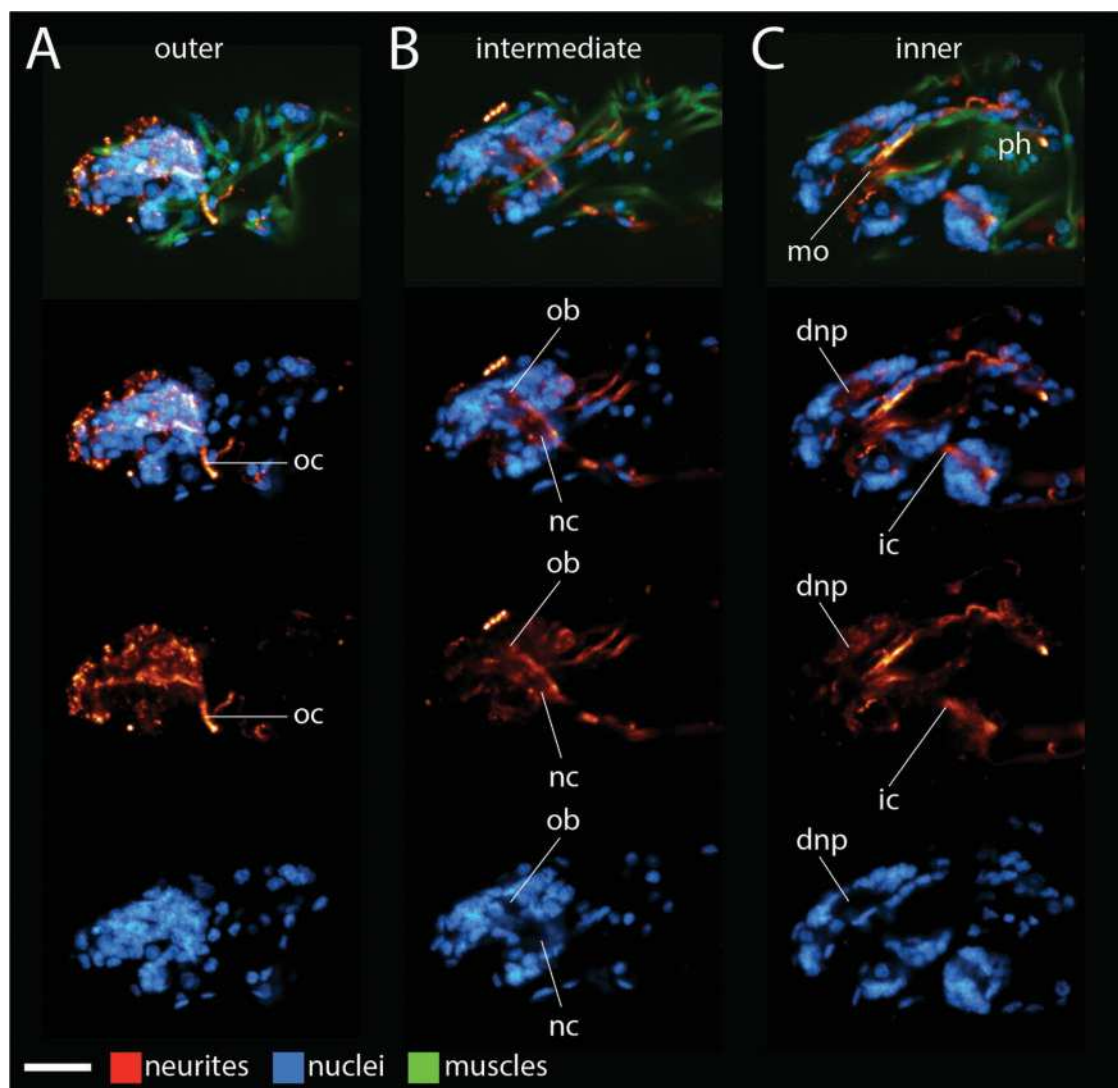Here we present a hypothesis for the composition of the tardigrade brain that relates the anterior nervous

Fig. 3 Lateral views of *H. dujardini* brain morphology. (**A–C**) Maximum projections of confocal *Z* series, from outer (left) to inner (right). Anterior is to the left. Dorsal is to the top. We use the top panels to label structures associated with the mouth and the foregut. Neurites are stained with a *β*-tubulin antibody (red), muscles are stained with phalloidin (green), and nuclei are stained with DAPI (blue). The top panels show neurites, nuclei, and muscles. Lower panels show a subset of these structures. The scale bar in the lower left hand corner equals 10 μm. Abbreviations: dnp, dorsal neuropil; ic, inner connective; mo, mouth, that is, the stomodeal nervous system; nc, neurite clusters; ob; outer brain; oc, outer connective; ph, pharynx.

system to the trunk ganglia. In our hypothesis, the neurite clusters that wrap around the foregut and give rise to part of the dorsal brain neuropil are serially homologous to the neuropil of trunk ganglia (Fig. 8A). Our hypothesis is based on structural and positional similarities between trunk ganglion neuropil and the neurite clusters (Fig. 7). The morphology of trunk ganglion neuropil and the neurite clusters appears to covary between species. Trunk ganglion neuropil and the neurite clusters are in regions of low nucleus density, a defining characteristic of ganglionic neuropil (Richter et al. 2010). Trunk ganglion neuropil and the ventral part of the neurite clusters are wider than the paired connectives that they give rise to. Furthermore, paired connectives extend directly between the ventral parts of the neurite clusters and the first trunk ganglion (the inner connectives in Figs. 2–6); paired connectives also extend directly between adjacent trunk ganglia (Zantke et al. 2008; Persson et al. 2012; Mayer et al. 2013a, 2013b; Schulze and Schmidt-Rhaesa 2013; Persson et al. 2014; Schulze et al. 2014; Smith and Jockusch 2014). Based on their structural and positional similarities to neuropil of trunk ganglia and their apparent direct connection to the dorsal brain neuropil (Figs. 2–6), we predict that the neurite clusters represent neuropil of the brain; therefore
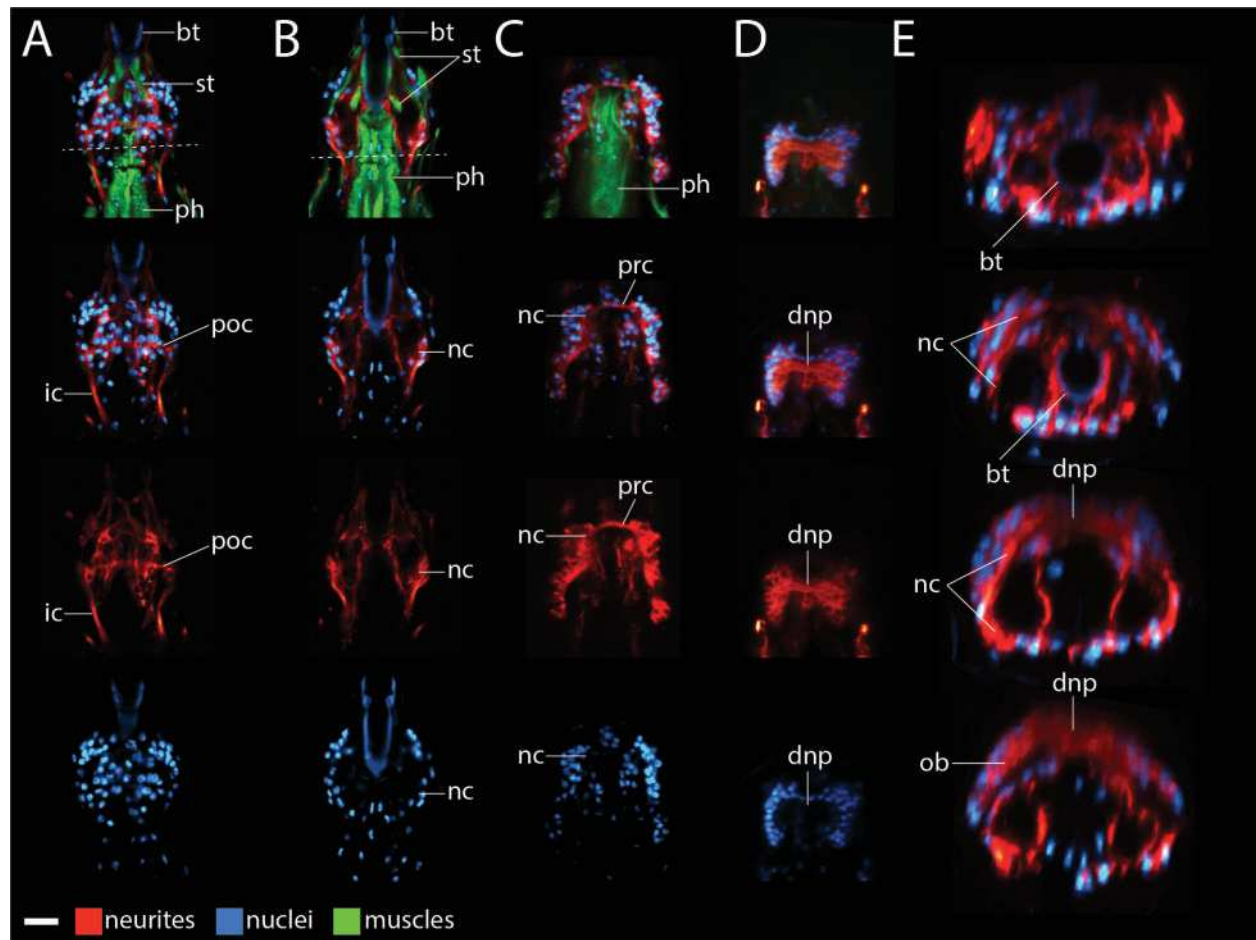
Fig. 4 *Milnesium* n. sp. brain morphology. (**A–D**) Ventral (left) to dorsal (right). Anterior is to the top. (**A–C**) Virtual slices. (**D**) Maximum projection. (**A, B**) Dashed line represents the boundary between the head and the trunk. Neurites are stained with a β-tubulin antibody (red), muscles are stained with phalloidin (green), and nuclei are stained with DAPI (blue). The top panels show neurites, nuclei, and muscles. Lower panels show a subset of these structures. The buccal tube was also visible in the blue channel. We use the top panels to label structures associated with the mouth and the foregut. The scale bar in the lower left hand corner equals 10 μm. (**E**) Virtual cross-section renderings of the specimen shown in A–D, from anterior (top) to posterior (bottom). Neurons are rendered red and nuclei are rendered blue. Images are not to scale with each other in E. Abbreviations: bt, buccal tube; dnp, dorsal neuropil; ic, inner connective; nc, neurite clusters; ob; outer brain; ph, pharynx; poc, post-oral commissure; prc, preoral commissure; st, stylet muscles.

in our model, the tardigrade brain nearly circumscribes the foregut (Fig. 8A).

Our hypothesis could be tested by investigating the expression patterns of genes that regulate trunk ganglion development during embryogenesis in *H. dujardini*. The conserved set of genes that regulates neurogenesis in euarthropods and other metazoans provides good candidates for this study (Stollewerk 2016). We predict that genes that pattern trunk ganglia in *H. dujardini* will also be expressed in the region of the developing head where neurite clusters will ultimately be positioned. Intriguingly, during *H. dujardini* embryogenesis, a Paired box antibody marks nuclei in presumptive trunk ganglia and nuclei near where neurite clusters will be positioned (Gabriel and Goldstein 2007; Smith and Goldstein 2017), which

supports our hypothesis. Alternatively, the neurite clusters we identified might not represent ganglionic neuropil. In this case, expression of genes that pattern ganglia in the trunk may be restricted to a dorsal region in the head, if the brain is restricted to a supraesophageal position, which has been suggested (Mayer et al. 2013a; Schulze et al. 2014). We consider this alternative to be unlikely, given that our results suggest that the structure of the neurite clusters and the structure of trunk ganglion neuropil appear to be evolving in concert in Tardigrada (Fig. 7), in accordance with serial homology.

Comparison with previous reports

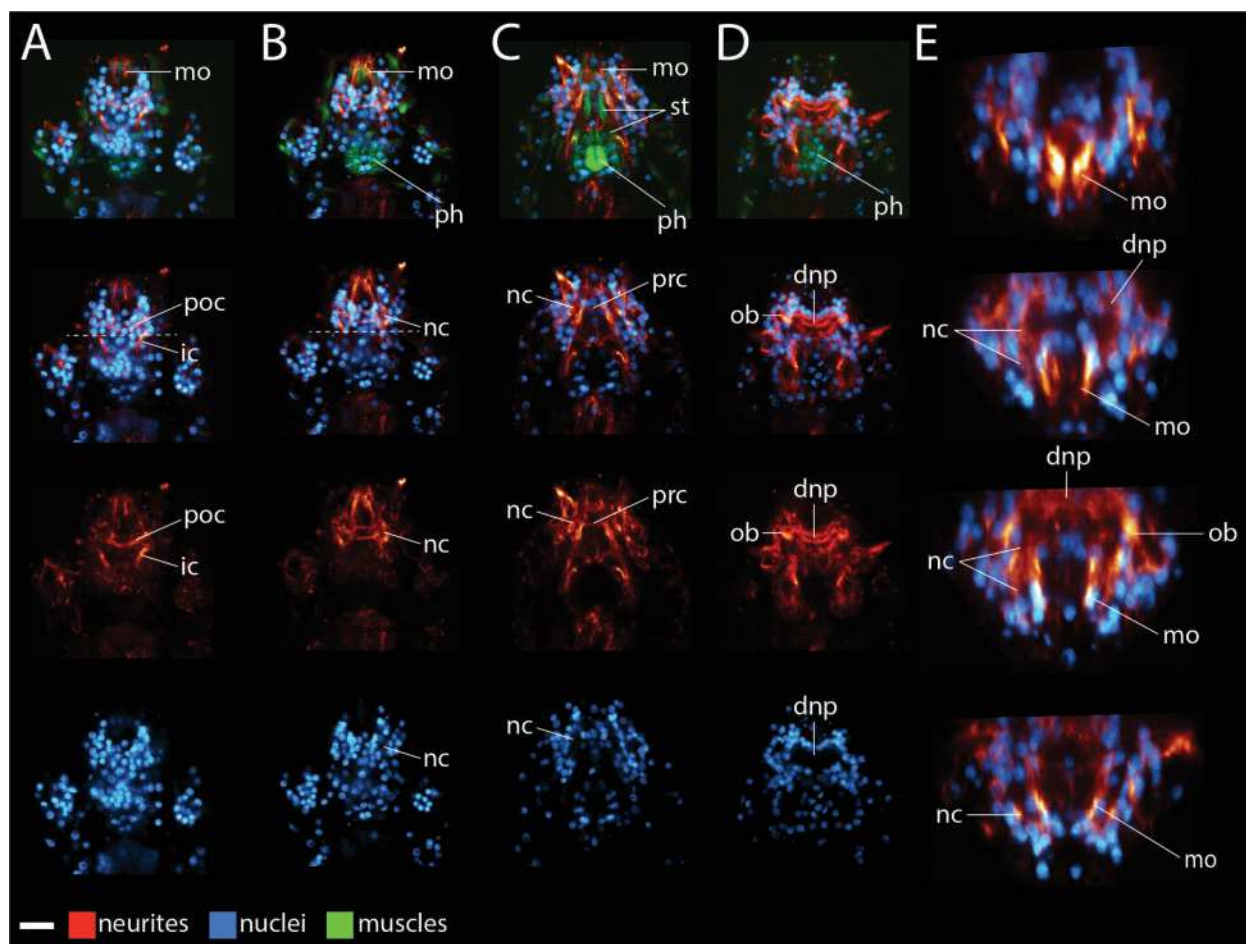Our results support the conclusions of previous studies, while expanding on them in interesting

Fig. 5 *Echiniscus* n. sp. brain morphology. (**A–D**) Maximum projections of ventral (left) to dorsal (right) Z-series slices. Anterior is to the top. (**A–B**) Dashed line represents the boundary between the head and the trunk. In the upper panels, neurites are stained with a β-tubulin antibody (red), muscles are stained with phalloidin (green), and nuclei are stained with DAPI (blue). The top panels show neurites, nuclei, and muscles. Lower panels show a subset of these structures. We use the top panels to label structures associated with the mouth and the foregut. The scale bar in the lower left hand corner equals 10 μm. (**E**) Virtual cross-section renderings of the specimen shown in A–D, from anterior (top) to posterior (bottom). Neurons are rendered red and nuclei are rendered blue. Images are not to scale with each other in E. Abbreviations: dnp, dorsal neuropil; ic, inner connective; mo, mouth, that is, the stomodeal nervous system; nc, neurite clusters; ob; outer brain; ph, pharynx; poc, post-oral commissure; prc, preoral commissure; st, stylet muscles.

ways. Zantke et al. (2008) suggested that the tardigrade brain exhibits circumesophageal morphology. Results of our study support this conclusion. The circumesophageal component of the brain in the model of Zantke et al. (2008) consists of paired circumbuccal connectives (cco) that are connected by a dorsal commissure (dco) above and a post-oral commissure (poc) below the foregut (modeled in Fig. 1B, top panel). We predict that the dorsal commissure, circumbuccal connectives, and post-oral commissure in the model presented by Zantke et al. (2008) correspond to the dorsal neuropil, neurite clusters, and post-oral commissure, respectively, of our model (compare Fig. 1B, top panel to Fig. 8A). In our model, we refer to the dorsal most part of the brain as the dorsal neuropil because it includes neurites

extending from two different positions—the outer and inner brain, that is, it represents outer *and* inner brain commissures that are not easily distinguishable. We refer to the circumbuccal connectives identified by Zantke et al. (2008) as neurite clusters because we predict that they represent a part of a ganglion—the neuropil; connectives refer to neurite bundles that connect ganglia, rather than referring to parts of ganglia (Richter et al. 2010). Unlike the model of Zantke et al. (2008), we identified a preoral commissure that directly connects to the neurite clusters in all species of our study. The model of Zantke et al. (2008) includes a preoral commissure, but it is not directly connected to the circumbuccal connectives in their model (the neurite clusters in our model).
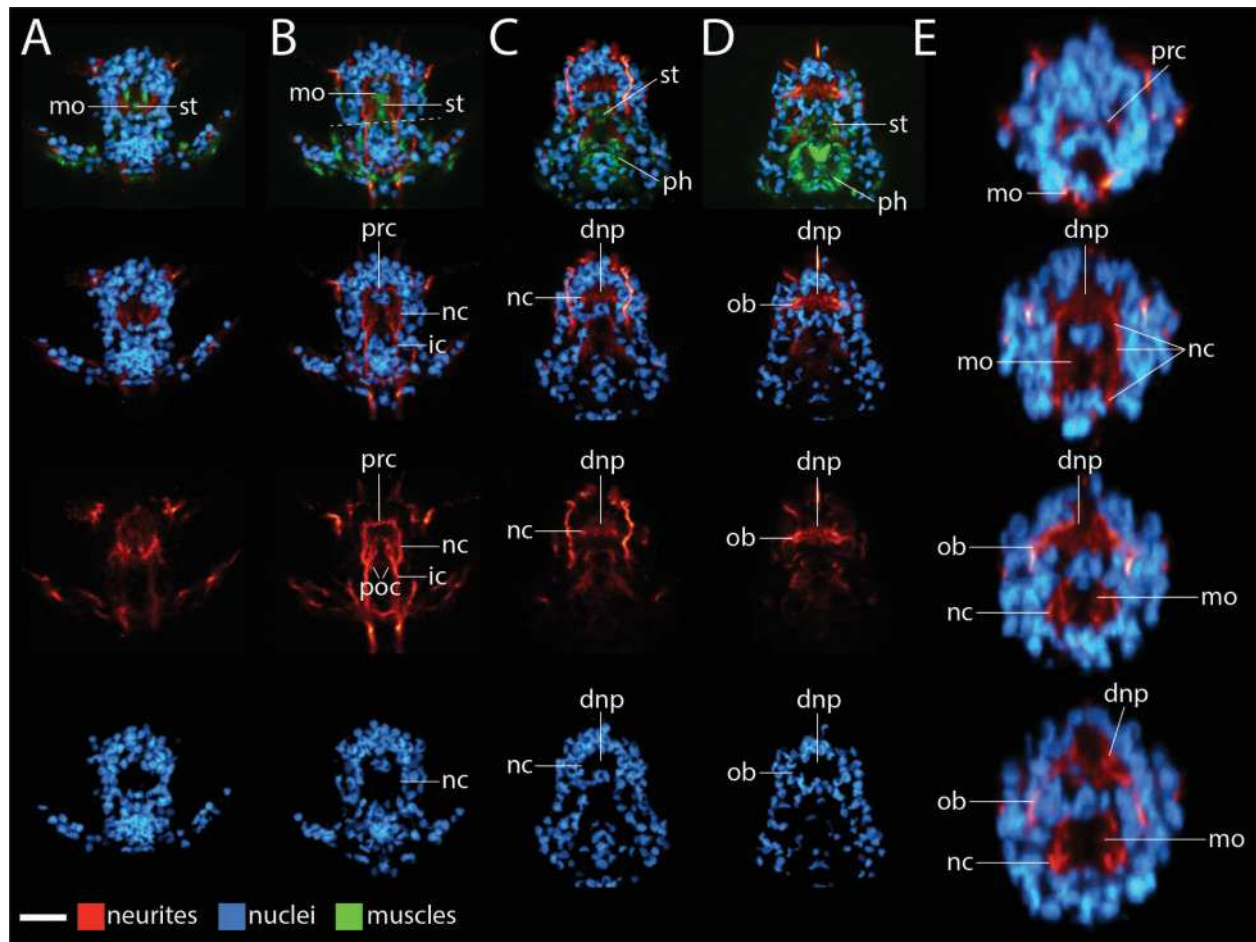
Fig. 6 *Batillipes* n. sp. brain morphology. The mouth faces ventrally in this species. (**A–D**) Maximum projections of ventral (left) to dorsal (right) Z-series slices. Anterior is to the top. In the upper panels, neurites are stained with a β-tubulin antibody (red), muscles are stained with phalloidin (green), and nuclei are stained with DAPI (blue). The top panels show neurites, nuclei, and muscles. Lower panels show a subset of these structures. We use the top panels to label structures associated with the mouth and the foregut. The scale bar in the lower left hand corner equals 10 μm. (**B**) Dashed line demarcates posterior boundary of the head. (**E**) Virtual cross-section renderings of the specimen shown in A–D, from anterior (top) to posterior (bottom). Neurons are rendered red and nuclei are rendered blue. Images are not to scale with each other in E. Abbreviations: dnp, dorsal commissure; ic, inner connective; mo, mouth, that is, the stomodeal nervous system; nc, neurite clusters; ob; outer brain; ph, pharynx; poc, post-oral commissure; prc, preoral commissure; st, stylet muscles.

Persson et al. (2012) predicted that the inner part of the tardigrade brain—the part adjacent to the foregut in their model—was composed of several lobes (modeled in Fig. 1B, middle panel). By contrast, we did not identify independent lobes in the presumptive inner brain region. We predict that the subesophageal ganglion (g0), ventrolateral lobes (vll), and the inner lobes (il) of Persson et al. (2012) represent neuronal somata that lie adjacent to the neurite clusters in our model (compare Fig. 1B, middle panel to Fig. 8A). Furthermore, we predict that the commissures connecting the paired inner lobes and paired outer lobes in the model of Persson et al. (2012) represent the dorsal neuropil of our model. It is unclear what parts of their model correspond to the pre- and post-oral commissures identified in our study. In agreement with the model of Persson et al. (2012, 2014), early descriptions of tardigrade nervous systems typically included a subesophageal ganglion (Marcus 1929; Kristensen 1983; Dewel and Dewel 1996; Dewel et al. 1999; Nielsen 2001). However, several recent studies that utilized immunohistochemical methods and confocal laser scanning microscopy could not identify a subesophageal ganglion (Zantke et al. 2008; Mayer et al. 2013a, 2013b; Schulze and Schmidt-Rhaesa 2013; Schulze et al. 2014; Smith and Jockusch 2014). Our model reconciles these different interpretations by suggesting that the subesophageal ganglion identified in earlier studies is actually paired ventrolateral extensions of the brain, as previously proposed (Mayer et al. 2013a).
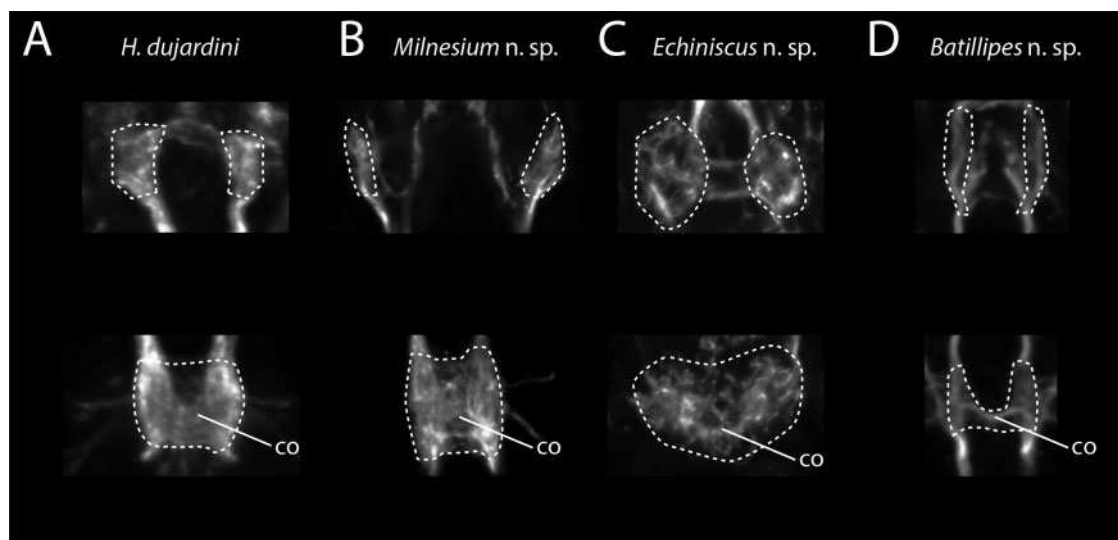
Fig. 7 Comparison between the ventral parts of the inner neurite clusters (top panels) to trunk ganglion neuropil (bottom panels). (**A**–**D**) β-Tubulin antibody stained neurites. All panels show 1.5 μm thick maximum projections besides the upper panel of B, which is a virtual slice. Neuropil is outlined in the bottom panel and predicted neuropil is outlined in the top panel. Within columns, images are to scale. (A) *Hypsibius dujardini*. Second trunk ganglion. (B) *Milnesium* n. sp. Second trunk ganglion. (C) *Echiniscus* n. sp. First trunk ganglion. (D) *Batillipes* n. sp. First trunk ganglion. Abbreviation: co, commissure.
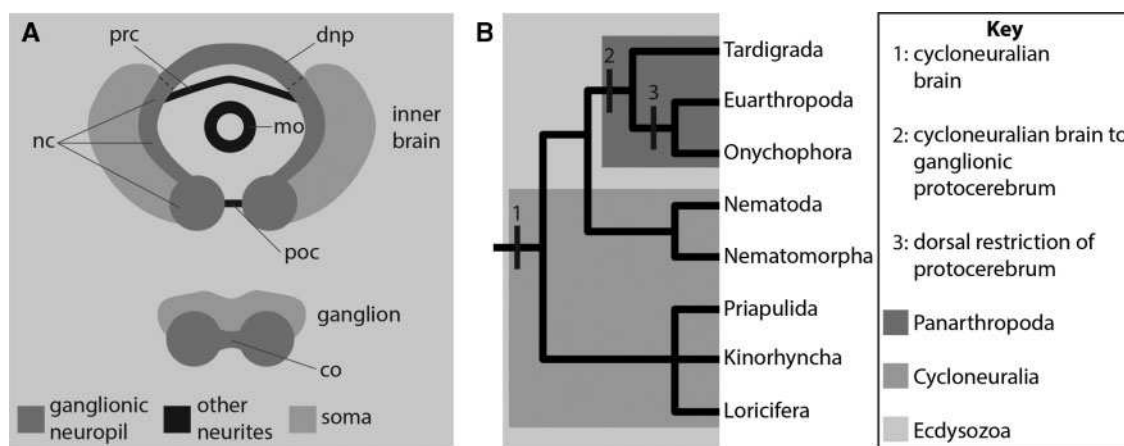

Fig. 8 Hypothesis for the structure of the tardigrade brain and its implications for panarthropod brain evolution. (**A**) Hypothesis for homology between the tardigrade inner brain (top) and trunk ganglia (bottom). We show models of cross-sections through the brain and a trunk ganglion. The dashed lines demarcate the boundaries between what we refer to as neurite clusters and what we refer to as the dorsal neuropil in the "Results" section. We only model the inner brain. Neurites extending from the outer brain also contribute to the dorsal brain neuropil. (**B**) Model of the evolution of the panarthropod protocerebrum. The phylogeny is based on Campbell et al. (2011) with the addition of Loricifera based on Laumer et al. (2015). Abbreviations: co, commissure; dnp, dorsal neuropil; mo, mouth, that is, the stomodeal nervous system; nc, neurite clusters; poc, post-oral commissure; prc, preoral commissure.

Mayer et al. (2013a) suggested that the circumesophageal component of the tardigrade nervous system—referred to as the nerve ring (nr) in their model—is part of the stomodeal nervous system, rather than part of the brain (Fig. 1B, bottom panel). In their model, the tardigrade brain is restricted to a dorsal position. We suggest that the nerve ring identified by Mayer et al. corresponds to the preoral commissure, neurite clusters, and post-oral commissure of our model (compare Fig. 1B, bottom panel to Fig. 8A). We agree with Mayer et al. (2013a) that these components innervate the stomodeal nervous system. However, in our model, the neurite clusters and the neuronal somata that give rise to them are part of the brain. This is based on our interpretation of the neurite clusters as ganglionic neuropil and the fact that part of the dorsal brain neuropil appears to be a direct extension of the neurite clusters. Therefore, we conclude that the tardigrade brain nearly circumscribes the foregut, rather than being restricted to a dorsal position.

Our model expands on the models of previous workers by suggesting that the neurite clusters within the head represent ganglionic neuropil. Other studies have not recognized the ganglion-like morphology of the neurite clusters in the head. It is possible that our combination of β-tubulin antibody and Cy3 labeled secondary antibody is more effective for labeling ganglion-like structures compared with the combinations of α-tubulin antibodies and secondary antibodies used in other studies. It also seems likely that the general morphology of the tardigrade brain is easiest to interpret in smaller specimens. For example, the boundary between the presumptive inner brain and the stomodeal nervous system is much clearer in *H. dujardini* hatchlings (Fig. 2) than it is at later stages (Smith and Jockusch 2014).

A model for the evolution of the panarthropod protocerebrum

In our interpretation of tardigrade brain morphology, brain neuropil nearly wraps around the foregut (Fig. 8A). Based on our interpretation and recent hypotheses regarding ecdysozoan phylogeny (Campbell et al. 2011; Rota-Stabelli et al. 2013; Dunn et al. 2014; Giribet 2016), we propose a model for the evolution of the protocerebral brain ganglion of Panarthropoda (Fig. 8B). In this model, the most recent common ancestor of Ecdysozoa exhibited a cycloneuralian-like brain (Fig. 8B, transition 1). A ganglionated protocerebrum evolved after the panarthropods diverged from their cycloneuralian relatives (Fig. 8B, transition 2). This step would have required two major changes in nervous system morphology. First, the clusters of neuronal somata that typically sit in anterior and posterior positions relative to the neuropil ring in cycloneuralian brains would have to have moved to primarily lateral positions immediately adjacent to the neuropil ring. Second, the ventromedial nerve cord that extends from the nerve ring in cycloneuralians would have to have split into two independent nerve cords that sit in ventrolateral positions. Interestingly, Schmidt-Rhaesa (1997/1998) suggested that the ventromedial nerve cord characteristic of Nematoda and Nematomorpha—which together form the proposed sister lineage of Panarthropoda (Campbell et al. 2011; Rota-Stabelli 2013)—has a paired origin. Therefore, the evolution of paired ventrolateral nerve cords in Panarthropoda could have simply involved the repositioning of ventromedial nerve cords that were already paired in the most recent common ancestor of Panarthropoda and Nematoda + Nematomorpha. If the ventrolateral paired nerve cords of tardigrades were forced to sit immediately adjacent to each other in a ventromedial position, neuropil would completely circumscribe the foregut in our model of the tardigrade brain, as it does in cycloneuralians. Lastly, in our model, dorsal restriction of protocerebral neuropil evolved in the euarthropod + onychophoran lineage, after it split from Tardigrada (Fig. 8B, transition 3). In this sense, a dorsally restricted protocerebral ganglion can be viewed as a synapomorphy of the euarthropod/onychophoran clade.

Our model is contingent on two conditions. First, the neurite clusters we identified must be part of the brain. Evidence supporting or refuting this condition could be collected via additional studies of brain development in tardigrades (see above). Second, our model is contingent on a particular view of ecdysozoan phylogeny. In this view, Tardigrada forms the sister group of a euarthropod + onychophoran clade (Campbell et al. 2011; Rota-Stabelli et al. 2013; Dunn et al. 2014; Giribet 2016). By contrast, some recent molecular analyses recover Tardigrada as a sister group to either Nematoda or Nematoda + Nematomorpha (Borner et al. 2014; Laumer et al. 2015; reviewed in Edgecombe and Giribet, this volume). Additionally, recent morphological analyses recover Tardigrada as the sister group of Euarthropoda (Smith and Ortega-Hernández 2014; Smith and Caron 2015; Murdock et al. 2016; Yang et al. 2016). Under these alternative phylogenetic hypotheses, when and how many times brains evolved ganglionated architecture or shifted to a supraesophageal position in ecdysozoan phylogeny would be less clear. However, the grouping of Tardigrada with Nematoda has been suggested to result from systematic error due to long branch attraction (Dunn et al. 2008; Campbell et al. 2011; Giribet and Edgecombe 2012). Furthermore, the Tardigrada + Euarthropoda clade recovered by recent morphological analyses is based on only a small number of similarities in nervous system architecture between these two lineages (Edgecombe and Giribet, this volume). Additional analyses are required to resolve ecdysozoan phylogeny.

Conclusions

While our hypothesis requires additional tests (see previous section), we find it interesting to speculate on its ramifications. Our hypothesis predicts that the most recent common ancestor of Panarthropoda exhibited a circumesophageal brain. If the tardigrade brain is homologous to the protocerebrum of euarthropods and onychophorans, as evidence suggests (Mayer et al. 2013a; Smith et al. 2016), then it may be possible to identify the regions of the euarthropod and onychophoran protocerebrum that evolved from the ancestral circumesophageal

component. The protocerebrum of euarthropods and onychophorans is composed of two regions—the archicerebrum, which includes the optic lobes, and the prosocerebrum, which includes neurosecretory centers (Siewing 1963; Scholtz and Edgecombe 2006; Steinmetz et al. 2010; Strausfeld 2012; Ortega-Hernández et al. 2017). It is possible that the circumesophageal component of the tardigrade brain includes cells from either or both of these regions. By contrast, the tardigrade brain may not be divided into regions with clear homology to either the archicerebrum or prosocerebrum. Any of these possibilities would be informative with regards to the evolution of the panarthropod protocerebrum. These possibilities could be tested by investigating the embryonic expression patterns of *orthodenticle* and *six3*—markers of the archi- and prosocerebrum, respectively (Steinmetz et al. 2010; Ortega-Hernández et al. 2017)—during tardigrade brain development. The evolutionary origins of the protocerebrum of Panarthropoda remain mysterious (Ortega-Hernández et al. 2017). Future studies of the tardigrade brain may contribute to unlocking this mystery.

Acknowledgments

Funding

Supplementary data

Supplementary data available at *ICB* online.

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