

The urbilaterian brain revisited: novel insights into old questions from new flatworm clades

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Abstract Flatworms are classically considered to represent the simplest organizational form of all living bilaterians with a true central nervous system. Based on their simple body plans, all flatworms have been traditionally grouped together in a single phylum at the base of the bilaterians. Current molecular phylogenomic studies now split the flatworms into two widely separated clades, the acoelomorph flatworms and the platyhelminth flatworms, such that the last common ancestor of both clades corresponds to the urbilaterian ancestor of all bilaterian animals. Remarkably, recent comparative neuroanatomical analyses of acoelomorphs and platyhelminths show that both of these flatworm groups have complex anterior brains with surprisingly similar basic neuroarchitectures. Taken together, these findings imply that fundamental neuroanatomical features of the brain in the two separate flatworm groups are likely to be primitive and derived from the urbilaterian brain.

Keywords Brain · Evolution · Acoel · Platyhelminth · Urbilateria

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Conserved developmental programs for diverse bilaterian brains

At the structural level, the brains of higher deuterostomes such as vertebrates and higher protostomes such as arthropods or annelids are strikingly different (Bullock and Horridge 1965; Nauta and Fertag 1986). Moreover, the embryological processes that give rise to these brains are also different in these two animal groups (Wolpert et al. 1998). The brain and dorsally located nerve cord of vertebrates derive from a dorsal neuroectoderm that invaginates to form a neural tube. In contrast, the brain and ventrally located ganglionic nerve cord of arthropods and annelids derive from a ventral neuroectoderm. In addition, the mechanism of neural progenitor proliferation shows significant differences. As shown for several arthropod taxa, but also probably true for other protostome phyla, asymmetrically dividing neural stem cells (neuroblasts) generate morphologically distinct lineages of neurons/glia cells which also form structural units of the brain (Hartenstein et al. 2008). By contrast, neural progenitors in vertebrates form a layer of symmetrically dividing cells. These cells eventually switch to asymmetric divisions when producing neurons, but no evidence exists to date that neurons descending from individual progenitors form structural units, such as individual brainstem nuclei, or cortical layers. These and other differences in CNS structure and development have been used as one basis for the classification of “vertebrate-like” notoneuralia versus “invertebrate-like” gastroneuralia types (Brusca and Brusca 1990). It is interesting to ask what the CNS of the common bilaterian ancestor looked like, and how one can envisage the evolutionary changes that led to the divergence of the two types of nervous systems.

A wealth of data obtained during the last two decades suggests that despite their different morphologies, both the “vertebrate-like” deuterostome brain and the “invertebrate-like” protostome brain types share many fundamental

characters at the molecular and genetic levels that involve homologous genes with comparable expression patterns and functional roles (for recent reviews see Lichtneckert and Reichert 2005; Denes et al. 2007; Reichert 2009). In particular, similar genetic patterning mechanisms underlie the anteroposterior and the dorsoventral regionalization of the insect and mammalian brain. Anteroposterior regionalization of the brain involves the cephalic gap genes *otd/Otx* and *ems/Emx* (in the anterior brain) and the *Hox* genes (in the posterior brain); dorsoventral regionalization of the brain involves the columnar homeobox genes *vnd/Nkx2*, *ind/Gsh*, and *msh/Msx*; and additional subdivisions in the brain involve *Pax2/5/8*, *unpg/Gbx*, *erm/Fezf*, and *mirr/Irx* expression domains (Fig. 1). Comparative gene expression studies

carried out in many other protostomes and deuterostomes confirm the fact that homologous genes are expressed with similar topology during brain development in all of these animals (Wada and Sato 2001; Puelles and Rubenstein 2003; Holland 2009). Noteworthy are an extensive series of gene expression studies that have been carried out on the developing brain of the protostome annelid *Platynereis* which suggest that not only complex brain patterning programs but even individual neural cell types from corresponding progenitor regions are conserved among protostomes and deuterostomes (reviewed in Arendt et al. 2008).

Taken together, these studies indicate that key developmental processes such as regionalization of the neural primordium and specification of certain cell types are conserved in brain

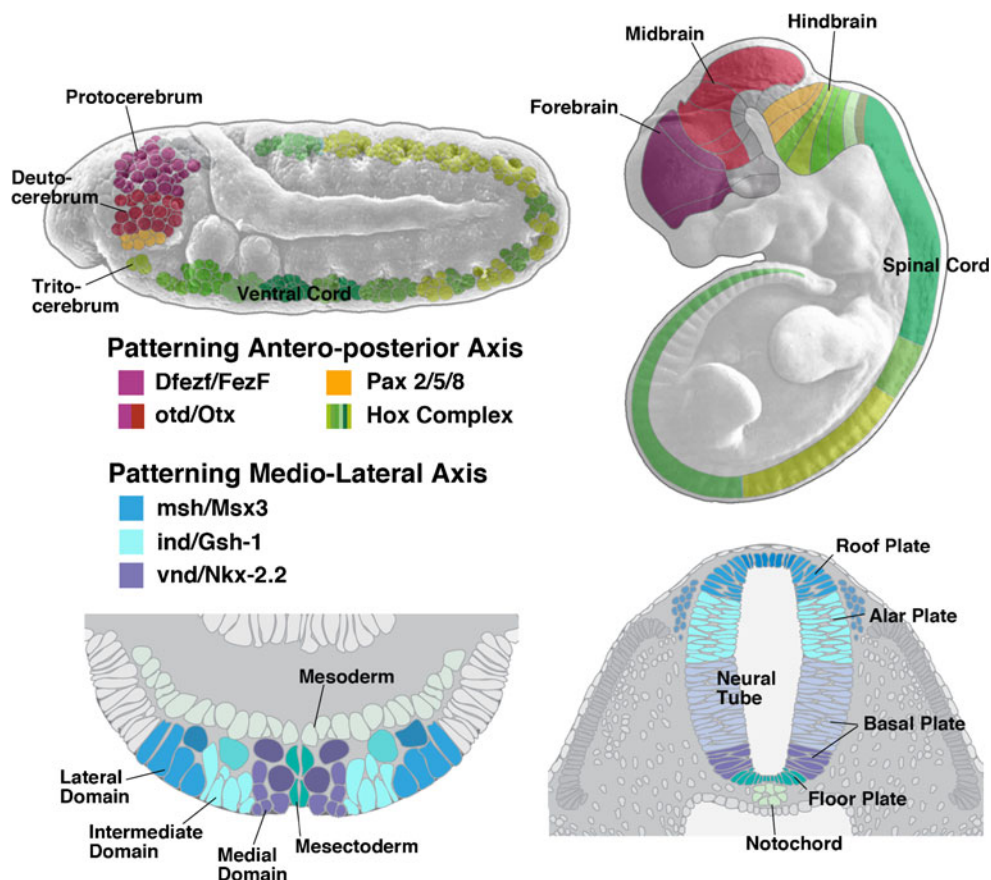


Fig. 1 Specification of regional identity of the neural primordium in *Drosophila* (left) and mouse (right) by conserved genes. *Upper panels*: genes of the *Hox* complex and other transcriptional regulators of the Homeobox family control identity along the anteroposterior axis. The neuroblast layer of the fly embryo is subdivided into segmentally repeated groups of cells called neuromeres; each neuromere produces one segmental ganglion. *Hox* genes are expressed in single neuromeres, or groups of neuromeres, located in the trunk of the embryo, which produces the ventral nerve cord and the posterior brain (tritocerebrum). The neuromeres of the anterior head (protocerebrum, deutocerebrum, tritocerebrum) express other homeobox genes: *DFezf* and orthodenticle (*otd*) in the protocerebrum and anterior deutocerebrum, and the *Pax2/5/8* homolog in the posterior deutocerebrum. In the mouse

embryo (*upper right*), homologs of all of these genes are found in groups of neuromeres ordered in the same anteroposterior sequence. *Lower panels* show schematic cross sections of the neural primordium in *Drosophila* (left) and mouse (right). Three homeobox genes, *msh/Msx3*, *ind/Gsh-1*, and *vnd/Nkx-2.2*, are expressed in longitudinal columns within the neuroblast layer of flies and the neural tube of mammals. The expression of *vnd/Nkx-2* defines a medial column of neuroblasts in *Drosophila*; in the mouse, it is expressed in the ventral neural tube (called basal plate), which produces motoneurons and ventral interneurons. *Ind/Gsh-1* is expressed in an intermediate column of neuroblasts in flies, and in the ventral alar plate in vertebrates. *Msh/Msx3* defines a lateral group of neuroblasts in *Drosophila* and the dorsal-most domain of the neural tube in vertebrates

development of protostomes and deuterostomes. This implies that the brains of all bilaterian animals are evolutionarily related and derive from an ancestral urbilaterian ancestor which may have already possessed a developmental genetic program for brain architecture of considerable complexity. While key conserved elements of the developmental genetic program that gives rise to the brains of extant deuterostomes and protostomes have now been uncovered, it nevertheless remains unclear which of the brains of extant bilaterians most closely reflects the organizational state of the primitive ancestral urbilaterian brain.

Phylogenetic evidence for a deep separation of two flatworm clades

Classically, flatworms are considered to have the simplest organization of all bilaterians which is characterized by a lack of coelom, respiratory system, circulatory system, skeletal system, and through-gut (Hyman 1940; Barnes et al. 1993; Hickman et al. 2004). Flatworms have been traditionally grouped together in a single phylum, the members of which are thought to have been least changed from the ancestral bilaterian form. Accordingly, many traditional phylogenies placed this classical platyhelminth monophylum in a group of “acoelomates” at a basal position in the bilaterian tree (Fig. 2a). Given this phylogenetic perspective, the flatworm brain might also be least changed from the ancestral form and hence most representative of the urbilaterian brain. This notion is in accordance with classical comparative neuroanatomical studies that considered flatworms to be the most primitive bilaterians possessing a true central nervous system (Bullock and Horridge 1965; Hanstroem 1968).

The flatworm CNS features characteristics intermediate between the diffuse nerve net of coelenterates and the nervous system of “higher” animals. Thus, whereas the “higher” protostome and deuterostome groups possess compact ganglia located either dorsally (as in chordates) or ventrally (as in many protostomes), flatworms have an anterior brain and a set of neural chords, called the orthogon, that are distributed all around the trunk at ventral, lateral, and dorsal levels (Fig. 3). With a look towards earlier evolutionary stages, i.e., to the medusa or polyp-like ancestor of flatworms, it was suggested that the orthogon derives from the diffuse nerve net whereby concentrations of neurons at certain places (e.g., around the mouth or tentacles) coalesce into coherent fiber tracts; looking forward towards “higher animals”, it was hypothesized that a further concentration and restriction of neural elements had ensued, in such a way that in the ancestors of protostomes the dorsal tracts of the orthogon were eliminated, and in

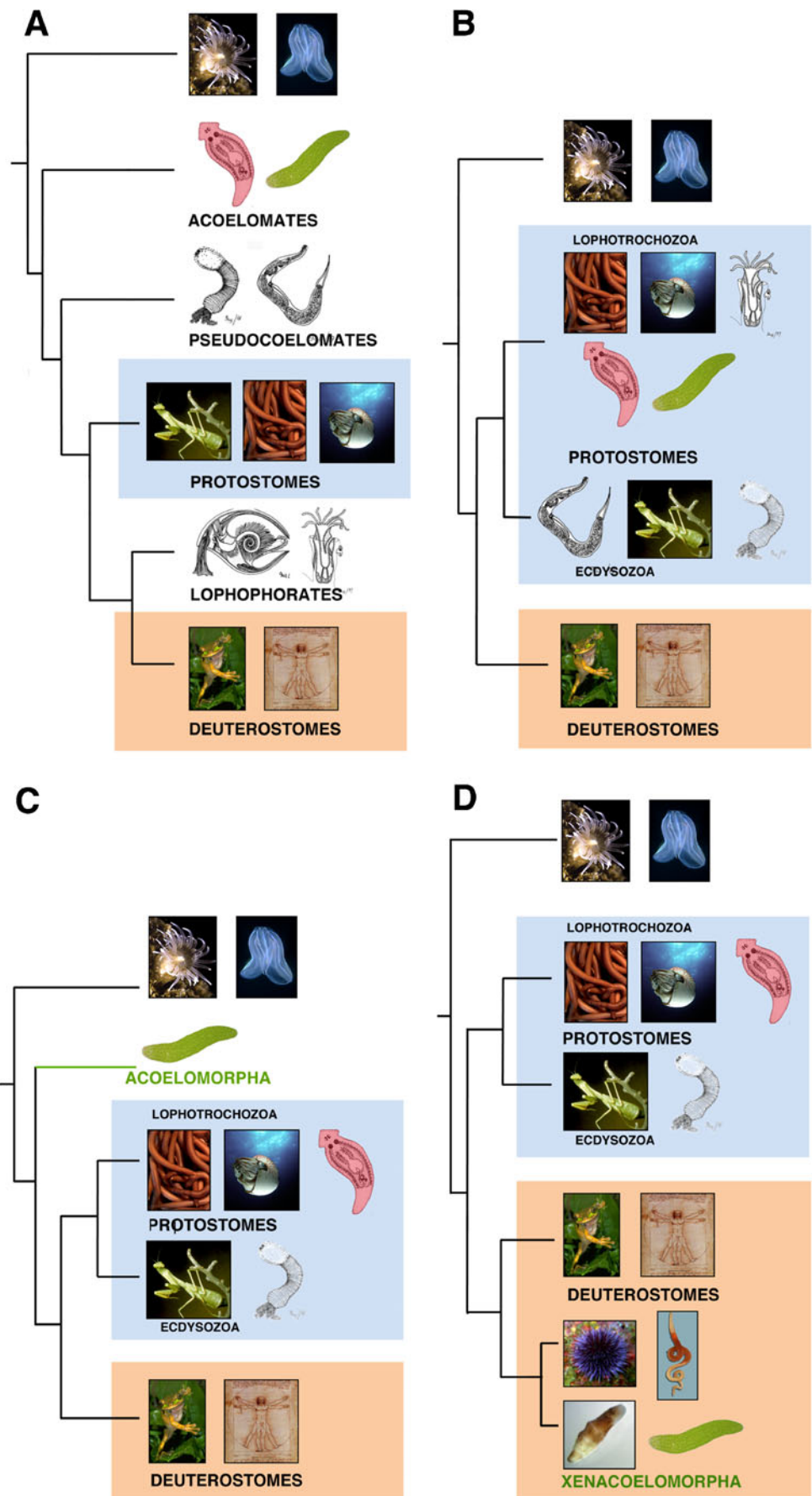
the ancestors of chordates the ventral ones (Reisinger 1925; Hanstroem 1968).

With the advent of molecular phylogenetics a decade ago, a major revision of the classical bilaterian phylogeny became necessary (Adoutte et al. 1999, 2000). In this revision, the entire platyhelminth phylum was removed from its basal position and firmly embedded within the lophotrochozoa, one of the two protostome superclades (Fig. 2b). As a result, the platyhelminth flatworms could no longer be considered to be more basal than any of the other lophotrochozoan phyla such as the annelids or molluscs. From this revised phylogenetic point of view, there is no a priori reason to assume that the flatworm CNS should manifest primitive features characteristic of the urbilaterian brain. Indeed, based on this revised phylogeny none of the living animals would correspond to intermediates between protostomes and deuterostomes, hence, making it more difficult to reconstruct the anatomical organization of the ancestral bilaterian brain from any extant species.

Strikingly, the most recent molecular phylogenomic studies have changed this situation once again, with profound consequences for our notion of the origin of the brain and we would like to draw attention to this fact. An entire subgroup of the classical platyhelminth phylum, the acoelomorph flatworms comprising the acoels and nemertodermatids, has now been removed from the remaining lophotrochozoan (protostome) platyhelminths. These recent studies now place the acoelomorph flatworms either at the base of the bilaterians preceding the protostome/deuterostome split or associated with the deuterostomes (Philippe et al. 2007, 2011; Hejnol et al. 2009; Hejnol and Martindale 2008; Mwinyi et al. 2010; Edgecombe et al. 2011). In all of these new phylogenetic scenarios, the acoelomorph flatworms are now widely separated from the platyhelminth flatworms which remain firmly embedded in the protostome supergroup (Fig. 2c, d). Importantly, in all of these cases, the last common ancestor of the two deeply separated flatworm clades corresponds to the last common ancestor of all bilaterians, urbilateria.

The new phylogenetic dissociation of the acoelomorph flatworms from the platyhelminth (protostome) flatworms indicates that these two animal groups are separated in evolution by at least half a billion years (Peterson et al. 2001, 2008). Nevertheless, in morphological terms, both of these groups manifest the same organizational features of the classical “flatworm” body plan implying that some of the architectural aspects of this body plan have been evolutionarily conserved. This in turn suggests that comparative studies of acoelomorph flatworms and platyhelminth flatworms might indeed reveal insight into the primitive organizational features of the common urbilaterian ancestor, including those of its brain.

Fig. 2 Simplified phylogenetic trees, showing placement of Acoels and Platyhelminths. **a** Traditional phylogeny, **b** modified from Adoutte et al. (2000), **c** modified from Hejnol et al. (2009), **d** modified from Philippe et al. (2011)



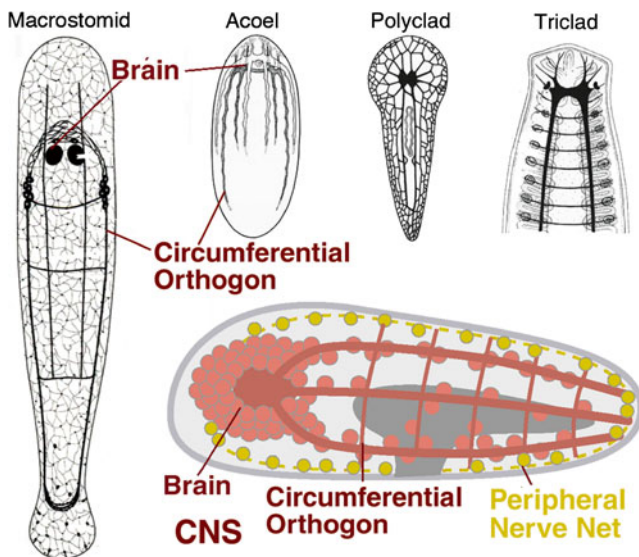


Fig. 3 Elements of the nervous system of flatworms, including platyhelminths and acoels. Line drawings (*top*) after Hanstroem (1928)

Common neuroarchitectural features of acoelomorph and platyhelminth brains

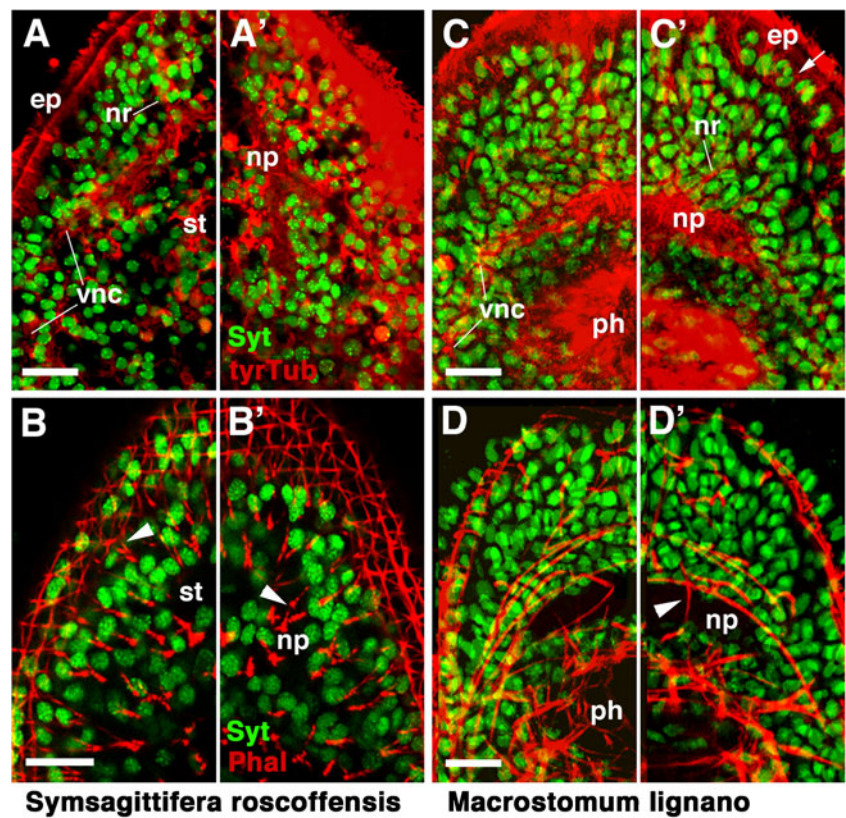
Despite this deep phylogenetic separation, the acoelomorph and the platyhelminth flatworms both have nervous systems that are remarkably similar. Notably, and in contrast to previous claims, recent neuroanatomical studies demonstrate that both types of flatworms have relatively complex anterior brains that consist of a cortex of neural cell bodies and a central neuropile with numerous commissural and longitudinal fiber bundles.

Early classical histological analyses of the central nervous systems of acoelomorph flatworms reported the presence of a bilobed central brain composed of numerous neuronal cell bodies associated with complex commissural and connective fiber bundles (e.g., von Graff 1891; Delage 1886). Many more contemporary studies of acoelomorph nervous systems have been based primarily on immunocytochemical methods using antibodies against neurotransmitters (e.g., Reuter et al. 1998; Gaerber et al. 2007; Kotikova and Raikova 2008). From these studies, it has been concluded that the acoelomorph CNS has only a simple anterior aggregate of transverse and longitudinal fibers corresponding to a diffuse “commissural brain”. However, since these types of immunolabeling studies display only small neuronal subsets, such as the populations of neurons expressing the peptide FMRFamide, and usually fail to reveal the nervous system as a whole, they are not very well suited to reveal comprehensive neuroarchitectural features. Recently, these problems have been resolved by the use of antibody markers combined with serial transmission electron microscopy and 3D reconstruction techniques to study the brain of the acoel *Symsagittifera roscoffensis* (Bery et al. 2010). This

investigation confirms early histological findings and clearly confirms the presence of a compact anterior brain in juvenile acoels that was claimed in classical studies, as well as more recent investigation of the developing acoel, *Neochildia fusca* (Ramachandra et al. 2002). The crescent-shaped *Symsagittifera* brain is composed of approximately 700 cells arranged in a cortical layer that surrounds a complex central neuropile. The two bilateral domains of the brain neuropile are linked by three commissural tracts, and the neuropile also contains a bilateral pair of simple pigment-cup eyes and surrounds an unpaired mechanoreceptive statocyst (Fig. 4a). Three pairs of longitudinal nerve cords, which are cross-connected by numerous commissures, emanate from the brain, extend the entire length of the animal, and are flanked by regularly spaced neural cell bodies. Most neurons appear to conform to the characteristic unipolar type of invertebrate ganglionic nerve cells, whereby a single neurite (“cell body fiber”) issues from the soma. Reaching the neuropile, the cell body fiber branches into multiple neurites which enter into pre- and postsynaptic relationships with neighboring neurons. Polarized synapses with differently shaped vesicles (suggesting excitatory and inhibitory synapses, as well as neurosecretory transmitter release) were identified in the *Symsagittifera* brain and nerve cords. Aside from the brain and nerve cords, scattered peripheral neurons form a diffuse nerve plexus around and in between the muscle fibers and epidermal cell bodies. Comparable findings have been reported in an independent study of neurogenesis in *Symsagittifera* by Semmler et al. (2010). In view of these findings, the notion that acoels have “diffuse” nervous systems and lack a “true” central brain is clearly untenable.

In all of the above features, the neuroarchitecture of the acoel brain is remarkably similar to that of the platyhelminth brain. The neuroanatomy of the platyhelminth flatworm brain has been described for numerous species and in all cases the architecture of the brain is comparable corresponding to a typical complex invertebrate ganglion (e.g., Lentz 1967; Keenan et al. 1981; Reuter and Gustafsson 1995; Agata et al. 1998; Younossi-Hartenstein et al. 2000, 2001; Gustafsson et al. 2002; Cebria 2008). Moreover, recently Morris et al. (2007) have used immunocytochemical analysis to construct a 3D model of the brain of the basal platyhelminth *Macrostomum lignano* so that a direct comparison with the 3D model of the acoel brain of *S. roscoffensis* is possible. This comparison confirms the common architectural features of both types of flatworm brain (Figs. 4c, d and 5e–g). Thus, the anterior brain of *Macrostomum* comprises approximately 1,000 neurons whose cell bodies are located in an outer cortical layer surrounding a sizeable crescent-shaped central neuropile. Numerous stereotyped commissural and longitudinal axon tracts subdivide the neuropile into discrete compartments and simple pigment-cup eyes are located in

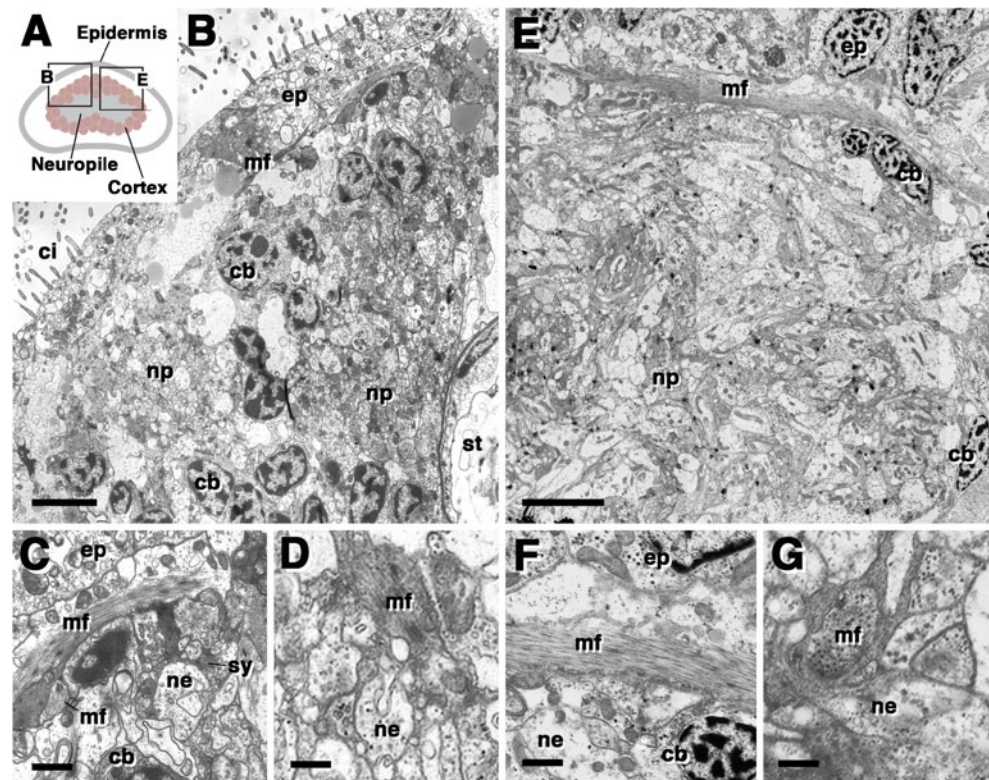
Fig. 4 Structure of the brain of juvenile acoel (*Symsagittifera roscoffensis*; **a–b'**) and juvenile platyhelminth (*Macrostomum lignano*; **c–d'**). All panels show z-projections of horizontal confocal sections of heads of juveniles; anterior is up. Cell nuclei are labeled green by nuclear dye Sytox (*Syt*); in upper panels (**a**, **c**), red label (anti-tyrosinated tubulin; *tyrTub*) represents cilia and nerve fibers; in lower panels (**b**, **d**), muscle fibers are labeled red by rhodamine–phalloidin (*Phal*). Each panel is split into two halves; right half (e.g., **a**) corresponds to more ventral projection, left half (e.g., **a'**) more dorsal projection. *ep* epidermis, *np* neuropile, *nr* nerve root, *ph* pharynx, *st* statocyst, *vnc* ventral nerve cord. *Bar*=10 μ m



the dorsolateral brain, similar to what has been described for planarians and other flatworm clades (Younossi-Hartenstein et al. 2000; Martín-Durán et al. 2012). One principle and

two accessory pairs of longitudinal nerve cords project from the brain posteriorly along the body wall; these are accompanied by regularly spaced neural cell bodies. A further

Fig. 5 Ultrastructural aspects of the brain of juvenile acoel *S. roscoffensis* (left; **b–d**) and *M. lignano* (right; **e–g**). Schematic cross section (**a**) indicates image frames of photographs shown in panels (**b**) and (**e**). Frames include part of the brain cortex with neuronal cell bodies (*cb*) as well as neuropile (*np*). Panels (**c**)–(**d**) and (**f**)–(**g**) show higher magnifications of cellular elements of the flatworm brain. *cb* cell body, *ci* cilia, *ep* epidermal cell, *mf* muscle fiber, *ne* neurite (axon or dendrite), *np* neuropile, *st* statocyst, *sy* synapse. *Bars*=3 μ m (**b**, **e**); 0.5 μ m (**c**, **f**); 0.25 μ m (**d**, **g**)



peculiarity seen in the brain of Platyhelminthes and Acoels and few, if any, other bilaterian phyla is the mingling of neurons with other cell types. Thus, in *Macrostomum* and *Symsagittifera* alike, a dense meshwork of muscle cells and glands penetrate the brain (Figs. 4c, d and 5e, g).

Neuroglia, which forms multilayered sheaths around neuronal cell bodies, neuropile compartments, and fiber tracts in vertebrates and all “higher” invertebrate taxa (Hartline 2011; Hartenstein 2011; Boyan et al. 2011; 2012), is absent in acoels and platyhelminths. Individual cells which, in single sections, appear to form lamellar “glia-like” processes do exist in *Symsagittifera*, as well as several parasitic platyhelminths species (Biserova 2000, 2008; Bery et al. 2010). However, the pronounced outer glial lamella that covers ganglionic surfaces in arthropods, annelids, or molluscs is absent in acoels and platyhelminths alike (Fig. 5b, e). The lack of glia in flatworms as well as several other “lower” protostome and deuterostome phyla (Hartline 2011) suggests that this cell type, which is so prevalent in the nervous system of higher animals, arose multiple times independently. The fact that early expressed fate determinants of glia are fundamentally different in *Drosophila* and vertebrates (Hartenstein 2011; Hartline 2011) lend further support to this idea.

Similarities are also observed in the development of the nervous system in acoels and rhabditophoran platyhelminths. In the platyhelminth *Macrostomum*, cleavage follows a modified spiral pattern and results in a solid embryonic primordium surrounded by an external yolk layer. Subsequently, cells at the anterior and lateral periphery of the embryo evolve into the embryonic primordium, a multilayered mesenchymal cell mass which gives rise to the body wall and nervous system (Fig. 6a, b). The future brain appears as a bilateral condensation at the anterior end of the embryonic primordium (Fig. 6a, b). Organ differentiation proceeds within the embryonic primordium during stages 5 and 6 when neurons extend axons that form a central neuropile, the outer cell layer of the somatic primordium turns into a ciliated epidermal epithelium, and muscle precursors extend myofilaments that are organized into a highly regular orthogonal network of circular, diagonal, and longitudinal fibers (Fig. 6c, d). Development of the acoels *Neochildia* and *Symsagittifera* follows a very similar path as that described above. Thus, following a modified spiral cleavage (“duet spiral cleavage”), the embryonic primordium consists of a solid, spherical mass of cells. With the onset of organogenesis, the outermost layer of cells transforms into a ciliated epidermal epithelium (Fig. 6e, f). The subepidermal, two to three cell diameter thick layer of densely packed cells comprises the progenitors of muscles and brain, and differentiates in the same way as noted above for *M. lignano* (Fig. 6g, h).

Neurogenesis as summarized above for flatworms and acoels is in marked contrast to that of “higher taxa”, where

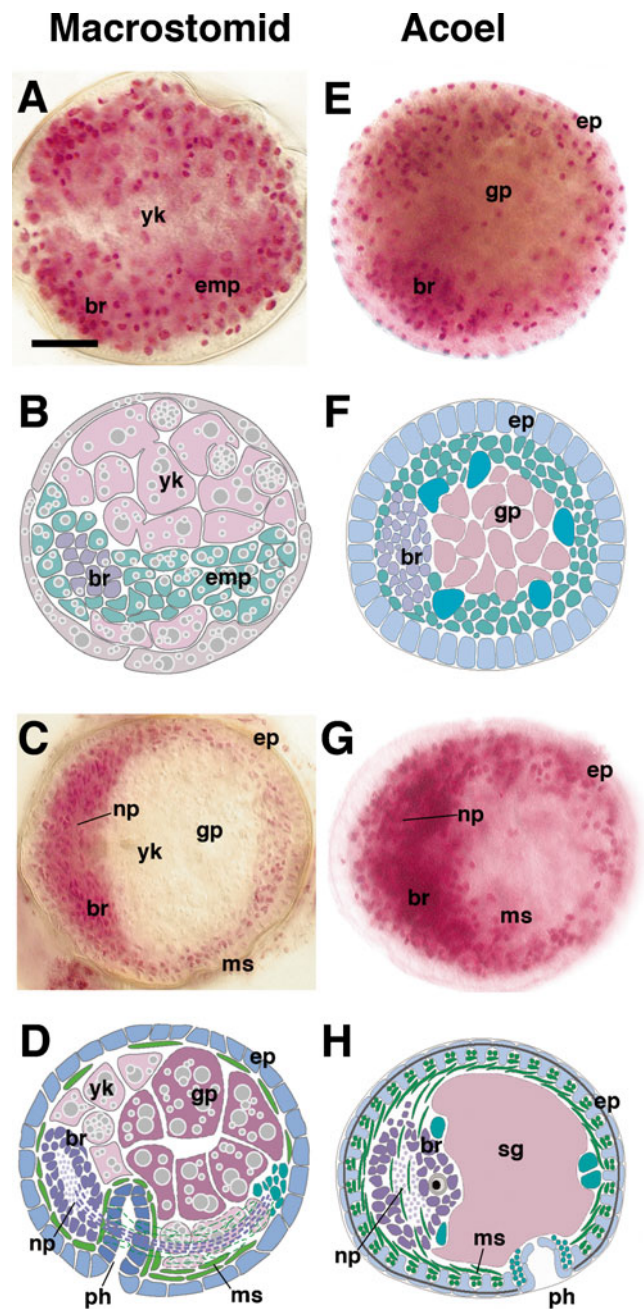


Fig. 6 Embryonic development of platyhelminth (*M. lignano*; panels a–d) and acoelomorph flatworm (*Neochildia fusca*; panels e–h). Panels of first row (a, e) and third row (c, g) show photographs of embryos labeled with nuclear marker fuchsin (red). Dorsal view, anterior to the left. (a) and (e) represent an early stage (stage 4; approximately 30 % of development) of organogenesis; (c) and (g) a late stage of organogenesis (stage 6; approximately 70 % of development). Second row (b, f) and fourth row (d, h) are schematic drawings of embryos represented in photographs above. br brain primordium, emp embryonic primordium, ep epidermis, gp gut primordium, ms muscle, np neuropile, ph pharynx, sg syncytial gut, yk yolk. Bar=20 μ m

a distinct neural primordium, separated from other embryonic tissues (e.g., the vertebrate neural tube arising through invagination of the neurectoderm; or the insect neuroblast

layer, formed by delamination of neuroblasts) appears long before neurons, or any other cell types, differentiate. In acoels and flatworms, no germ layers or spatially separable organ primordia are apparent before onset of cellular differentiation. Neurons, muscle cells, and gland cells form a “mélange” within the deep layers of the embryonic primordium; one might speculate that, to a large degree, local cell–cell interactions within the embryonic primordium decide over the fate of individual cell types. It is interesting to note that despite of this morphogenetic differences separating flatworms/acoeles from other bilaterians, molecular determinants of cell fate and signaling pathways are nonetheless highly conserved (Brindley 2005; LoVerde et al. 2007; Newmark et al. 2008; Riddiford and Olson 2011; Almuedo-Castillo et al. 2012).

Taken together, these findings demonstrate that acoelomorph flatworms and the (protostome) platyhelminth flatworms both have central nervous systems that include ganglionic anterior brains. Furthermore, they indicate that both types of brains manifest similar neuroarchitectural and developmental features despite the deep phylogenetic separation of the two flatworm clades. These results suggest that the morphological features shared by the brains of the two flatworm clades are likely to be representative of the brain of their urbilaterian ancestor.

Outlook: desperately seeking the urbilaterian

The comparative neuroanatomical analysis of protostome versus deuterostome flatworms supports the notion of a common evolutionary origin of bilaterian brains that has been derived from developmental genetic analysis. To investigate this further, it will be important to study the molecular mechanisms of brain development in acoelomorph and platyhelminth flatworms. A comparison in these two flatworm clades of the expression and function of the suite of conserved developmental control genes that underlie brain development in higher bilaterians should provide further insight into the primitive versus derived features of the brain in protostomes and deuterostomes as well as in the elusive urbilaterian ancestor.

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