Hemichordata

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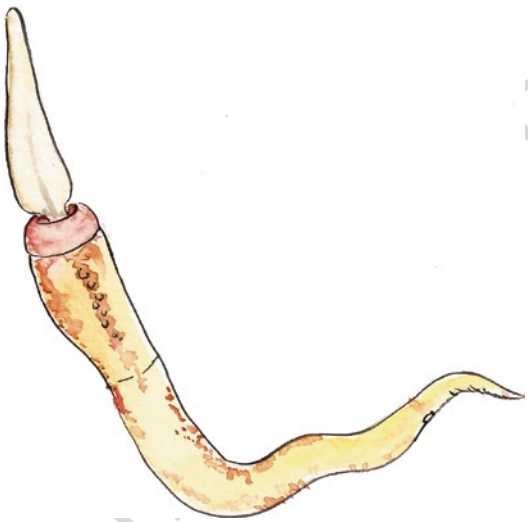

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INTRODUCTION

Synopsis

Hemichordata is a group of exclusively marine animals, consisting of two subgroups, the sessile and small colonial pterobranchs and the solitary, vermiform enteropneusts (acorn worms) (Fig. 2.1; van der Horst 1939; Hyman 1959; Benito and Pardos 1997). With about 130 described species, Hemichordata comprises a relatively small taxon of benthic animals (http://www.marinespecies.org/index.php; Cameron 2005). They are distributed worldwide and inhabit shallow coastal areas but are also found in the deep sea. For a long time, pterobranchs have been known only from deep waters, whereas enteropneusts were thought to burrow mainly in shallow waters. However, within the last five decades, about a dozen of different enteropneusts have been documented in the deep sea (Osborn et al. 2012). In contrast, pterobranchs have been found in intertidal zones of tropical waters only recently (Lester 1985) and might have been overlooked previously due to their minute size and superficial similarities in their gross morphology with other tube-dwelling animals, such as polychaetes and bryozoans.

Enteropneusts as well as pterobranchs exhibit a tripartite body organisation divided into an anterior prosoma, a mesosome and a posterior metasomal region (Fig. 2.1). In enteropneusts, the prosoma is called "acorn" or "proboscis", while in pterobranchs, it is termed "mouth shield". The middle body region encompasses the anteroventral mouth opening and is referred to as the "collar" or "mesosome", respectively. The mesosome of pterobranchs holds a tentacular crown for filter feeding (van der Horst 1939; Benito and Pardos 1997).

Both hemichordate groups have a characteristic excretory system in the prosoma. It is composed of a contractile pericardium that encloses the heart sinus that anteriorly continues into the glomerulus. The glomerulus is a highly dilated blood plexus lined by podocytes that are capable of mediating ultrafiltration into the protocoel. The accumulated ultrafiltrate then leaves the protocoel via the proboscis pore (Balser and Ruppert 1990). Indeed, such an excretory complex is also present in echinoderms (axial complex), thus representing a synapomorphy for Ambulacraria, yet in hemichordates, the unique stomochord is incorporated into this complex (Dohle 2004). The stomochord is an endodermal protrusion filled with vacuolated cells surrounded by a

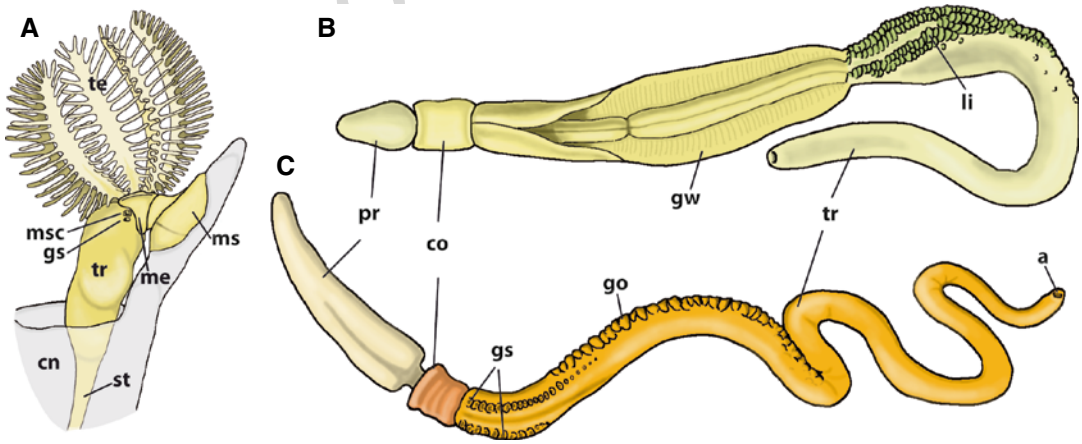

Fig. 2.1 Overview of the general morphology of hemichordates. (**A**) Habitus of an adult pterobranch, *Cephalodiscus* sp (Modified from Lester 1985), 2.5 mm. (**B**) Adult specimen of *Balanoglossus clavigerus* (Ptychoderidae), 25 cm. (C) Adult specimen of *Saccoglossus mereschkowskii* (Harrimaniidae), 4 cm (**B**, **C** modified from Goldschmid 2007). *a* anus, *cn* coenecium, *co* collar, *go* gonads, *gs* gill slit, *gw* genital wing, *li* liver sacs, *me* mesosome, *ms* mouth shield, *msc* mesocoelic pore, *pr* proboscis, *st* stalk, *te* tentacles, *tr* trunk

thickened extracellular matrix that serves as a supportive structure for the pericardium-glomerulus complex (Balser and Ruppert 1990; Mayer and Bartolomaeus 2003; Kaul-Strehlow and Stach 2011; Merker et al. 2013). A possible homology between the stomochord and the notochord of chordates is still debated.

Enteropneusta

Enteropneusta comprises less than 110 described species of worm-shaped animals (Cameron 2005) ranging from 0.5 mm to more than 2 m in size (Hyman 1959; Worsaae et al. 2012). Acorn worms are usually ground dwellers that live in muddy or sandy sediments; only few are known to be associated with the undersides of rocks (Spengel 1893; Hyman 1959; Cameron 2005). The body of acorn worms is vermiform and can be subdivided into three regions, each of which is supported internally by a corresponding coelomic cavity (Spengel 1893; Morgan 1894). The anterior proboscis differs in shape from long and slender (Fig. 2.1C) to conical and short (Fig. 2.1B; van der Horst 1939). Posteriorly, the proboscis connects to the collar region (mesosome) by a robust stalk. The highly muscular proboscis is the main locomotory organ and is used for the search for food and burrowing (van der Horst 1939; Benito and Pardos 1997). The short and barrel-shaped collar region surrounds and overlaps the posterior part of the proboscis including the proboscis stalk (Fig. 2.1B, C). The mouth opening is positioned ventrofrontally between the constriction separating proboscis and collar region (Bateson 1884, 1885; Spengel 1893). The middle collar region is followed by the long trunk region that forms the largest part of the enteropneust body. The trunk is functionally differentiated into the anterior branchio-genital region, the middle hepatic region and the posterior abdominal (intestine) region (Fig. 2.1B, C; Spengel 1893; Hyman 1959). A bilateral row of numerous dorsolateral gill slits accompanies the branchio-genital region. In many large species in particular, the specialised hepatic region is characterised by numerous liver sacs that are projections of the intestine and body wall (Fig. 2.1B). The tubular abdominal region is comparatively long and bears the anus at its terminal end. The nervous system of enteropneusts is developed as a basiepidermal nerve net throughout the body (Silén 1950; Bullock and Horridge 1965). More condensed areas are present in the trunk region, where they constitute a dorsal and a ventral median longitudinal nerve cord, respectively (Bullock 1946; Knight-Jones 1952). Furthermore, the dorsal nerve cord continues anteriorly into the collar region to form the subepidermal, hollow collar cord that is reminiscent of the neural tube present in chordates (Morgan 1894; Kaul and Stach 2010; Miyamoto and Wada 2013). The collar cord links to the basiepidermal plexus at the base of the proboscis, that is, the proboscis stem. Given that enteropneusts mainly consist of soft body parts, they are bad candidates for leaving fossilising remains, explaining their poor fossil record. Nevertheless, a recent finding of a tube-dwelling enteropneust dates their origin back to at least the Cambrian (Caron et al. 2013).

Pterobranchia

Pterobranchia is a small taxon consisting of roughly 25 species, of which all are colonial and semi-sessile animals (Cameron 2005; Nielsen 2011). They are tube dwellers that secrete the so-called coenecium from the anterior mouth shield (Fig. 2.1A). The body size of the individual zooid lies between 1 and 5 mm and an entire colony is built from a single larva that reproduces asexually by budding (Anderson 1907; van der Horst 1939). The mesosome is equipped with tentacles that are used for filter feeding and probably also serve respiratory function. The bulbous trunk region harbours most of the U-shaped digestive tract including the posterior pharynx, stomach and intestine. The anus opens on the dorsal side of the anterior trunk region, just behind the mesosome. The nervous system of pterobranchs constitutes a basiepidermal nerve net. Additionally, a prominent dorsal brain is present at the base of the mesosomal tentacles (Dilly et al. 1975; Rehkämper et al. 1987; Stach et al. 2012). The brain features an anterior neuron-rich area composed of serotonergic and giant neurons as well as a posterior neuropil. Further concentrated parts of the peripheral nervous system are tentacle

nerves, a ventral stalk nerve and a pair of branchial nerves in *Cephalodiscus gracilis* (Stach et al. 2012). Pterobranchs mainly live associated with hard substrates since their secreted tubes build encrusting aggregates on rocks and shells. The majority of described species have been collected in the deep sea and only few species are known from shallow waters. These include *Rhabdopleura normani* and *C. gracilis* that can easily be accessed by snorkelling in shallow waters on the Bermuda Islands (Lester 1985, 1988a, b) or *Rhabdopleura compacta* that lives off the south coasts of England (Sato et al. 2008). Due to their secreted tubes, pterobranchs are associated with the fossil group Graptolithina that are known from the Cambrian through the Carboniferous. Graptolithina composes a quite diverse group of fossils of pelagic tube-dwelling colonies that are supposed to be closely related to the extant genus *Rhabdopleura* (Sato et al. 2008).

Within the last couple of years, Sato and colleagues have begun to develop *Rhabdopleura compacta*, a species that can be found in the south of England, as an emerging model organism that can be used for developmental studies (Sato et al. 2008, 2009; Sato and Holland 2008).

Historical Background

The very first mentioning of an enteropneust can be traced back to the work of Friedrich Eschscholtz in 1825, who described a wormlike animal named *Ptychodera* that he interpreted as a new species of holothuroid echinoderms (Eschscholtz 1825). Only a few years later, and without knowledge of Eschscholtz' description, Stefano Delle Chaje documented a sand-dwelling worm that he named *Balanoglossus clavigerus* (Delle Chaje 1829). However, both reports were only short notes showing sketchy drawings, and it was not before 1866 until the first detailed anatomical description of an enteropneust was published by Alexander Kowalevsky (1866). At that time, phylogenetic relationships of enteropneusts were controversially discussed. Because of a vermiform body, a closer relationship to either nemerteans, annelids or other "worms" was suggested, but also holothurians were considered as relatives. Interestingly, a conspicuous larva, first documented around 1850, played a more important role in resolving this issue than initially thought. In 1849, Johannes Müller found a peculiar larva in the plankton near Marseille, France. He named this larva "tornaria" and placed it in a closer connection to the bipinnaria of sea stars, because of similarities in the arrangement of the ciliary bands (Müller 1850). At the same time, a similar larva was found by August Krohn, but this one had a much more sinuously running ciliary band and he suggested this to be a different species (Krohn 1854). In those days, it was not known that these varying larvae actually display successive developmental stages. Nowadays, this particular larval stage with secondary lobes and saddles of the ciliary feeding band (neotroch) is called "Krohn stage", referring to its original describer. In fact, all of the succeeding larval stages have later on been given specific names, being in connection to the historical background of their discovery. This will be discussed more precisely in the paragraph dealing with late development. Nevertheless, it was Elias Metschnikoff who made a promising finding in 1870, when he collected a larva that showed similarities with the worm of *Balanoglossus* (Metschnikoff 1870), yet his colleagues did not believe in a connection between echinoderm-like tornaria and the wormlike enteropneust. It took three more years until Alexander Agassiz successfully documented the metamorphosis of a tornaria larva into a juvenile *Balanoglossus*, thus finally unravelling the larva's unknown affiliation (Agassiz 1873). In the following years, a number of descriptive studies were added to the existing list. Bateson published a study of direct development of *Saccoglossus kowalevskii* without a tornaria larva and at the same time he was the first who observed early cleavage stages in enteropneusts (Bateson 1884, 1885, 1886). He suggested a close relationship of enteropneusts with chordates, because of shared characters such as gill slits, stomochord and a neurulated collar cord.

Since then, consecutive studies on various aspects of enteropneusts have significantly increased our understanding of the group and were summarised in several classical textbooks and treatises such as Spengel's monograph on enteropneusts (1893), van der Horst's "Enteropneusta" in *Bronn's Klassen und Ordnungen des Tierreichs* (1939) or Hyman's *The Invertebrates* (1959), to name but a few. Strangely, the interest in enteropneust research declined within the last half of the twentieth century and relatively few works were published. Only recently, with upcoming immunocytochemical as well as molecular techniques and the new field of "EvoDevo" research, people have rediscovered the potential and importance of enteropneusts in unravelling evolutionary developmental questions (Tagawa et al. 1998a, b; Lowe et al. 2003, 2006; Röttinger and Martindale 2011; Röttinger and Lowe 2012; and references therein).

Systematics and Phylogenetic Relationships

Morphologically, hemichordates are well supported as deuterostome animals, because the main coelomic cavities originate from the archenteron by enterocoely and the mouth is formed secondarily during development. However, the exact position of Hemichordata within the Deuterostomia and its putative sister group has been controversially discussed within the last decade, due to incongruent morphological as well as molecular data. On the one hand, shared chordate features such as gill slits, stomochord and neurulated collar cord in enteropneusts lead already Bateson (1885) and Barrington (1965) to suggest a closer relationship of Hemichordata and Chordata. Indeed, phylogenetic analyses based on morphological characters strongly support this view (Young 1962; Maisey 1986; Schaeffer 1987; Ax 2001). However, more recent molecular phylogenetic analyses consistently reveal a sister group relationship between Hemichordata and Echinodermata, comprising the Ambulacraria (Fig. 2.2; Bourlat et al. 2006; Cannon et al. 2009; Hejnol et al. 2009; Edgecombe et al. 2011). At present, Ambulacraria seems to be widely accepted and is also morphologically supported by a shared larval type (dipleurula with specialised neotroch ciliary band) as well as similar coelomic systems and excretory organs. Pterobranchs and enteropneusts are classically treated as sister groups, yet molecular phylogenetic analyses strongly support the position of pterobranchs as a sister group to Harrimaniidae (an enteropneust subclade), thus rendering Enteropneusta paraphyletic (Fig. 2.2; Cameron et al. 2000; Cannon et al. 2009; Osborn et al. 2012). The possibility that pterobranchs evolved a sessile and colonial lifestyle secondarily from a solitary, wormlike enteropneust ancestor has further been supported by the recent discovery of a tubicolous enteropneust from the Cambrian (Caron et al. 2013).

There are three main subclades to which the majority of enteropneust species belong to, namely, Ptychoderidae (Spengel 1893), Spengelidae (Willey 1898) and Harrimaniidae (Spengel 1901) (Fig. 2.2). A fourth taxon has recently been described, the Torquaratoridae (Holland et al. 2005), comprising enteropneusts from the deep sea (Osborn et al. 2012). Members of the Torquaratoridae are characterised by lacking a stomochord as adults. Moreover, the proboscis skeleton is either reduced to a small plate or completely absent. The Ptychoderidae includes the first enteropneust species described, *Ptychodera flava*, and is characterised by the presence of gill-slit synapticles and a distinct trunk region featuring liver sacs and genital wings (Figs. 2.1B and 2.2). Members of the Spengelidae do not exhibit genital wings at the trunk region and liver sacs were only described for the genus *Schizocardium* (Spengel 1893; Cameron 2005).

Ptychoderidae and Spengelidae possess a biphasic life cycle and develop indirectly via a pelagic tornaria larva. Harrimaniidae includes the smallest representative of an acorn worm described so far, *Meioglossus psammophilus* (Worsaae et al. 2012), with less than 1 mm in

length. This taxon possesses the simplest anatomy, as their members lack liver sacs as well as genital wings within the dorsal trunk region (Fig. 2.1C). Also, the gill slits in harrimaniids are not supported by synapticles as in other enteropneust subclades (van der Horst 1939; Cameron 2005). Harrimaniid enteropneusts such as *Saccoglossus* develop directly, not passing through an extended pelagic stage. The hatchlings resemble young adults and soon settle in the sediment to grow into a juvenile enteropneust.

Pterobranchs are grouped into two genera, *Cephalodiscus* (M'Intosh 1882) and *Rhabdopleura* (Allman 1869) (Fig. 2.2). A putative third genus is monotypic with *Atubaria heterolopha* (Sato 1936) from deeper Japanese waters, yet the validity of this genus is questionable (Mierzejewski 2004). Many *Cephalodiscus* species live in fingerlike branched coenecia and have individual zooids, although in some species, the zooids are linked to each other by the posterior stalk (Lester 1985). *Cephalodiscus* spp. are characterised by a globular metasomal region, one pair of gill slits, two gonads and mesocoel pores and between five and nine pairs of tentacles on the mesosome (Hyman 1959). A muscular stalk is present at the posterior end of the metasomal region and aids in its withdrawal into the coenecium. Species of the genera *Rhabdopleura*, in contrast, bear only a single pair of tentacles on the mesosome, have only one gonad and lack gill pores altogether (Schepotieff 1907; van der Horst 1939). The zooids of one colony live in tubular coenecia and stay interconnected by the stalk throughout lifetime (Lankester 1884; Hyman 1959).

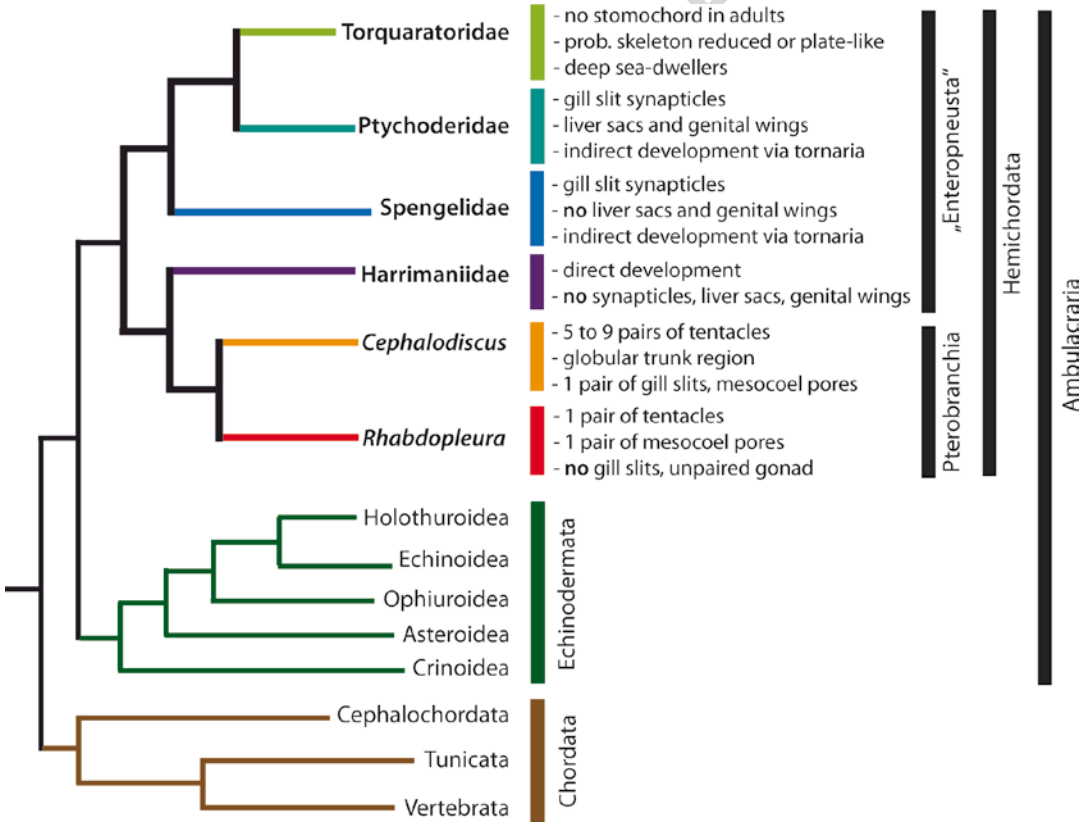

Fig. 2.2 Deuterostome phylogeny. Cladogram compiled from recent phylogenetic analyses (Osborn et al. 2012; Worsaae et al. 2012). The position of Xenacoelomorpha is debated and not shown here (Hejnol et al. 2009; Philippe et al. 2011)

EARLY DEVELOPMENT

Development in Pterobranchs

Pterobranchs are less frequently encountered as enteropneusts, which is why knowledge on the reproduction and development of these minute animals is still fragmentary. So far, it is known from *Rhabdopleura* that the fertilised, yolk-rich egg undergoes holoblastic, radial and equal cleavage, eventually leading to a uniformly ciliated larva (Fig. 2.3A–G; Stebbing 1970; Dilly 1973; Lester 1988a). The larva is of elongated shape with a tapering posterior end (Sato et al. 2008). Its colour is opaque and yellowish. A brown spotty pigmentation is present over the body and the larva exhibits a conspicuous ventral depression (Fig. 2.3F, G). After a short pelagic period, the larva tests the substrate and eventually settles with the ventral side secreting a cocoon. Inside the cocoon, the metamorphosing larva develops the anlagen of the tentacles, the mouth shield and the metasome; thus, the future tripartite body organisation is already established (Fig. 2.3H–K; Lester 1988b). After a few days, the cocoon breaks and the zooid starts to form its coenecium, thereby founding a new colony (Fig. 2.3L). In *Cephalodiscus*, even less is known about development, although a few accounts on single developmental stages are present (Harmer 1905; Anderson 1907; Schepotieff 1907; Dilly 2013). A recent ultrastructural study shows an elongated, three-layered embryonic stage of *C.*

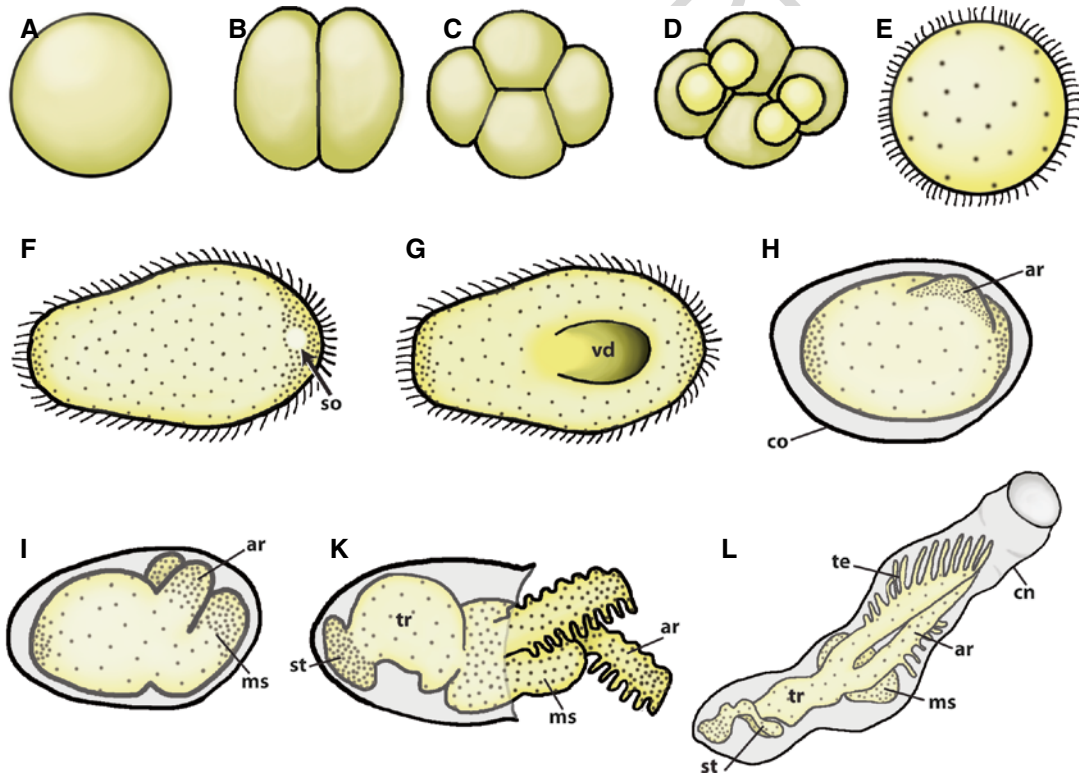

Fig. 2.3 Development and metamorphosis of *Rhabdopleura normani*. (**A**) Zygote. (**B**) Two-cell stage. (**C**) Four-cell stage. (**D**) Eight-cell stage. (**E**) Spherical, ciliated embryo. (**F**) Swimming larva from dorsal. (**G**) Swimming larva from ventral. (**H**) Metamorphosing larva in secreted cocoon, approx. 12 h after settlement. Lateral view. (**I**) Same as in **H**, approx. 33 h after settlement. (**K**) Hatching juvenile, approx. 100 h after settlement. (**L**) Zooid in secreted coenecium. Drawings not to scale (Modified from Lester 1988a, b). *ar* arm holding tentacles, *cn* coenecium, *co* cocoon, *ms* mouth shield, *so* sensory organ, *st* stalk, *te* tentacle, *tr* trunk, *vd* ventral depression

gracilis (Stach 2013). The prospective meso- and metacoels are already separated from the endoderm, whereas the prospective protocoel is still continuous with the endoderm. The one pair of gill pores seems to develop asynchronously, in favour of the left side. The creeping larva lacks any planktonic specialisations and might develop directly into a young zooid without passing through a pelagic stage.

Development in Enteropneusts

Fertilisation is external in all studied enteropneusts and spawning is usually dependent of the species' habitat and correlated with seasons, whereby temperature and light intensity play a major role (Hadfield 1975). As outlined before, two different modes of development have been observed in enteropneusts. Whereas members of Ptychoderidae and Spengelidae develop a pelagic larval stage, the tornaria, harrimaniid enteropneusts develop directly from yolk-rich eggs. Early development including cleavage patterns has been described for several enteropneust species (Bateson 1884; Stiasny 1914a; Burdon-Jones 1952; Colwin and Colwin 1953; Tagawa et al. 1998a; Urata and Yamaguchi 2004). In all studied enteropneusts, cleavage is radial, holoblastic and nearly equal.

Embryology in Direct Developing Enteropneusts

In direct developing enteropneusts such as *Saccoglossus kowalevskii*, a gravid female spawns between 200 and 1.000 oocytes with a diameter of about 300 µm. After fertilisation, a thick vitelline membrane is formed around the zygote. Subsequently, the fertilised egg undergoes radial cleavage of which the two first cleavages are meridional and the third is latitudinal (Bateson 1884; Colwin and Colwin 1953). The fourth cleavage results in a single animal tier of eight cells (Fig. 2.4), whereas the vegetal cells divide latitudinally to yield an upper tier of four larger cells as well as a lower tier of four smaller cells. Cell labelling in *Saccoglossus* showed that the cells of the animal tier give rise to the anterior ectoderm, while the upper tier of vegetal cells will form the middle and posterior ectoderm (Colwin and Colwin 1951; Darras et al. 2011). Only the cells of the lower vegetal tier will differentiate into endo- as well as mesoderm (Fig. 2.4). Continuous cleavages lead to a rounded coeloblastula. Before gastrulation, the blastula becomes cup-shaped by flattening of the animal-vegetal axis, while at the same time, the thickened vegetal pole invaginates circumferentially (Colwin and Colwin 1953). After gastrulation of the prospective endomesoderm, the blastopore closes and the embryo again elongates along the anterior-posterior axis (Fig. 2.5A). At this time of development, the opisthotroch (ciliary band), composed of numerous long compound cilia, is visible demarcating the postanal field. The embryos start to spin around within the vitelline membrane as soon as the cilia start to beat. Only a few hours later, a circumferential groove starts to subdivide the embryo in an anterior proboscis region and a posterior region constituting the future collar and trunk (Fig. 2.5B). At this stage, the anterior part of the invaginated archenteron separates as the first coelomic cavity, thereby forming the future protocoel (Figs. 2.4 and 2.6A). The paired meso- and metacoels are present as separated evaginations from the middle and posterior regions of the archenteron (Figs. 2.6A), yet are still connected to the archenteron. Around 4 days after fertilisation, the embryos are of elongated shape with a perianal field that is bent ventrally (Fig 2.5C). A second circular groove forms the border between the collar and trunk region. By this stage, the meso- and metacoels are pinched off and enclose the endoderm almost completely. A sixth, yet small coelomic cavity is situated at the posterodorsal base of the proboscis and will later differentiate into the pericardium or heart vesicle. In contrast to the pro-, meso- and metacoels that originate from the endoderm by enterocoely, the pericardium develops by schizocoely from the ectoderm in *S. kowalevskii* (Kaul-Strehlow and Stach 2011). Schizocoely is typical for formation of the mesoderm in various protostomes and only rare cases in deuterostomes are known (Technau and Scholz 2003). The endoderm connects to the exterior on the ventral side

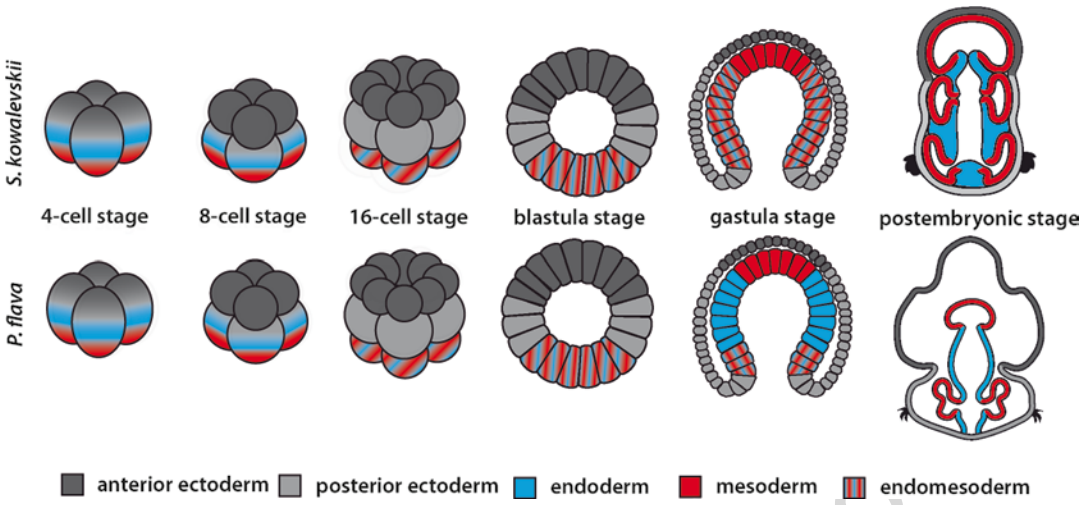

Fig. 2.4 Early embryology of direct and indirect developing enteropneusts. *Upper row*: the direct developing harrimaniid *Saccoglossus kowalevskii*. *Lower row*: the indirect developing ptychoderid *Ptychodera flava*. The exact timing of separation of endo- and mesoderm remains unclear

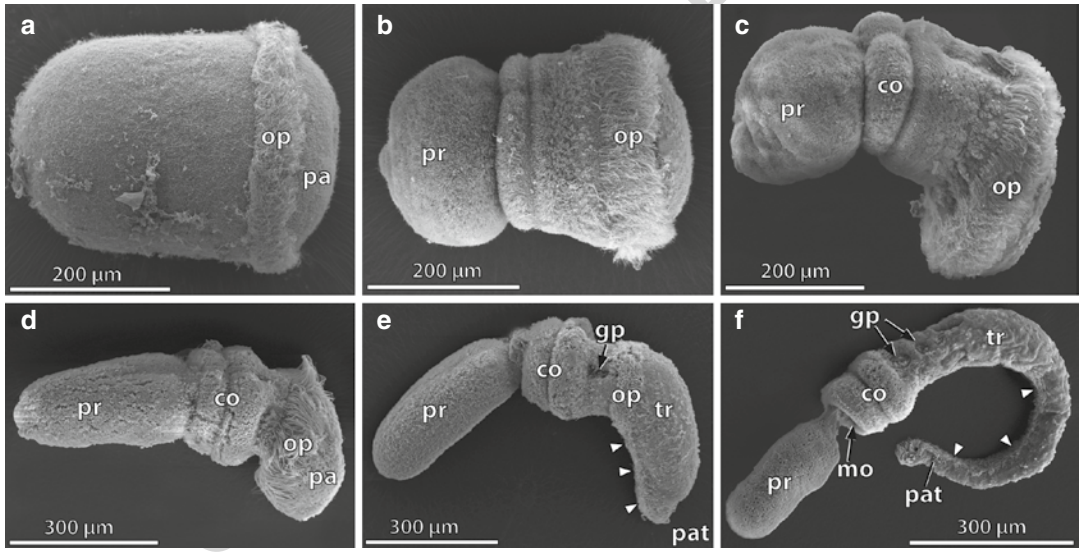

Fig. 2.5 Scanning electron micrographs of developmental stages of *Saccoglossus kowalevskii*. (**A**–**C**) Fertilisation membrane removed. (**A**) Late gastrula. (**B**) Early kink stage. (**C**) Dorsal flexure stage. (**D**) One-gill-slit hatchling. (**E**) Early settling juvenile. Note the ventral creeping sole (*arrowheads*) and the postanal tail. (**F**) Three-gill-slit juvenile with adult-like gross morphology. *co* collar, *gp* gill pore, *mo* mouth opening, *op* opisthotroch, *pa* perianal field, *pat* postanal tail, *pr* proboscis, *tr* trunk (© Sabrina Kaul-Strehlow 2015. All Rights Reserved)

just between the proboscis and collar region, forming the mouth opening, whereas the anus is still closed. The developing stomochord protrudes into the proboscis base as a short, rod-like extension from the anterodorsal roof of the endoderm. Closely behind the posterior margin of the collar region, the endoderm pierces through the metacoel of the trunk region and establishes contact with the ectoderm, thereby forming the anlagen of the first pair of gill pores (Fig 2.6C). Embryogenesis is usually complete at around 5 days after fertilisation in embryos

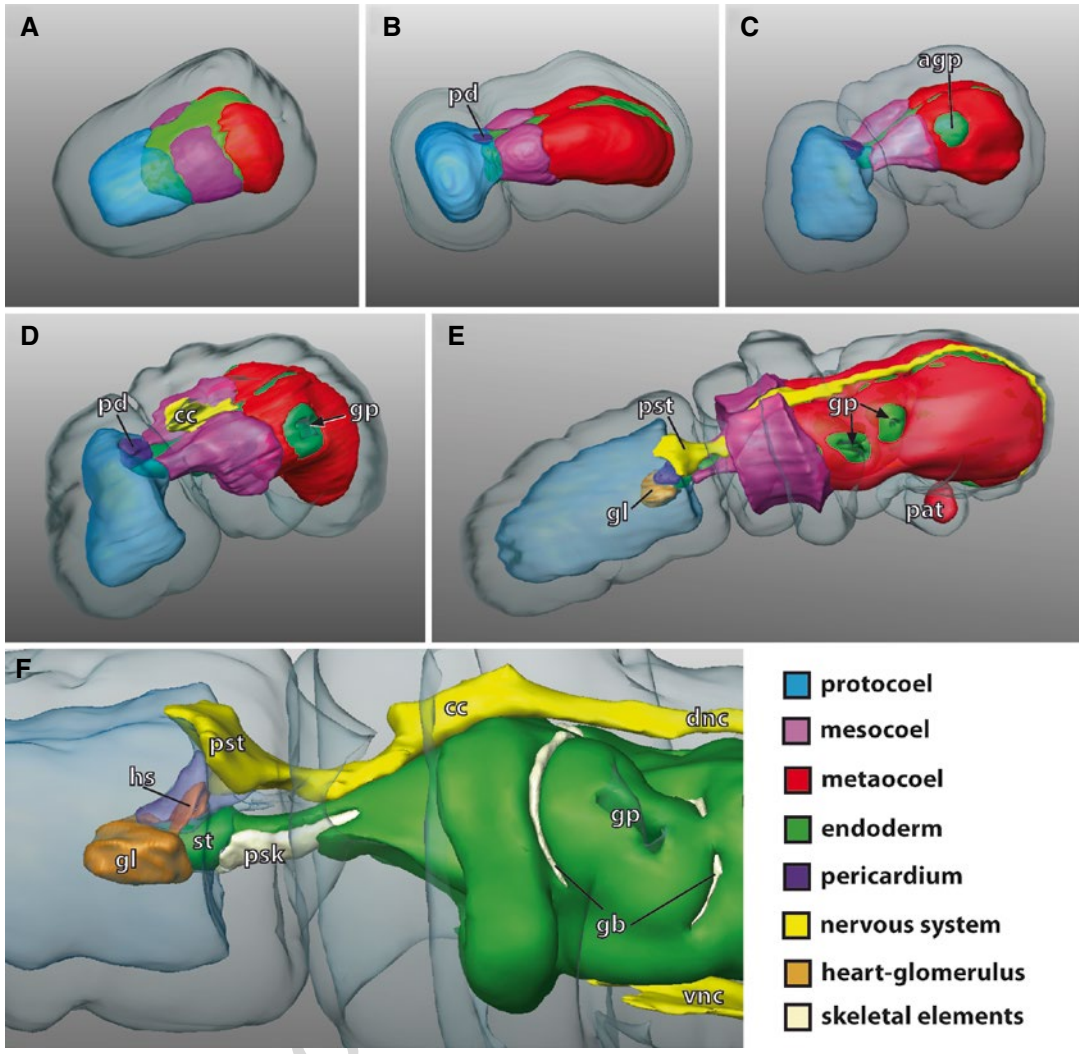

Fig. 2.6 3D reconstructions of major organ systems in different developmental stages of *Saccoglossus kowalevskii* based on complete serial semithin sections. Note, only the centralised parts of the nervous system are reconstructed in (**D**–**F**); the nerve net is not shown. Anterior is (almost) to the left in all images. (**A**) Late gastrula with anlagen of the prospective meso- and metacoels. The anterior protocoel has already pinched off of the endoderm. (**B**) Early kink stage. Meso- and metacoels are separated from endoderm. The pericardium emerges at the dorsal base of the protocoel. (**C**) Dorsal flexure stage showing anlagen of the first pair of gill pores. (**D**) One-gill-slit hatchling with opened pair of gill pores and neurulating collar cord in the mesosome. (**E**) Two-gill-slit juvenile with resembling adult morphology. (**F**) Close-up showing the inner organisation of the proboscis, collar and anterior trunk region in detail. *agp* anlage of gill pore, *cc* collar cord, *dnc* dorsal nerve cord, *gb* gill bar, *gl* glomerulus, *gp* gill pore, *hs* heart sinus, *pat* postanal tail, *pd* pericardium, *psk* proboscis skeleton, *pst* proboscis stem, *st* stomochord, *vnc* ventral nerve cord

cultured at 22 °C and hatch from the fertilisation membrane (Colwin and Colwin 1953; Lowe et al. 2004). At that time, *Saccoglossus* exhibits an elongated body shape measuring ~500 µm in length with a ventrally bent perianal field (Figs. 2.5D and 2.6D). One pair of dorsolateral gill pores is present in the anterior part of the trunk region. After hatching, the animals swim in the water column for a couple of hours and soon start to burrow and feed in the sediment.

Embryology in Indirect Developing Enteropneusts

The ontogeny including metamorphosis of indirect developing enteropneusts has been studied in species of the genera *Balanoglossus* and *Ptychodera* (Morgan 1891; Stiasny 1914a, b; Tagawa et al. 1998a; Urata and Yamaguchi 2004; Nielsen and Hay-Schmidt 2007; Miyamoto et al. 2010). Only recently, the first study of the development of a member of the Spengelidae, *Glandiceps hacksi*, has been investigated (Urata et al. 2014). Although the later development of indirect developers differs considerably from direct developers such as *Saccoglossus*, cleavage patterns and fate maps are identical and moreover show strong resemblance to sea urchins (Henry et al. 2001; see Chap. XX). The 16-cell stage of *Ptychodera flava* comprises an animal tier of eight cells (primordial anterior ectoderm), an upper vegetal tier of four cells (primordial posterior ectoderm) as well as a lower vegetal tier of four cells (primordial endomesoderm) (Fig. 2.4; Tagawa et al. 1998a). Subsequent cleavages give rise to a coeloblastula. Cleavage speed varies greatly between enteropneust species. Whereas in *P. flava* it takes about ~18 h post fertilisation (pf) until gastrulation starts (cultured at 22–24 °C) (Tagawa et al. 1998a), it begins at around ~13 h pf in *Balanoglossus clavigerus* (cultured at 20 °C) (Stiasny 1914a), and in *B. misakiensis*, this stage is already reached within ~9 h pf (cultured at 26 °C) (Urata and Yamaguchi 2004). At the end of gastrulation, the blastopore is closed and the protocoel is pinched off from the anterior region of the archenteron. The protocoel soon attaches to the epidermis of the animal pole and fuses with the dorsal ectoderm to form the hydropore. After ~45 h pf, embryonic development is completed in *P. flava* and the larva hatches from the fertilisation membrane to instantly start swimming (Tagawa et al. 1998a; Nielsen and Hay-Schmidt 2007). The early larva is of more or less spherical shape and soon develops into the typical tornaria larva. The first tornaria stage is called Müller stage and is characterised by a closed mouth as well as anus, open hydropore and a developing neotroch (circumoral ciliary band). The digestive tract is tripartite and composed of pharynx, stomach and intestine. In contrast to *P. flava*, which has a comparably long larval development for enteropneusts, the Japanese species *B. misakiensis* exhibits a shortened larval cycle and its larva hatches already at 24 h pf, thereby skipping the Müller stage (Urata and Yamaguchi 2004). Early hatched larvae of *B. misakiensis* have already developed an opisthotroch that typically characterises the Heider (second) stage of tornaria larvae (Fig. 2.7A). In all enteropneusts studied so far, the hydropore opens only after the protocoel has separated from the endoderm (Spengel 1893; Hyman 1959). However, in the recently studied spengelid *Glandiceps hacksi*, the hydropore forms prior to this event. This "precocious hydropore formation" is so far unique for the enteropneust *G. hacksi*, yet it is known also from various holothuroid echinoderms (Urata et al. 2014 and references therein).

LATE DEVELOPMENT

Late Development in Direct Developing Enteropneusts

When direct developing enteropneusts such as *Saccoglossus kowalevskii* hatch, the first pair of gill pores is open and the animals swim actively by the propelling opisthotroch. As soon as the animals start burrowing in the sand, the opisthotroch is remodelled and extends on the ventral side of the trunk in order to serve as a creeping sole for the juvenile worms (Fig. 2.5E; Burdon-Jones 1952; Stach and Kaul 2012). As the worms grow older, gill pores are added successively and the body size gains considerably in length. The juveniles measure up to a few millimetres at this point and resemble adult worms in many aspects, except for the still present postanal tail and the lower number of gill pores (Figs. 2.5F and 2.6E). The protocoel within the proboscis region is lined by a myoepithelium that forms the body wall musculature, composed of an outer layer of circular muscles and an inner layer of longitudinal muscles. A single proboscis pore is located posterodorsally on the left side and opens to the

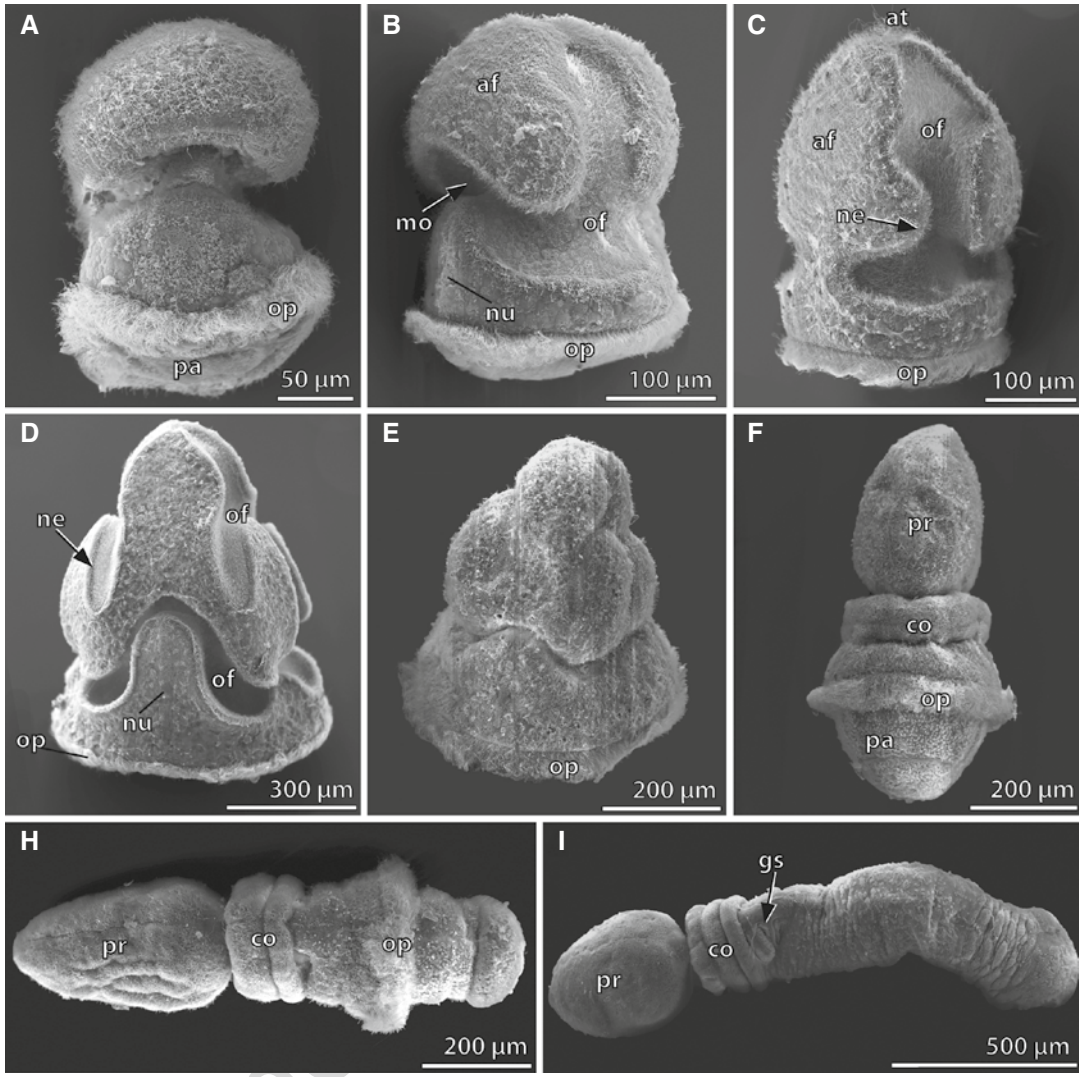

Fig. 2.7 Scanning electron micrographs of developmental stages of *Balanoglossus misakiensis*. (**A**) Early hatched Heider stage larva (30 h pf). (**B**) Tornaria at late Heider stage (48 h pf) with ventral neurotroch and oral field developed. (**C**) Early Metschnikoff stage (120 h pf) showing the beginning of primary lobe formation of the ciliary band of the oral field (neotroch). (**D**) Fully grown tornaria of *B. misakiensis* at late Metschnikoff stage (10 days pf) with deep primary lobes. (**E**) The Spengel stage (13 days pf) designates the beginning of metamorphosis and is characterised by the fusion of the neotroch, eventually obliterating the oral field and reducing the size of the body. (**F**) Agassiz stage tornaria (14 days pf) with acorn-shaped preoral region (future proboscis). This stage is competent to undergo settlement. (**G**) Early settled juvenile (12 h post settlement) with elaborated collar and elongated trunk region. The larval opisthotroch is still present. (**H**) Two-gill-slit juvenile (3 days post settlement) that already resembles a minute adult enteropneust. *af* aboral field, *at* apical tuft, *co* collar, *gs* gill slit, *mo* mouth opening, *ne* neotroch, *nu* neurotroch, *of* oral field, *op* opisthotroch, *pa* perianal field, *pr* proboscis (© Sabrina Kaul-Strehlow 2015. All Rights Reserved)

exterior. The number and position of the proboscis pores in enteropneusts is species dependent. Accordingly, in some species, bilateral pores are present, while in others only a single one is found on the right side. The pericardium is situated posteriorly within the protocoel and is dorsally attached to the stomochord. The pericardium is a small coelomic cavity that is lined ventrally by

epithelial muscle cells overlying the heart sinus, which is in principle an enlarged area within the extracellular matrix (ECM) filled with colourless blood fluid (Balser and Ruppert 1990). By contraction of the pericardial epithelial muscle cells, the blood fluid is forwarded into the anterior glomerulus. The glomerulus is a highly ramified enlargement of the ECM that is lined with podocytes on the protocoelic side. These podocytes have fingerlike extensions and are involved in filtration of the blood fluid. Together with the efferent proboscis pore, the heart-glomerulus complex represents the excretory system of hemichordates. The digestive tract in these juvenile worms is already subdivided into the typical regions, the anterior buccal cavity followed by the pharyngeal region harbouring the dorsolateral gill pores, connected to the stomach by a short and thin tubular esophagus ending in a short hindgut region that opens into the anus. The paired meso- and metacoels are lined by a single layer of epithelial cells that contain basal myofilaments. Within the metacoels, these myofilaments constitute a substantial longitudinal musculature, in particular on the ventral side. The mesocoels send a pair of extensions anteriorly through the proboscis stalk into the base of the proboscis. These mesocoelic protrusions flank the stomochord and contain longitudinal muscle strands that are involved in moving the entire proboscis. The few gill pores situated dorsolaterally at the anterior trunk region are kidney-shaped with the depression facing dorsally (Fig. 2.5F). Only later a dorsal tongue bar grows ventrally to eventually give the gill pores their slitlike U shape. Tongue bars are supported internally by a collagenous bar that forms within the ECM. It is the same material of which the proboscis skeleton is made of. The proboscis skeleton supports the stomochord ventrally and bifurcates within the collar region to flank the buccal cavity on either side. As the juveniles grow, subsequent development primarily involves increase of size, particularly trunk elongation, and addition of gill slits. The number of gill slits in adult *S. kowalevskii* varies greatly as new pairs seem to be added continuously throughout lifetime.

Neurogenesis in Direct Developing Enteropneusts

Neurogenesis in direct developing enteropneusts such as *Saccoglossus* has been studied thoroughly by molecular genetic analyses, yet morphogenetic data are still scarce. At late gastrula stage (Fig. 2.5A), serotonergic neurons form throughout the future proboscis region and project neurites posteriorly (Cunningham and Casey 2014). In stages close to hatching, a considerable basiepidermal nerve net is developed throughout the entire embryo (Kaul and Stach 2010). Before hatching, the collar cord at the dorsal midline of the collar region neurulates gradually from anterior to posterior to finally occupy a subepidermal position underneath the epidermis (Figs. 2.6D–F and 2.8). Just after neurulation, the collar cord comprises a large area of neuronal precursors that surround a central lumen (central canal) and small ventral areas filled with neurites (Fig. 2.8C). In older juvenile worms, a circumferential basiepidermal nerve net is present within the proboscis and collar region. Within the trunk region, the majority of neurites seems to run within the longitudinal nerve cords, i.e., the dorsal and the ventral cord (Kaul and Stach 2010), whereas only scattered neurites are present laterally. The dorsal nerve cord extends posteriorly until the anus and is anteriorly continuous with the collar cord. The ventral nerve cord is usually broader and runs along the midline of the trunk region to end in front of the postanal tail. Within the collar region, the collar cord is differentiated into a dorsal sheath of somata including unipolar giant neurons as well as smaller ependymal cells lining the central canal. About two-thirds of the collar cord are filled with numerous neurites that form a ventral neuropil (Fig. 2.8D). The collar cord continues anteriorly into the proboscis stem, a thickened area of neurites located at the dorsal base of the proboscis (Fig. 2.6E, F).

Late Development in Indirect Developing Enteropneusts

After hatching, the larvae of the indirect developer *Balanoglossus misakiensis* are of slightly

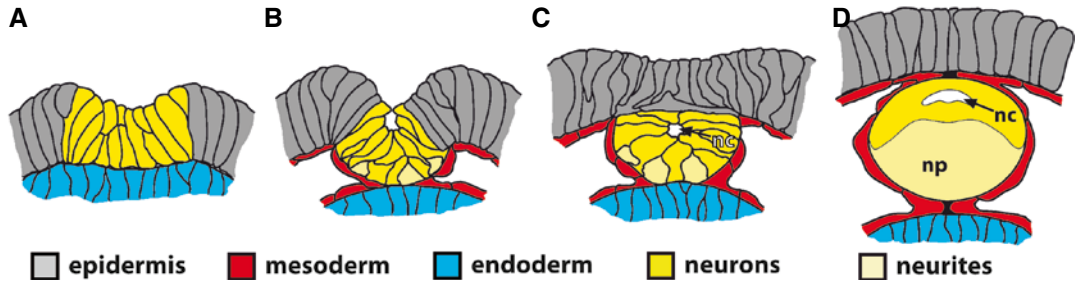

Fig. 2.8 Semi-schematic illustration of the neurulation process in *Saccoglossus kowalevskii* as revealed by transmission electron microscopy. (**A**) Early stage showing dorsal neural plate. (**B**) the neural plate invaginats ventrally. (**C**) The neural plate has formed a subepidermal tubular nerve cord. (**D**) The collar cord comprises a dorsal sheath of soma and a ventral neuropil. (Modified from Kaul and Stach 2010). *nc* neural canal, *np* neuropil

elongated shape and feature an apical hood below which the mouth opening will soon break through on the ventral side. A less pronounced perianal ciliary ring and the opisthotroch are present near the posterior end of the larvae (Heider stage) (Fig. 2.7A). Approximately 1 day after hatching, a simple neotroch is developed, separating the aboral field from the oral field that is used for food collection and transport (Fig. 2.7B). On the ventral side, another longitudinal yet short ciliary band is present, the neurotroch. The larvae swim actively in the water column and feed on phytoplankton as soon as the mouth and anus have opened. A few days later, the tornaria has increased in size and the neotroch develops a more complex pattern of ciliary bands on the anterior half of the larva by forming primary lobes (Fig. 2.7C). The beginning of primary lobe formation is characteristic for the early Metschnikoff stage. At the anterior tip, a pair of dark eye spots is visible alongside the central ciliary tuft. During subsequent development, the tornaria of *B. misakiensis* grows to a remarkable size of 1.5 mm and the primary lobes of the neotroch form deep protrusions to enlarge the oral field considerably (Fig. 2.7D). At the Metschnikoff stage, adult structures such as the proboscis vesicle (pericardium) and the meso- and metacoels become apparent. The proboscis vesicle forms a small coelomic cavity close to the hydropore on the right side (Fig. 2.9A). Its origin seems to be species dependent and has been reported from the ectoderm (Spengel 1893; Stiasny 1914b) or mesoderm by pinching off of the protocoel (Dawydoff 1907; Morgan 1894; Ruppert and Balser 1986). The meso- and metacoels in *B. misakiensis* form as a lateral pair of protrusions from the intestine region (Figs. 2.4 and 2.9A). They elongate anteriorly to subsequently constrict in the middle to subdivide into the anterior mesocoel and posterior metacoel. This mode of development has also been reported from *B. clavigerus* (Bourne 1889; Spengel 1893; Stiasny 1914a) and *Glandiceps* sp. (Rao 1953). In tentaculated tornaria of, e.g., *Ptychodera*, however, the meso- and metacoels form from multiple clusters of mesenchymatic cells within the blastocoel (Morgan 1894). After the Metschnikoff stage, indirect developers typically enter the so-called Krohn stage by developing secondary lobes and saddles on the neotroch without obvious changes of their internal anatomy. In this stage, the tornaria exhibits a more compact shape with a nearly planar perianal field. The Krohn stage larva may differ morphologically between species and exhibit species-specific characters. For instance, the Krohn tornaria of *Ptychodera flava* develops a highly sinuous neotroch eventually resulting in small tentacles (Hadfield 1975; Nielsen and Hay-Schmidt 2007). In contrast, the neotroch in *B. clavigerus* never develops tentacles on the secondary lobes, and in *B. misakiensis*, the Krohn stage is skipped completely by proceeding directly into the Spengel stage (Figs. 2.7E and 2.9B). An elaborated neotroch with tentacles as found in *P. flava* is likely to result in a more efficient food uptake and correlates with the extended pelagic period of up to 5 months in this species.

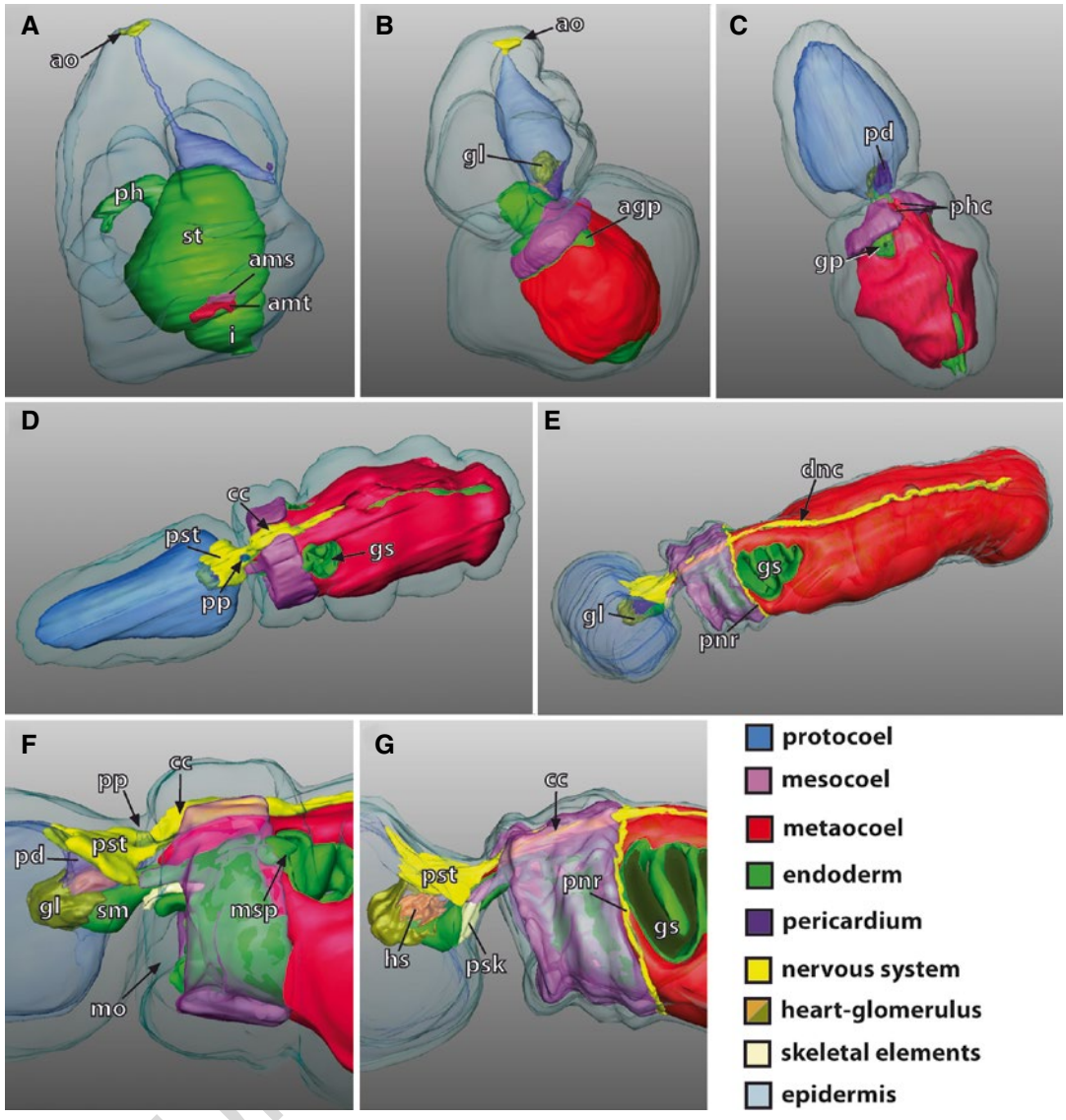

Fig. 2.9 3D reconstructions of major organ systems in different developmental stages of *Balanoglossus misakiensis* revealed from complete serial semithin sections. Opisthotroch and ciliary bands are omitted. Anterior to the top in (**A**–**C**) and to the left in (**D**–**G**). (**A**) Overview of the inner anatomy of a typical tornaria at the Metschnikoff stage. (**B**) Spengel stage. All coelomic cavities have enlarged considerably. The digestive tract is pulled backwards. Anlagen of the first gill pores are visible. (**C**) Agassiz stage with first pair of gill pores opened. (**D**) Early settled juvenile. The gill slit is U-shaped, because of a dorsal tongue bar. The central nervous system forms at this stage. (**E**) Two-gill-slit juvenile (3 days post settlement). (**F**) Close-up of D showing the inner organisation of the proboscis, collar and anterior trunk region in detail. (**G**) Close-up of E showing the inner organisation of the proboscis, collar and anterior trunk region in detail. *agp* anlage of the gill pore, *ams* anlage of the mesocoel, *amt* anlage of the metacoel, *ao* apical organ, *cc* collar cord, *dnc* dorsal nerve cord, *gl* glomerulus, *gp* gill pore, *gs* gill slit, *hs* heart sinus, *i* intestine, *mo* mouth opening, *msp* mesocoelic pore, *pd* pericardium, *ph* pharynx, *phc* perihaemal cavity, *pnr* peribranchial nerve ring, *pp* proboscis pore, *psk* proboscis skeleton, *pst* proboscis stem, *sm* stomochord, *st* stomach

All the preceding larval stages (Müller, Heider, Metschnikoff, Krohn) comprise the so-called progressive larval development where the larvae increase in size. The following Spengel stage marks the beginning of the regressive larval development and is characterised by a decrease in size of the larva and remodelling of the preoral part by fusion of the ciliary bands of the neotroch (Stiasny 1914b; Agassiz 1873; Nielsen and Hay-Schmidt 2007). As a result, the future proboscis gets a more and more smooth surface and the position of the neotroch is indicated by grooves. At the same time, a number of internal modifications occur. The protocoel enlarges significantly and begins to fill up the preoral part of the larva, thereby subsequently reducing the blastocoel (Fig. 2.9B). The proboscis vesicle has increased in size and is now situated dorsally onto the developing stomochord. The mouth opening and pharynx have shifted backwards and the anlagen of the gill pores are visible as paired, lateral evaginations from the posterior pharyngeal region (Fig. 2.9B). The meso- and metacoels have extended anteriorly as well as medially and surround the digestive tract almost completely. The Spengel stage in *Balanoglossus misakiensis* lasts only for a couple of hours and marks the transition from the fully grown tornaria into the competent Agassiz stage. The Agassiz stage is the last stage before the animals settle and grow into a juvenile acorn worm. This stage is characterised by the complete absence of the neotroch and potential tentacles. The larvae of *B. misakiensis* are of elongated shape and the future three body regions can be distinguished (Figs. 2.7F and 2.9C). The anterior proboscis region is conical and separated from the posterior part by a deep constriction where the mouth opens into on the ventral side. The collar region is short and subdivided from the posterior trunk region by a shallow circular depression. The former planar perianal field is now highly convex as it has started to grow out posteriorly. The opisthotroch is still well developed and continues to propel the larva through the water. The protocoel has completely extended and opens to the exterior through the proboscis pore on the left side of the dorsal base of the proboscis (Fig. 2.9C). The glomerulus spans the anterior tip of the protruding stomochord and is posterodorsally adjoined by the pericardium. In *B. misakiensis*, one pair of dorsolateral gill pores is present at this stage at the anterior margin of the trunk region (Fig. 2.9C). Time and number of formation of gill pores is species specific, since reports from other enteropneust species show competent Agassiz larvae with several pairs of gill pore anlagen (Agassiz 1873; Morgan 1894). The meso- and metacoels progressively reduce the blastocoel to the dorsal and ventral midline by which the haemal system is formed. The paired metacoel sends anterodorsal projections into the base of the proboscis, that is, the perihaemal cavities. Larvae at the Agassiz stage usually stop swimming in the upper water column and instead begin to visit the bottom more frequently. The pair of apical eye spots degenerates at this stage and the larvae are now competent for settlement. After settlement, the larva grows into a young juvenile worm mainly by elongation of the trunk region. In settled juveniles of *B. misakiensis* approximately 12 h post fertilisation, the opisthotroch is still present in the middle of the trunk region in the majority of specimens (Fig. 2.7G). The overall morphology shows only minor changes compared to the competent Agassiz stage which concern the collar region, shape of the gill pores and the coelomic cavities. The collar region is subdivided into an anterior and a posterior part by a circular constriction. A dorsal tongue bar grows ventrally and gives the gill slit its final U shape. Moreover, paired mesocoel ducts open into the first gill slit on both sides and connect the mesocoel to the exterior (Fig. 2.9F). Within the posterior part of the protocoel, the stomochord-heart-glomerulus complex is almost completely developed. The coelomic system gets more and more intricate as the animals grow and aside from the perihaemal cavities, that are extensions from the trunk coelom (metacoel), the collar coelom (mesocoel) also sends bilateral projections anteriorly into the base of the proboscis, thereby flanking the stomochord (Fig. 2.9G). At ~3 days post settlement, the juvenile worms of *B. misakiensis* have completely lost the opisthotroch (Fig. 2.7H). The proboscis is short and conical and the collar region

exhibits a three-lobed shape (Figs. 2.7H and 2.9E, G). The proboscis skeleton is present and supports the fragile neck region by underlying the stomochord (Fig. 2.9G).

Approximately 1 week after settlement, formation of a premature juvenile featuring a distinct hepatic region and three pairs of gill slits is completed in *Balanoglossus misakiensis* (Urata and Yamaguchi 2004).

Neurogenesis in Indirect Developing Enteropneusts

In particular, the broad usage of antibody stainings to visualise specific parts of the nervous system has contributed significantly to our knowledge of neurogenesis in indirect developing enteropneusts. Several papers are available that describe the nervous system in single tornaria stages, yet only two detailed studies documenting a complete developmental series have been published so far (Nielsen and Hay-Schmidt 2007; Miyamoto et al. 2010). In particular, the exact mode of neural remodelling from metamorphosis through juvenile stages is still unclear.

The nervous system in tornaria larvae develops gradually from anterior to posterior. In early hatched larvae, the nervous system comprises a small apical organ of few synaptotagmin-like immunoreactive (LIR) as well as serotonin-LIR cells with neurites projecting posteriorly (Fig. 2.10A, B) (Nielsen and Hay-Schmidt 2007; Miyamoto et al. 2010). As the neotroch develops neurite bundles, neurons form along the ciliary band. In addition, the pan-neuronal marker synaptotagmin reveals a nerve net throughout the oral field of the larvae (Miyamoto et al. 2010). In older larvae, the apical organ usually consists of numerous serotonin- as well as FMRFamide-LIR neurons and a pair of eye spots situated laterally within the apical organ (Fig. 2.10C, D) (Nezlin and Yushin 2004). The exact ultrastructure of the eyes has not been investigated in detail. The scarce data available describe a mixed photoreceptor cell with a rhabdomere as well as a modified cilium (Brandenburger et al. 1973). If true, tornaria larvae would feature a photoreceptor cell type that is unique in the animal kingdom, i.e., a combined rhabdomeric and ciliary photoreceptor. However, further studies including serial sections for TEM and characterisation of the molecular signature of the eyes in tornaria larvae are necessary in order to substantiate or reject this postulation. For instance, the composition of the sea urchin eye was also discovered only recently (Ullrich-Lüter et al. 2011) and revealed a solely rhabdomeric photoreceptor.

In older tornaria larvae, serotonin-LIR as well as FMRFamide-LIR neurons of the apical organ are arranged in two clusters of cells, one situated in the preoral part of the neotroch and the other in the postoral part of the neotroch (Fig. 2.10C, D). Both clusters of neurons are interconnected by a comprehensive central neuropil (Nezlin and Yushin 2004). The opisthotroch nerve ring contains synaptotagmin and tyrosine hydroxylase in early stages and later also serotonin (Nielsen and Hay-Schmidt 2007; Miyamoto et al. 2010). The neural arrangement in the tornaria shows strong congruence to that in echinoderm larvae, further supporting the assumption that both larval types are homologous and evolved from a common ambulacrarian ancestor (Byrne et al. 2007). At the time of metamorphosis when tornariae reach the Agassiz stage, the larval nervous system degrades and the adult nervous system starts to develop (Miyamoto et al. 2010). It could be shown that the majority of nervous cells of the ciliary band degrade and contribute little to the adult nervous system. During settlement, a basiepidermal nerve net within the proboscis and collar region becomes apparent and the nerve cords develop along the ventral and dorsal midline of the trunk region. The collar cord within the collar region neurulates in a similar way as in *Saccoglossus kowalevskii* (Fig. 2.8) and eventually becomes situated subepidermally (Morgan 1894; Miyamoto and Wada 2013). From approx. 3 days post settlement in *Balanoglossus misakiensis*, all main parts of the centralised nervous system are present (Fig. 2.9E, F), that is, anterior proboscis stem, neurulated collar cord, circumferential peribranchial nerve ring and a dorsal as well as a ventral longitudinal nerve cord within the trunk region. Unfortunately, almost nothing is known about the formation and distribution of specific neurotransmitters such as serotonin or

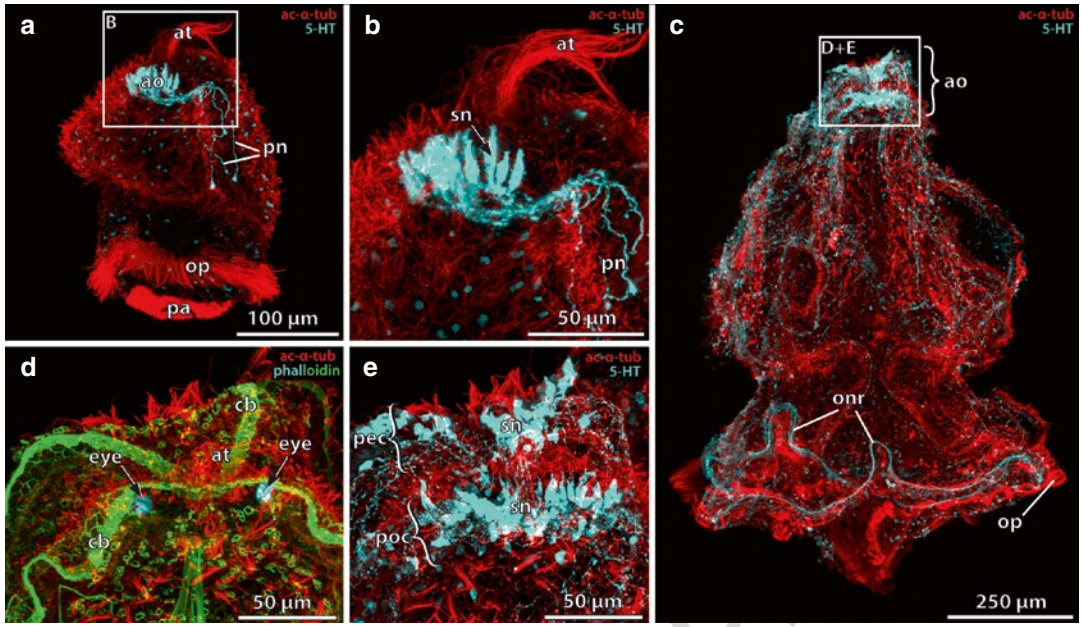

Fig. 2.10 Antibody stainings of the developing nervous system in *Balanoglossus misakiensis*. (**A**) Early tornaria (Heider stage). (**B**) Same stage as in (**A**). The apical organ consists of about 18 serotonin-LIR (5-HT) sensory cells. (**C**) Fully grown tornaria (late Metschnikoff). Dorsal view. (**D**) Close-up of the apical region of fully grown tornaria showing the lateral pair of eye spots and the course of the ciliary bands. (**E**) Same region as in (**D**). The apical organ consists of numerous sensory cells (5-HT), subdivided into a ventral and a dorsal cluster. *ao* apical organ, *at* apical tuft, *cb* ciliary band, *eye* eye spot, *onr* opisthotroch nerve ring, *op* opisthotroch, *pa* perianal field, *pec* preoral cluster, *poc* postoral cluster, *pn* posteriorly neurites, *sn* sensory cell

FMRFamide in juvenile enteropneusts. One sole immunocytochemical study has been published on the nervous system of the miniaturised species *Meioglossus psammophilus* (Worsaae et al. 2012). The adult nervous system of *M. psammophilus* comprises several serotonergic sensory neurons within the anterior and middle part of the proboscis. A basiepidermal nerve net extends throughout the proboscis and is most prominent at the dorsal base (proboscis stem). Posterior to the proboscis stem, individual serotonergic neurites pass through the collar cord. A circumferential ring of serotonergic neurons is present in the collar region. These sensory neurons project with a single neurite at first posteriorly, until the end of the collar region, and then ventrally into a median nerve cord. Serotonin-LIR reveals a comparably short ventral nerve cord that bifurcates at the level of the midgut into a pair of ventrolateral neurite bundles that further extend posteriorly until the end of the trunk region. Individual serotonergic neurons are scattered throughout the trunk epidermis and project into one of the two ventral nerve cords.

Taken together, the few available data on neurogenesis of the adult nervous system in enteropneusts make it considerably difficult to compare it to other deuterostomes and accordingly render testing of homology hypotheses difficult.

Comparative Aspects of Hemichordate Development

Hemichordates and particularly enteropneusts seem to have retained a number of ancestral deuterostome traits, such as radial cleavage, enterocoely and bilateral symmetry. Early development including cleavage and cell fates is highly conserved among hemichordates (Colwin and Colwin 1951, 1953; Tagawa et al. 1998a). The radial, equal and holoblastic cleavage leads to a

coeloblastula that subsequently undergoes gastrulation. Hemichordates share this cleavage pattern with echinoderms (Henry et al. 2001; McClay [AU2] 2011; Chap. XX) and, depending on the yolk content, also with chordates (Bertrand and Escriva 2011; Lemaire 2011; Chap. XX). Radial cleavage is a plesiomorphic feature for Ambulacraria and Chordata and was most likely inherited from an early ancestor (Ax 2001; Nielsen 2011).

The five main coelomic cavities (single protocoel, paired meso- and metacoels) in hemichordates originate from the endoderm by enterocoely. While the protocoel derives from the anterior end of the archenteron in all species studied, the formation of the meso- and metacoels varies considerably. For instance, the meso- and metacoels in *Saccoglossus kowalevskii* and *Glandiceps hacksi* (Urata et al. 2014) develop from separate evaginations of the middle and posterior endodermal region (Bateson 1884; Kaul-Strehlow and Stach 2013), whereas in *Balanoglossus clavigerus* and *B. misakiensis*, they emerge from a single pair of evaginations that eventually subdivide into the more anterior mesocoels and posterior metacoels (Stiasny 1914b; Spengel 1893; Urata and Yamaguchi 2004). A closer look at other enteropneust species and echinoderms reveals enormous intraphyletic variation of coelom formation (for review, see Nielsen 2011). Although an ancestral deuterostome pattern may be hard to reconstruct, it seems that at least in all cases, the mesoderm is formed from the endoderm. Moreover, in echinoderms as well as hemichordates, the mesoderm forms as three successive pairs of coelomic cavi- [AU3] ties (proto-, meso- and metacoels) (Chap. XX). During the development of the cephalochordate amphioxus, the larva passes a similar tricoelomate stage, before additional coelomic pouches are added (Stach 2002) (Chap. XX). The fact that three pairs of coelomic cavities are present in members of all main deuterostome groups, at least at a certain developmental stage, leads to the conclusion that this is an ancestral condition for deuterostomes. As mentioned before, the development of the protocoel seems to be rather conserved among ambulacrarians in being always the first coelomic cavity that pinches off very early from the anterior end of the endoderm. In this aspect, the precocious hydropore formation documented in some holothurians and the spengelid enteropneust *G. hacksi* (Urata et al. 2014) is very interesting. The mode of development is so strikingly similar that an independent evolution can hardly be assumed. It shows that irrespective of the derived phylogenetic position of holothurians within Echinodermata, they nevertheless may have retained more ancestral traits than previously thought. It should be repeated here that it was already Eschscholtz (1825) who compared and related the first described enteropneust *Ptychodera flava* to holothurians.

The sixth coelomic cavity, the pericardium, is part of the heart-glomerulus complex and thus indirectly involved in excretory function. Homology of this heart-glomerulus complex of hemichordates with the axial complex of echinoderms is widely accepted, because of a number of functional and structural similarities (Balser and Ruppert 1990; Mayer and Bartolomaeus 2003; Kaul-Strehlow and Stach 2011; Nielsen 2011; Merker et al. 2013). These include a contractile pericardium (pulsatile vesicle in echinoderms), filtrating podocytes on the protocoelic (axocoelic) side, an excretory hydropore and a glomerulus. However, despite the unquestioned homology of the differentiated structures, the ontogenetic origin shows considerable variations between enteropneust species as well as echinoderm species. For instance, the pericardium in *Saccoglossus kowalevskii* develops from the ectoderm by schizocoely (Kaul-Strehlow and Stach 2011), a mode of development that is usually associated with protostomes (Technau and Scholz 2003). On the other hand, a mesenchymatic (Morgan 1891; Rao 1953) and further enterocoelic origin (Dawydoff 1907) of the pericardium has been reported from other enteropneust species. The same holds true for echinoderms (for review, see Hyman 1955) and demonstrates that homologous structures indeed may have different ontogenetic origins. However, a general or even ancestral mode of pericardial formation for Ambulacraria is thus hard to infer. The situation is even more complicated by the fact that corresponding counterparts of the nephridial complex of ambulacrarians may be present in

chordates (Chap. XX). In particular, homology of the pericardium with Hatschek's left diverticulum or Hatschek's pit in cephalochordates (Goodrich 1917; Franz 1927; Nielsen 2011) or with Hatschek's nephridium in cephalochordates (Stach 2002) has been suggested earlier. In any case, if homologous structures are present in chordates, then the nephridial complex of Ambulacraria may represent a plesiomorphic character within deuterostomes rather than constituting a synapomorphy of Echinodermata and Hemichordata.

The nervous system of the tornaria larva consists of an apical organ comprising different types of neurons and nerves along the ciliary bands (Hay-Schmidt 2000; Nezlin and Yushin 2004; Miyamoto et al. 2010). Serotonin-LIR cells in the apical organ are arranged in bilateral clusters interconnected by a median neuropil (Nezlin and Yushin 2004). During ontogeny, the nervous system develops gradually from anterior to posterior, and at metamorphosis, the larval nervous system degrades and the adult nervous system is formed (Miyamoto et al. 2010). The nervous system of the different echinoderm larvae develops likewise and features in principle the same components. Of course, taxon-specific traits are present, but the general neural body plan of an apical organ with sensory cells resting in the apical ciliary band and nerves along the neotroch is present (Hay-Schmidt 2000; Burke et al. 2006; Byrne et al. 2007). As in hemichordate tornariae, the echinoderm larval nervous system contributes little if anything to the pentameral nervous system of the juveniles (Byrne and Cisternas 2002; Cisternas and Byrne 2003; Nakano et al. 2006). Because of numerous resemblances between the morphology of hemichordate tornariae and echinoderm larvae, they have been grouped together under the term dipleurula-type larvae (Metschnikoff 1881). However, since the sister group of Ambulacraria, that is, Chordata, do not have primary larvae, it remains uncertain if a dipleurula larva was already present in the last common ancestor of Deuterostomia. Thus, the dipleurula larva with its specific neotroch is likely to be a synapomorphy uniting Hemichordata and Echinodermata (Nielsen 2011).

GENE EXPRESSION

The pivotal phylogenetic position of hemichordates, the shared fate map during ambulacrarian embryonic development (Colwin and Colwin 1951; Cameron et al. 1987, 1989; Cameron and Davidson 1991; Henry et al. 2001) and the above-mentioned classical and modern morphological descriptions have suggested homologies between various hemichordate, echinoderm and chordate features. Hence, hemichordates are particularly appealing to investigate the evolution of deuterostome developmental mechanisms, and in the past decades, the growing community working on hemichordates has developed a basic toolset to gain insight into the molecular mechanisms that drive embryonic development, the patterning of the larval and adult body plan as well as the molecular signature of particular structures, i.e., gill slits (Rychel and Swalla 2007; Gonzalez and Cameron 2009; Gillis et al. 2011).

As for most "non-model" organisms, classical degenerative PCR approaches (Tagawa et al. 1998b) or, more recently, the analysis of transcriptomic data sets were used to identify genes and characterise their expression in hemichordates (Lowe et al. 2003; Röttinger and Martindale 2011; Chen et al. 2014). Lately, the genomes of *Ptychodera flava* and *Saccoglossus kowalevskii* have been sequenced and used for genome comparisons between these two species (Freeman et al. 2012). This will provide an important resource to identify the genetic toolkit and regulatory elements of acorn worms. Protocols have been developed and optimised for whole mount or section in situ hybridisation or immunocytochemistry and are now routinely applied on several hemichordate species (Tagawa et al. 1998b; Okai et al. 2000; Lowe et al. 2003; Smith et al. 2003; Sato et al. 2009; Miyamoto et al. 2010; Miyamoto and Wada 2013). In order to determine relative spatial gene expression for a set of genes, double fluorescent in situ hybridisation has been developed in *S. kowalevskii* (Pani et al. 2012).

During the reproductive season, controlled spawning and fertilisation produce large numbers of synchronously developing embryos and larvae

that are amenable for pharmacological drug or recombinant protein treatments to analyse the effects of perturbing signalling pathways on the developmental process (Lowe et al. 2006; Darras et al. 2011; Röttinger and Martindale 2011; Pani et al. 2012; Green et al. 2013). However, specific gene knockdown experiments using siRNA and mRNA that are microinjected into fertilised oocytes have so far been reported only from *Saccoglossus kowalevskii* (Lowe et al. 2006; Darras et al. 2011; Pani et al. 2012; Cunningham and Casey 2014; Green et al. 2013). *S. kowalevskii* appears also to be the most suitable acorn worm species for classical embryological experiments (Colwin and Colwin 1950) that have recently inspired researchers to combine blastomere isolation and grafting experiments with molecular analysis to investigate the inductive capacities of individual blastomeres or animal-vegetal explants (Darras et al. 2011; Green et al. 2013).

Endomesoderm Formation and the Posterior Organiser

In metazoans, canonical ß-catenin/Wnt (cWnt) signalling plays crucial roles during various aspects of embryonic development such as embryonic polarity, germ layer specification, posterior growth and anterior-posterior axis patterning (Croce and McClay 2006; Lee et al. 2006; Martin and Kimelman 2009; Cho et al. 2010; [AU5] Niehrs 2010; Chaps. XX, YY and ZZ). A recent study in *Saccoglossus kowalevskii* has dissected the role of cWnt signalling during enteropneust development (Darras et al. 2011). Combining classical embryology, gene-specific knockdown experiments and gene expression analysis, the authors showed that ß-catenin is accumulated at the vegetal pole, the future site of gastrulation, which is required for endomesoderm specification. In addition, the endomesoderm secretes yet undefined signals that determine the posterior fate of the adjacent ectoderm, as the ectoderm will adopt default anterior fates when the endomesoderm is removed (Darras et al. 2011). This mechanism is very similar to the one observed in echinoderms (Angerer et al. 2011) and vertebrates (Niehrs 2010), suggesting a conserved function of cWnt signalling at the base of deuterostomes in germ layer specification and the formation of a posterior organiser (Darras et al. 2011).

In hemichordates, mesoderm forms by enterocoely (Bateson 1884), a process that is shared with echinoderms and basal chordates such as amphioxus and ascidians. The FGF signalling pathway plays a crucial role in mesoderm induction in vertebrates and basal chordates (Slack et al. 1989; Kim et al. 2000; Imai et al. 2002; Fletcher et al. 2006; Kimelman 2006; Bertrand et al. 2011; Chap. XX). In order to investigate the [AU6] evolution of mesoderm formation, a recent study has examined the role of FGF signalling during mesoderm formation in *Saccoglossus kowalevskii* (Green et al. 2013). Expression of the FGF ligand *fgf8/17/18* is restricted to ectodermal regions overlying sites of mesoderm specification within the archenteron, while the regions that will form mesoderm express the receptor *fgfr-B*. The resulting suggestion that mesoderm induction in the archenteron requires contact with the ectoderm to allow FGF/FGFR signalling is confirmed by embryological experiments that are combined with gene expression analysis of the downstream target *snail*. Gene-specific knockdown and gain-of-function experiments show that FGF8/17/18 is required and sufficient for mesoderm induction in *S. kowalevskii* and support the idea that FGF signalling played an ancestral role in deuterostome mesoderm formation (Green et al. 2013; Chaps. XX and YY). [AU7]

Dorsoventral Patterning

BMP, a ligand of the TGFß family, and its antagonist Chordin play a central role in establishing the dorsoventral axis and the specification of the central nervous system (CNS) in bilaterian animals (Arendt and Nübler-Jung 1996; De Robertis and Sasai 1996; Holley and Ferguson 1997; De Robertis et al. 2000; De Robertis and Kuroda 2004; Chaps. XX, YY and ZZ). Gene expression [AU8] analysis in *Saccoglossus kowalevskii* and *Ptychodera flava* has shown expression of *bmp2/4* and its potential downstream target *dlx* in dorsal

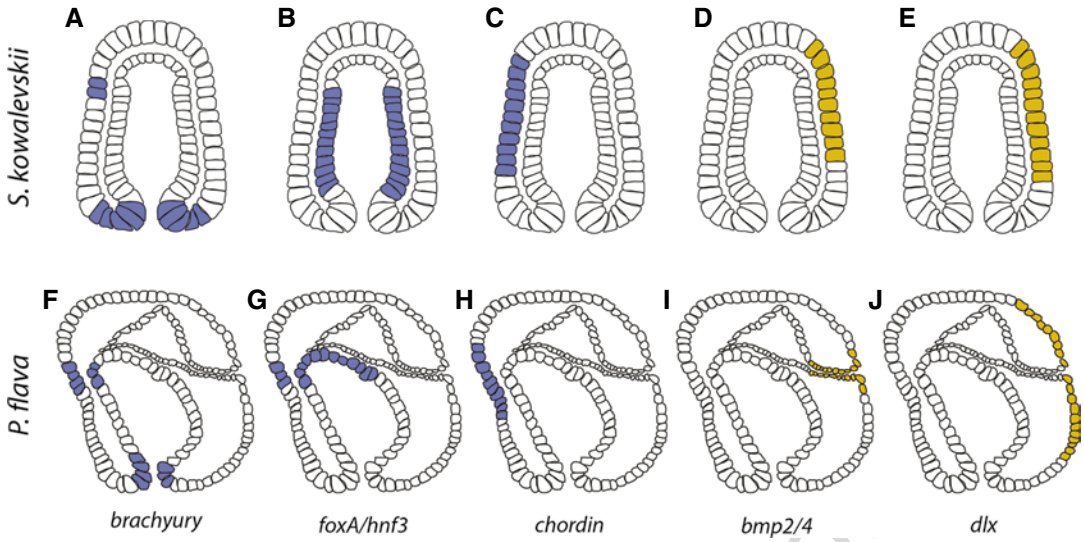

Fig. 2.11 Comparison of dorsoventral gene expression patterns in hemichordates. Illustrations of ventral (*purple*) and dorsal (*yellow*) gene expression patterns in late gastrulae of *Saccoglossus kowalevskii* (**A**–**E**) and *Ptychodera flava* (**F**–**J**). All illustrations are based on published data: **A**, **B and E**, Lowe et al. (2006); **C**, Röttinger and Lowe (2012); **D**, Darras et al. (2011); and **F**–**J,** Röttinger and Martindale (2011)

territories (Fig. 2.11D, E, I, J), while *chordin* transcripts (Fig. 2.11C, H) are localised in the ventral ectoderm, suggesting that these proteins are also involved in dorsoventral patterning in hemichordates (Harada et al. 2001, 2002; Lowe et al. 2006; Röttinger and Martindale 2011). Functional studies in *S. kowalevskii* demonstrate the implication of BMP signalling in this process, as overexpression and knockdown of BMP2/4 result in dorsalised or ventralised embryos, respectively (Lowe et al. 2006). Based on functional studies in other bilaterian animals, the prediction would be that overactivating BMP signalling represses neural fates (De Robertis and Kuroda 2004). However, this is not the case in *S. kowalevskii* embryos treated with recombinant BMP4 protein, suggesting that BMP signalling in hemichordates is involved in dorsoventral patterning but not in neurogenesis (Lowe et al. 2006).

The molecular mechanisms underlying dorsoventral patterning have been extensively studied in echinoderms (Angerer et al. 2000; Duboc et al. 2004; Su and Davidson 2009; Saudemont et al. 2010; Chap. XX) and are represented in a simplified version in Fig. 2.12A (for a more comprehensive version, see Chap. XX). To date, the only functional molecular studies that have been performed in harrimaniid (*Saccoglossus kowalevskii*) and also in ptychoderid (*Ptychodera flava*) enteropneusts aim to describe the dorsoventral patterning event in hemichordates (Lowe et al. 2006; Röttinger and Martindale 2011). This enables comparing the molecular mechanism controlling dorsoventral patterning within ambulacrarians (Fig. 2.12).

While *bmp2/4* expression in enteropneusts is restricted to dorsal structures (Fig. 2.11D, I; Harada et al. 2002; Lowe et al. 2006; Röttinger and Martindale 2011), *bmp2/4* expression in echinoderms is localised on the opposite site in the ventral ectoderm (Angerer et al. 2000; Duboc et al. 2004). Interestingly, functional studies in *Saccoglossus kowalevskii* and echinoderms have shown that regardless the expression domain of the diffusible ligand *bmp2/4*, its activity is always confined to the dorsal ectoderm (Duboc et al. 2004; Lowe et al. 2006). In *S. kowalevskii* and *Paracentrotus lividus* (echinoderm), *dlx* is an indirect downstream target of BMP signalling (Lowe et al. 2006; Saudemont et al. 2010). In *Ptychodera flava*, *dlx* transcripts are detected in the dorsal ectoderm (Harada et al. 2001; Röttinger and Martindale

[AU9]

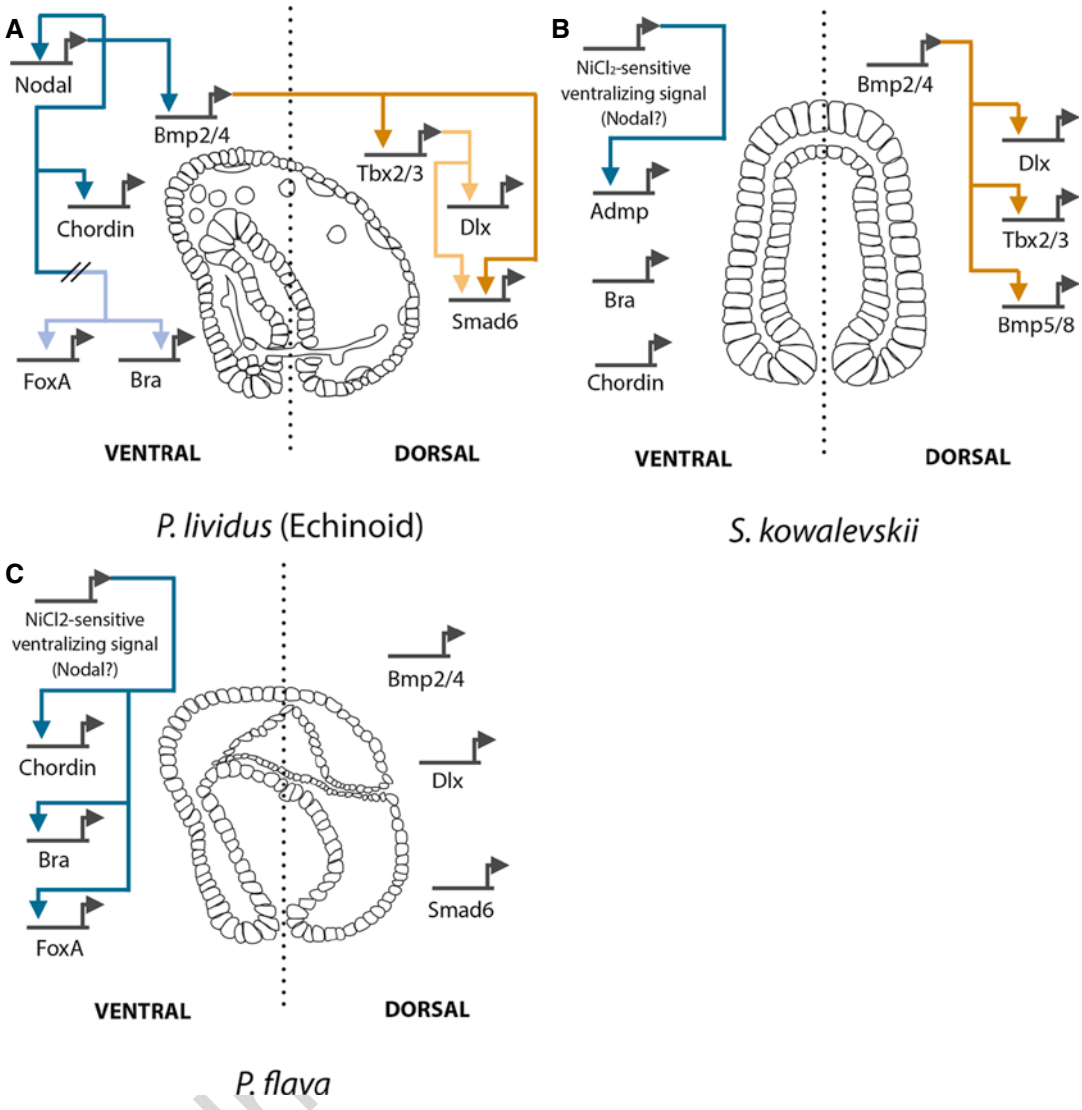

Fig. 2.12 Comparison of dorsoventral patterning mechanisms in ambulacrarians. In echinoderms (**A**), Nodal signalling is essential to determine ventral fates and induce expression of *bmp2/4* that acts on the dorsal side to specify dorsal ectoderm. In *Saccoglossus kowalevskii* (**B**), BMP signalling is crucial for specifying the dorsal ectoderm as well, which might potentially also be the case for *Ptychodera flava* (**C**). While a $NiCl_2$-sensitive signal is involved in specifying ventral fates in enteropneusts (**B**, **C**), the relation to Nodal signalling remains unknown. (**A**) *Paracentrotus lividus* (echinoderm), simplified diagram based on data from Saudemont et al. (2010). (**B**) *Saccoglossus kowalevskii* (harrimanid enteropneust), diagram based on data from Lowe et al. (2006), Saudemont et al. (2010) and Röttinger and Martindale (2011). (**C**) *Ptychodera flava* (ptychoderid enteropneust), diagram based on data from Tagawa et al. (1998b), Taguchi et al. (2000), Harada et al. (2001, 2002) and Röttinger and Martindale (2011)

2011), suggesting that BMP signalling is active in this territory as well. However, additional experiments are required to confirm this hypothesis.

The Nodal signalling pathway plays crucial roles in various deuterostome developmental processes such as endo- and mesoderm formation and axial patterning events along the anterior-posterior, dorsoventral and left-right axis (Whitman 2001; Hamada et al. 2002; Morokuma et al. 2002; Stainier 2002; Yu et al.

2002; Chaps. XX, YY and ZZ). In echinoderms, Nodal signalling is not only crucial for establishing left-right asymmetries in the larva (Duboc et al. 2005) but plays also an essential role in the establishment of the dorsoventral axis during embryonic development (Duboc et al. 2004; Chap. XX). In fact, *nodal* expression in the ventral ectoderm induces ventral expression of *bmp2/4* which in turn diffuses to the dorsal ectoderm to induce expression of its downstream targets (Fig. 2.12A; Duboc et al. 2004; Lapraz et al. 2009; Su and Davidson 2009; Saudemont et al. 2010). Among the downstream targets of Nodal signalling in echinoderms are the ventrally expressed genes *chordin*, *foxA* and *bra* (Saudemont et al. 2010; Chap. XX). With the exception of the strictly endodermal expression of *foxA* in *Saccoglossus kowalevskii* (Darras et al. 2011; Fritzenwanker et al. 2014), *bra* and *chordin* transcripts are also detected in ventral domains in *S. kowalevskii* and *Ptychodera flava* (Fig. 2.11; Tagawa et al. 1998b; Röttinger and Lowe 2012), suggesting that a Nodal-dependent mechanism may be required to define ventral domains in hemichordates.

$NiCl_2$ treatments in echinoderms ventralise the embryos and induce radialised expression of *nodal* (Duboc et al. 2004). Interestingly, $NiCl_2$ treatments in *Saccoglossus kowalevskii* and *Ptychodera flava* also ventralise the embryos and radialise expression of *bra* and *foxA* in *P. flava* (Röttinger and Martindale 2011), further strengthening the idea that a $NiCl_2$-sensitive and potentially Nodal-dependent mechanism is involved in dorsoventral patterning in hemichordates. However, the potential molecular link between $NiCl_2$ and Nodal and the molecular connection between the ventralising $NiCl_2$-sensitive signal and dorsalising BMP effects in hemichordates remain unclear, and additional work is required to understand the degree of conservation to the mechanism of dorsoventral patterning in echinoderms.

Anterior-Posterior Patterning

The bulk of molecular studies in hemichordates have been carried out in the direct developing species *Saccoglossus kowalevskii*, the indirect developer *Ptychodera flava* and, more recently, in another ptychoderid hemichordate species, *Balanoglossus simodensis*. The recent sequencing and comparison of the *S. kowalevskii* and *P. flava* genomes has revealed the identical genomic organisation of their 12-gene Hox clusters (Freeman et al. 2012), which is reminiscent of the Hox cluster organisation of *B. simodensis* (Ikuta et al. 2009). With the exception of differences at the posterior end of these clusters, the hemichordate organisation is strikingly similar to that of chordates, supporting the idea that the ambulacrarian ancestor possessed minimally a 12-gene Hox cluster with at least nine genes organised and oriented the same as their chordate orthologs (Freeman et al. 2012).

In vertebrates, expression of Hox genes as well as other transcription factors such as *barH*, *engrailed*, *pax2/5/8*, *six3*, etc., is restricted to the central nervous system (CNS). To gain insight into the origin of the chordate CNS, previous studies in *Saccoglossus kowalevskii* have analysed the expression patterns of these genes and showed their circumferential epidermal expression during early development that potentially reflects the broad and diffuse distribution of neurons in these stages (Lowe et al. 2003; Aronowicz and Lowe 2006; Lemons et al. 2010; Pani et al. 2012). In adult tissue, however, gene expression analysis of neuronal markers such as *Elav*, *synaptotagmin*, *VAChT*, *serotonin*, *Hb9*, *Drg11* and *GABA* has shown the existence of a centralised ventral as well as dorsal nerve cord that is internalised at the level of the enteropneust worm's collar into the collar cord (Nomaksteinsky et al. 2009). Intriguingly, the observed centralisation of the enteropneust nervous system was in contrast to previous studies that described the presence of a diffuse nerve net in hemichordates (Lowe et al. 2003). One idea to explain these fundamental differences was that the developing ectoderm may represent a transient diffuse nerve net unrelated to that of the adult (Nomaksteinsky et al. 2009). In order to gain a better understanding of the relation between the embryonic and adult nervous systems, a recent study has analysed a broad range of genes associated with neurogenesis during early *S. kowalevskii* development (Cunningham and Casey 2014).

This analysis has revealed that already during embryonic development, expression of most of the analysed genes transitions from a circumferential to a dorsal and ventral midline localisation. Hence, this observation suggests that developmental centralisation of the nervous system in hemichordates occurs earlier than initially anticipated (prior to hatching) (Cunningham and Casey 2014).

Perturbation of BMP signalling in *Saccoglossus kowalevskii* affects dorsoventral patterning but not the distribution of neurons in the analysed embryos (Lowe et al. 2006). This observation has led to the idea that nervous system formation in hemichordates may be insensitive to a BMP/Chordin gradient that is crucial for the formation of the CNS in protostomes and vertebrates. However, this analysis was carried out on embryos that presented a diffuse expression pattern of neuronal markers (Lowe et al. 2006). With the recent observations of a ventral and dorsal CNS in hemichordates (Nomaksteinsky et al. 2009; Cunningham and Casey 2014), it would be crucial to re-analyse the effects of perturbing BMP signalling on the centralisation of the nervous system at adequate embryonic stages.

Taken together, the studies described above do not contradict the classical idea that portions of the hemichordate central nervous system may be homologous to the chordate CNS (Knight-Jones 1952; Lowe et al. 2003; Nomaksteinsky et al. 2009). However, the current data make it impossible to unequivocally settle this issue at present (Holland et al. 2013).

The tubular organisation of the collar cord of enteropneusts has been proposed to be homologous to the chordate neural tube (Morgan 1894; Bateson 1886; Ruppert 2005; Kaul and Stach 2010; Luttrell et al. 2012). However, gene expression data of *bmp* and *chordin* (see above) support the theory of dorsoventral inversion of body axes at the base of chordates (Lowe et al. 2006). According to this, the chordate neural tube would be homologous to the ventral nerve cord of enteropneusts, yet it is the dorsal collar cord that neurulates in enteropneusts. Moreover, genes such as *pax6*, *nkx 2.2* and *msx* that have similar domains in the chordate neural tube and the protostome nerve cord (Denes et al. 2007) do not at all have resembling domains in *Saccoglossus kowalevskii* (Lowe et al. 2006). Hence, because of the lack of clear molecular data, its homology remains controversial (Ruppert 2005; Nomaksteinsky et al. 2009; Kaul and Stach 2010; Holland et al. 2013).

Recent work in the ptychoderid enteropneust *Balanoglossus simodensis* has analysed the expression of genes known to be crucial of formation and patterning of the chordate neural tube (Miyamoto and Wada 2013). This study reports expression of *bmp2/4*, *dlx*, *pax3/7* and *soxE* in dorsal regions of the collar cord but failed to observe expression of a potential ventral marker, *pax6*, thus suggesting a partially conserved patterning mechanism between the hemichordate collar cord and the chordate neural tube (Miyamoto and Wada 2013). In chordates, Hedgehog (hh) signalling emitted from the notochord and received by the neural plate (via the Hedgehog receptor patched (ptc)) is essential for patterning the neural tube along the dorsoventral axis (Echelard et al. 1992). In *B. simodensis*, expression of *hh* appears restricted to the stomochord and the anterior endoderm, which lies beneath the collar cord, during metamorphosis. In contrast, *ptc* is expressed in the mesoderm surrounding *hh*-expressing endoderm as well as the midline of the neural plate (Miyamoto and Wada 2013). These results suggest that Hedgehog signalling from the underlying endoderm may be received by the collar cord. However, gene-specific functional assays are required to determine if Hedgehog signalling is required for dorsoventral patterning of the neural tube in hemichordates.

In summary, the molecular studies currently available show striking similarities between vertebrates and enteropneusts in regard to the spatial deployment of transcription factors (Fig. 2.13) and signalling centres involved in neuronal anterior-posterior and dorsoventral patterning of the neural tube. The implications of these observations in establishing potential homologies between enteropneust and vertebrate body plans and on the evolution of the chordate CNS are currently highly debated (Holland et al. 2013) and additional work is required on hemichordates as well as invertebrate chordates (ascidians and amphioxus) to gain more insights into this long-lasting question (see Chaps. XX and YY). [AU12]

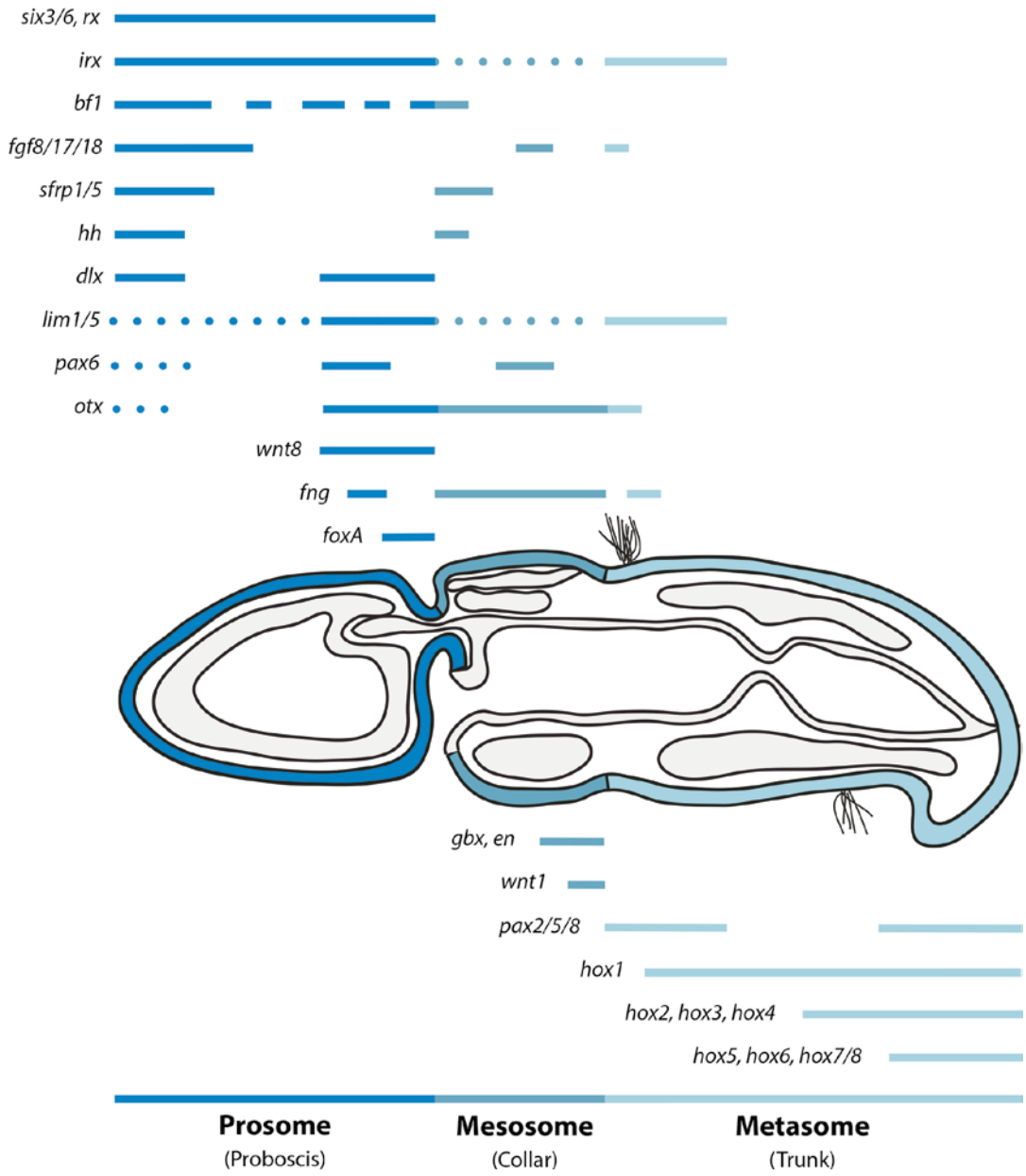

Fig. 2.13 Ectodermal anterior-posterior gene expression in hemichordates. Diagram summarising anterior-posterior regionalisation of gene expression domains in the ectoderm of the harrimanid enteropneust hemichordate *Saccoglossus kowalevskii* (Represented gene expression patterns based on data from Lowe et al. (2003), Aronowicz and Lowe (2006), Pani et al. (2012))

OPEN QUESTIONS

- Neurogenesis of the adult nervous system
- Function of the gill slits
- Investigations and characterisation of the light sense organs in tornaria larvae and adult enteropneusts
- Development of the muscular system in direct and indirect developing enteropneusts
- Roles of canonical Wnt and FGF signalling in ptychoderids and of Nodal signalling in direct and indirect developing enteropneusts
- All aspects of pterobranch embryogenesis and development

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