

Cephalopod development: what we can learn from differences

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Abstract

Introduction

The molluscan neuro-muscular system shows extreme diversity. Cephalopods present an original body plan, a derived neuro-muscular complex and a development with drastic changes in the antero-posterior/dorso-ventral orientation. How it took place during evolution is an unresolved question that can be approached by the study of developmental genes. Studying the expression of conserved transcription factors (Pax and NK families, otx, apt) and morphogen (hedgehog) during development is a good test of the conservation of their functions. We underline here unexpected expression patterns during cephalopod development, and we aim to suggest that these patterns may be, at least partly, in relation to morphological novelties in this clade.

Conclusion

The expression patterns observed point out the diversity of molecular pathways recruited during evolution and the necessary carefulness regarding generalization of results obtained from a very small set of model organisms. Studying different species, with a large diversity of morphology, could help to have a better understanding of the variety of the genes roles and/or of the plasticity of networks.

Introduction

Molluscs represent a very surprising metazoan clade. Including Polyplacophora, Bivalvia, Gastropoda, and Cephalopoda, they show clear synapomorphies (shell, mantle, muscular foot) but present a high

diversity of body plan organisation (Figure 1).

Cephalopods are the most intriguing group, presenting not only an original body plan among molluscs, particularly regarding the locomotory system, but also obvious convergences with vertebrates, especially regarding the nervous system. The contribution of cephalopods in neurosciences, from cellular physiological mechanisms to behaviour in vertebrates, is doubtless - by the famous nobel work on giant axons of the squid but also by their learning and memory abilities regarded as surprising for an "invertebrate"¹. But cephalopods can also be studied for themselves to address evolutionary questions: how it is possible to evolve from an ancestral mollusc through an ancestral cephalopod and which mechanisms have led to these innovations is a crucial point in the understanding of cephalopod lineage characteristics and also of neuromuscular complex evolution.

In this context, an evo-devo approach seems pertinent. These studies aim to characterize genes controlling development, which are involved in modifications of body axes and patterning or morphological novelties. By comparative analyses, these studies allow inferences about the evolutionary process but they also reveal the evolutionary plasticity of developmental genes and gene networks.

Based on the peculiar case of cephalopods, and the knowledge on the most conserved and known transcription factors and morphogens, this review aims to point out the diversity of molecular pathways recruited during evolution and the necessary carefulness regarding generalization of results obtained from a too small set of model organisms. Several aspects must be considered in the study of the

molecular control of the development in an evolutionary perspective.

Discussion

The authors have referenced some of their own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. Animal care was in accordance with the institution guidelines.

Cephalopods are morphological innovators

By contrast with the typical molluscan design, coleoids (octopus, squids, cuttlefish) show a derived and specialized neuro-muscular complex. The brachial crown and the funnel, both presumptively derived from the molluscan foot, and the highly muscular mantle are used in the characteristic jet propulsion locomotion of cephalopods.

Histological studies have shown that these locomotor muscles are oblique striated muscles, a muscle type also identified in nematodes, annelids or other molluscs². The nervous system of cephalopods is very different from that of other lophotrochozoans³. They have no ventral nerve cord and their nervous system is highly specialized. It includes a peripheral nervous system (PNS) with stellate ganglia, nervous cords of the arms/tentacles and several sparse ganglia (Figure 1) and, above all, a very complex central nervous system (CNS) with a brain enclosed in a cartilaginous capsule and associated to voluminous optic lobes. Described in detail in numerous coleoid cephalopods, the brain comprises of supra- and sub-oesophageal masses (SPM and SBM, Figure 1) disposed between the eyes around the oesophagus and divided into 25 major lobes, with specific different functions⁴.

The movements and behaviour are under the control of the brain receiving and analysing visual input from the camerular eyes, which are convergent

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structures with vertebrates. The giant fibre system allows very fast answers (e.g. fast jet propulsion) to environmental cues.

It is generally assumed that these muscular and nervous systems have been selected concomitantly to the loss of the molluscan external protective shell during evolution. But the arguments sustaining this assumption are quite elusive justifying attempts to develop new approaches such as evo-devo studies.

Development of cephalopods: an axis imbroglia

Unlike other molluscs, cephalopods have a direct development without neither visible larval stage nor metamorphosis event. Nevertheless, drastic changes in embryonic orientation occur during development within the protecting egg capsule.

The development of cephalopods is well described since the early 20th century and the masterwork of Naef⁵.

All cephalopods have telolecithal eggs and present a discoidal cleavage unlike other molluscs in which a total and spiral segmentation occurs. In *Sepia officinalis*, the model we work on in the lab in an eco-evo-devo perspective, cleavage leads to a flat, disk-shaped embryo until stage 14 (based on Lemaire's system) where organogenesis starts (Figure 2)^{6,7}.

Until stage 20, the flat embryo shows a clear bilateral symmetry axis, parallel to the yolk surface, sometime described as antero-posterior due to the position of the future mouth. Progressively, the embryo takes volume (stages 19 to 21) and stick up perpendicularly to the yolk with a flexion of all the body (Figure 2A). The future adult antero-posterior axis is marked by the cephalopodium (head and arms) in the anterior and the visceropallium (mantle, funnel and viscera) in the posterior (stages 22 to hatching at stage 30) (Figure 2B). Importantly, at the same time, the future adult dorsal and ventral parts differentiate (e.g. all skin components allowing camouflage⁸). Thus, these changes cannot be interpreted as a simple flexion of the preexisting embryonic axis. Contrary to other

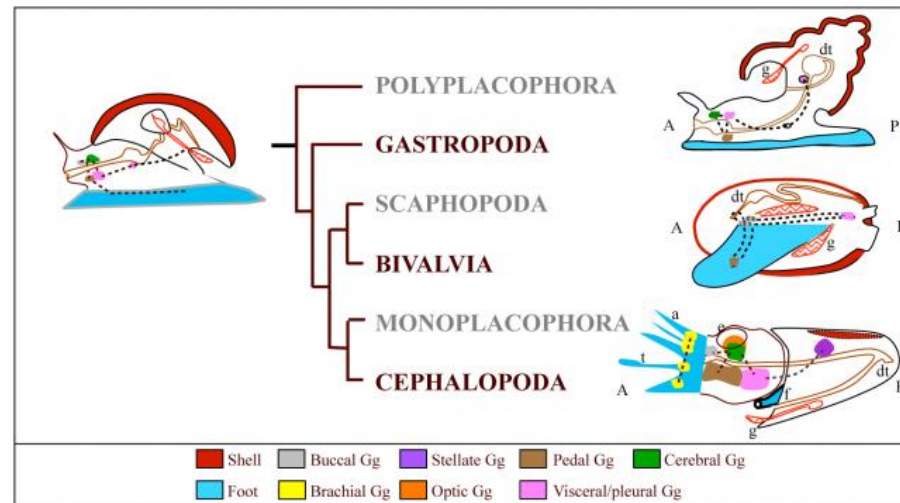


Figure 1: Cephalopod organisation versus mollusc organisation. Foot and shell as well as the anterior triangle of ganglia (cerebral, pedal, visceral) are synapomorphies of molluscs and are present in the ancestor. The foot is conserved in Bivalvia and Gastropoda but is modified in Cephalopoda in peribuccal appendages (8 arms and 2 tentacles in *Sepia*) and funnel. The shell is regressed in Cephalopoda and the nervous system is modified. Brachial ganglia are novelties in relation to the arm appearance, stellate ganglia are unique and most of the ganglia are divided in several lobes and concentrated into a brain, composed of a supraoesophageal mass, SPM with issued from cerebral ganglia, and a sub-oesophageal mass, SBM issued from pedal and visceral ganglia. The optic lobes, connected to the eyes, represent 90% of the CNS. A : anterior ; P : posterior ; a : arm ; e : eye ; g : gill ; Gg : ganglion ; dt : digestive tube ; t : tentacle. Puncted line : nerve and/or connectives.

classical developmental models, especially vertebrates, the adult antero-posterior axis is very different from the embryonic axis underlying bilateral symmetry and runs from the embryonic oral side (yolk side) to aboral side, which correspond respectively to the future anterior and posterior part. This is reflected in the vocabulary used in the field, which describes an “embryonic orientation” and an “adult/physiological orientation”⁹ (Figure 2).

This imbroglia points out that, even based on classical orientated structures (the mouth in an anterior and ventral position), morphology is not sufficient to understand the orientation of the body plan during development in cephalopods.

Hox genes are described as general antero-posterior organizers in Bilateria, showing a colinear pattern of expression, with anterior-class genes expressed in more rostral domains than posterior-class ones (aka the well-known “hox colinearity”). But cephalopods should be added to the list of « bizarre » species with no clear correspondence

between regionalization and hox genes expression; in fact, cephalopod hox genes expression seems to correlate better with morphological novelties¹⁰.

Interestingly, the hypothesis that hox colinearity is only the result of an exaptation has been presented in vertebrates¹¹. As in other clades (echinoderms, tunicates)¹², the recruitment of hox genes in other functions than the specification of an antero-posterior axis may provide an explanation to the innovative body architectures found in cephalopods.

The question of the existence and the nature of “master organizing genes” in cephalopods remains unanswered.

Development of neuro-muscular complex

In our lab, we focus on the neuro-muscular complex. Developmental studies have identified a molluscan basic design (bauplan) in the cephalopod nervous system, including a classical set of paired ganglia: cerebral, pedal and visceral-pleural ganglia (Figure 1). The nervous system emerges as distant ganglia, some of them merging and fusing later to form

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the cephalopod CNS (Figure 2C). This development of the brain is similar between cephalopod species although the timing of lobe formation and rearrangement seems to be related to the mode of life at hatching¹³. In *Sepia officinalis*, at the beginning of organogenesis (stages 15 to 20), the cerebroid ganglia emerge on both sides of the future mouth, the visceral ganglia develop on both sides of the mantle and the pedal ganglia emerge between arms 4 and 5¹⁴. All these ganglia begin condensing and merging to each other from stage 23 to form the brain in the embryo's head (Figure 2C). As for the PNS, both stellate ganglia begin to develop on the left and right sides of the mantle at the edges of the presumptive shell sac from stage 19 and intrabrachial ganglia take place all along the arms' crown and develop into the arms from stage 17. At the same time the muscular system develops: the mantle growth as a disk in the centre of the embryo; the shell sac invaginates and is closed at stage 20, the two paired elements of the funnel are disposed in lateral parts of the mantle for the pouches and posteriorly for the tube (Figure 2B); the arms appear as buds in the periphery of the embryo, they grow and the differences between arms and tentacles are already visible at stage 20.

Thus, the development of the nervous system is a very complex and highly coordinated process. How do ganglia develop, how do they migrate and merge as lobes and how do brain lobes mature are essential questions.

Different genes, transcription factors and morphogens have been characterized in lophotrochozoans (including molluscs) as playing a major role in the developing nervous system. We explored these genes in *Sepia officinalis*.

Genes and the neuromuscular complex

Otx/orthodenticle is a transcription factor known to have a highly conserved role in the differentiation of anterior neural structures in metazoans from vertebrates to brachiopods and annelids¹⁵. In *Sepia*

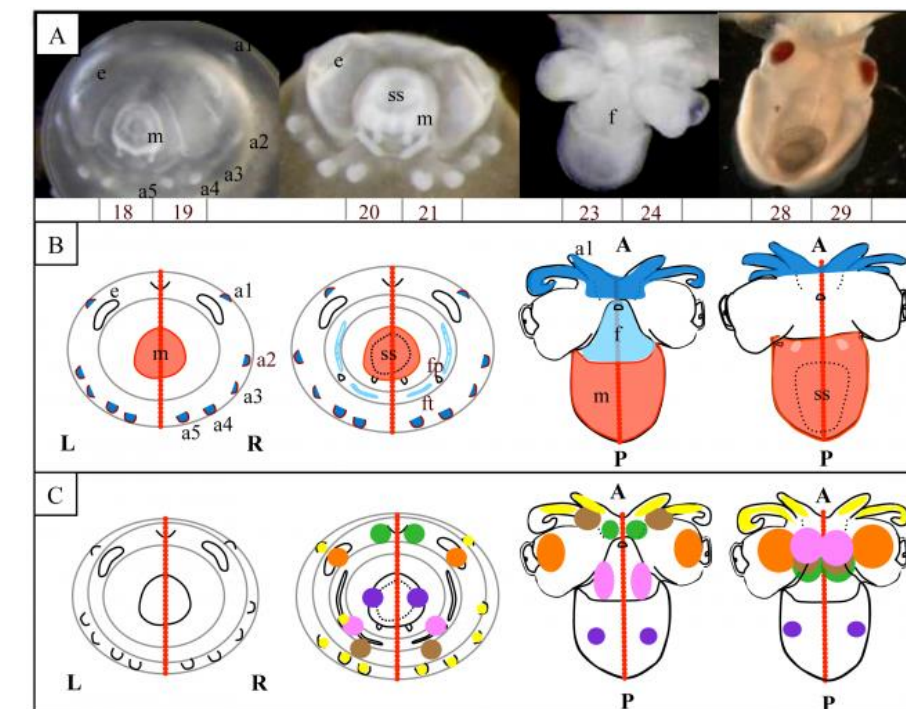


Figure 2: Development of *Sepia officinalis* and orientation. A: General organisation of the embryo from stage 14 to stage 30 at hatching. Stages are indicated below. B: Development of the muscular structures involved in locomotory functions. The first and second drawings are in aboral view, the third in ventral view, the fourth in dorsal view. C: Development of the nervous system from sparse ganglia to the brain. The two last drawings are in ventral view. L: left R: right. m: mantle; fp: funnel pouch; ft: funnel tube; e: eye; a1 to a5: arm 1 to arm 5; ss: shell sac; Color code of ganglia: see figure 1.

officinalis embryos, otx expression is observed in cerebral and pedal ganglia, which will constitute anterior parts of the adult central nervous system (all the SPM and aSM/mSM (anterior and medial) parts of the SBM (Figure 1)¹⁶.

otx seems to have conserved its role for anterior CNS determination, although the territories implicated are sparse ganglia, and not a unique central nervous tissue as in vertebrates or insects. In this context, studies in *Euprymna* have suggested that Hox genes do not play their classical role: Lab, Hox3, and Scr, considered as anterior genes, are expressed in visceral ganglia, corresponding to the future posterior part of the SBM (pSM), whereas the most posterior Hox genes, Ant, Lox4, Post 2 are expressed in pedal ganglia corresponding to aSM/mSM¹⁰. It is not yet clear how many genes and which ones are expressed in the cerebroid ganglia in *Euprymna*. This questions the regionalization of the NS versus the body. Otx2 is expressed in the SPM

very late during the development¹⁶ suggesting that the regionalization could be accurate only for the SBM; the SPM in the dorsal position could then be considered as anterior. This point needs further comparative studies to be solved.

Among the different genes of the hedgehog pathway identified in vertebrates, shh is the most studied and has a key role in organogenesis and in dorso-ventral organisation of the vertebrate brain by acting upstream on several genes (transcription factors) such as Pax6 and Pax 3/7^{17,18} (Figure 3A).

In ecdysozoans *Drosophila* hedgehog is necessary for proliferation initiation of larval neuroblasts and in some lophotrochozoan trochophore larvae, hh is expressed in neural cells of the ventral cord/chain or in the midline territory and sensorial cells^{19,20}.

In vertebrates, Shh acts upstream Pax3 and Pax7, which have a role in neural crests specification and in muscular progenitors determination in somites. In *S. officinalis*, Pax3/7 is

expressed only in the pedal ganglia (thus linked to the arms) and later in the aSM but not in a dorsal position of the nervous system, and *hh* is not expressed within the neural ganglia areas (Figure 3B)²¹. *Pax6* is expressed in cerebroid, optic and pedal ganglia, all of them leading to SPM and aSM/mSM.

Moreover, the expression of *hh* is exclusive of *Pax6* expression: *hh* could have a role in the limitation of *Pax6* nervous expression in *Sepia officinalis* as it is expressed in the border of *Pax6* expression areas. Unlike vertebrates, *Pax6* is expressed all along the development and the expression is not restricted to a dorsal area²¹ (Figure 3B).

It has been suggested in *Euprymna scolopes*, that apterous, a LIM-homeodomain transcription factor could share a role with *Pax6* in the patterning of CNS, but expression patterns of apterous do not suggest any role in the early dorso-ventral patterning as it is observed in other groups²². Cephalopods are likely to use unconventional pathways to regionalize their nervous system along the dorso/ventral axis.

In most developmental models, locomotor muscles setting up is under the control of myogenic regulating factors (MRFs), downstream *Pax3* and *Pax7* at least in vertebrates²³. MRFs proteins have been putatively localized in *S. officinalis* in muscular cells of arms and tentacles²⁴ but this observation has not been confirmed at the molecular level, questioning their role (unpublished results). An *hh*-like expression has been reported in a specific sub-population of myoblasts in *S. officinalis* juveniles²⁵, but no *hh* expression has been evidenced in mesodermal structures leading to muscles in the embryo and during all the main organogenesis processes²⁵.

A contrario, a major expression of *NK4* in locomotory muscle territories (arm, funnel, mantle) has been evidenced²⁶. This gene is known to have an essential role in the cardiac muscle setting up in other metazoans. These results suggest a secondary recruitment of *NK4* for the morphological innovations in

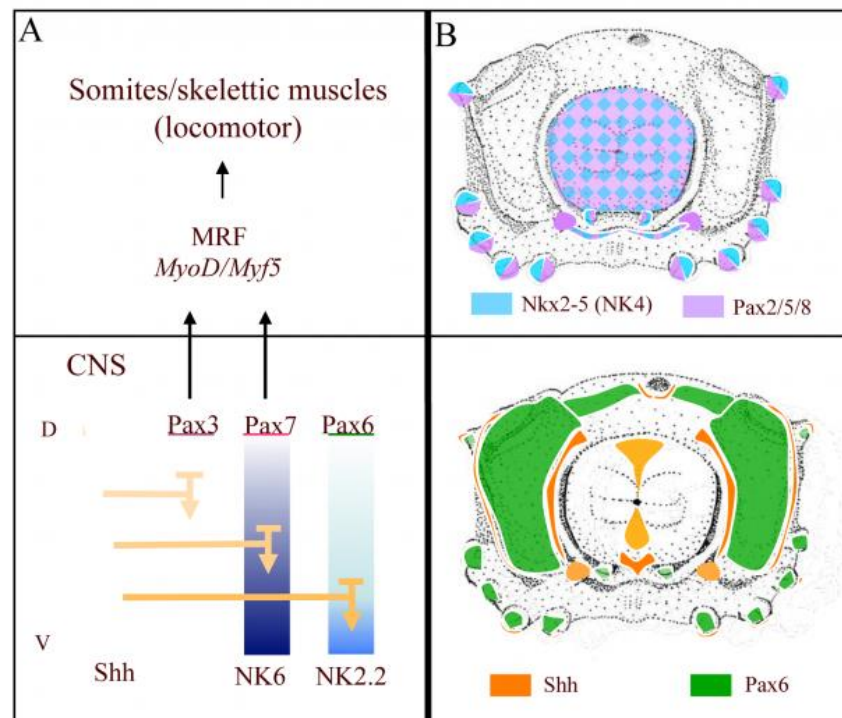


Figure 3: Neuromuscular development in vertebrate (A) and in *Sepia officinalis* (B). A below: molecular pathways of genes involved in the dorso-ventral setting up of the CNS where the expression of *Pax* genes depends of the morphogen *shh* produced with a dorso-ventral gradient (inspired from the neural tube position). A above: factors involved in the muscular differentiation and controlled upstream by *Pax* genes. B below: aboral view of *Sepia* embryo at stage 21. *hedgehog* is not expressed in the CNS and *Pax6* is expressed in ganglia whatever their dorso-ventral position. B above: all muscular territories, mantle, arms and funnel express *NK4* and *Pax258*, not *Pax37*. D: dorsal side, V: ventral side.

cephalopods. Moreover unlike vertebrates or *Drosophila*, *Pax3/7* is not expressed in muscular progenitors; it appears more involved in ectoderme differentiation than in muscular differentiation (or at least mesodermic area)²⁷.

Because it was not possible to characterize myogenic factors in all the ESTs database available in cephalopods and because of the involvement of other genes than expected, we suggest that unconventional pathways have been selected for the development of muscular derived structures. These pathways are likely linked to the nervous system evolution.

Conclusion

Shh is a key gene in vertebrates in the setting up of dopaminergic and serotonergic systems, very important for the movements, the locomotion and the cognitive abilities.

With cephalopods being the only non-vertebrates to produce an important quantity of dopamine and serotonin, the knowledge of the molecular control of the development of the dopaminergic system could help to understand its evolution and the putative role of *hedgehog*.

Actually, the orientation of the nervous system and the setting up of associated muscular structures do not seem determined by the same gene network: *Pax 3/7* and *hh* roles remain to be clarified in the embryo development; *Otx*, *Pax6*, possibly with *apterous*, and several *Hox* genes intervene in the nervous system development (setting up, maturation, differentiation), and *NK4* has a role in locomotory muscle development.

The subsequent recruitments of highly conserved (in protostomes and deuterostomes) transcription factors have extended their contributions in the appearance of morphological novelties. These results highlight the

diversity of molecular pathways recruited during evolution and the necessary carefulness regarding generalization of results obtained from a very small set of model organisms.

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