The evolution of sensory and neurosecretory cell types in bilaterian brains

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The evolution of sensory and neurosecretory cell types in bilaterian brains

To my parents and my husband for their encouragement and patience

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1 INTRODUCTION

1.1 Defining homology relationships across Bilateria

During the last decades, molecular and genetic tools added a wealth of molecular information on the development and function of many different structures, such as eyes or brain neurosecretory systems in many species. These data help to describe and understand the function of one particular structure in one particular species (and maybe its close relatives), but they do not explain by themselves how the existing similarities between very distant species, such as insects and vertebrates have to be interpreted. Indeed, the insect and vertebrate brains both contain various cell types that are remarkably similar in function and molecular setup. On the other hand, there are many dissimilarities in the cell types and organs between the different species. We often face the situation that some aspects of a given structure are seemingly conserved across distant species, but other aspects are not. So, what do these similarities mean, especially in evolutionary terms? Did similar structures occur accidentally several times in independent evolutionary lines- implying that the molecular similarities are only caused by similar functional acquirements? Or do they share common ancestry from the last common ancestor, that all truly primarily bilateral symmetric species (including echinoderms) share? These last common ancestors are called Urbilateria (De Robertis and Sasai, 1996). In the classical terminology, to ask this question means to try to determine homology of structures, such as sensory or neuroendocrine systems, between e.g. flies and annelids and humans. This means that in order to really understand development or evolution of structures such as eyes or the hypothalamus one has to elucidate their common heritage. Homology for sensory or neurosecretory systems across Bilateria would imply that Urbilateria already possessed a rudimentary version of these systems. In this case, we can address a second question: how were these rudimentary systems subsequently modified during the evolution of specific model organisms, such as mouse or fruit fly or humans?

In order to reconstruct the features of Urbilateria, one has to compare the morphological and molecular data available from bilateral symmetric animal species that are evolutionary far apart. Bilateria are grouped into two main groups,

Deuterostomia and Protostomia (Grobben, 1908 and see Figure 1). According to recent analysis of 18S rRNA and Hox genes, the Protostomia are subdivided into Ecdysozoa (cuticle-moulting animals including most notably arthropods and nematodes) and Lophotrochozoa (mostly animals displaying a spiral cleavage of the egg and a trochophore-like larva, including annelids and molluscs; Aguinaldo *et al.*, 1997, de Rosa *et al.*, 1999, de Rosa, 2001, Peterson and Eernisse, 2001, Mallatt and Winchell, 2002 and see Figure 1).

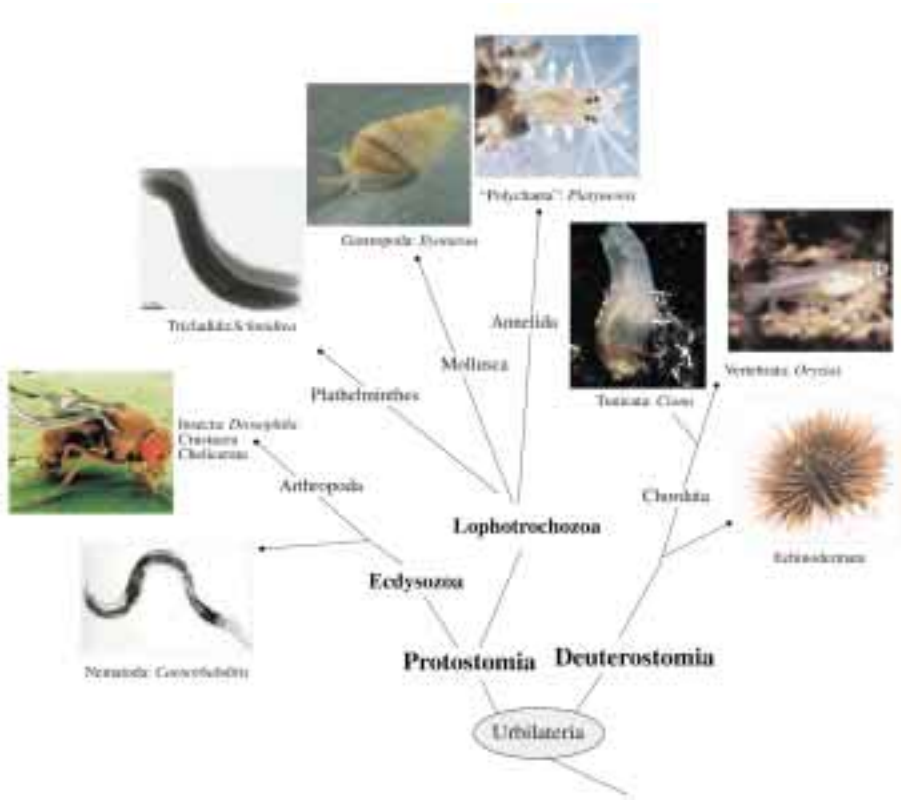


Figure 1. Phylogenetic tree of the Bilateria.

This tree shows the currently most commonly accepted view of the phylogenetic relationships among the 'triploblastic' animals. Note that the position of all current invertebrate model organisms is either in the ecdysozoan or deuterostomian branch. *Platynereis dumerilii*, as a polychaete, belongs to the lophotrochozoan branch of Protostomia. Modified from Arendt and Wittbrodt, 2001.

Many morphological and molecular data are available for the mostly vertebrate model organisms on the deuterostomian side, as well as for the protostomian *C.elegans* and *Drosophila melanogaster*, both belonging to the Ecdysozoa. However, these latter systems are disadvantageous for comparative purposes, because they are extremely derived, probably owing to the ecological niches they occupy, prompting the restructuring of the body, organ, and cellular morphologies, as well as an extensive shortening of their generation time. This concomitantly leads to changes in the molecular networks controlling the animal's development. A derived molecular network is characterized by sequence derivation and, importantly, loss of genes. Many examples indicate now that this is true for *Drosophila* and *C.elegans* (Raible and Arendt, 2004; Tessmar-Raible and Arendt, 2003). As a consequence of this gene loss, many genes only found in vertebrates have been considered to be vertebrate – specific features– and thus were the cell types and structures these genes are expressed in.

In order not to draw wrong conclusions, we need to compare extant species that have deviated little from their Urbilaterian ancestors — in terms of morphology, and thus also in terms of molecules. According to fossil records, the earliest bilaterians found were marine worms of considerable size, with a morphology somewhat in between the body plans of today's polychaete annelids, molluscs, and brachiopods (Conway Morris, 1998). A long standing discussion on the basis of morphological arguments points in a similar direction, that urbilaterians may have resembled annelids (Arendt and Nübler-Jung, 1994; Dohrn, 1875; Nübler-Jung and Arendt, 1994). Additionally, growing molecular evidence (Knoll and Carroll, 1999) supports the idea that they were complex animals. Therefore, I chose to investigate the polychaete annelid *Platynereis dumerilii*.

1.2 Platynereis dumerilii – an ancestral organism for the comparative study of animal development and genome evolution

Platynereis dumerilii (Lophotrochozoa/Spiralia, Annelida, 'Polychaeta', Nereidida) is a medium-sized marine species (5-6 cm) and displays a development, canonical for polychaetes (Figure 2): oocytes are released freely into the water in large numbers

(several hundreds/female), and fertilized by the male. The whole life cycle can be as short as three months under lab conditions. Its morphology exhibits several features considered to be ancestral, as supported by fossil records from the early cambrium (Conway Morris, 1998 and see Figure 3).

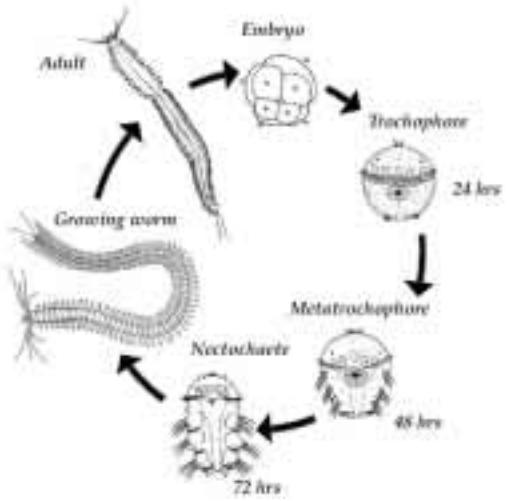


Figure 2. The life cycle of the annelid *Platynereis dumerilii*

The egg undergoes spiral cleavage, an amphistomous gastrulation that gives rise to a trochophore larva that finally metamorphoses to the juvenile animal that displays most of the adult body plan. Developmental times are valid for 18°C.

(scheme courtesy of G. Balavoine with original pictures taken from Dorresteijn *et al*., 1993; Hauenschild and Fischer, 1969)

It has a homonomously segmented body axis (Patel, 2003; Prud'homme *et al*., 2003; Stollewerk *et al*., 2003) and appendages that are the prototypical polychaete parapodia, that were already present in the first cambrian annelid like fossils (Conway Morris, 1998). *P.dumerilii,* like most annelids, has a prototypic closed circulatory system with a large dorsal pulsatile vessel (heart), the basic architecture of which is remarkably conserved.

Figure 3. Fossil records supports an ancestral status of polychaetes.

Two fossils **(a and b)**, considered to be annelids from the early Pre-/Cambrium, are compared to a mature *Platynereis dumerilii* female **(c)**. a) Cambrian explosion fossil of Aysheaia, taken from www.pbs.org/kcet/shapeoflife/ animals/annelids4.html; b) *Dickinsonia* taken from www.ucmp.berkeley.edu/vendian/dickinsonia.html; c) Platynereis dumerilii, EMBL culture, picture courtesy of C. Burgtorf and P. Steinmetz

The central nervous system (CNS) of *Platynereis* exhibits the classical rope–ladder–like organisation viewed to be of ancestral type: "From the standpoint of comparative neurology there could hardly have been a better choice of a polychaete as the common classroom type than *Nereis*" (Bullock and Horridge, 1965 p. 735). Its paired larval eyes are of simple pigment-cup organisation, resembling the proposed 'Protoeye' of Urbilateria (Arendt *et al.*, 2002; Arendt and Wittbrodt, 2001; Gehring and Ikeo, 1999). Besides these ancestral morphological features *Platynereis dumerilii* also exhibits primitive developmental features. Its blastopore gives rise to both the mouth and the anus (amphistomous gastrulation; Arendt and Nübler-Jung, 1997), and it develops indirectly via a primary ciliated larva (in spiralians called trochophora), as many of the present-day lophotrochozoans do. These primary ciliated larvae are also considered an ancestral feature for Bilateria (Arendt *et al.*, 2001). A schematized drawing of such a larva, along with the definition of views and body regions used in the study, is shown in Figure 4.

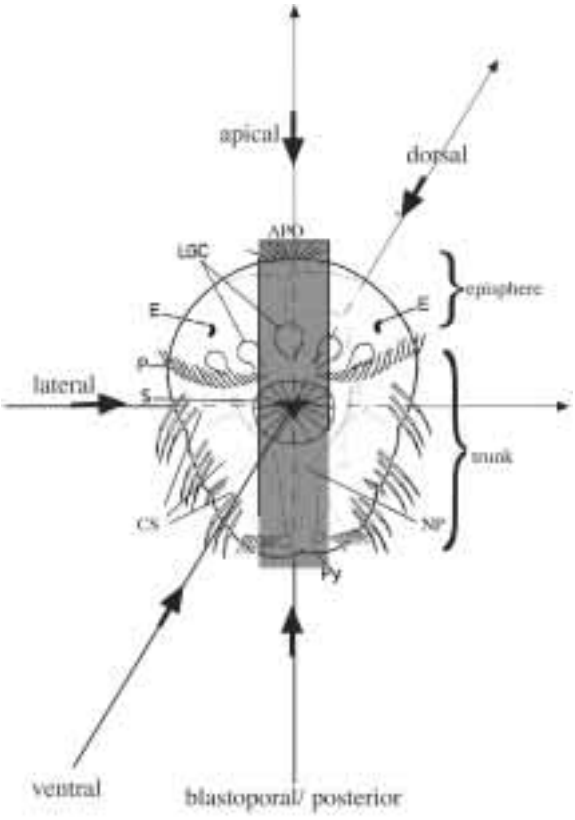


Figure 4. Definition of views and body regions of the *Platynereis* larva used in the study.

The picture shows a schematized 48hpf embryo from the ventral side. Approximate orientations and views are also true for younger and older stages. APO apical organ, CS chaetal sacs, E larval eye, LGC larval gland cells, NP neural plate, P prototroch, Py pygidium, S stomodeum. Black arrows along the lines of the coordinate system indicate the corresponding views (e.g. ventral). Accordingly, the body surface visible by this view is referred to by the same term (e.g ventral episphere). In addition, the grey box indicates the body area referred to as median (e.g. ventral median episphere vs. dorsal median spisphere). The terms 'proximal' and 'distal' are used according to standard usage, as 'proximal' being closest to the body surface and 'distal' being distant from the body surface. Picture modified from Dorresteijn *et al.*, 1993.

Consistent with the assumption that *Platynereis* never left its original marine habitat and thus has a rather original set of genes, its genome has a size of approximately

900-1000Mb (Gambi, 1997; Gregory, 2001) and genes tend to be present in single copies only. In addition, the sequences of these single copy genes tend to be prototypical to those of other taxa, in that the sequences are evolving at a steady rate rather than rapidly changing or diverging. This steadiness is reflected in molecular phylogenetic trees by relatively short branch lengths for *P.dumerilii* sequences compared with some other model organisms (*Drosophila, C.elegans* and *C.intestinalis*) that are known to be evolving and diverging quickly at the molecular level (Figure 5.). Thus, the genome of *Platynereis dumerilii* shows no signs of strong reduction, expansion or derivation.

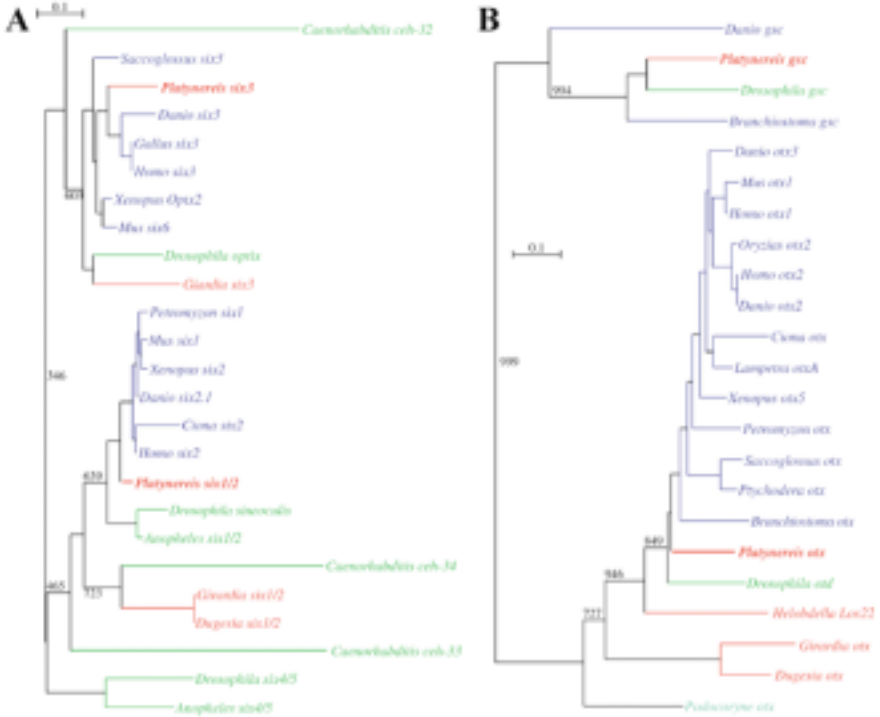


Figure 5. *Platynereis dumerilii* genes are molecularly little derived.

Molecular genetic trees of the **(A)** *six1/2* and *six3* gene family; **(B)** *otx* and *gsc* gene family. Gene representatives of Deuterostomia are represented in blue, Ecdysozoa in green, Lophotrochozoa in red, and the single cnidarian species in turquoise. The branches of the respective *P.dumerilii* genes are represented in bold. Trees were calculated using conceptual translations of gene sequences. Numbers at the branch points are bootstrap values. *P.dumerilii* genes are less derived than other protostomian genes. This becomes apparent by their relatively short branch lengths and their close association to vertebrate genes. The latter is an artifact. Phylogenetic trees of single genes should reflect the phylogenetic relationships. They are currently viewed as depicted in Figure 1. However, if some sequences differ much more from the average than the others, they cause a phenomenom known as 'long branch attraction' (Felsenstein, 1978), resulting in an artifical clustering of these derived sequences. In return, sequences that have changed less will be artificially brought together, as seen here for the sequences of *Platynereis dumerilii* and vertebrates. The accession numbers belonging to these phylogenetic trees can be found in the Appendix.

In summary, a unique combination of ancestral features on morphological, developmental and molecular/ gene level, contrasting with the derived characteristics of all major molecular model systems previously established for Protostomia, makes *Platynereis dumerilii* an essential reference point for comparative studies across Bilateria. This concerns all aspects of comparative development. During recent years whole genome sequencing has been performed for several Bilateria, creating another source of an exponentially growing amount of data. Once more, these genome sequencing projects suffer from the fact that the so far sequenced Protostomia are derived, leaving the question about the nature of ancestral genes and genomic organisation largely open. Again, *Platynereis dumerilii* is elementary to fill this gap.

An important strength of conventional model organisms lies in their amenability to molecular techniques. In this aspect, *Platynereis* has a similar potential as conventional model organisms. It has been reared successfully in the lab for the last 30 years, starting in the laboratory of C. Hauenschild in Mainz, Germany. Embryos can be obtained all year round, sperm freezing has been established. *Platynereis dumerilii* thus qualifies for RNA/ DNA injections, transgenesis, as well as for reverse genetic methods, such as gene kock-out, e.g. via **t**argeted **i**nduced **l**ocal **l**esions **in** **g**enome/tilling (Colbert *et al.*, 2001) or gene replacements.

1.3 The concept of homologous cell types

Classically, the concepts of homology and analogy are applied on structures and organs, such as eyes, nose or brain nuclei or appendages, heart and inner organs. The comparison of large structures is certainly possible for closely related species, however those comparisons become increasingly difficult with increasing phylogenetic distance between organisms. The reason is that many structures and organs are compound structures, meaning they are composed of different cell types. These cell types can originate at different places and can thus have different evolutionary/ developmental histories. This implies that some of the cell types making up the compared organs might be homologous while others are not. A good example is the adrenal gland of vertebrates: the cells of the adrenal cortex derive from the mesodermal coelomepithelium, whereas the cells of the adrenal medulla are modified neurons. In this case, the different origin of these cells is obvious, because

the tissues of origin are far apart and thus the migration is obvious. The adrenal glands of mammals as a whole structure have no homologous structure in fish, because the different cell types are still separated in the latter (Romer and Parsons, 1991, p.539-540). Therefore, the concept of homology can only be applied if one takes the distinct origin of the cells into account and compares on the level of cell types. Then, the cells of the adrenal cortex of mammals are homologous to the interrenal organ cells of fish, and the cells of the mammalian adrenal medulla can be directly compared to the chromaffine cell groups between the kidneys of fish (Romer and Parsons, 1991, p.539-540).

Structures composed of cell types with different origins might in fact be much more common. What is valid for the adrenal gland, has as well to be considered for other places in the animals. A second place to look at is the vertebrate brain. The functional units are known as brain nuclei, which are aggregates of different types of cells. With the example of the adrenal gland in mind, it should be clear that nuclei are probably not the proper units for evolutionary cross-comparisons, even if at first glance all the cell types seem to originate from a somewhat similar region. Distances in the brain are often small, thus cell types originating ontogenetically in adjacent though distinct areas might be wrongly considered a homogenous population. Besides a spatially close distance, there can be an additional problem that may make it difficult to detect the different origins of cell types. The cell movements (such as the movement of the adrenal cortex and medulla cells) that help us to trace the different origins of cells are usually remnants of an evolutionary earlier adult state, that was modified further and resulted in the current state we find today. In the words of Haeckel's biogenetic law: ontogeny recapitulates phylogeny (Haeckel, 1874; Haeckel, 1886). However, ontogeny itself can be subject to modifications, e.g. in order to shorten developmental processes. This might result in the abolishment of time-consuming developmental migration processes that could have given us a hint on the comparability of a structure.

Taking into account that organs/ tissues/ brain nuclei represent composite structures of distinct cell types of separate origin, the mentioned examples imply that at least in cases of larger evolutionary distance it is more suitable to perform the determination of homology/ analogy across species on cell type level, avoiding the aforementioned

problems. This is the concept of comparative molecular cell biology (Arendt, 2003; see Figure 6.). A cell type is a homogeneous population of cells expressing the same genes during specification and differentiation stages that implement a defined cellular phenotype. The concept of comparative cell biology is not only useful to understand homology relationships of tissues and organs existing today. It also contains the key to understand how these different tissues and organs can evolve. The creation of paralogous genes (and associated enhancer regions) via gene duplication events, and their subsequent differential expression and subfunctionalisation (Ohta, 2003; Van de Peer *et al*., 2001), allows the divergence of the cell type that previously expressed the common precursor of the duplicated gene.

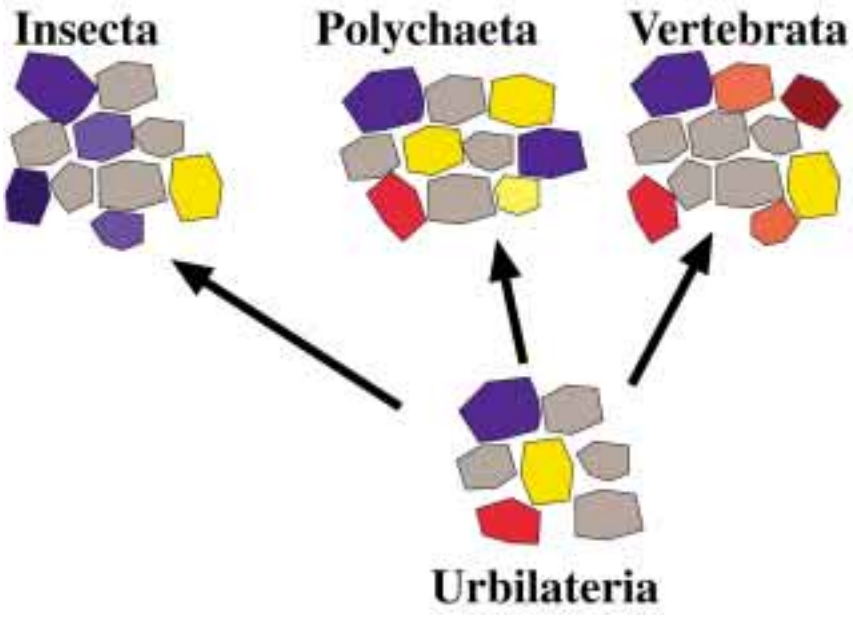


Figure 6. Schematized cell type relationships in different animal groups.

Each cluster depicts a group of different cells in different animal groups. Different basic colors represent distinct cell types. Cell types can get lost (red in insect group) or slightly modified in their molecular components (slight color variations, e.g. light and dark red in vertebrate group). All blue cells within the insect group are sister cell types, as are all blue cells in the polychaete group. Cells of different colors are non-homologous across Bilateria, because they derive from different cell types already present in Urbilateria. All cells of one main color (e.g. all blue cells) can be considered homologous across Bilateria, because they all derive from a common precursor present in Urbilateria.

Examples for this are vertebrate *nk2.1/4* genes (in zebrafish *Zfnk2.1a* and *Zfnk2.1b*, see Figure 22 A; Rohr *et al.*, 2001; Small *et al.*, 2000), several zebrafish paralogs (Volff and Schartl, 2003) or the different *opsins* in the photoreceptor cells of *Drosophila melanogaster* (Chou *et al.*, 1996; Cook and Desplan, 2001; Papatsenko *et al.*, 1997; Pollock and Benzer, 1988; Salcedo *et al.*, 1999). The resulting cell types are sister cell types. Homologous cell types can thus be regarded as the total of sister cell types in the compared species, which diversified from the same evolutionary precursor cell type in the last common ancestor of the compared groups (see Figure 6.)

Following the argumentation of the above examples, the concept of comparative molecular cell biology thus proposes that a similar morphology and molecular set-up of distinct cell types argues for a phylogenetic relatedness of the cells sharing these similarities (Arendt, 2003). The antithesis to this is that molecularly and morphologically similar cell types exhibit similarities not because of common ancestry, but rather because they independently activated the same molecular cascade, e.g. via the spontaneous activation of one master gene at a novel expression site. However, although this latter possibility always has to be taken into account, the various developmental examples in which cells migrate over distances for no apparent reason (see below) can be seen as an indication that cell types do not easily change identity during evolution. It often seems simpler to diversify and rearrange existing cell populations, than to newly create the same cell type again directly at different places. Why else would there be at least four different cell migration streams in the mammalian forebrain (Corbin *et al.*, 2001)? Why should the different cell types of the adrenal gland migrate together from very different tissues of origin? Why do the cells of the vertebrate adenohypophysis untertake a long migration in order to end underneath the hypothalamus and don't develop in place (e.g. see Kawamura *et al.*, 2002)? Why do the forebrain GnRH positive neurons and part of the populations of FMRF and dopaminergic neurons originate in (or close by) the olfactory placode, and need to migrate into the brain to reach their functional positions (Schwanzel-Fukuda and Pfaff, 1989, reviewed in Wirsig-Wiechmann *et al.*, 2002)? To cite the words of Rallu (Rallu *et al.*, 2002a): "Until relatively recently, the different regions of the forebrain were thought to develop as independent

compartments. However, it has become clear that extensive mixing of cells occurs, perhaps because different progenitor zones generate specific subsets of neural cell types, which subsequently become widely distributed throughout the telencephalon." All these examples are indications that it is apparently not so simple to 'just' switch on a cell type specification cascade at the right time and place. If so, this makes the single cell types a suitable unit for long-range evolutionary comparisons, and strengthens the concept of cell-type homology.

In summary, the concept of comparative molecular cell biology covers two important evolutionary points. First, it enables us to define homology relationships for tissues and organs across that were previously of unclear comparability. Second, it links molecular events (gene/ genome duplications) with morphological events (duplications, gradual changes and possible migrations of cell types), and by this helps significantly understanding the proceeding of evolution.

1.4 The bilaterian nervous system

The central nervous system is subdivided into two main parts, a nerve strand and an anterior cerebral center. Although the nerve strand (ventral nerve cord, VNC, in Protostomia and spinal cord, SC, in vertebrates) is located on opposite body sides across Bilateria (a feature reflected by the terminological distinction between *gastroneuralia* and *notoneuralia*), molecular and morphological data strongly suggest that the nerve strand developed only once in Bilateria, reasoning for a dorsal-ventral axis inversion in the deuterostomian branch (Arendt and Nübler-Jung, 1994; Arendt and Nübler-Jung, 1999; De Robertis and Sasai, 1996; Dohrn, 1875).

In contrast to the nerve strand, the anterior cerebral nerve center (brain) is located at a similar position in all Bilateria. Probably due to the very similar location of a cerebral center in almost all species (except some exceptions in clearly derived ones, such as Bivalvia or Echinodermata) a general homology of brains is broadly considered a reasonable hypothesis, at least in the "molecular field". Besides the striking similarity in location, there are two more arguments in favor of this possibility. First, it is very plausible that a center that governs all basal body processes is highly conserved. Secondly, the expression domains of genes like *otx*/*otd*, *emx*/*ems* or *six3*/*6* can be interpreted as molecular support for a general,

though ill-defined, homology of brains (see e.g. Reichert and Simeone, 2001; D. Arendt, unpublished and Figure 7). However, it is quite astonishing that the question of what precisely should be evolutionary conserved in the bilaterian brain is largely unanswered. The analysis on this level is important, since there are clear differences between the brains of vertebrates and Protostomia. One example is the mid-hindbrain boundary organizer (MHBO), which is currently assumed to be vertebrate specific (Rhinn and Brand, 2001). Certainly, similarities on the cell type level do exist between vertebrates and Protostomia. For example, they share cell types with similar functions, such as light detection or chemosensation or neurosecretion. In this study I provide evidence that at least several similar cell types shared between polychaetes and vertebrates are homologous, meaning they trace back to Urbilateria. Several of these cell types will be concentrated in, or locate close to a distinct apical region, the apical organ of primary ciliated larvae (such as the trochophora larvae of polychaetes).

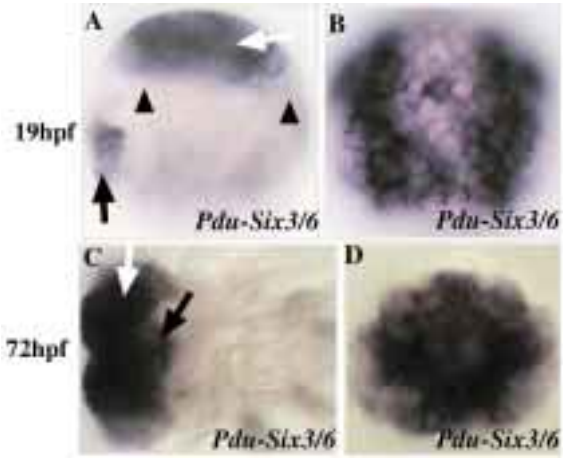


Figure 7. Expression of *six3/6* in *Platynereis dumerilii.*

(A-D) Expression of *Pdu-six3/6* as first determined by D. Arendt by WMISH. Black arrows indicate expression in the stomodeum, white arrow expression in the episphere. Black arrowheads indicate position of the prototroch. B,D) apical views onto the episphere. C) Lateral and D) apical view at 19hpf. E) ventral and F) apical view at 48hpf. Note that the weaker staining in the episphere is still true staining, as it is the case at 19hpf. G) Ventral and H) apical view at 72hpf. The *Pdu-six3/6* expression got strongly reduced at the lateral regions of the brain. The slight blue color in the parapodia is background staining.

1.4.1 The apical organ and its cell types

An identifiable apical thickening, termed apical organ, is a common feature of marine primary ciliated larvae across Bilateria (for location of this organ in the animal see Figure 4). These larvae are considered ancestral for Bilateria (Arendt *et al.*, 2001) and homology of this organ based on cell morphologies and immunocytochemistry has been suggested by several authors starting in the 19th century (Conklin, 1897; see detailed discussion and references in Kempf *et al.*, 1997). The apical organ (APO) consists of a varying number of cells, of which some bear cilia, and an apical plexus formed by basal processes of the APO cells (see Figure 8A,B). This apical plexus is highly neurosecretory (Lacalli, 1984). In molluscs it has been speculated that the apical neuropil releases its substances in a hormonal/ endocrine fashion directly into the hemocoel (Kempf *et al.*, 1997). In the mollusc veliger larva, 4 different types of cells have been described: ciliary tuft cells, type I and II parampullary neurons and ampullary neurons. The ciliary tuft cells give rise to the long cilia that form the prominent tuft of the organ. They usually lack neurites. The latter three cell types exhibit a flask-shaped apearance. Ampullary and parampullary cells type I extend their distal dendrites through the overlaying pre-trochal epithelium. Each dendrite gives rise to a single cilium or several cilia. Ampullary neurons are particularly distinctive, because they have a deep internal pocket formed by an invagination of the distal dendritic terminal, the walls of which give rise to the cilia. The cilia are either located entirely within the dendritic invagination or extend to the outer surface. The parampullary cells type II lack these types of ciliated dendrites (Bonar, 1978; Chia and Koss, 1984; Kempf *et al.*, 1997 and references therein). In polychaete trochophorae, as well as in planarian larvae, cells partly reminiscent of the veliger APO cells have been described. Apical tuft cells can exist, and ampullary cells have been found in *Phyllodoce* (polychaeta) and in the dorsal brain region of *Pseudoceros* (polycladida). Almost all cells have slender, tapering surface processes, bearing one/two cilia or a cilium–like stub. Several of these cells appear ‘flask-shaped’ (Heimler, 1981; Heimler, 1988; Lacalli, 1981; Lacalli, 1982; Lacalli, 1984).

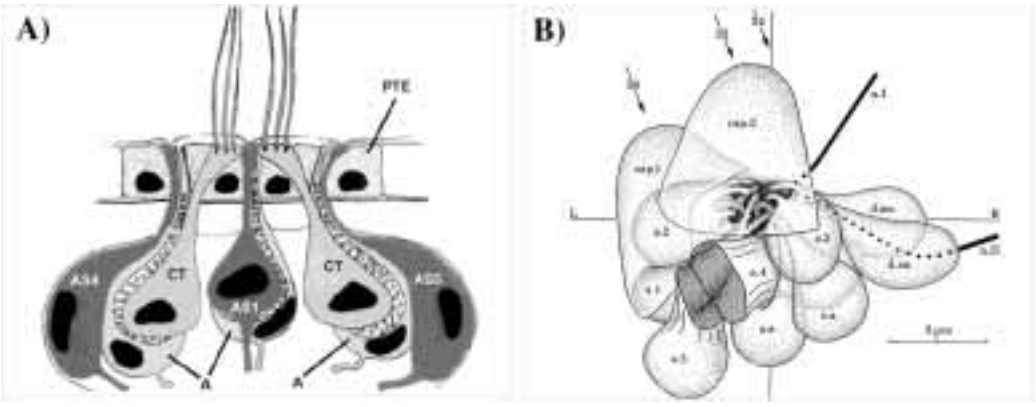


Figure 8. Two schematics of the APO of molluscs and polychaetes.

(A) shows the general organization of the APO of trochophore-type larvae from the ventral or dorsal side. The scheme included type I parampullary neurons (AS1, AS4, AS5), ampullary neurons (A), and ciliary tuft cells (CT). Three dendritic bundles that extend through the pretrochal epithelium (PTE). Multiple type I parampullary neurons extend dendrites in each of these bundles; however, for clarity, the diagram shows only one of these neurons associated with each bundle. Figure **(B)** shows the typical neurosecretory complex of a trochophore-type larvae. Note the interdigitating processes. Cilia were not drawn in. (dorsal to the bottom, ventral up; L=left, R=right; the different abbreviations stand for the different types of APO cell types, described in the paper) A taken from Kempf *et al.*, 1997. B taken from Lacalli, 1984.

One of the major characteristics of the APO is the presence of neurosecretory cell types. RFamide(s) and enkephalin-like neuropeptides, as well as serotonin (5-Hydroxytryptamin/5HT) have been reported to have their starting point of epispheral expression in the apical organ region of primary ciliated larvae (Bisgrove and Burke, 1987; Dickinson and Croll, 2003). 5-HT is an especially interesting case, since its early expression in the apical organ region is highly conserved for primary ciliated larvae across Bilateria (reviewed in Hay-Schmidt, 2000; Lacalli, 1994).

Importantly, the apical organ is a larval organ. Its function in the larva has not been clearly defined. However, a chemosensory or mechanosensory function with an influence on motility and/ or metamorphosis of the larvae has been proposed, based on cell morphology and cell ablation studies (Bonar, 1978; Conklin, 1897; Kempf *et al.*, 1997; Voronezhskaya and Khabarova, 2003 and references therein). The APO might be a transient, larval organ, since the cell fate of its cells is mostly unclear. However, for polychaetes, T. Lacalli describes that the cells of the APO change little of their overall organisation and structure also during late larval stage. He reports that the apical tuft is lost and that the cells become incorporated into the cerebral ganglia. (Lacalli, 1981; Lacalli, 1984). Whether or not they are maintained at post-

larval stages is still highly controversial, based on data from several other authors in other organisms (see e.g. Chia and Rice, 1978; Dickinson and Croll, 2003; Page, 2002).

In summary, the APO is an association of strongly conserved cell types that are probably involved in sensory functions, movement and metamorphosis. The APO is found in primary ciliated larvae across Bilateria, but has not been observed in chordates. A possible scenario could be that chordates also possess at least some of the ancient APO-like cell types, but that these cell types have largely lost their larval specific characters. A speculation into this direction, based on morphological data, has been put forward by T.Lacalli. He suggested an evolutionary relationship of the APO of primary ciliated larvae with the frontal eye complex of *Branchiostoma* (Lacalli, 1994).

Finally, it is very interesting to note that long ciliated and/ or other sensory cells reminiscent of those present in the apical organ regions of bilaterian primary ciliated larvae are present already in the planula larvae of cnidaria, as well as in adult ctenophores (Harrison and Westfall, 1991).

1.4.2 Neurosecretory cells

The majority of vesicles in a neuron are usually the synaptic vesicles. These are vesicles directly produced and loaded with neurotransmitter at the presynaptic terminal ensuring fast synaptic transmission. This is in contrast to the default secretory pathway that transports proteins from the Golgi apparatus to the plasma membrane. If these vesicles carry many proteins or peptides, they appear electron dense, and are thus called dense–core vesicles (DCV). Neurosecretory cells are neurons that contain many DCVs. Neuroendocrine cells are neurosecretory cells that pour the content of their vesicles into the body fluid via which it becomes distributed to more distant effector organs (Hall, 1992; Kandel *et al.*, 2000).

Besides the quantity of DCVs, neurosecretory neurons also differ from other neurons by the quality, i.e. the cargo that is carried by the DCVs. These cargos, i.e. the substances that are found in these DCVs are of different kind and function. They are usually specific peptides, steroids bound to a transport peptide, and also include serotonin and catecholamins, the latter of which can as well be found in synaptic

vesicles (Andrews *et al*., 2002; Bruns *et al*., 2000; Hall, 1992; Kandel *et al*., 2000; Nirenberg *et al*., 1995). (Note that the molecules secreted by the neurosecretory cells are not completely bound to the DCV.)

The term 'neurosecretory cells' thus refers to a large collection of different cell types. Neurosecretory cells can be found in various regions of the brain. Particular cell types, however, tend to cluster in delimited regions. In vertebrates, neurosecretory cell types occur in several regions throughout the adult brain. However, at least in the forebrain, the vast majority is located in or originates from the median/ ventral areas (Norris, 1997). This is especially apparent from the early origin and location of neurosecretory cell types in the brains of lower vertebrates, such as lamprey or teleosts (Brodin *et al*., 1990; Holzschuh *et al*., 2001; Kaslin and Panula, 2001; Varga *et al*., 1999; Weigle and Northcutt, 1999; Yanez *et al*., 1992), from the early brain fate map of the chick (Cobos *et al*., 2001), and the often highly specific early expression of several neuropeptide genes (Fiorentino *et al*., 2001; Moore and Lowry, 1998; Pritchard *et al*., 2002; Rastogi *et al*., 2001; Wichterle *et al*., 2001). Several examples of cell migration are known that only later in development spread different types of neurosecretory cell to other regions of the brain (as known for FMRF, GnRH, somatostatin, AVP/O family (see citations above and Arnold-Aldea and Sterritt, 1996; Schwanzel-Fukuda and Pfaff, 1989; Wirsig-Wiechmann *et al*., 2002). The different neurosecretory cell types often assemble into different brain nuclei, of which the majority is located in the hypothalamus and preoptic area (Norris, 1997). Several functions have been connected to the different neurosecretory cell types in vertebrates. They are involved in various aspects of reproduction (gonad development, sexual maturation), as well as of metabolism (energy and salt homeostasis, growth; Norris, 1997). Even the density of bones is under the control of hypothalamic cells (Olney, 2003).

Neurosecretory control centers with similar widespread functions exist in various protostomian species, e.g. the sinus gland and lateral cephalic nerve plexus of crustaceans, the pars interecerebralis and ring gland of insects (Campos-Ortega and Hartenstein, 1997; Hartenstein, 1997; Siga, 2003; Thorndyke and Goldsworthy, 1988). A major neurosecretory organ, the infracerebral gland, which contains a widespread set of neurons with neurosecretory material, also exists in the brain of

adult nereids. (Baskin, 1976; Golding, 1967; Golding, 1974; Golding *et al.*, 1968; Hauenschild and Fischer, 1961; Porchet *et al.*, 1985; Scharrer, 1936).

Despite the fact that these cells fulfill similar control functions in Protostomia and Deuterostomia, the similarities on molecular level seemed to be so far rather restricted. Orthologous enzymes for the synthesis of histamine, serotonine and dopamine are present in the neurons of both Protostomia and Deuterostomia ((Lundell and Hirsh, 1994; Mantzouridis *et al.*, 1997; Melzig *et al.*, 1998; Monastirioti, 1999 and see molecular phylogenetic trees in results part of this thesis). Additionally, several 'vertebrate-like neuropeptide' receptors have been found in the genome of insects (*Anopheles* and *Drosophila*), such as *the gnrh-receptor*, *growth hormone secretagogue receptor* (*ghs-r*), *growth hormone releasing hormone receptor* (*ghrh-r*) and the *vasopressin/oxytocin-receptor* (Hewes and Taghert, 2001; Hill *et al.*, 2002; Iversen *et al.*, 2002; Park *et al.*, 2002). However, several biochemical studies indicate that all of the (above mentioned) orthologous insect receptors tested, do not bind the orthologous 'vertebrate-like' peptides in these species (Iversen *et al.*, 2002; Park *et al.*, 2002; Staubli *et al.*, 2002). Additionally, with few exceptions (for example RFamide(s), NPY-related peptide, insulin and possibly PACAP), almost no 'vertebrate-like' neuropeptides could be found in the fully sequenced genomes of *Drosophila melanogaster* and *C.elegans* ((Baggerman *et al.*, 2002; Li *et al.*, 1999; Nathoo *et al.*, 2001; Vanden Broeck, 2001 and including own BLAST searches). A similar scenario is apparent on the transcription factor side. Genes involved in the development of the median forebrain region in vertebrates are either absent (as *vax* in *Drosophila*) or exhibit an expression pattern, that is not obviously comparable with the vertebrate brain expression pattern (as *sco/nk2.1* and *otp*; Acampora *et al.*, 2000; Simeone *et al.*, 1994; Zaffran *et al.*, 2000). If *Drosophila* and *C.elegans* have only very few cell types reminiscent to the vertebrate neuroendocrine system, what was the situation in Urbilateria? Did the neuoendocrine cells present in today's vertebrate forebrain develop in the deuterostome line only, or were they already (at least partly) present in Urbilateria, but got lost somewhere on the evolutionary way leading to *Drosophila melanogaster* and *C.elegans*? Clearly, the investigation of an independent and more ancestral

protostomian organism, such as *Platynereis dumerilii* should provide more insight into this problem.

1.4.3 The vertebrate hypothalamus

Because the hypothalamus is doubtlessly the most important neurosecretory and neuroendocrine structure of the vertebrate forebrain, it should be given some additional introduction. The ventro/ median region, which is located below the tracts of the postoptic commissures (see Figure 9 and Figure 10) equates to what we usually consider as the embryonic hypothalamus in lower (i.e. evolutionarily more basal) vertebrates, such as fishes (Wilson *et al.*, 2002). The ventral/median forebrain includes additional regions, such as the preoptic area, which shares many characteristics with the hypothalamus. The most anterior portion of the median region of the neural plate give rise to the adenohypophysis, the major secretory system that later forms a functional unit with parts of the hypothalamus, the hypothalamic-hypophysal axis (see Figure 9, Figure 10 and Kawamura *et al.*, 2002). In adult animals, the hypothalamus and the other structures are composed of a variety of different nuclei. The comparison of these nuclei across species is very difficult, even between lower and higher vertebrates. Although it is clear that a neurosecretory/ neuroendocrine system exists in all of these species, the comparisons are complicated by the fact that this system comprises varying numbers of nuclei that in addition undergo extensive migrations (Norris, 1997). Thus the evolution of the hypothalamus is enigmatic. As an example, lamprey possesses a preoptic area with two nuclei and a hypothalamus, separated into three nuclei. In non-mammalian tetrapods, the hypothalamus alone consists of more than 10 nuclei already (Norris, 1997). This emphasizes, that the anatomy of the hypothalamus is highly dynamic. Therefore the concept of homologous cell types is particularly useful in this context (see above). Not only does it provide the only plausible level for homology comparisons, it also permits the comparison of the neuroendocrine system beyond vertebrates. Figure 9 and Figure 10 contain the data on the basis of which I drew the schematic shown in Figure 10C. This schematic will become important in the results and discussion parts of this thesis, and has thus been explained in more detail here. It should also be noted that the ventral region of the closed neural tube corresponds to

the median region of the open neural plate. In order to take both into account I refer to these regions as ventral/ median regions or the vertebrate brain. One should be aware that the equivalent term for the *Platynereis* larva is 'median brain' (ventro-median is only a subregion of this, for better understanding compare to Figure 4).

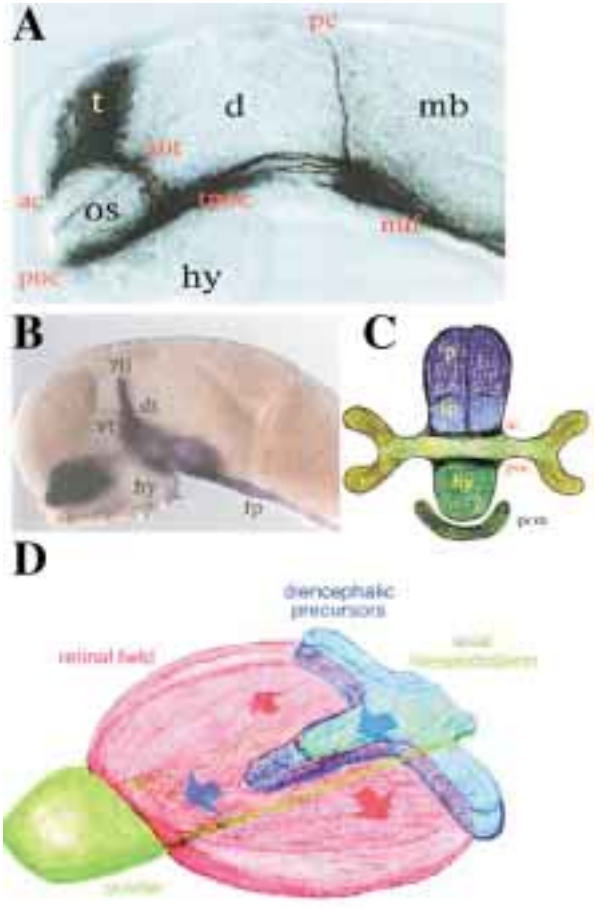


Figure 9. Movements and architecture of the vertebrate hypothalamus.

This figure imparts a current overview of the origin and movement of vertebrate ventro-median forebrain cells. Abbreviations: ac-anterior commissure, d- diencephalons, dt-dorsal thalamus, e- eye, fp- floor plate, mb- midbrain, mlf- medial longitudinal fasciculus, os optic stalk, p pallium, pcm- prechordal mesoderm, poc postoptic commissuire, r retina, sot supraoptic tract, sp subpallium; t telencephalon, tpoc tract of the postoptic commissure, vt ventral thalamus, zli zona limitans intrathalamica; **(A)** Lateral view onto an 1d old zebrafish brain stained with an antibody against acetylated tubulin, showing the early axon tracts. **(B)** Zebrafish brain of an embryo at the same stage and from the same perspective, showing the morphology of the regions and a staining of *Drshh* (blue). **(C)** Cartoon of a highly schematized forebrain viewed from anterior, showing the hypothalamus in relation to the telencephalon (i.e. pallium and subpallium) and the pcm. **(D)** Proposed model for the separation of a single retinal field by median posterior cells. Ventral diencephalic precursors (blue) move anterior (blue arrow) and ventral to the retinal field (red) and form the primordium of the hypothalamus. In late neural plate stages, they occupy a position ventral to the retinal field and dorsal to the axial mesendoderm (green). Retinal precursors move laterally (red arrows) to form the bilateral eyes. Telencephalic cell fates at the anterolateral periphery of the neural plate are not shown here. Anterior to the lower left, posterior to upper right, dorsal to the top.
Pictures A-C taken from Wilson *et al.*, 2002, picture D taken from Varga *et al.*, 1999.

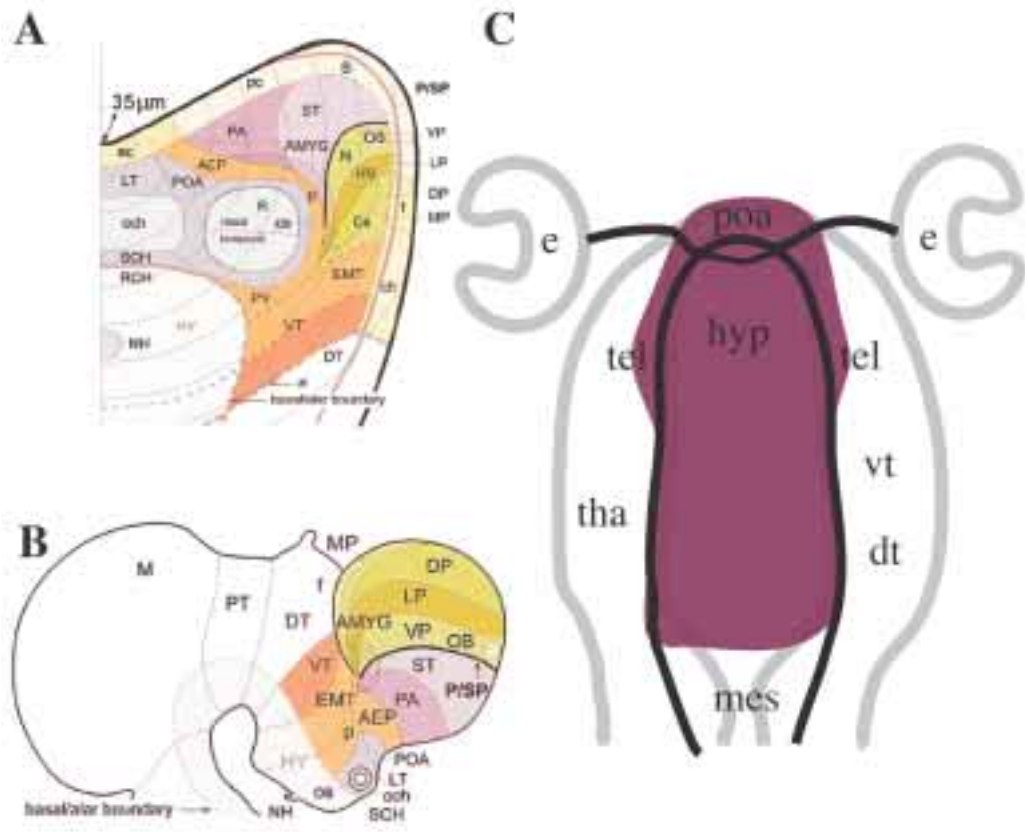


Figure 10. Fatemaps, movements and schematics of the vertebrate hypothalamus.

Abbreviations see Figure 9, important additional abbreviations: hy hypothalamus, lt lamina terminalis, nh neurohypophysis, och optic chiasm, poa preoptic area **(A)** Fate map of the chicken anterior forebrain anlage at stage HH8 (four somites). The prospective primordia of the main components of the avian forebrain are represented in a schematic drawing of the partially flattened anterior neural plate. **(B)** Final location of the mapped forebrain territories after closure of the neural tube and initial evagination of the telencephalic vesicles (approx. stage 28). **(C)** Highly schematized drawing of an opened vertebrate brain summarized from the pictures Figure 9 A-D and this figure E, F (cut-open older brain and early fate maps). Axon tracts that cross over the dorsal (in the neural plate stage or cut-open equals lateral) have been omitted. Light black line are the major axon tracts (compare to Figure 9 A). Bold black lines are borders of brain areas. The median/ ventral region containing the majority of neurosecretory cells is red. (hyp hypothalamus, mes mesencephalon, tel telencephalon).
Pictures E and F taken from Cobos *et al*., 2001.

1.4.4 Cell types of photosensory structures

Photosensory cells exist either alone or in combination with cells containing a shading pigment. The latter enables the animal to detect the direction of light, which is the definition of the main function of an eye. The minimal eye or "protoeye" is thus composed of one photoreceptor cell and one pigment cell, and is present in today's planarians and trochophora-type larvae (Arendt, 2003; Arendt *et al*., 2002; Arendt and Wittbrodt, 2001; Gehring and Ikeo, 1999). The more elaborate eyes, e.g.

in vertebrates, contain additional cell types, such as retinal ganglion, horizontal, amacrine, bipolar and Mueller – glia cells.

The non-photosensitive pigment cells contain melanin or pterins to absorb the light and often have the capacity to secrete lens-forming material (Eakin and Westfall, 1965; Eakin and Westfall, 1964; Fischer and Brökelmann, 1966). Lenses are one of the best examples for convergent recruitment of molecules, since even closely related species use completely unrelated molecules for this structure (Piatigorsky, 2003; Wistow, 1993). Photoreceptor cells contain a photopigment, rhodopsin, consisting of a proteinaceous, and non-proteinaceous part, opsin and retinal, respectively. This photopigment is usually stored in membranous surface enlargements that originate either from the apical membrane, that folds into actin – based microvilli or lamellae, or arise around a stereocilium and are based on tubulin. The first type is called rhabdomeric and the second type ciliary photoreceptor (see also Figure 15A, B). Importantly, this morphological cell type distinction is corroborated on the molecular level: The morphological different photoreceptors employ non-orthologous opsins, Gα-subunits, rhodopsin kinases and arrestins (Arendt and Wittbrodt, 2001). The rhabomeric photoreceptors (rhPRCs) are commonly found in protostomian eyes and in lower deuterostomian larval eyes (with few exceptions). *Platynereis* has three pairs of eyes: one pair of larval eyes, and two pairs of adult eyes. All of them are composed of rhabdomeric photoreceptor cells (Arendt *et al*., 2002; Fischer and Brökelmann, 1966). The larval eyes have the 'protoeye' composition of one photoreceptor and one pigment cell, whereas the adult eyes consist of several photoreceptor and pigment cell, continue to grow during adulthood, and also contain a lens (see Figure 11).

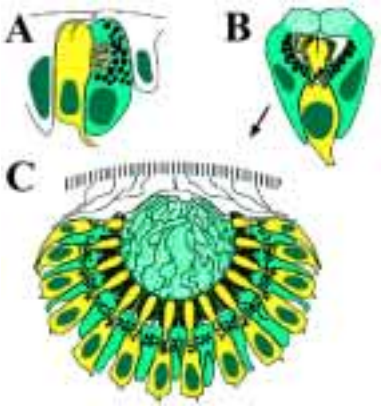


Figure 11. The larval and adult eyes of *Platynereis dumerilii*.

(A) larval eye **(B)** adult eye at app. 72hpf, **(C)** grown adult eye of *Platynereis dumerilii*.. Yellow are the rhabdomeric PRCs and green are the pigment cells. Figure taken from Arendt *et al*., 2002.

The ciliary photoreceptors (ciPRCs) are the photoreceptors in the eyes of chordates. Additionally, based on morphology ciliary photoreceptors have been described as non-pigment cell associated photoreceptors in the brain of various species across Bilateria (reviewed in Arendt and Wittbrodt, 2001). Their function in the brain is unknown.

On the one hand, the differences in the cellular setup of eyes in Protostomia vs. Chordates strongly indicate that eyes as compound structures are non-homologous. On the other hand, several orthologous transcription factors, such as *pax6* and *six3*/6 have been implicated in eye development in vertebrates as well as in *Drosophila melanogaster* (see e.g. Jordan *et al*., 1992; Loosli *et al*., 1999; Quiring *et al*., 1994; Seimiya and Gehring, 2000; Seo *et al*., 1999), making it difficult to believe that these molecular similarities are purely the product of independent recruitment. How can this contradiction be understood?

1.5 Aim of this thesis

In summary, photosensory and neurosecretory structures are found across Bilateria. However, from the current view, when mainly Ecdysozoa and Vertebrata are compared, extensive differences between the respective protostomian and deuterostomian structures exist and make their evolutionary relationships uncertain. In principle, photosensory and neurosecretory systems in different species could result from different phylogenetic scenarios: a) In the first situation, the scenario of analogy, both photosensory and neurosecretory systems evolved independently in the two evolutionary lineages leading to Protostomia and Deuterostomia. b) In the alternative case, the scenario of homology, these systems existed already in Urbilateria, but were extensively modified during evolution at least in the lineage leading to nematodes and insects.

In this study, I chose a comparative molecular approach to distinguish between these possibilities. My aim was to use molecular and histochemical tools to identify brain areas or cell types of the photosensory and neurosecretory system that are comparable between Protostomia and Deuterostomia and thus likely to be ancestral for Bilateria. The main experimental system for my studies was the larva of *Platynereis dumerilii*, following the rationale that the aforementioned ancestral

features of this novel Protostomian model species make it an ideal reference point for Bilaterian-wide comparisons. I concentrated on a number of genes that encode key developmental regulators, enzymes, receptors or transmitters, because the combined expression of these molecules is uniquely characteristic for specific cell types. With the help of these molecular 'fingerprints', together with histochemical studies and the analysis of cellular morphologies, I performed comparative studies mainly on the level of cell types, as a new level for comparative molecular studies. By this I tried to determine the extent of the relationship between the photosensory structures of polychaetes and vertebrates, and between the median- ventral forebrains (the brain centers of neurosecretory activity) of both groups. Finally, I extended the comparative analysis to a functional level by investigating possible ancestral roles of a major signalling pathway (the Hedgehog-signalling pathway) in the development of these structures across Bilateria.

In more general words, Darwin's theory of evolution (Darwin, 1859) and Haeckel's biogenetic law (Haeckel, 1874; Haeckel, 1886) imply a continuity of cells or structures throughout the animal kingdom. Genes, cells and structures will be modified, giving rise to modified genes, cell types and structures. Every gene, cell type or structure has its ancestry. So, which components of the vertebrate eye and hypothalamus date back to urbilaterian times?

2 RESULTS

2.1 Indications for a conserved molecular organisation of Bilaterian brains

The vertebrate brain is subdivided into three main regions- the forebrain (prosencephalon =tel- and diencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). One gene family demarcating almost the entire vertebrate forebrain is the *six3/6* subfamily of the *sine oculis/ six* family. The ancestral bilaterian *six3/6* underwent a gene duplication in vertebrates, resulting in the *six3* and *six6* genes, that both exhibit an evolutionarily highly conserved expression. They demarcate early the anterior–most portion of the neural plate, and are later maintained (alone or together) in the CNS in the tegmentum, posterior pretectum, whole visual system, broad regions of the hypothalamus, the pineal, the ventral telencephalon (LGE, MGE, septum, preoptic area) and thalamus (Bovolenta *et al.*, 1998; Ghanbari *et al.*, 2001; Jean *et al.*, 1999; Loosli *et al.*, 1998; Oliver *et al.*, 1995; Seo *et al.*, 1998). As mentioned in the introduction, a *six3/6* ortholog is also active in the anterior-most region of protostomian brains, and thus can serve as a molecular indication for the comparability of the brains across Bilateria (see Figure 7 and Figure 12). In other words, are the contiguous anterior-most *six3/6* regions of vertebrates and polychaetes the demarcations of an ancestral Bilaterian brain? If the answer is yes, more similarities should be evident between vertebrate and polychaete *six3/6*-expressing brain regions.

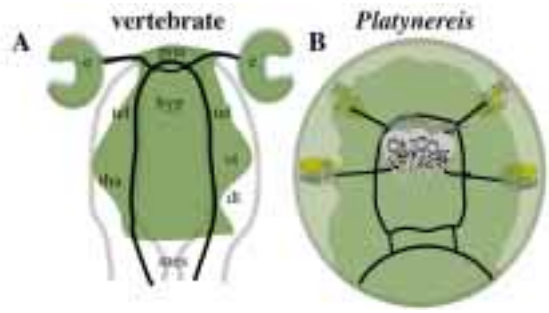


Figure 12. Schematized *six3/6* expression in the forebrain of vertebrates and *Platynereis dumerilii.*

(A) Highly schematized overview of vertebrate *six3/6* expression (green) in the vertebrate forebrain (see introduction and Figure 9 and Figure 10 for references and abbreviations). **(B)** Highly schematized apical overview of the expression of *Pdu-six3/6* (green; see introduction and Figure 7), dorsal is to the top, ventral down. Major axon tracts in black, grey cell in the middle represent the apical organ, more ventral yellow cells represent the larval PRCs, more dorsal yellow cells the developing adult eyes. The schematic is closest to the 48hpf stage.

To explore on this, I first investigated the expression of two additional genes involved in forebrain regionalisation and development in the vertebrates by WMISH. These two genes delimit the lateral/ dorsal versus median/ ventral aspects of the forebrain in vertebrates. One of them is *pax6* that beside its role in eye development (see below) marks the lateral/dorsal aspects of the vertebrate brain, the other one is *nk2.1* that demarcates the median/ventral aspects (Corbin *et al.*, 2003; Murakami *et al.*, 2001; Stoykova *et al.*, 2000; Wilson and Rubenstein, 2000). *nk2.1* is especially interesting, since it very specifically marks the ventral forebrain in every bilaterian species looked at so far (Lazzaro *et al.*, 1991; Lowe *et al.*, 2003; Ogasawara *et al.*, 2001; Rohr *et al.*, 2001; Small *et al.*, 2000; Venkatesh *et al.*, 1999), except in *Drosophila melanogaster* (Zaffran *et al.*, 2000). It has been stated, based on expression and mouse knock-out data, that:

> "*Nkx2.1* was not localized to specific nuclei, but rather distributed over a wide area of the ventral region of the hypothalamus. … (It is) not essential for the formation of specific neural nuclei but is important for the ventral hypothalamic area." (Nakamura *et al.*, 2001)

In the ventral forebrain of mammals *nk2.1* is present in the medial ganglionic eminence, preoptic region, basal ganglia (pallidum) and septum. In the hypothalamus of adult mouse it is maintained in the ventromedial hypothalamic nucleus, medial tuberal nucleus, arcuate nucleus and mammillary body, as well as in the posterior lobe of the pituitary. These regions are also effected in nk2.1-/- mice (Kimura *et al.*, 1996; Nakamura *et al.*, 2001).

2.1.1 Pdu-nk2.1 *is expressed in the median brain anlage complentary to* Pdu-pax6

I therefore investigated the expression of *nk2* in relation to the expression of *pax6* in the *Platynereis* forebrain (*Pdu-pax6*, cloning and initial characterization in the context of eyes, antennae and palpae by D.Arendt). As previously noted by D. Arendt, very lateral brain expression of *Pdu-pax6* starts to be clearly visible at 15hpf (Arendt *et al.*, 2002). By looking at earlier stages, I found that there might be some staining at 12hpf already, but this was very weak and fuzzy, and difficult to distinguish from background (data not shown). The lateral brain domains leave a gap

in the median region above the developing stomodeum. (Blue dots in the more median episphere above the oil droplets of the macromeres at 19hpf or 24hpf are likely to be background stain.) This gap narrows over time (see Figure 13 D, F). As first noted by D. Arendt, few *Pdu-pax6* positive cells in the dorsal region of the median episphere are visible at later stages. The rest of the median region appears to stay devoid of *Pdu-pax6* staining (see Figure 13F). I have found that this *Pdu-pax6* free median region is the place where *Pdu-nk2.1* appears. *Platynereis dumerilii* Nk2.1 *Pdu*-Nk2.1 (cloned by G. Balavoine) clusters together with the vertebrate Nk2.1 group, and apart from the outgroup containing the vertebrate Nk2.3 genes in a phylogenetic tree (Figure 22). As I determined by WMISH analysis, clear expression of *Pdu-nk2.1* is visible at 15hpf. It already demarcates the most median aspect of the brain and continues as one domain across the prototroch, but does not continue much further. Weak staining can be detected in 2-3 cell in the very middle of the episphere, where the apical organ is forming (Figure 14A). These cells enhance their staining at 24hpf and successive stages, when the staining spreads further over the median portion of the trochophoran episphere (see Figure 13C, E). Additional expression is detected in the stomodaeum and at the posterior end of the larva. In the trunk *Pdu-nk2.1* is weakly present at 15hrs at the ventral surface, and additionally stronger staining is present at the posterior end. The staining along the trunk midline is very weak and difficult to discern from background (Figure 14B). During subsequent development it can be found in a restricted group of cells in the stomodeum, as well as within the middle of the embryo (Figure 14C,F,H). Probably, this thin stripe correspond to endoderm, as judged by position. At 48hpf it is very apparent that *Pdu-nk2.1* is expressed in at least some of both the 'flasked-shaped' crescent and center cells of the APO (Figure 14D,E). During subsequent development its median brain expression spreads more and more to the dorsal, until it spans the entire median brain (compare Figure 13C,E, Figure 14A,D, G). I have established a protocol for double wholemount in situ hybridisation (WMISH) for *Platynereis* and by this was able to determine that the *Pdu-nk2.1* brain staining exactly fills the gap left by *Pdu-pax6* (Figure 13G). Judged by WMISH stainings the topology of both expression domains largely persists until later stages. The more lateral ‘wings’ of the *nk2.1* staining spread further lateral and overlap with the *pax6* positive domain during later development (Figure 13 C,E, G, Figure 14D,G).

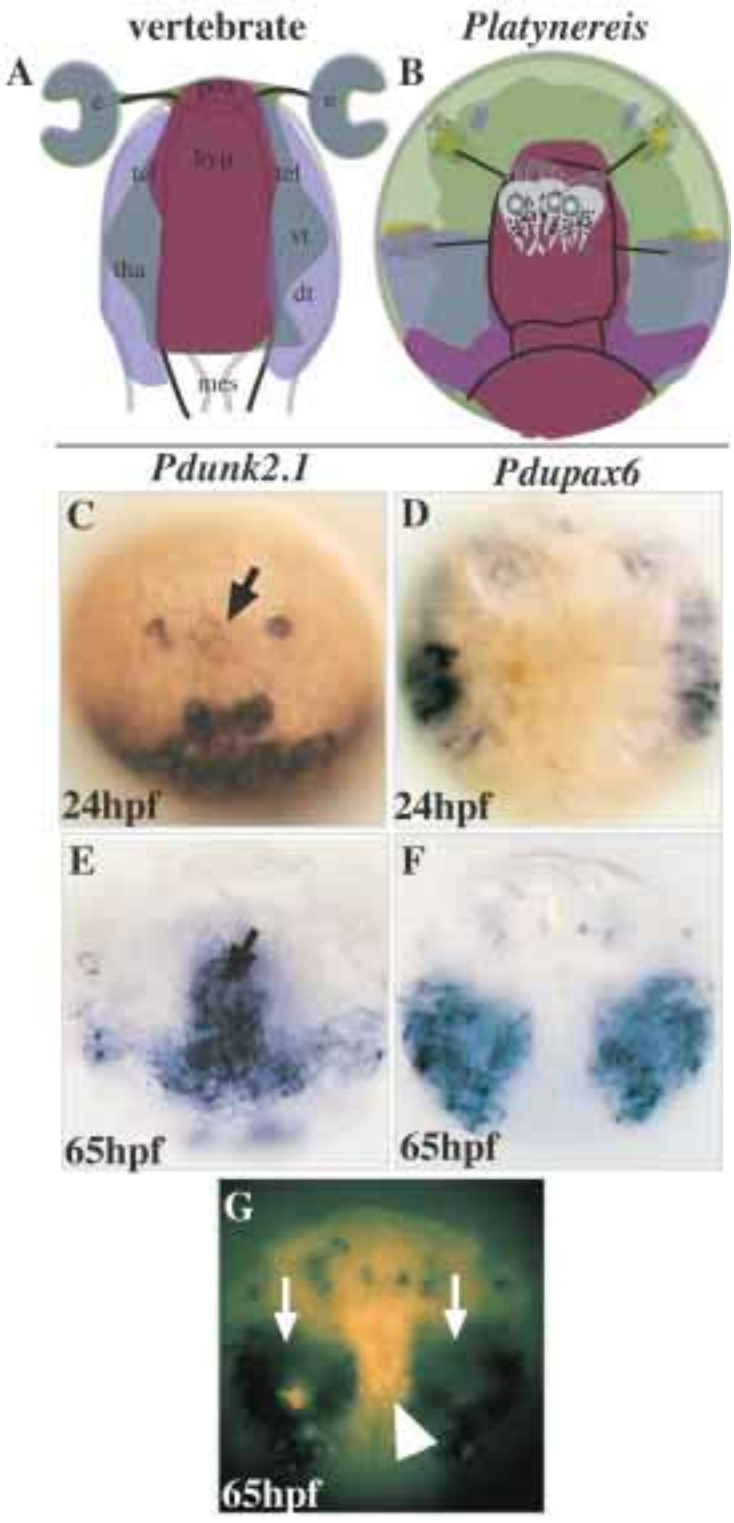


Figure 13. Complementary expression of *pax6* and *nk2.1* in vertebrates and *Platynereis dumerilii*..

(A) and **(B)** highly schematized brain of vertebrates and *Platynereis dumerilii* episphere, respectively. Compare to Figure 9, Figure 10, Figure 12 for more detailed descriptions and abbreviations. *six3/6* expression schematized in green, *nk2.1* in red and *pax6* in blue.

(C-G) Selection of expression patterns of *Pdu-nk2.1* and *Pdu-pax6*. For all apical pictures: dorsal to the top, ventral down. C), E) apical view of *Pdu-nk2.1* expression. Note the expression in the apical organ (black arrows). D), F) apical view of *Pdu-pax6* expression. D) Courtesy of D. Arendt. G) Double staining of *Pdu-pax6* (dark staining, small arrows) and *Pdu-nk2.1* (fluorescent red color, arrowhead). The animal shown in this picture is identical to the animal in picture F.

Taken together, as summarized in Figure 13 A and B, there are strong similarities in the molecular set-up of the vertebrate forebrain and the trochophora-type larvae. The

most anterior region of both brains is marked by *six3/6* expression, and subdivided into lateral vs. median domains by *pax6* and *nk2.1*, respectively.

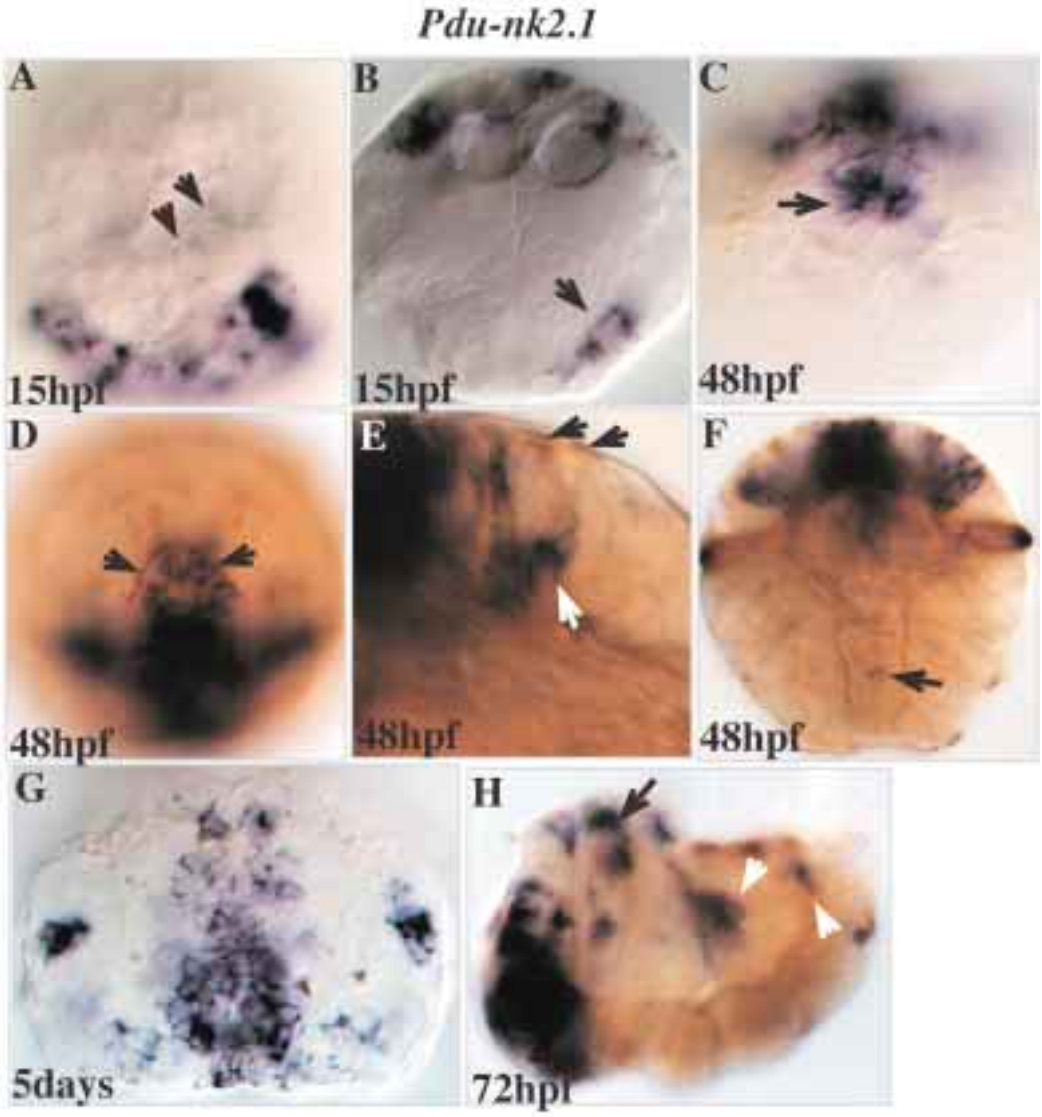


Figure 14. Expression of *Pdu-nk2.1* at different stages.

Blue staining of *nk2.1* (detected by WMISH), brown staining with an anti-actylated tubulin antibody, revealing stable microtubule strictures, such as axons of cilia. Also compare to Figure 13C,E. A, B) Expression at 15hpf. **(A)** Apical view, dorsal up. Black arrowsheads point to first weak staining occurring in the APO region. **(B)** Ventral view, black arrow points to posterior staining. **(C, F)** Ventral views at 48hpf. Apical is up, right to the left. (C) Arrow points to stomodeal staining. (F) Slightly deeper focal plane, arrow points to presumptive endodermal staining within the embryo. **(D)** Apical view at 48hpf, dorsal up. Black arrowheads point to the 'flasked-shaped' crescent cells of the APO. **(E)** Ventral view of the same specimen, magnification of the APO area. Black arrowheads point to the cilia of the 'flask-shaped' crescent cells, white arrow to the *Pdu-nk2.1* staining within them. Note how the cells span through the episphere. The long cells left to the 'flask-shaped' crescent cells belong to the 'flask-shaped' center cells of the APO and express *Pdu-nk2.1* as well. For more detailed descriptions of the APO cells, see below. **(G)** Apical view (dorsal up) at 5 days of development. The *Pdu-nk2.1* expression extends now through the entire median episphere. The only staining left from

the former lateral wings are two prominent lateral groups of cells. **(H)** Lateral view (apical to the left, ventral down) at 72hpf. Black arrow points to expression in the stomodeum, white arrowheads to presumptive endodermal staining within the embryo.

2.2 Molecular characterisation of the ciliary and rhabdomeric photoreceptor cell types in *Platynereis dumerilii*

The first part of the forebrain comparison between vertebrates and *Platynereis dumerilii* dealt with the question if broader brain areas are similar between these groups. My analysis of the distribution of three different gene expression domains strongly suggests that the overall regions are indeed assimilable. Consequently, the next step in my brain comparison has been to investigate these regions further by the molecular characterisation of the inherent cell types. My first focus has been on the light-sensitive cell types.

Platynereis dumerilii is a fortunate choice for the comparative study of the photosensitive system in Protostomia, in that both rhabdomeric and ciliary PRCs are present. Rhabdomeric PRCs (by morphological and molecular means) are the photoreceptor cell type present in the larval and adult eyes of *Platynereis* (Arendt *et al.*, 2002; Arendt and Wittbrodt, 2001; Fischer and Brökelmann, 1966 and see below).

2.2.1 Ciliary photoreceptor cell types

The *Platynereis* ciliary PRCs have been ultrastructurally characterized for the larval brain by Detlev Arendt and Adrian Dorresteijn (unpubl., see Figure 15F and G). Importantly, these cells do not express the previously described opsin molecule expressed in the rhabdomeric PRCs of the *Platynereis* eyes. For further characterization, and in order to molecularly compare the ciliary PRCs of *Platynereis* with those (i.e. rods and cones of the retina, deep brain PRCs) of vertebrates, I have identified and characterised the opsin photopigment specific for these cells.

2.2.2 *The opsin active in* Platynereis *ciliary PRCs closer relates to vertebrate rod and cone opsins than to canonical invertebrate opsins*

First, in order to confirm that the cells that by EM optics had been identified as ciliary photoreceptor cells are indeed photosensory, I set out to clone the *opsin* molecule expressed in these cells. A PCR screen yielded two novel *Platynereis opsins*. Their AA sequences are highly similar to the *opsin* of the vertebrate ciliary photoreceptor cells (rod, cone and deep brain ciPRCs), with which they also group together in phylogenetic trees. They are clearly distinct from the *opsin* present in the 'normal' rhabdomeric protostomian eye photoreceptor cells, and thus also distinct from the already known rhabdomeric *opsin* of *Platynereis* (*Pdu-r-opsin*; Arendt *et al*., 2002, Figure 16, Figure 17, Figure 18). However, I was only able to obtain a whole mount in situ pattern for one (*Pdu-ci-ops1*) of the genes. Probes of *Pdu-ci-ops2* never attained a pattern, despite extensive attempts. At 24hpf *Pdu-ci-ops1* can be detected in one single cell in the apical organ region (see Figure 15H). No additional staining is visible anywhere else. 24hpf is prior to the stage when the ciliary photoreceptors can be discerned by staining with an anti-acetylated tubulin antibody (Figure 15D, E). Acetylated tubulin is characteristic for stable microtubules, and thus can be used to stain cilia (including the branched cilia of the ciliary photoreceptors) and axon tracts (Arendt *et al*., 2002; Lodish *et al*., 1995 and see e.g. Figure 15D, Figure 23F). At 48hpf, a cluster of several cells in the apical organ region and dorsal to it expresses *Pdu-ci-ops1*. This includes the cells by EM characterized as ciliary PRCs, as apparent in double stainings of *Pdu-ci-ops1* with an anti-acetylated tubulin antibody (see Figure 15I, K). Importantly, I never detected any staining of *Pdu-ci-ops1* in the rhabdomeric PRCs that make up the larval and adult eyes of *Platynereis*. Two weakly staining cells could be detected as possibly sitting at the base of the developing adult eyes at 48hpf. However, it could be excluded by cell morphology and location that they belong to the population of cells that express the *Pdu-r-opsin* (data not shown). From this it can be concluded that *Platynereis dumerilii* has two truly different types of PRCs, the rhabdomeric type, expressing *Pdu-r-opsin* and constituting the eyes, and the ciliary type, located in the center of the brain and expressing *Pdu-ci-ops1*.

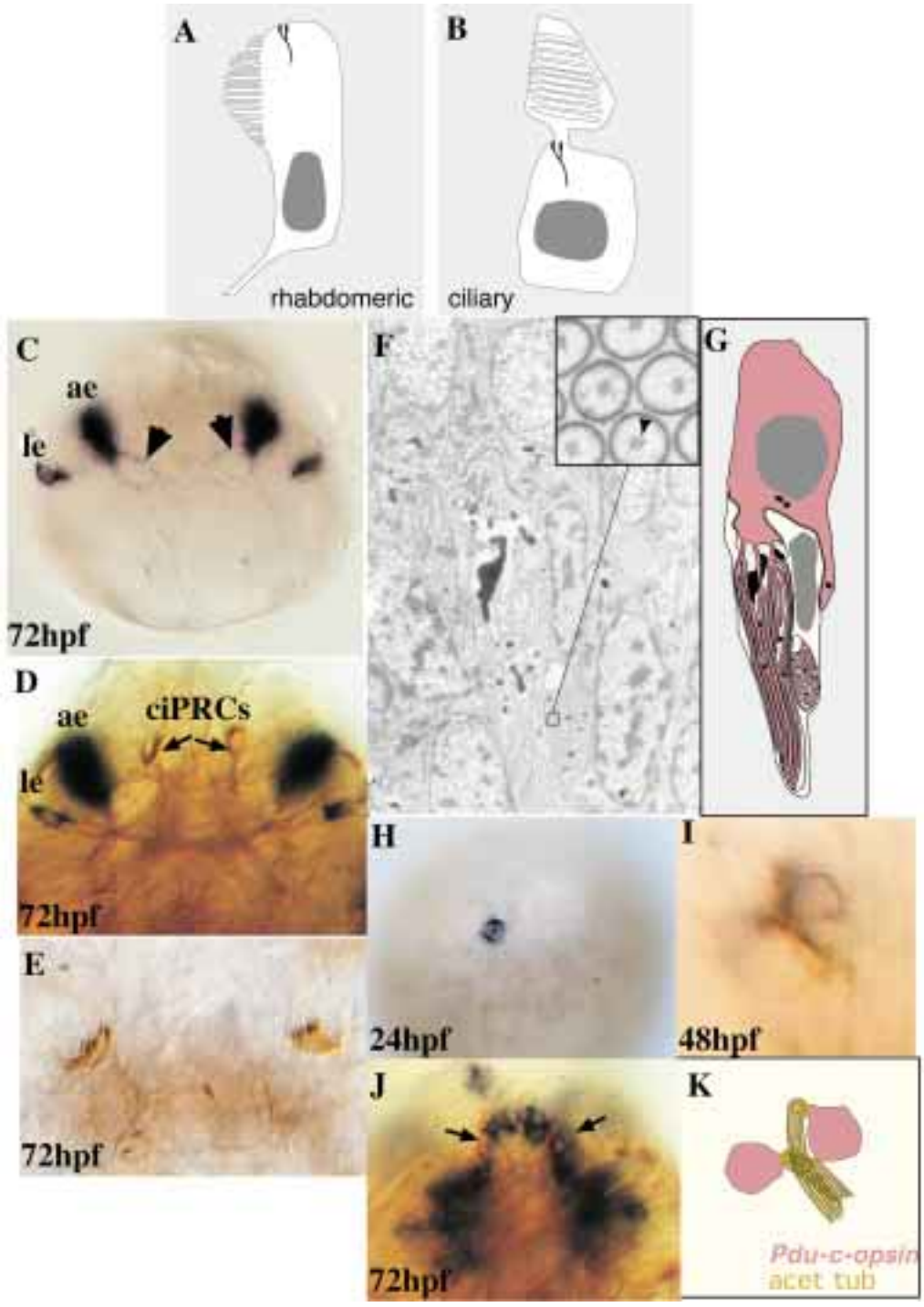


Figure 15. Rhabdomeric and ciliary photoreceptor cells in *Platynereis dumerilii*.

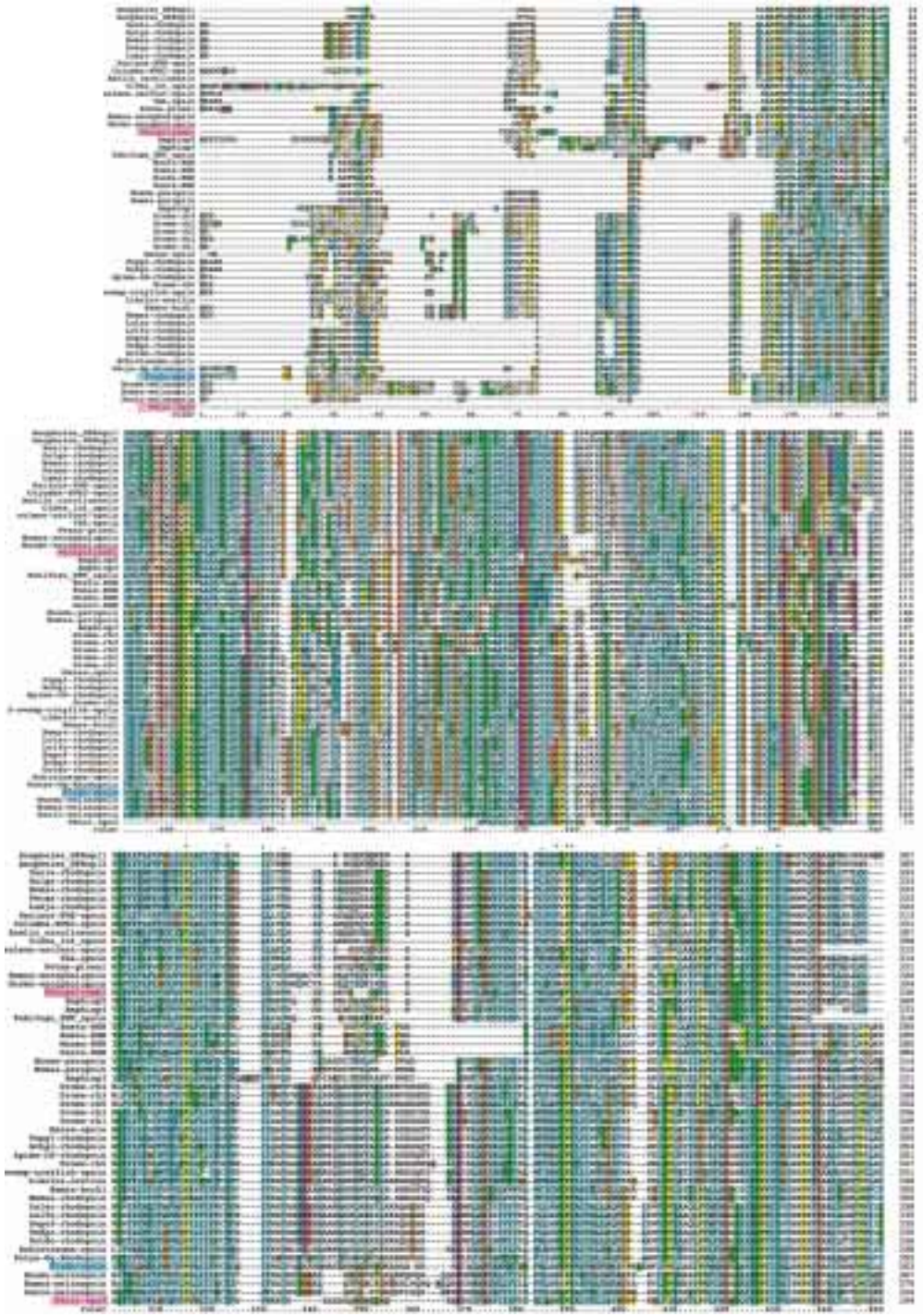
(A, B) schemes of a rhabdomeric and ciliary PRC, respectively. Pictures taken from Arendt *et al*., 2004. Note that the ciliary extensions do not need to be so prominent, as this is the case in the photosensitive CSF-contacting neurons in vertebrates (see below). **(C)** Apical view onto a 72hpf brain, dorsal up and ventral down. The embryo wasstained with an antisense probe against *Pdu-r-opsin*, which stains the larval (le) and adult (ae) eyes. (The adult eyes develop from one precursor, see Fischer and Brökelmann, 1966.) The black arrowheads point to the projection of the adult eyes, as can

be discerned by this staining. **(D)** Apical view onto 72hpf embryo, dorsal up, ventral down. In addition to the *Pdu-r-opsin* staining of C, this embryo was also stained with an antibody against act.tubulin (brown). The black arrows point to the ciPRCs that can already by discerned with an anti-act.tubulin antibody. Picture was taken together with D. Arendt. **(E)** Magnification of the ciPRCs of an embryo that had not been treated with proteinase K, and thus has a better conservation of the structures, that can be stained. The axon scaffold stains only very moderately, the ciPRC stain strongly. **(F)** EM picture of the larval ciPRCs of *Platynereis*. (courtesy of Drs..A.Dorresteijn and D.Arendt). The inset shows the microtubules in the branches of this cell (small arrowhead). **(G)** Schematized drawing of the EM picture (courtesy ofD.Arendt). **(H, I)** Staining with an antisense probe against *Pdu-ci-opsin1*. Embryos in apical view, dorsal up and ventral down. (H) At 24hpf the staining is visible in one single cell within the apical organ region. (I) More cells stain at 48hpf, but all are located around the apical organ region. The embryo is counterstained with anti-acet. tubulin (brown). This picture shows a magnification of two cells were the blue staining of the *Pdu-ci-opsin1* exactly co-localizes with the brown staining of the act. tubulin, staining the differentiated cilia of the ciPRCs. **(K)** Schematized version of I (courtesy of D. Arendt). **(J)** 72hpf embryo in apical view, dorsal up, ventral down. Picture and co-localization together with D.Arendt. Co-localisation of the ciPRCs (as discerned by anti-act. tub. staining in brown, black arrows) with the *Platynereis rx* gene (*Pdu-rx*), blue WMISH. As a confirmation, co-localisation was also confirmed by two-color WMISH at 48hpf (data not shown).

2.2.3 *The* Platynereis *ciliary photoreceptor cells express* rx*, a transcription factor specific for the differentiating vertebrate rods and cones*

In order to further extend the comparison of ciliary photoreceptor cells between vertebrates and *Platynereis* I determined the co-expression *Pdu-ci-ops1* with *Pdu-rx*. In vertebrates, *rx* has been shown to be essential for the development of the entire eye (Loosli *et al*., 2001; Mathers *et al*., 1997; Zhang *et al*., 2000). Most remarkable, it is long present in the ciliary PRCs in the eye, pineal and hypothalamus, but is switched off early in almost all other cell types in the developing retina (Chuang *et al*., 1999; Deschet *et al*., 1999; Kimura *et al*., 2000; Ohuchi *et al*., 1999). Conversely, although an *rx* gene has been cloned from *Drosophila melanogaster* and planarians these genes could not be found associated with or play a role in the development of the eyes (that consist of rhabdomeric PRCs) in these animals (Davis *et al*., 2003; Salo *et al*., 2002). Previous studies had shown that expression of the *Platynereis rx* gene does not cover the rhabdomeric photoreceptors, but instead the region of the ciliary photoreceptors (D. Arendt, unpublished results). By co-staining with an anti-act.tub antibody, as well as with double whole mount in situ stainings of *Pdu-rx* with *Pdu-ci-ops1*, I have determined that *Pdu-rx* is indeed co-expressed with *Pdu-ci-ops1* positive cells (Figure 15J and data not shown). Thus, the ciPRCs of *Platynereis dumerilii* and the vertebrate ciPRCs do not only share a similar morphology and

orthologous *opsin* molecules, but also the specific expression of the developmental control gene *rx*.



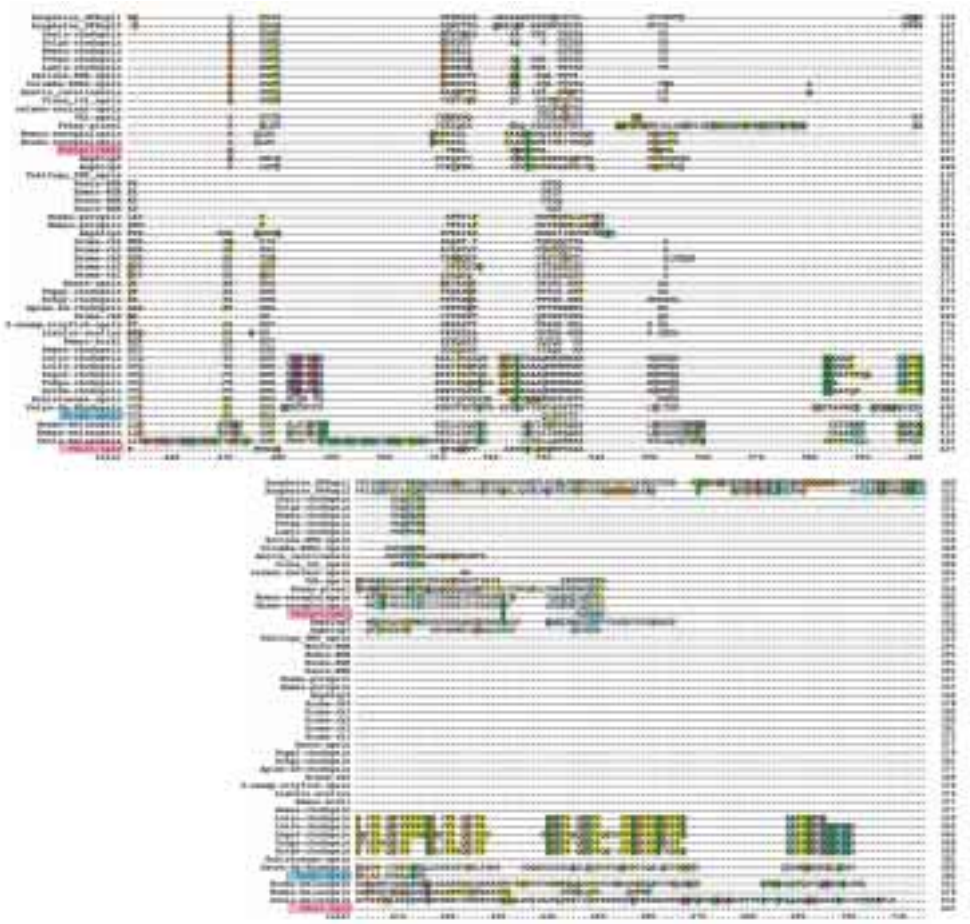


Figure 16. Sequence alignment of bilaterian Opsin proteins.

The alignment shows a clustalX alignment of various opsin molecules. *Pdu-ci-opsins* are underlayed in red, *Pdu-r-opsin* in blue. The species nomenclature uses the common species abbreviations in most cases. The alignment was used to construct the tree shown in Figure 18. The accession numbers belonging to this alignment can be found in the Appendix

The second light-sensitive cell type in *Platynereis* larvae are the rhabdomeric photoreceptor cells of the larval and developing adult eyes. If the vertebrate rods and cones rather relate to the ciliary photoreceptor cells of *Platynereis*, are there additional photosensitive cell types in the vertebrate retina that might correspond to the invertebrate rhabdomeric photoreceptors? Molecular comparions indicate that indeed there are.

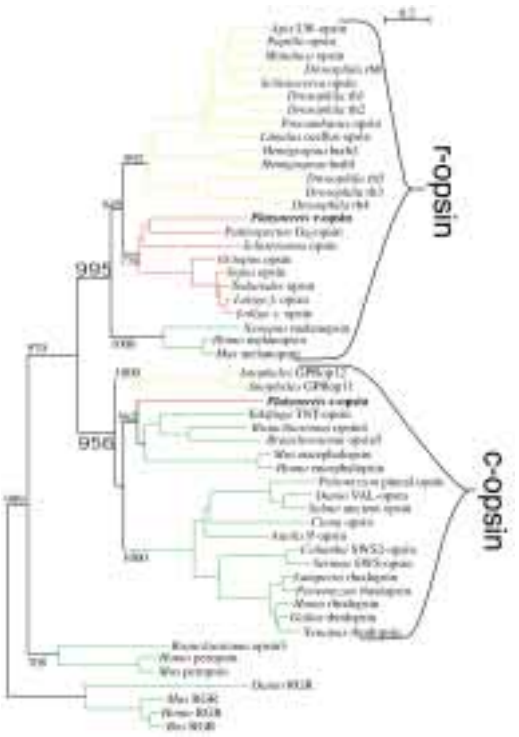


Figure 17. Phylogenetic tree for ciliary and rhabdomeric Opsin proteins.

This tree of bilaterians Opsins shows that there are at least two Opsin branches ancient for Bilateria. Ecdysozoan branches are depicted in yellow, deuterostomian branches in green and lophotrochozoan branches in red. Note that members of all three groups occur in both the ciliary and the rhabdomeric Opsin group. Tree was calculated using conceptual translations of gene sequences. Numbers at the branch points are bootstrap values. The accession numbers belonging to this phylogenetic tree can be found in the Appendix

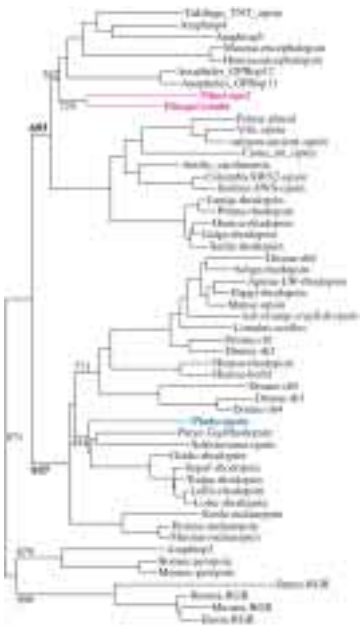


Figure 18. Phylogenetic tree for ciliary and rhabdomeric Opsin proteins, including both Pdu-Ci-opsins.

This tree originates from the alignment shown in Figure 16. The two Ci-opsins of *Platynereis* are shown in red, the R-opsin is shown in blue. Both Pdu-Ci-opsins cluster together in the ciliary Opsin group. The AA sequence of Pdu-Ci-ops2 is not fully known, and thus the alignment and tree formation relies on less AA residues, than the tree of Figure 17. This is the likely reason for the lower bootstrap values, however the principle is the same for both trees.

2.2.4 Rhabdomeric photoreceptor cell types

As it becomes apparent from the phylogenetic tree of the Opsins, vertebrates also possess an *opsin* of *rhabdomeric opsins* group (see Figure 17). This gene, *melanopsin,* is also present in the vertebrate retina. Remarkably, the *melanopsin* positive cells are not the photoreceptor cells, but the retinal ganglion, amacrine and horizontal cells. In addition it is present in the retinal pigment epithelium and the hypothalamus (cells of the preoptic nucleus (*Xenopus laevis*) and suprachiasmatic

nucleus; Berson *et al*., 2002; Drivenes *et al*., 2003; Hattar *et al*., 2002; Provencio *et al*., 1998; Provencio *et al*., 2000; Provencio *et al*., 2002). Does this mean that the melanopsin positive cell types in vertebrates are comparable to the rhabdomeric photoreceptor cells? In *Platynereis dumerilii* cells of the larval and adult eyes are of rhabdomeric type and express *Pdu-r-opsin* (observation by D.Arendt, see Arendt *et al*., 2002 and Figure 15D, 19A,B). In order to further characterize the rhabdomeric PRC–type and to compare it to the ganglion cells of the vertebrate retina, I investigated three transcription factors involved in retinal ganglion cell development in vertebrates for their expression in *Platynereis* rhabdomeric eyes.

2.2.5 Platynereis atonal homolog/Pdu-ath *is transiently expressed in the larval eye precursors*

In *Drosophila melanogaster atonal* is required for the specification of the R8 photoreceptor cells, which in turn induces the R1 – R7 PRCs (Frankfort and Mardon, 2002; Jarman *et al*., 1994). *atonal* is also required for the formation of the fly's ocelli and the first photoreceptor precursors of the Bolwig organ (the larval eye; Daniel *et al*., 1999; Jarman *et al*., 1994). In vertebrates, two orthologs of *atonal*, *ath5* (***at****onal* ***h****omolog*) and *ath1*, exist (see Figure 19G). *Ath5* is transitorily activated in retinal ganglion cell precursors just before RGC genesis in fish, frog, chick and mouse, and has been shown to promote retinal ganglion cell fate in *Xenopus laevis* lipofection experiments (Brown *et al*., 1998; Kanekar *et al*., 1997; Masai *et al*., 2000; Matter-Sadzinski *et al*., 2001). A mutation of *ath5* in zebrafish, as well as a targeted disruption of the murine gene leads to the specific absence of this cell type (Kay *et al*., 2001; Wang *et al*., 2001). The vertebrate paralog *ath1* has been discussed to be present in amacrine cells in mouse (see pers. comm. in Tessmar *et al*., 2002). I cloned the N-terminal fragment of a *Platynereis atonal homolog* (*Pdu-ath*) gene. Its expression is highly dynamic and described in detail in Arendt *et al*., 2002 (see also Figure 19). Most importantly, it is expressed in population of cells that, by position and time point, can be considered to correspond to the larval and adult eye precursors, as it was revealed with the help of D. Arendt (Figure 19).

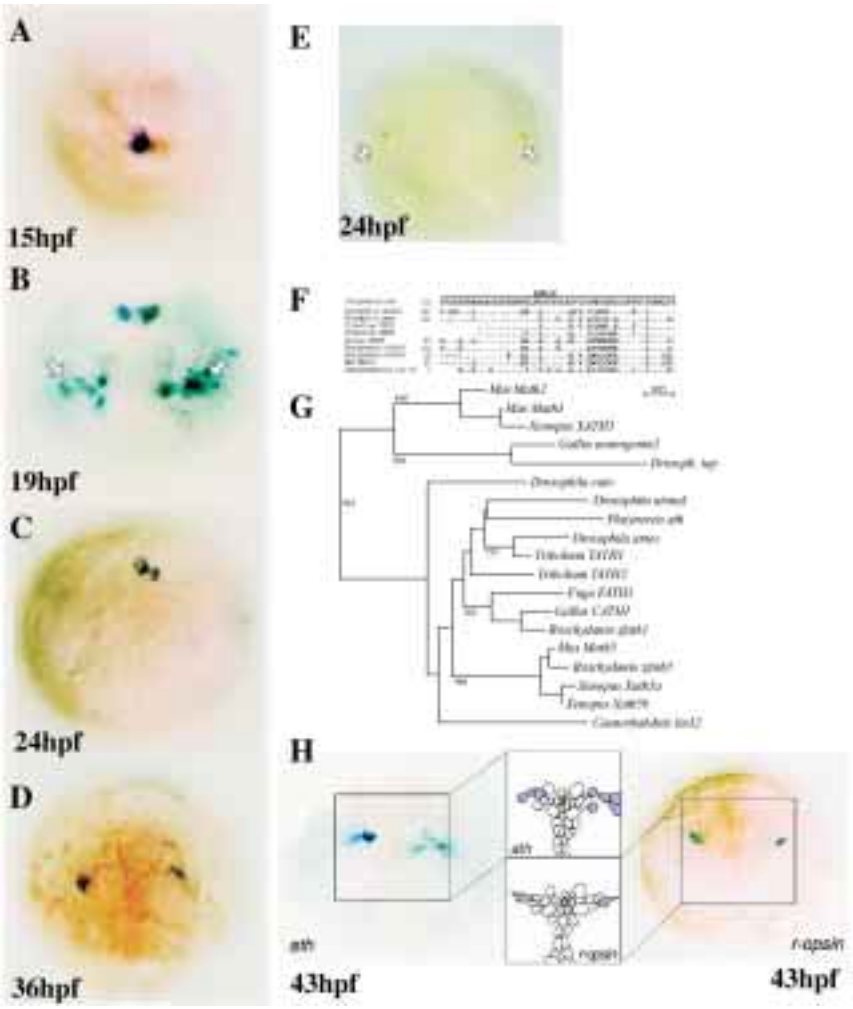


Figure 19. *Platynereis atonal homolog (Pdu-ath)* expression prefigures the formation of rhabdomeric PRCs.

(A-D) Apical view onto the highly dynamic *Pdu-ath* expression in the episphere of *Platynereis* larvae at different stages. Dorsal up, ventral down. (A) The first spot of *Pdu-ath* expression can be found in the apical organ indicating its partly sensory character (see below). (B) Expression broadens at 19hpf, were *Pdu-ath* is also strongly expressed in the region were the larval eye will be formed shortly thereafter (white arrows). (C, D) expression ceases again at later stages. **(E)** Location of the fully differentiated larval eyes, as can be seen by the associated pigment cells (white arrows). **(F)** Alignment of deuterostome and protostome Atonal-homolog bHLH domain sequences. Identical amino acids are abbreviated by a dot in the alignment, gaps are represented by '-'. **(G)** Phylogenetic tree of Atonal-homologs that reveals that Pdu-Ath is, by sequence, a true Atonal ortholog. Tree was calculated using conceptual translations of gene sequences. Numbers at the branch points are bootstrap values. **(H)** WMISH of *Pdu-ath* compared to *Pdu-r-opsin* at 43hpf, apical view. As schematized, *Pdu-ath* (left) ceases in the cells that start to express *Pdu-r-opsin* (right) (Pictures A-D taken and localisation to the rhabdomeric PRCs done together with D. Arendt, E, F and H courtesy of D. Arendt.) Accession number of *Pdu-ath*: AJ316543.

2.2.6 Pdu-brn3 *demarcates the larval eye region*

Two genes downstream of *ath1/5* that are also present in retinal ganglion cell (but not in rod and cone photoreceptor cells) in vertebrates are members of the *barH* and *brn3* families (Bermingham *et al*., 2001; Hutcheson and Vetter, 2001; Liu *et al*., 2001; Poggi *et al*., 2003).

The *brn3* family is a subfamily of the pou (***p****i**t1*, ***o****_ct1*, ***u****nc-86*)/ homeobox transcription factors. *Brn3d* lipofection experiments in *Xenopus laevis* resulted in an increase of the percent of retinal ganglion cells in the frog retina (Hutcheson and Vetter, 2001). Conversely, mutations and targeted disruption of *brn3* genes in human and mouse, respectively, leads to cell death of especially RGCs (Erkman *et al*., 1996; Gan *et al*., 1996). This death is a likely consequence of the abnormal/ lacking axon formation of RGCs (Erkman *et al*., 2000; Gan *et al*., 1999; Wang *et al*., 2000). In *Drosophila acj6* (*abnormal chemosensory jump 6*) is neither present in nor required for photoreceptor cell (R cells) development, but is instead required for proper synaptic connectivity of interneurons in the optic lobe that receive R cell synaptic inputs (Certel *et al*., 2000). However, the *brn3* gene of the gastropod *Haliotis asinina* is present in the eyes (O'Brien and Degnan, 2002).

I cloned the *Platynereis brn3* (*Pdu-brn3*) gene as part of a larger screen for *pou/ homeobox* genes. As a result of this screen I was able to determine at least 5 different subclasses of *pou* genes that are ancestral for Bilateria. Two of them, *pit1* and *rdf* subclasses had not been recognized as ancient classes previously (see Figure 20A). Note that in the case of the *rdf* subfamily, adding the Platynereis rdf ortholog helped to resolve the family tree, clarifying that the *brn5* and *rdf1* groups are distinct. The *brn3* subclass is clearly distinct and most distant among these five (Figure 20A). As I determined by WMISH and anti-acetylated tubulin staining, *Pdu-brn3* is present in the larval eye region from early stages onwards (Figure 21E). Its pattern gets very reminiscent to the *Pdu-pax6* expression at later stages (compare Figure 13F and Figure 21F, note that the animal in Figure 21F is 7hrs older). At 72hrs it demarcates the entire lateral brain domains, but is only sparsely present in the median region and, importantly is not expressed in the ciliary PRCs (Figure 21F). It is interesting to note, that this gene as well could not be detected in the region of adult eye photoreceptors (see Figure 21D-F), again very reminiscent of *pax6* (description of

Pdu-pax6 by D.Arendt, see in Arendt *et al*., 2002). In the head cells of the palpae also start to stain (data not shown), at 72hpf both antennal and palpal cells are covered (see Figure 21F). In addition to the episphere expression, *Pdu-brn3* is present in the VNC of the 48hpf larvae in two bilateral stripes flanking the outside of the major connectives, as revealed by co-staining with an anti-act.tub. antibody (see Figure 21G), and in two very posterior cells (Figure 21H). At 72hpf, these stripes resolve into bilateral symmetric pairs in the larval trunk segments (see Figure 21I). This might indicate that sensory neurons are located at these positions, because the *brn3* family in vertebrates is rather specifically present in the precursors of virtually all sensory neurons of the brain and spinal cord, except the ciliary PRCs (Andersen and Rosenfeld, 2001; Eng *et al*., 2001; Erkman *et al*., 1996; Fedtsova and Turner, 1995; Gan *et al*., 1996; Gerrero *et al*., 1993; Hutcheson and Vetter, 2001).

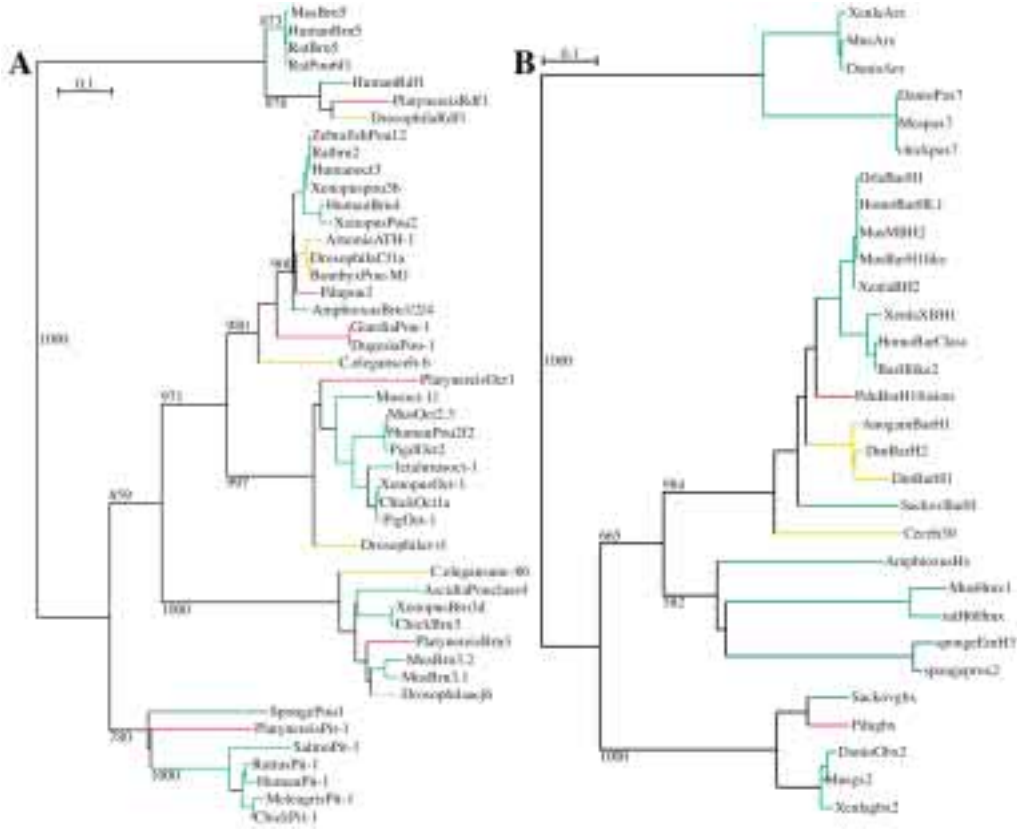


Figure 20. Phylogenetic trees of *Platynereis pou* and *barH* genes.

Color code: Blue- sponge, green- Deuterostomia, yellow- Ecdysozoa, red- Lophotrochozoa.

Trees were calculated using conceptual translations of gene sequences. Numbers at the branch points are bootstrap values. The protein alignments and accession numbers belonging to these phylogenetic trees can be found in the Appendix. **(A)** Phylogenetic tree including the five different *pou* genes of *Platynereis*. **(B)** Phylogenetic tree including the *Platynereis barH1* gene.

2.2.7 *Cloning of* Pdu-barH1 *as additional rhabdomeric PRC marker gene*

BarH is a homeobox gene that is expressed in retinal ganglion cells of *Xenopus laevis* (termed *Xbh1*) and the Medakafish *Oryzias latipes* (Patterson *et al.*, 2000; Poggi *et al.*, 2002). *Xbh1* lipofection into *Xenopus laevis* eyes leads to an increased amount of retinal ganglion cell (Poggi *et al.*, 2003). In *Drosophila* the two fly paralogs exhibit two different functions during eye development. On the one hand, *Dm-bar* represses *Dm-atonal* in undifferentiated cells underneath of differentiating PRCs behind the morphogenetic furrow, by this preventing additional recruitment of R8 photoreceptors from this undifferentiated population (Lim and Choi, 2003). On the other hand, *Dm-bar* genes are also present in and required for the R1 and R6 PRCs (Higashijima *et al.*, 1992a; Higashijima *et al.*, 1992b). I cloned a fragment of the *Platynereis barH1* gene (*Pdu-barH1*, see Figure 20B). Its expression has not yet been investigated at enough stages to be conclusive.

In summary, both *Pdu-ath* and *Pdu-brn3* can be detected in the rhabdomeric PRCs of the larval eyes of *Platynereis dumerilii* trochophora larvae, consistent with the idea that they are phylogenetically related to the in the retinal ganglion cells of vertebrates. A third candidate gene for expression in the *Platynereis* rhandomeric photoreceptors, *Pdu-barH1*, has been cloned.

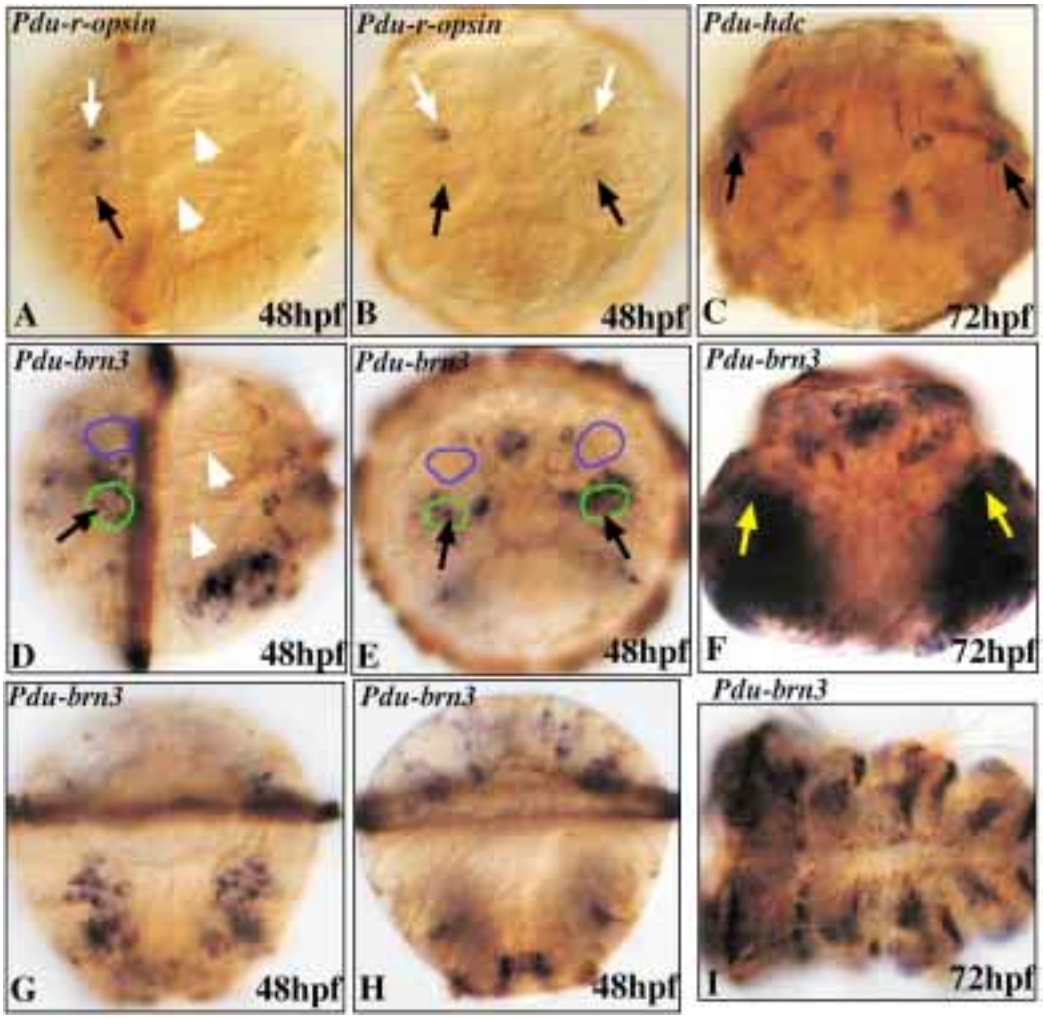


Figure 21. *Pdu-brn3* and *Pdu-hdc* in the larval eyes of *Platyneris dumeriliii*.

(A) Lateral view (apical left, ventral down) onto *Pdu-r-opsin*. expression. White arrowheads point to the first pair of parapodia for spatial orientation. Brown staining demarcates the developing axon tracts and ciliated structures, as elucidated with antibody staining against act.tub. **(B)** Apical view (dorsal up, ventral down) onto expression of *Pdu-r-opsin* at 48hpf. Black arrow points to the larval eye, white arrow to the developing adult eyes. **(C)** Apical view (dorsal up, ventral down) onto a 72hpf larva. Blue staining results from WMISH with an antisense riboprobe against *Pdu-hdc*. Black arrows point to the position of the larval eyes, brown staining are developing axon tracts, as demarcated by anti-act.tub. staining. Compare to Figure 15D for orientation. **(D)** Lateral view (apical left, ventral down) and **(E)** apical view (dorsal up, ventral down) onto expression of WMISH staining against *Pdu-brn3* (blue). Orientation is the same as in A) (for D) and B) (for E) to compare to the location of the larval eyes at this stage (indicated by black arrows and green circles for the larval eye area). *Pdu-brn3* is present in the larval eyes, but very likely not in the adult eyes (adult eye area indicted by the blue circle). The same conclusion emerges from **(F)**, an apical view (dorsal up, ventral down) onto a 72hpf larva. Blue *Pdu-brn3* WMISH staining, brown anti-act.tub. staining. Location of larval eyes indicated with yellow arrows. Compare also to Figure 12B, Figure 15D. **(G-I)** Expression of *Pdu-brn3* (blue) and anti- act. tub. (brown, axon tracts and cilia) as seen from the ventral side. G, H) 48hpf, apical is up, left is to the right. Note the *Pdu-brn3* staining in the VNC. This is an indication of the potential location of sensory neurons, since vertebrate *brn3* orthologs are expressed in virtually all sensory neurons. H) is focused slightly deeper in the embryo. I) 72hpf, apical is left, right is down.

2.2.8 Larval, but not adult eye regions are histaminergic

Finally, one characteristic of the eyes of arthropods is the use of histamine as one of their neurotransmitters (Nassel, 1999). The specific rate-limiting enzyme for histamine biosynthesis is *histidine decarboxylase* (Michal, 1999). I cloned and analyzed the expression of this gene, *Platynereis dumerilii histidine decarboxylase* (*Pdu-hdc*). The AA sequence of Pdu-Hdc I cloned can be found in the alignment depicting the phylogenetic relationship of this protein in the Appendix. Its expression further corroborates the unexpected distinction between larval and adult eyes in *Platynereis* larvae, by being expressed in the area of the larval, but not adult eye photoreceptor cells (Figure 21C). I checked if the adult eyes might express *Pdu-hdc* at later stages. However, also up to 6 weeks of development there was no detectable sign of *Pdu-hdc* in the adult eye region (Figure 29J). Unfortunately, nothing is known about the presence or absence of histamine in retinal ganglion cells of vertebrates, making the comparison to the vertebrates impossible so far.

In addition to the expression larval eye area, *Pdu-hdc* is present in cells that highly likely tbelong to the antennae by location (data not shown), as well as in more median brain cells that will be described below.

In summary, the two PRC types, ciliary and rhabdomeric, differ not only by morphology, but also by the expression of non-orthologous *opsin* molecules and of distinct sets of transcription factors during their development. In addition, the rhabdomeric cell type might further separate into distinct subtypes given that several genes (*pax6*, *brn3*, *hdc*) appear to be restricted to the rhabdomeric PRCs of the larval, but not of the adult eyes of *Platynereis*.

2.3 Molecular characterisation of neuroendocrine regions and cell types in *Platynereis*

In the sections above I described evidence that several features of the forebrain of vertebrates and the episphereal region of the *Platynereis* trochophora larva share common ancestry. On the one hand, this is likely true for the lateral vs. median subdivision of the brains by the genes *pax6* and *nk2.1*, respectively, in an overall *six3/6* positive forebrain area. On the other hand, I showed that this comparability

extends to the level of cells types present in the brains of vertebrates and *Platynereis*. Two types of PRCs, the ciliary and rhabdomeric PRCs are conserved and present in the anterior *six3/6* positive brain regions of both groups. In order to further extend this brain comparison, I focused for the next part in detail on the median brain regions of both the vertebrate and *Platynereis* brains. As mentioned in the introduction, the median/ventral region of the vertebrate forebrain itself, with the hypothalamus as its center, hosts the circuit of neurosecretory and neuroendocrine cell types that have an influence on all essential body functions. Given the prominent role of this neurosecretory control center, I then addressed the question to which extent this system can be traced back to an ancestral precursor. The strategy I employed was very similar to the one successfully followed in the analysis of the photosensory system, namely to compare distinct cell types present in the vertebrate neuroendocrine region to cells in the brains of invertebrate animals. I conducted my analysis in four different steps, moving from the comparison of larger brain areas to distinct cellular features. First, I selected a set of transcription factors that are specifically expressed in the median/ ventral *nk2.1* expressing forebrain anlage of vertebrates, and analysed if homologs of these region-specific genes are co-expressed in any area of the *Platynereis* larval brain. Second, I turned towards factors that are present in the ventral/ median region of the vertebrate forebrain, but less specific for this region, to further explore the similarity in the overall combinatorial transcription factor code of the compared brain regions. Third, I extended this analysis to the level of cell type differentiation markers (such as neural transmitters or receptors). Finally, I performed a brief analysis of the structural organisation and potential functional aspects of the cells of the apical organ, in order to compare also these aspects to the vertebrate ventral/ median brain region.

2.3.1 *The* Platynereis *median episphere expresses homologs of transcription factors that are highly specific for the median/ventral forebrain in vertebrates*

In vertebrates, the early ventral/median brain is molecularly characterized by the combined expression of several transcription factors that are thought to mediate regional identity, translating into the formation of distinct cell types in this area at

later stages. *Nk2.1* is one of the transcription factors that display a highly specific expression in the ventral/median vertebrate forebrain, as well as in the *Platynereis* larval median brain (see above and Figure 13), indicating evolutionary conservation.

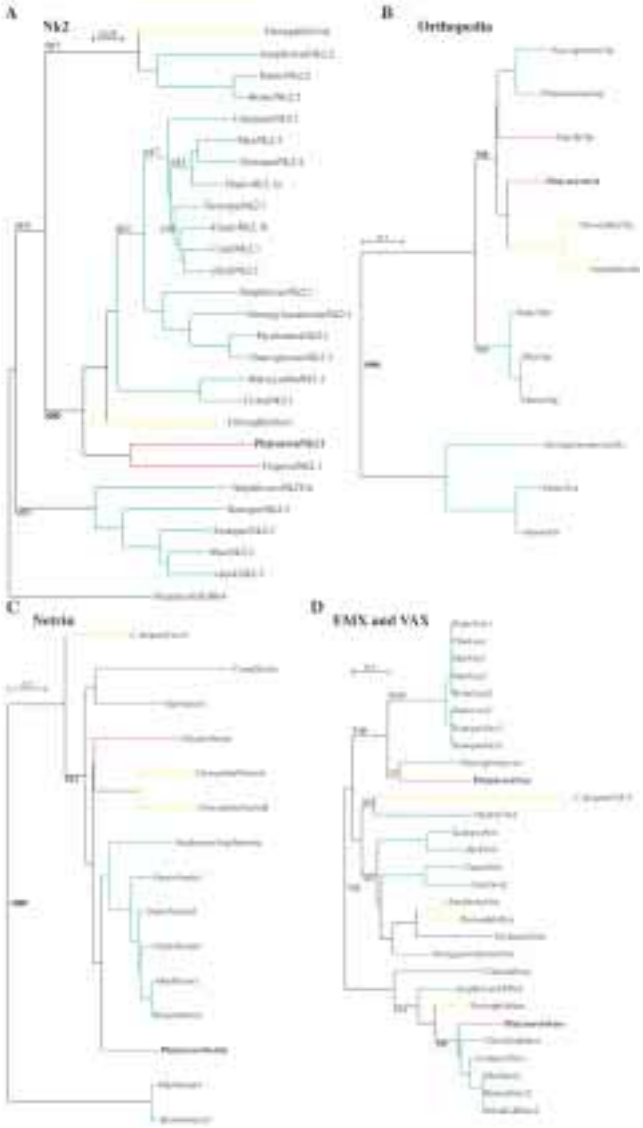


Figure 22. Position of *Platynereis* Nk2.1, Otp, Netrin, EMX and VAX in phylogenetic trees.

Trees were calculated using conceptual translations of gene sequences. Numbers at the branch points are bootstrap values. Color code: green Deuterostomia, yellow Ecdysozoa, red Lophotrochozoa, blue placozoa, sponges, cnidaria The alignments and accession numbers belonging to these phylogenetic trees can be found in the Appendix.

(A) Phylogenetic tree of the Nk2.1 family. Pdu-Nk2.1 clusters clearly within this group. The likely molecular sister group of the Nk2.1 family is the Nk2.2 family. **(B)** Phylogenetic tree of the Orthopedia family. *Pdu*-Otp clusters together with the ecdysozoan and echinoderm Otp members. The Aristaless family was taken as an outgroup. **(C)** Phylogenetic analysis of the Netrin family. *Platynereis* Netrin clusters together with the vertebrate Netrins. **(D)** Phylogenetic trees of the EMX and VAX families. Both families are related to each other, and each one can thus serve as outgroup for the respective other group. *Platynereis* Vax is a clear Vax ortholog and clusters together with the enteropneust Vax. Note that it is unclear if the *C.elegans* CEH-5 protein is rather a Not or a Vax familiy member, since in Blast analyses it always first groups with Vax orthologs. *Platynereis* EMX clusters together with the vertebrate EMX proteins.

To further corroborate this, I decided to study members of three other gene families that exhibit a highly specific expression in the vertebrate ventral/ median forebrain. These genes are *retinal homeobox* (*rx*), *ventral anterior homeobox* (*vax*), and *orthopedia* (*otp*).

2.3.1.1 *Platynereis rx* is present in the *Pdu-nk2.1* positive area, particularly in apical organ cells

In the previous section, *Pdu-rx* has been introduced as a marker for ciliary photoreceptor cells in the vertebrate eye, ventral forebrain and pineal (see above and Figure 15). Notably, the expression of *rx* family members is not restricted to ciliary PRCs in vertebrates, but it is also specifically present in additional cells located in a broader region of the hypothalamus (Chuang *et al.*, 1999; Deschet *et al.*, 1999). In addition to the suggestive expression pattern, mutants in the *rx3* gene of the Medakafish *Oryzias latipes* show a very disorganized hypothalamic area as well as behavioral defects (S. Winkler, personal communication). These observations are consistent with an important role of *rx* genes in the specification of cell types in the hypothalamus, that has however not been investigated in more detail.

I have investigated the spatial and temporal features of *Pdu-rx* expression in the *Platynereis* developing brain. By WMISH, *Pdu-rx* transcripts cannot be detected at 11hrs of development, but are apparent at 15hpf. Four hrs later, 4 groups of cells can be clearly discerned at the dorsal and ventral episphere, in addition to a few bilaterally symmetric cells in the more median region (Figure 23 I, J). *Pdu-rx* expression remains almost entirely restricted to the larval episphere (note however the occasional expression at the posterior end of the embryo of the 19hpf larva as well as in two cells flanking the prospective upper lateral side of the stomodeum at 24hrs (Figure 23 I and data not shown)). In addition to the aforementioned expression site in the ciPRCs, *Pdu-rx* is in later stages (48hpf) also present in a small group of cells exactly in the apical organ (2 cells in average, see Figure 23 K, L). Moreover, *Pdu-rx* transcripts are also present in two dorso-laterally located cell groups at this stage, which likely reflects the corresponding persistence of expression from the19hpf stage, as judged from the unique morphology and position of these cells. These *Pdu-rx*-expressing cells correspond to the anlagen of the nuchal organs (Figure 23 J,L). Nuchal organs are presumptive chemosensory organs of polychaetes (Ax, 1988). Between 48hpf and 72hpf, the overall expression pattern of *Pdu-rx* prevails, except for the fact that the ventral and more lateral epispheral expression vanishes, leaving only 2 longitudinal stripes running across the episphere, the apical organ and nuchal organ. Importantly, initial *Pdu-rx* expression possibly overlaps

with *Pdu-nk2.1* only in the dorsal-median episphere in one or two cells at best (Figure 13C, Figure 23J), if at all. In contrast, the later ventro-median *Pdu-rx* staining is clearly located within the *Pdu-nk2.1* domain (Figure 14, Figure 23K, L). Since most of the medially expressed *Pdu-rx* transcripts vanish at 72hpf (1-2 cells in the APO region remain usually visible), the two expression domains continue to overlap mainly in some cells in the more dorsal-median region (data not shown). In summary, the comparison of the expression of *Pdu-rx* to its vertebrate counterparts and to the domain demarcated by *nk2.1* in both animal systems further strengthens the notion that the median brain of *Platynereis* comprises conserved *nk2.1*/*rx*-positive brain regions, giving rise to specific cell types with a similar molecular setup as in the corresponding ventromedial brain regions of the vertebrates.

2.3.1.2 The expression of the *Platynereis vax* gene is highly specific for the apical organ and the median episphere

I turned towards a third group of transcription factors characteristic for the vertebrate ventral forebrain, the products of the *vax* genes. Two *vax* genes exist in vertebrates (*vax1* and *vax2*) that are highly specifically expressed in the optic chiasm, ventral eye and preoptic area across vertebrates (Barbieri *et al*., 1999; Bertuzzi *et al*., 1999; Hallonet *et al*., 1999; Hallonet *et al*., 1998). Importantly, no *vax* gene expression has been described outside the forebrain. Mouse knock-outs of *vax* genes lead to defects in the formation of retinal ganglion cell axon tracts, because the optic nerve glia cells fail to associate with RGC axons, and instead repulse them (Barbieri *et al*., 1999; Bertuzzi *et al*., 1999). In addition, *vax1* knock-out mice lack the entire preoptic area and the optic chiasm (Hallonet *et al*., 1999). No *vax* gene has so far been annotated for *Drosophila melanogaster* (based on the Flybase database: http://www.flybase.org/ and on my own sequence searches). A single possible *C.elegans vax* gene exists (*ceh-5*), but it has not been analysed apart from its cloning (see Wormbase: http://worm.imbb.forth.gr/, but also note the uncertainty of its positioning in phylogenetic trees: Figure 22D). As a consequence, *vax* was another good candidate for a gene whose presence and expression in *Platynereis* might provide a hint on the ancestrality of the vertebrate anlage of the neurosecretory system. *Platynereis dumerilii* has a single *vax* gene (cloned by D. Arendt, unpublished). In a phylogenetic tree, the derived protein sequence for *Platynereis*

Vax falls into a single cluster with its vertebrate counterparts, while Not and Emx proteins form the outgroups (Figure 22D). I decided to investigate the expression of *Pdu-vax* in more detail to determine if it overlaps with the *nk2.1* expression, during the development of the *Platynereis* median brain, as it would be suggested by the specific expression of the vertebrate family members. By WMISH I found that *Pdu-vax* transcripts are strikingly restricted to the median region (in and around the apical organ) of the *Platynereis dumerilii* larval episphere from 24hpf onwards (Figure 23G). This expression is maintained during the second day of development, becoming less restricted at 72hpf (Figure 23H and data not shown). The epispheral expression of *Pdu-vax* overlaps highly specifically and to a large extent with the *Pdunk2.1* expression (compare Figure 13C, D, Figure 14, Figure 23C, D, E, F). Outside the episphere, *Pdu-vax* is present in two bilaterally symmetric pairs of cells (data not shown) that are possibly part of the spinning glands, judged by their location and by the absence of staining in the first larval segment. (The larval spinning glands are the only segmental structures known to exist in only the rear two of the three larval segments.)

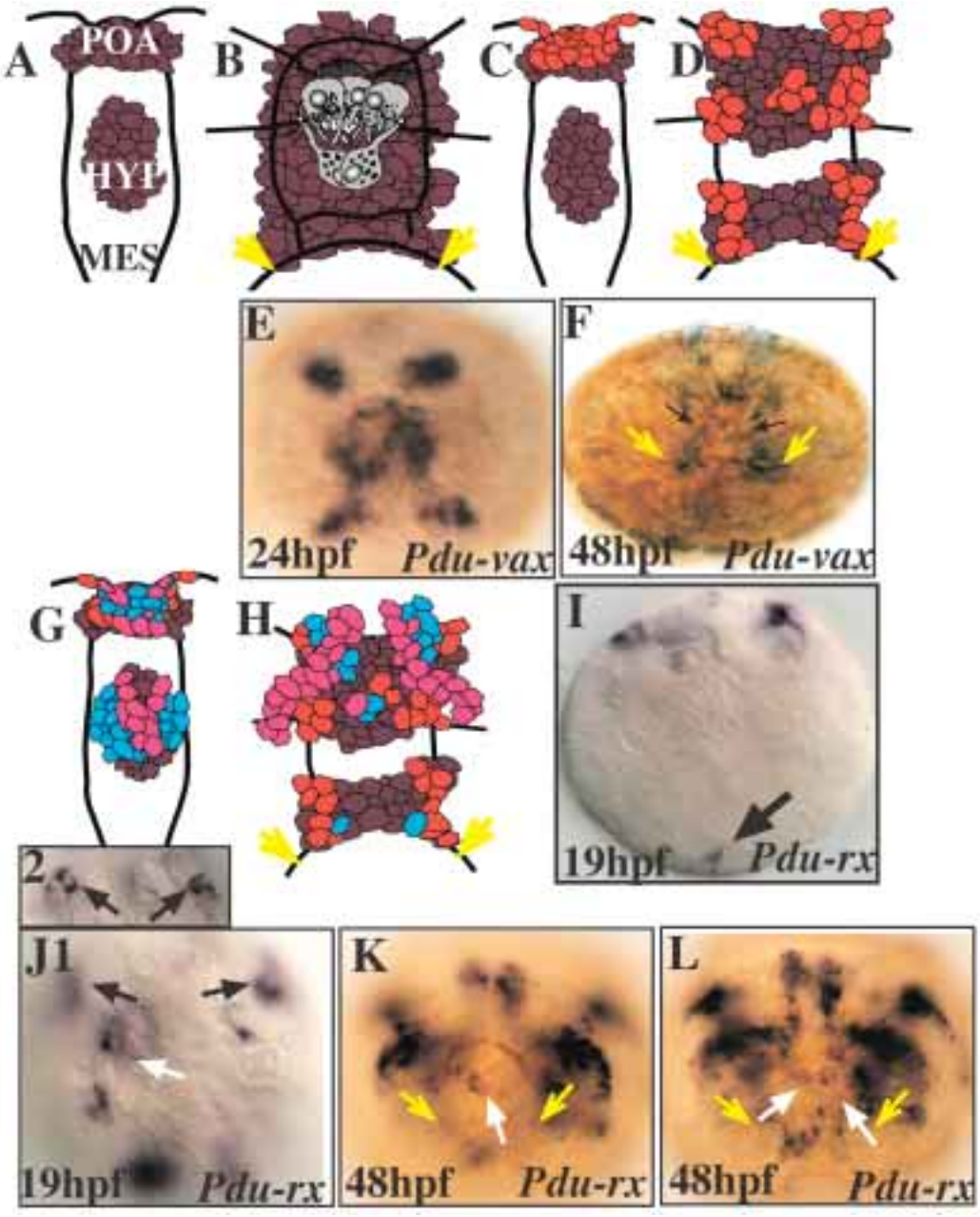


Figure 23. Expression of *Platynereis vax* and *rx* genes in comparison to vertebrates.

The figure shows pictures of the expression domains detected by WMISH along with simplified schemes to facilitate the comparison between *Platynereis* and vertebrate expression patterns. **(A, C, G)** simplified schemes of the respective consensus expression in the vertebrate ventral/ median forebrain. The schemes represent the median region of the scheme already introduced in Figure 4,Figure 10, Figure 12, Figure 13, which is the *nk2.1* positive region between the prospective major axon tracts (indicated in black). Similarly, **(B, D, H)** show schematized apical views (ventral down, dorsal up) of the median region (*nk2.1* positive) of the *Platynereis* episphere, between the major axon tracts (black). Grey cells in the middle of the scheme represent the apical organ. **Color code:** dark red: *nk2.1;* lighter red: *vax*; blue: *otp*; pink: *rx*. Yellow arrows are used for orientation and indicate the position of the stomodeal commissures in the schematics as well as on the WMISH pictures. **Abbreviations:** poa preoptic area, hyp hypothalamus, mes mesencephalon. Note that all schemes that are used in this study are only meant to give a rough impression. In particular, to facilitate the scheme,

the expression of vertebrate and *Platynereis nk2.1* genes is only partially depicted. Vertebrate *nk2.1* expression extends over the entire ventral/ median forebrain, Likewise, the *Pdu-nk2.1* expression is slightly broader than depicted here. **(E, F, I-L)** Gene expression patterns (detected in blue) and counterstains against acetylated tubulin (detected in brown) that demarcates the ciliated structures and axon tracts. **(E, F)** *Pdu-vax* expression, apical view (dorsal up, ventral down) at 24hpf (E) and 48hpf (F). Note the expression in the apical organ (E) as well as the exact correlation with the median axon tracts (F, marked by black arrows). **(I-L)** *Pdu-rx* expression. **(I)** 19hpf ventral view (apical up, right to the left), black arrow points to the occasoinal staining in the posterior region. **(J2)** Apical view (dorsal up) as in J1 with an enlargment of and focus on the *Pdu-rx* expression in the nuchal organ precursors. **(J1)** Apical view (dorsal up, ventral down). Black arrows point to the expression in the apical organ precursor, as focused on in J2). White arrow points to the most median expression at this time point. **(K)** and **(L)** Apical views (dorsal up, ventral down) onto 48hpf larvae. White arrow in K points to a cell in the APO expressing *Pdu-rx*. Picture in L is taken from the same view, but at a slightly deeper focus, the white arrow points to median brain expression. Yellow arrows for comparison to H. See Figure 15 for *Pdu-rx* expression at 72hpf. Picture in panel F courtesy of D. Arendt.

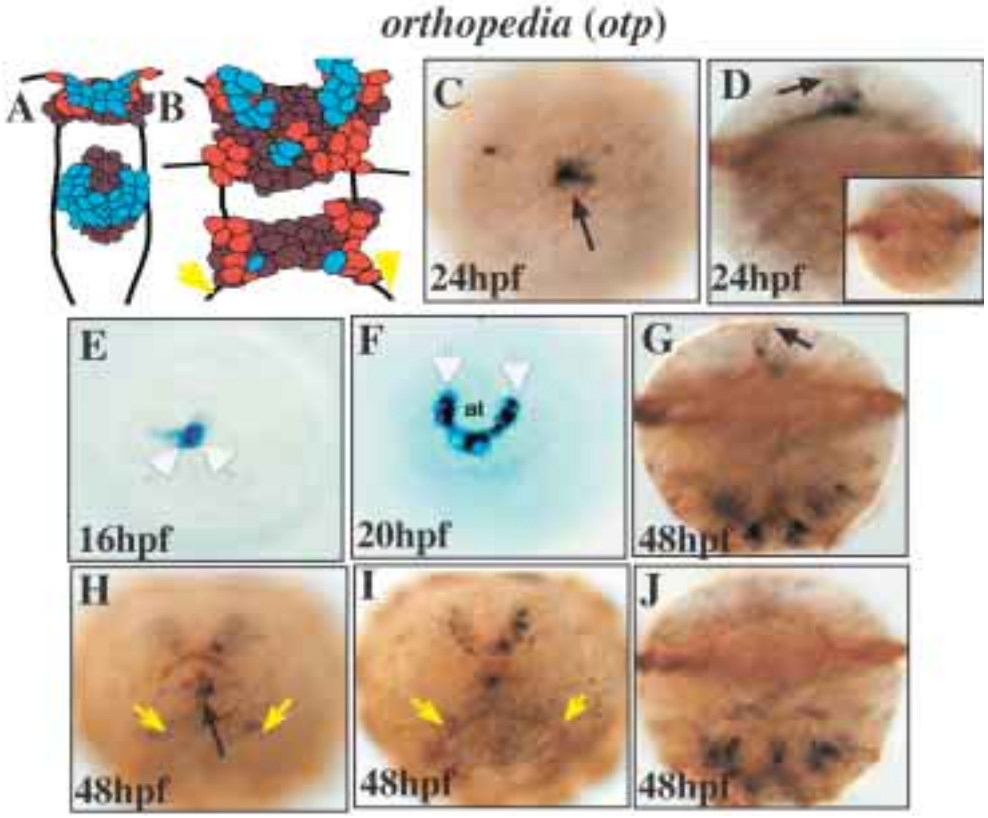


Figure 24. Expression of *Pdu-otp* compared to the expression in *Patella vulgata* and vertebrates.

(C, D, G – J) pictures of embryos processed with WMISH against *Pdu-otp* transcripts (detected in blue), counter-stained with an antibody against acetylated tubulin (see above, detected in brown). **(C)** 24hpf apical view, black arrow point to APO. **(D)** 24hpf ventral view, black arrow points to APO cells, note the brown extensions (stained with act. tub.) to the surface. Small inset: Upper plane of focus- start of expression in the trunk. **(E, F)** Highly specific expression of a mollusc *otp* homolog in the apical organ of *Patella vulgata* (white arrows). Pictures taken from (Nederbragt *et al*., 2002). **(G)** Ventral view of a *Platynereis* larva at 48hpf. The *Pdu-otp* expression is in a flask-shaped cell with ciliated extensions to the surface (black arrow). **(J)** as G, but focussed more on the surface. **(H)** 48hpf apical view, black arrow points to APO. Note the crescent of APO cilia (act. tubulin staining) that surrounds the *Pdu-otp* staining. Yellow arrows permit comparison to B. **(I)** Slightly deeper focus than

H. **(A)** and **(B)** summarize the expressions as before (*otp* expression marked in blue). For further explanations see Figure 23.

Co-staining of acetylated tubulin by an antibody staining reveals that even at the onset of axon outgrowth (at 24hpf), *Pdu-vax* expression partly prefigures the developing axon tracts surrounding the apical organ (Figure 23G,H). Later, expression demarcates these axon tracts and the epispheral part of the stomodeal commissure (first localisation to the axon tracts by D. Arendt, unpublished). One should note its remarkable specificity for the epispheral median axon tracts, since it does not demarcate or is expressed close to the various axon bundles running on the lateral side, as well as the axon tracts of the ventral nerve cord. This is a remarkable detail, because one prominent function of the vertebrate *vax1* gene is the proper formation of the median aspect of a highly specific proportion axon tracts (formation of optic chiasm, anterior commissure, corpus collosum) in the vertebrate ventral/ median Bertuzzi *et al*., 1999. The only expression in the trunk is not visibly associated with any axon.

In conclusion, there are two aspects in which *Pdu-vax* remarkably resembles the vertebrate *vax* expression. First, in both vertebrates and *Platynereis* it is highly specific for a median region in which it overlaps during all time points with the highly localized *nk2.1* expression region. Second, in both cases it demarcates axon tracts specifically in this region, while it does not associate with other tracts elsewhere in the animal (Figure 23 G, H). In summary, *Pdu-vax* is the third transcription factor whose expression lends further support to the notion that protostomian and deuterostomian ventral median brains share extensive molecular similarities with each other that suggest an ancestral origin of this region.

2.3.1.3 The expression of the *otp* gene is highly specific for cells of the apical organ across Lophotrochozoa

The fourth transcription factor that I decided to analyse was the *orthopedia*/*otp* gene. Vertebrate homologs of *otp* are expressed along the vertebrate spinal cord as well as in the forebrain, where it is highly specific for the ventral region (Acampora *et al*., 2000; Simeone *et al*., 1994). In mouse, the *otp* expression in the forebrain has been analyzed in detail. It is expressed in the optic stalk, preoptic area, and the periventricular, paraventricular, suproptic and arcuate nuclei of the hypothalamus. In

addition, it is present in the amygdaloid complex and the *stria terminalis* (a slender, compact fibre bundle, originating from the amygdala, that connects the amygdala with the hypothalamus and other basal forebrain regions). Functionally, *otp* has been shown to be required for the terminal differentiation of the neuropeptidergic neurons of the hypothalamic nuclei. These neurons include a particular subset, the neurosecretory magno- and parvocellular neurons. Molecularly, these neurons are characterized by the presence of the peptides Argenine/Vasopressin (AV), Oxytocin (O), corticotropin releasing hormone (CRH), somatostatin (SS), tyrotropin releasing hormone (TRH), as well as growth hormone releasing hormone (GHRH). The neurons containing GHRH are the only neurons of this category that are not affected by the *otp* disruption (Acampora *et al*., 2000; Acampora *et al*., 1999; Simeone *et al*., 1994; Wang and Lufkin, 2000). In *Drosophila, otp* is present in the brain and VNC, as well as in the hindgut. In the latter tissue it is under direct control of *brachyenteron*, the *Drosophila brachyury* ortholog. Its further expression or function in the CNS, however, has not been investigated.

I obtained a *Platynereis* EST clone with high sequence similarity to *otp* from the 48hpf cDNA library (constructed by me with the help of H. Snyman) in the course of the WMISH project (see below). The predicted protein encoded by this EST clearly groups together with the insect *otp* genes and their vertebrate counterparts, so that I refer to this clone as *Platynereis otp (Pdu-otp;* Figure 22B). Next, I performed a WMISH analysis to determine the expression of this clone in the developing *Platynereis* larva. I found *Pdu-otp* to be expressed in specific groups of cells of both the episphere and the trunk (Figure 24). At 24hpf, transcripts can be detected in very few cells of the apical organ (likely to belong to the flask-shaped center cells, see below) and slightly lateral to it in the median region of the brain. Additionally, two cells can be detected directly below the prototoch, to the left and right side of the stomodeum (Figure 24 C, D). Transcription in the apical organ persists over time, while additional cells become positive for this gene that are located in the ventral and dorsal median region of the brain (Figure 24 H, I). In contrast, no transcripts were detected in the lateral aspects of the episphere (Figure 24). Importantly, this highly specific expression in APO cells has also been detected for the trochophora larvae of the mollusc *Patella vulgata* ((Nederbragt *et al*., 2002), Figure 24 E, F), and is thus a

conserved feature across Lophotrochoazoa.

In the trunk, *otp* positive cells were found mostly along the connectives and the midline at the posterior end of the larva. The staining also detected several cells in the area where the first and second post-stomodeal commissure meet with the main trunk connectives, as well as additional cells that are not visibly associated with the axon tracts (Figure 24 G, J). The epispheral staining extends up to 72hpf (data not shown).

In conclusion, the *Platynereis otp* ortholog is likely present in 'flask-shaped' cells in the center of the APO. Its expression in the episphere is highly specific for the median brain region. Previous data from molluscs indicate that the expression in the APO is a feature highly conserved across Lophotrochozoa. The median brain expression of *Pdu-otp* is a further illustrative correlation between the median brain of trochophora-type larvae and the vertebrate ventral/ median forebrain.

To summarize the results obtained from the comparison of the four transcription factors *nk2.1, rx, vax* and *otp*, I found strong correlations between the expression of these four genes in the median regions of the *Platynereis* and vertebrate brains.

It is important to note, that the four transcription factors chosen for this comparison constitue the limited set of those transcription factors known to be most specific for the ventral/ median forebrain of vertebrates (to my knowledge). Importantly, co-expression of these genes in the vertebrate brain is highly specific for the ventral/ median forebrain region, and in particular the hypothalamus. Likewise, the *Platynereis* orthologs of these genes show early, pronounced, and spatially restricted expression in the median region of the *Platynereis* larval episphere that includes the apical organ.

2.3.2 *Orthologs of* sim, pou3 *and* gsx *transcription factors, essential for the formation of hypothalamic neuropeptidergic neurons, are also present in the apical organ region of* Platynereis *larvae*

To further explore the extent of molecular similarity between the vertebrate ventral/ median forebrain and the *Platynereis* median episphere, I investigated the expression

of three additional genes – *Pdu-sim*, *Pdu-pou3* and *Pdu-gsx* – in the episphere of *Platynereis dumerilii* larvae. Deviating from the previous examples, the vertebrate counterparts of these three genes are not as regionally specifically expressed. The set of transcription factors known to be involved in the formation of ventral/ median forebrain cell types is limited. The aforementioned factors belong to the few that have been shown to be essential for the formation of particular cell types in the vertebrate hypothalamus. Therefore, they are additional candidates to address the question whether the genetic programs involved in cell type specification downstream of regional marker genes is likely to be shared between the *Platynereis* and vertebrate neuroendocrine systems.

2.3.2.1 The *Platynereis sim* gene is present in cells of the apical organ and the median brain

The transcription factors encoded by *single-minded*/*sim* genes are characterized by the presence of both a bHLH and a PAS domain. Vertebrates have two *sim* genes that share ancestry with one ancestral pre-vertebrate *sim* gene. These two *sim* genes are expressed in a broad region of the ventro-/median CNS: in the forebrain (anterior hypothalamic nuclei, mammillary nuclei), midbrain, and the spinal cord (lateral floorplate). In addition, *sim* gene expression is found at the vertebrate brain borders (mid–/ hindbrain, pros–/ mesencephalic boundary and *zona limitans*), the cerebral cortex and the olfactory bulb. Outside the CNS, *sim* genes are transcribed in paraxial and intermediate mesoderm, the former of which gets restricted to the dermatome and the presumed limb myoblast precursors, while the latter gives rise to the developing cells of the kidney. In addition, *sim* gene expression can be found in the oral epithelium. Importantly, in the hypothalamus, *sim* is present in the neuroendocrine cells of the paraventricular, supraoptic and the anterior periventricular nuclei (for vertebrate expression patterns, refer to: Ema *et al*., 1996; Fan *et al*., 1996; Goshu *et al*., 2002; Wen *et al*., 2002). Consistent with a functional role of *sim* genes for the differentiation of cells in these nuclei, a knock-out of the murine *sim1* gene causes a lack of differentiation of the neurons containing AV, O, SS, CRH and TRH (abbreviations see above; Michaud *et al*., 1998).

Platynereis contains one *sim* homolog (*Pdu-sim*, sequence by H. Snyman, D. Arendt, unpuplished). As I determined by WMISH, *Pdu-sim* transcripts are present in cells

within the apical organ of the 48hpf larva, as well as around it (see Figure 25 C, D). Part of the *Pdu-sim* expression in the APO localizes exactly below the cilia of the 'flask-shaped' crescent cells of this organ, making it very likely that at least some *Pdu-sim* expressing cells belong to this cell type (Figure 25 C). In addition, the gene is expressed in cells surrounding the larval optic nerve (Figure 25D, white arrow). Moreover, transcripts were found in the posterior midline of the VNC, as well as in the proximal stomodeum and two lateral/ventral cell groups of the distal stomodeum. In addition, transcripts could be detected in some paired lateral cells (Figure 25 F, G). In the 72hpf larva, *Pdu-sim* expression forms a crescent in the median region of the brain (Figure 25 E). Later, expression is also maintained in the stomodeum and in the VNC (data not shown).

To resume these observations, the *Platynereis* ortholog of the vertebrate *sim* family is expressed in the median brain, although it is not specific for this region. Particularly, transcripts are found in (likely 'flask-shaped' crescent) cells of the APO. Again, this is consistent with the assumption that these *Platynereis* median brain cells compare to the vertebrate ventral/ median forebrain cells.

2.3.2.2 *Pdu-pou3* is expressed in cells of the median episphere of *Platynereis* larvae, partly overlaping with *Pdu-sim*

One gene acting downstream of *sim* family members in the murine hypothalamus is *brn2*, a *pou/homeobox* gene. It belongs to the *pouIII* family, which in vertebrates includes *brn1*, *brn2, brn4,* and most frequently one ortholog in Protostomia (see Figure 20 A). This *pou* subfamily is very broadly expressed in the vertebrate brain (Alvarez-Bolado *et al*., 1995). In particular, it is co-expressed in a subgroup of neuroendocrine neurons of the hypothalamus. Importantly, a *brn2* knock-out in mice revealed that this gene is essential for the activation of CRH, AV, O, as well as for the axon outgrowth and finally survival of these neurons (Nakai *et al*., 1995; Schonemann *et al*., 1995). Additionally, *brn2* is genetically downstream of *sim1* in a subset of the *sim1* positive nuclei (Michaud *et al*., 1998). I cloned *Platynereis pou3* (*Pdu-pou3*) in the course of the *pou* class gene screen (see above). To determine where *Pdu-pou3* is active, I performed WMISH on different stages of development. In the 24hpf larva, *Pdu-pou3* is present in three groups of cells (two bilateral dorsal groups and the apical organ (data not shown). This expression domain broadens

towards the ventral and dorsal side at 48hpf, but it stays mainly restricted to the median region (Figure 26 C). At 72hpf, *Pdu-pou3* is only expressed in some cells in the apical organ region and its surroundings. *Pdu-pou3* transcripts are also present in the stomodeum, in a broad domain in the ventral region, as well as in the chaetal sacs (data not shown). Importantly, the expression of *pou3* along the epispheral connectives at 48hpf, as well as the ventral domains at 72hpf is very reminiscent of the expression of *Pdu-sim* (Figure 25 B, D; Figure 26 B, C and data not shown). It is thus highly likely that both genes are co–expressed in these cells, consistent with the vertebrate situation.

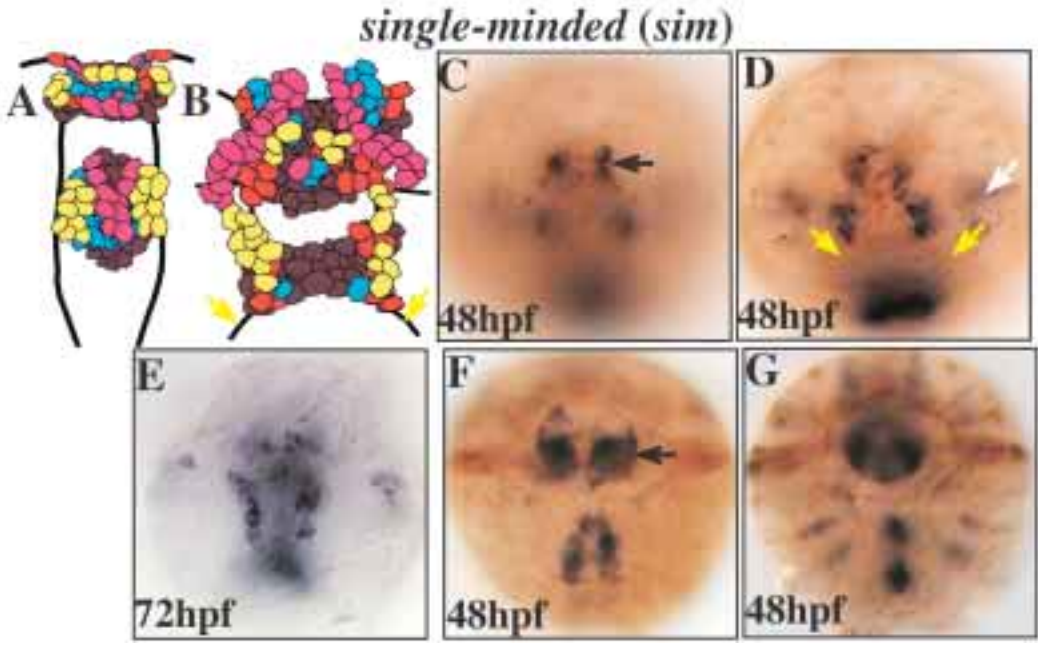


Figure 25. *single-minded/sim* expression in *Platynereis* and its comparison to vertebrates.

(C-G) As in the previous figures, brown staining results from anti-acetylated tubulin detection that demarcates ciliated structures and axon tracts. Blue: WMISH signal detecting transcripts of *Pdu-sim* at 48hpf (C, D, F, G) or 72hpf (E). **(C)** and **(D)** Apical view (dorsal up, ventral down). In C, the level of focus is at a higher plane. Black arrows in C and F point to *Pdu-sim* expression in the apical organ, white arrow in D to a staining that is still in the episphere, but out of focus and directly above the larval eye tract. **(F, G)** Ventral view at 48hpf, anterior is up, left to the right. Black arrow in F points to the transcripts in the stomodeum, the blue signal below is in the midline of the VNC. In G, the level of focus is below the one in F. **(E)** Apical view (dorsal to the top). See text for further details.

Like in the previous panels, **(A)** and **(B)** summarize the expressions in vertebrates and in *Platynereis*, respectively. For explanations see Figure 23. Color code: dark red: *nk2.1*; lighter red: *vax*; blue: *otp*; pink: *rx*.; yellow (cells): *sim*; green: *pou3*; lilac: *gsx*. As before, yellow arrows indicate the position of the stomodeal commissures in the schematics as well as on the WMISH pictures.

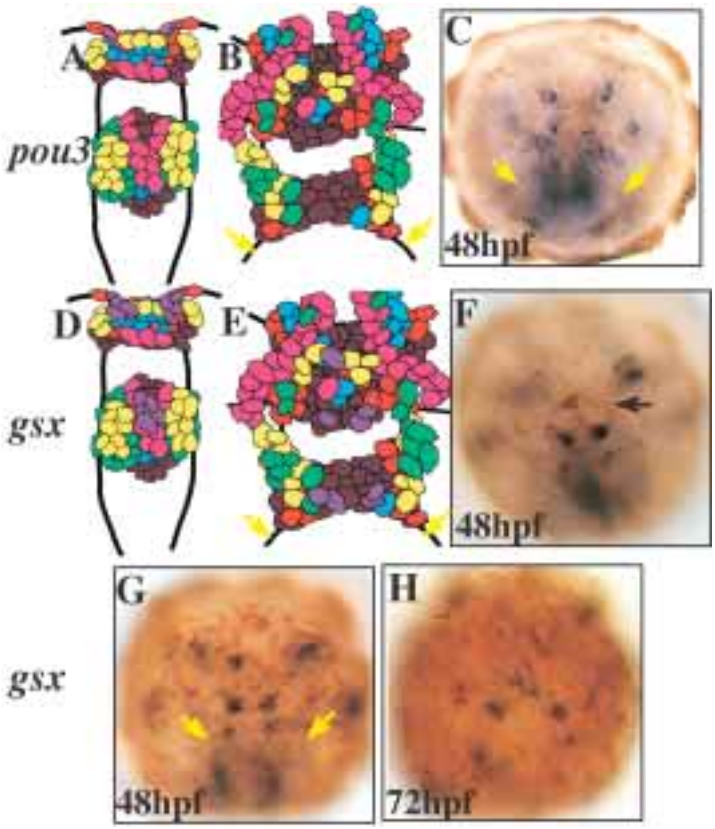


Figure 26. Expression of *pou3* and *gsx* in *Platynereis* and comparison to vertebrates.

(A, B, D, E) Like in the previous figures, schematized summaries of the expression in vertebrates (A, D) and *Platynereis* (B, C). For color code, see Figure 25

(C, F-H) Combined WMISH (blue) and anti-acetylated tubulin detection (brown), as before. **(C)** Expression of *Pdu-pou3* at 48hpf; apical view (dorsal up). Note the overlap with the *Pdu-sim* expression in the median region when compared to Figure 25. **(D, F-H)**. Expression of *Pdu-gsx;* apical view (dorsal up) at 48hpf (F, G) and 72hpf (H). Black arrow in F points to expression in the APO.

2.3.2.3 *Pdu-gsx* transcripts are present in cells of the apical organ

Finally, I included *Platynereis gsx* (*Pdu-gsx*) in my comparison. In vertebrates, two *gsx/gsh* genes exist, originating from one ancestral precursor in Bilateria. Their homeobox is related to the antennapedia class homeobox, and they are members of the parahox group. In vertebrates, *gsx* genes have so far been found to be expressed in the central nervous system only, where they are present in many regions. They occur in the spinal cord (where it specifically demarcates the intermediate colum descendants; reviewed in Arendt and Nübler-Jung, 1999), rhombencephalon, optic tectum and forebrain. In the forebrain, it is present in the telencephalon (olfactory bulb, median and lateral ganglionic eminence in mouse), the thamalus and the hypothalamus (in medaka fish and mouse). *gsh2* is required for the formation of dopaminergic neurons in the olfactory bulb and proper development of the hindbrain. Important for the context of this study, Gsh2 binds to the promoter of growth hormone releasing hormone (*ghrh*). The cells producing this peptide in the hypothalamus are the major population of parvocellular neurons not visibly affected

by a knockout of members of the *otp*, *sim*, or *pou3* family (see above). Importantly, targeted knock-out of the murine *gsh-1* gene abolishes these *ghrh*-positive parvocellular neurons in the arcuate nucleus, however not in the ventromedial hypothalamus (references for vertebrate expression and function: Deschet *et al.*, 1998; Hsieh-Li *et al.*, 1995; Li *et al.*, 1996; Mutsuga *et al.*, 2001; Szucsik *et al.*, 1997; Toresson and Campbell, 2001; Valerius *et al.*, 1995).

I investigated the expression of a *Platynereis* homolog of *gsx* (*Pdu-gsx*, gift of D. Ferrier, unpublished) by WMISH. As far as I could determine, *Pdu-gsx* expression is restricted to the central nervous system and two bilateral symmetric stripes in the stomodeum at all stages examined (up to 72hpf**).** In the ventral nerve cord, transcripts appear in two stripe-like domains that fuse at the posterior and reside between the two main connectives. Additionally, two spots of expression can be detected in the peristomal region (data not shown). The epispheral expression shows two lateral and one median domain(s) (Figure 26F,G). The ventro-lateral domains partly follow the axon tract that is joined by the larval eye axon (data not shown). The cells of the ventral part of the median domain are located above the stomodeal commissure, the most dorsal cells are located directly within the crescent of the 'flasked-shaped' crescent cells of the apical organ, to which at least some of them belong (Figure 26F,G). At 72hpf, the VNC expression is restricted to the third segment and the border between the second and third segment. Few cells (one/two) are staining at the border between the peristome and the first segment at this stage. The stomodeal expression changes only little (data not shown). The epispheral expression is restricted to the median region, with the exception of two cells staining dorso-laterally and most likely belonging to the nuchal organs (Figure 26H). Therefore, *Pdu-gsx* is present in the *Platynereis* larval brain, where its expression is present in the *Pdu-nk2.1* positive region, the median brain and cells of the APO. However, *Pdu-gsx* is not specific for the median brain and APO (as is also the case for the vertebrate orthologs, which are not excusively expressed the hypothalamus).

In summary, I have shown that (1) *nk2.1*, *rx*, *vax* and *otp,* highly specific for the vertebrate ventral/ median forebrain, are expressed specifically, and partly overlap, in the median *Platynereis* brain including the APO. In addition, I have shown that (2) the *Platynereis* orthologs of the *sim*, *pou3* and *gsx* families, members of which have

been shown to be essential for development of peptidergic cell types of the hypothalamus, also localize to the median region of the larval *Platynereis* brain including the apical organ. Notably, not all of the expression domains are located within the median region, but there is at least a substantial mutual overlap between these genes and with the *pdu-nk2.1* expression in this region of the larval brain. Thus, all available information so far strongly supports homology of the median region of the prospective *Platynereis* brain with the embryonic anlage of the vertebrate hypothalamus (see final schematics in Figure 40 A, B, and compare to Figure 13A, BFigure 23 A-D, G, H, Figure 24 A, BFigure 25 A, B Figure 26 A, B, D, E), inferring that this region already had an equivalent in the urbilaterian ancestor. In the following sections, I explored how this equivalence is reflected on the level of differentiated cell types.

2.3.3 Analysis of markers that indicate specific differentiated cell types and exist in the median/ventral forebrain of vertebrates

In the preceding analysis, I primarily focused on molecular regulators that are characteristic for early developmental phases and specify either whole brain regions or larger populations of precursor cells. My major conclusion was that the *Platynereis* larval median brain region (including the apical organ) shares extensive similarities on the level of the transcription factors with the median ventral forebrain of vertebrates (including the hypothalamus), inferring that these areas derive from the same region in the hypothetical ancestral brain of Urbilateria. I then tried to address to which extent these areas also display similarities in the differentiation of particular cell types, implying functional equivalence. I chose to analyse neuropeptides and their precursors, rate-limiting enzymes involved in the synthesis of histamine and dopamine, as well as morphological and cell physiological criteria.

2.3.3.1 Three neuropeptide precursors are highly conserved across Bilateria

The general picture from the analyses of the genomes of the so far sequenced Ecdysozoa is, that many neuropeptides, such as AV, O, CRH, TRH or GHRH, do not exist outside deuterostomian groups (see introduction). Inportantly, these are among

the best characterized downstream targets of several transcription factors analysed above. If my hypothesis about the homology of the median part of the *Platynereis* episphere and the vertebrate ventral/ median forebrain is correct, one would strongly expect these neuropeptides to be present in *Platynereis*. I therefore analyzed additional available sequences and literature from protostomian species to determine if orthologs of these neuropeptides exist in protostomian species other than the common invertebrate model organisms. This is in fact the case. My analyses of the available sequences showed that, by the principle of evolutionary parsimony, *Platynereis* should possess orthologs of at least three neuropeptide precursors whose vertebrate counterparts are downstream targets of the transcription factors analysed above: First, Arginine-Vasopressin (AV) and Oxytocin (O) are among the peptides absent in the mouse knock-outs of the transcription factors *otp*, *sim1* or *brn2* (see above). AV, O, as well as the Isotocin are likely vertebrate paralogs that are encoded in one precursor together with the vertebrate Neurophysin I and Neurophysin II paralogs. Therefore, this group is probably equivalent to a single ancestral bilaterian precursor molecule, which contained at least the Vasopressin-related peptides together with the Neurophysin- related peptides (Satake *et al*., 1999; van Kesteren *et al*., 1992 Figure 27B). Consistent with a conserved role of AV/O peptide family, the receptors, which sense these peptides in vertebrates are well conserved in Lophotrochozoa (Figure 27A). Second, the vertebrate peptide GHRH, absent in the mouse knock-out of the *gsx1* gene, is a member of the multiple peptide precursor PACAP (*pituitary adenylate cyclase–activating polypeptide*)/ glucagon superfamily (Sherwood *et al*., 2000), that is even conserved in the cnidarian *Hydra magnipapillata (*accession#: BAC21156). The third pro-peptide, POMC, contains (among other peptides) alpha-MSH. POMC and its cleavage product alpha-MSH are present in the arcuate nucleus of the vertebrate hypothalamus, a nucleus strongly affected in the *otp* and *nk2.1* knock-out mouse, (see above and Pritchard *et al*., 2002). A POMC peptide highly similar to the vertebrate peptide has been recently isolated from the leech (Salzet *et al*., 1997), and is thus likely to be present in *Platynereis*, too.

In summary, this theoretical analysis strongly suggests that Urbilateria already possessed a complex set of neuropeptides, ancestral to the set found in today's

vertebrates. This makes it conceivable that not only the transcription factors of the vertebrate ventral/ median brain are present in the median brain of the *Platynereis* larva, but also at least some of their downstream targets.

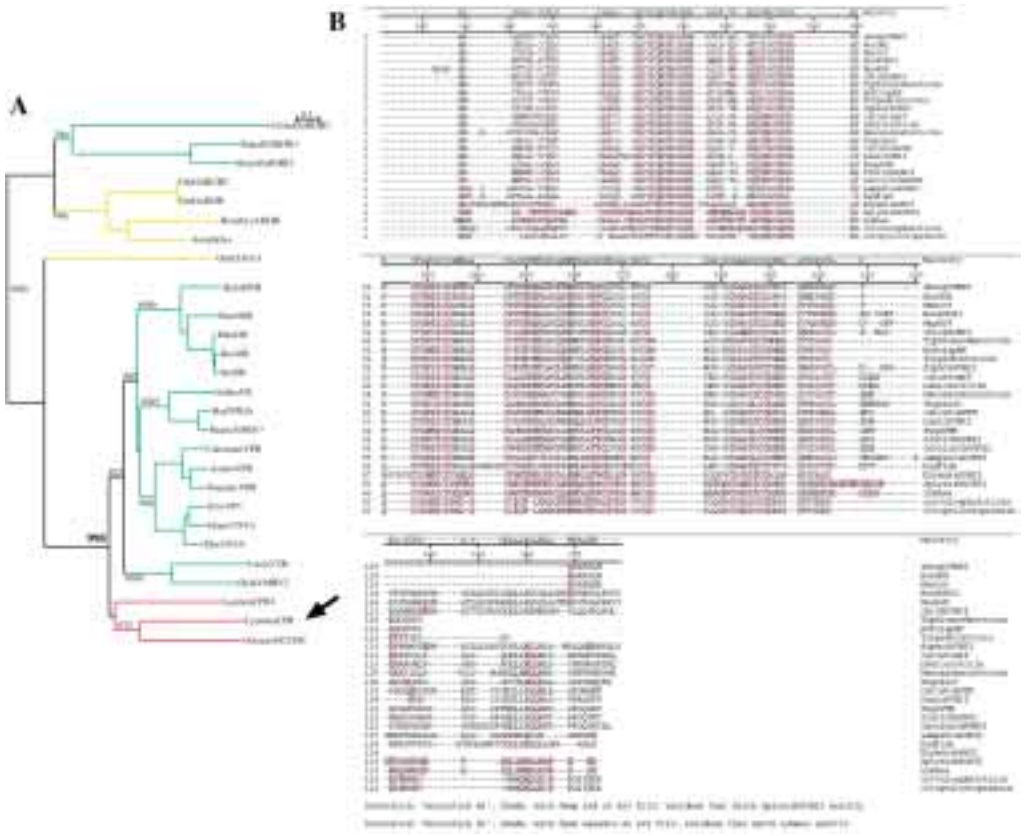


Figure 27. Conservation of the VPOneurophysin and its receptor across Bilateria.

(A) Phylogenetic tree of the VPO receptor, a member of the 7TMR subfamily. The tree was calculated using AA sequences. Numbers at the branch points are bootstrap values. Its closest relative, the GnRH receptor was taken as an outgroup (for the *Platynereis* member of this group see Figure 29). Yellow- Ecdyzoa, green- Deuterostomia, red- Lophotrochozoa (arrow). The clustering together of lophotrochozoans and vertbrate sequences is artifical, because of the derivedness of the insect receptor. **(B)** AA alignment and comparison of VPOneurophysin precursors that have already been cloned in Bilateria. Color code: all AA residues identical to the AA of the *Lymnea stagnalis* VPOneurophysin precursor appear in pink, all those that are not identical to the *Lymnea stagnalis* protein, but to the precursor of *Aplysia californica* are red. No ecdysozoan ortholog has been identified yet, despite the high conservation between Lophotrochozoa and vertebrates.

The alignments and accession numbers belonging to these phylogenetic trees can be found in the Appendix.

To further continue my brain comparison on the cell type level, I focused next on four molecules, demarcating distinct post-mitotic differentiating cell types present within the ventral/ median forebrain of vertebrates. Two of the four molecules, RFamide(s) and *histidine decarboxylase* (the rate limiting enzyme for the production of histamine) are highly specific for this region in vertebrates. Two others, serotonin

(as well as one of the rate limiting enzymes for the production of serotonin, *dopa decarboxylase*) and the *gonadotropin releasing hormone receptor* (*gnrh-r*) are less specific in the vertebrate brain, but nevertheless useful for my brain comparison, because their major expression sites in vertebrates are in the ventral/ median forebrain, and especially in the hypothalamus. Unfortunately, for none of the four molecules any transcription factor controlling their presence in the vertebrate ventral/ median forebrain has been described.

2.3.3.2 RFamide(s) is present in 'flask-shaped' center cells of the *Platynereis* apical organ

RFamides comprise a whole family of small carboxy– terminally alpha amidated peptides, which exhibit the c-terminal sequence –Phe (or Tyr) –Met (or Leu) –Arg –Phe-NH_2 for their biological functions. This family has cardioexcitatory effects and is believed to counteract opiates (Mercier *et al*., 2003; Raffa, 1988). The peptides are very ancient, since members of this family (most commonly FMRFamide) are already present in neurons of Placozoa and Cnidaria, but can equally well be detected in the nervous systems of every bilaterian species investigated so far (Schuchert, 1993; Thorndyke and Goldsworthy, 1988). Besides the presence of FMRFamide positive neurons in the spinal cord, only two additional populations of FMRFamide positive neurons exist in the vertebrate CNS. One population originates in the region of the olfactory placode, and migrates into the basal forebrain along the nervus terminalis. The other population first appears in the circumventricular organs of fish and in the preoptic area and hypothalamus of tetrapods, where it is maintained. In contrast to the first, this second population does not derive from the migratory placodal cells. FMRF positive neurons of the latter population have been identified as CSF-contacting neurons in the poa and hypothalamic region of fish and amphibians (Fiorentino *et al*., 2001; Jirikowski *et al*., 1984; Krishna and Subhedar, 1992; Rastogi *et al*., 2001; Wicht and Northcutt, 1992; Wirsig-Wiechmann *et al*., 2002). Important for my comparison with the *Platynereis* RFamide(s) positive cells, RFamide(s) in the vertebrate hypothalamus is present in cells of the arcuate nucleus (and its corresponding structures in lower vertebrates), where it overlaps with the expression of *nk2.1*. Moreover, this nucleus is completely absent in the *nk2.1*-/- mouse (Chen *et al*., 1989; Krishna and Subhedar, 1992 and see above about *nk2.1*).

Additionally RFamide(s) positive cells are present in the preoptic nucleus of the hypothalamus of non-mammalian vertebrates (see references three sentences above).

I determined the expression of *Platynereis* RFamide(s) by antibody stainings. No staining could be detected at 15hpf and 19hpf. At 24hpf little blue dots could be detected at the posterior pole at the larvae, that in account of their absence in control embryos represent true staining. Very rarely, one single cell in the apical organ region can be detected at this time point (data not shown). At 48hpf a cluster of app. 8 cells stains in the apical organ and adjacent ventro-median brain area. The cells of the apical organ have a peculiar shape. An apical process extends to the apical surface, where it splits into several little extensions (Figure 28L,N, compare also to C). These little extensions appear to hang into the surrounding outside (Figure 28L,N, compare also to C). Because of their special shape I call these'flask-shaped' center cell of the APO (green and yellow cell in the middle of the schematic in Figure 28 M), distinct from the 'flask-shaped' crescent cell of the APO (grey cells in the middle of the schematic in Figure 28 M). The latter comprise the crescent of cells, the cilia of which are made visible by the anti-acetylated tubulin antibody staining (green arrows in Figure 28C). The'flask-shaped' center cell of the APO also exhibit high transcript levels of *Pdu-nk2.1* (Figure 14 D, E). During subsequent stages more cells are added to the median brain domain. These cells are maintained, since they can be followed to one month of development, where they finally locate in between the adult eyes (see Figure 29 I and data not shown) In addition, RFamide(s) positive cells can be detected in the VNC, starting from app. 72hpf onwards, and in the nuchal organ region, starting from app. 65hpf onwards (data not shown).

In summary, RFamide(s) are highly specifically localized to few cells of the median brain area of *Platynereis* larvae. These include a specific group of APO cells, the 'flask-shaped' center cell, also positive for the *Pdu-nk2.1* gene. This situation is very reminiscent to the vertebrate hypothalamus, where RFamide(s) positive cells localize into a nucleus expressing and depending on the vertebrate *nk2.1* gene. This indicates that not only transcription factors orchestrating the development of cells of the median forebrain areas are conserved between vertebrates and *Platynereis* larvae, but that these transcription factors appear to specify homologous cell types.

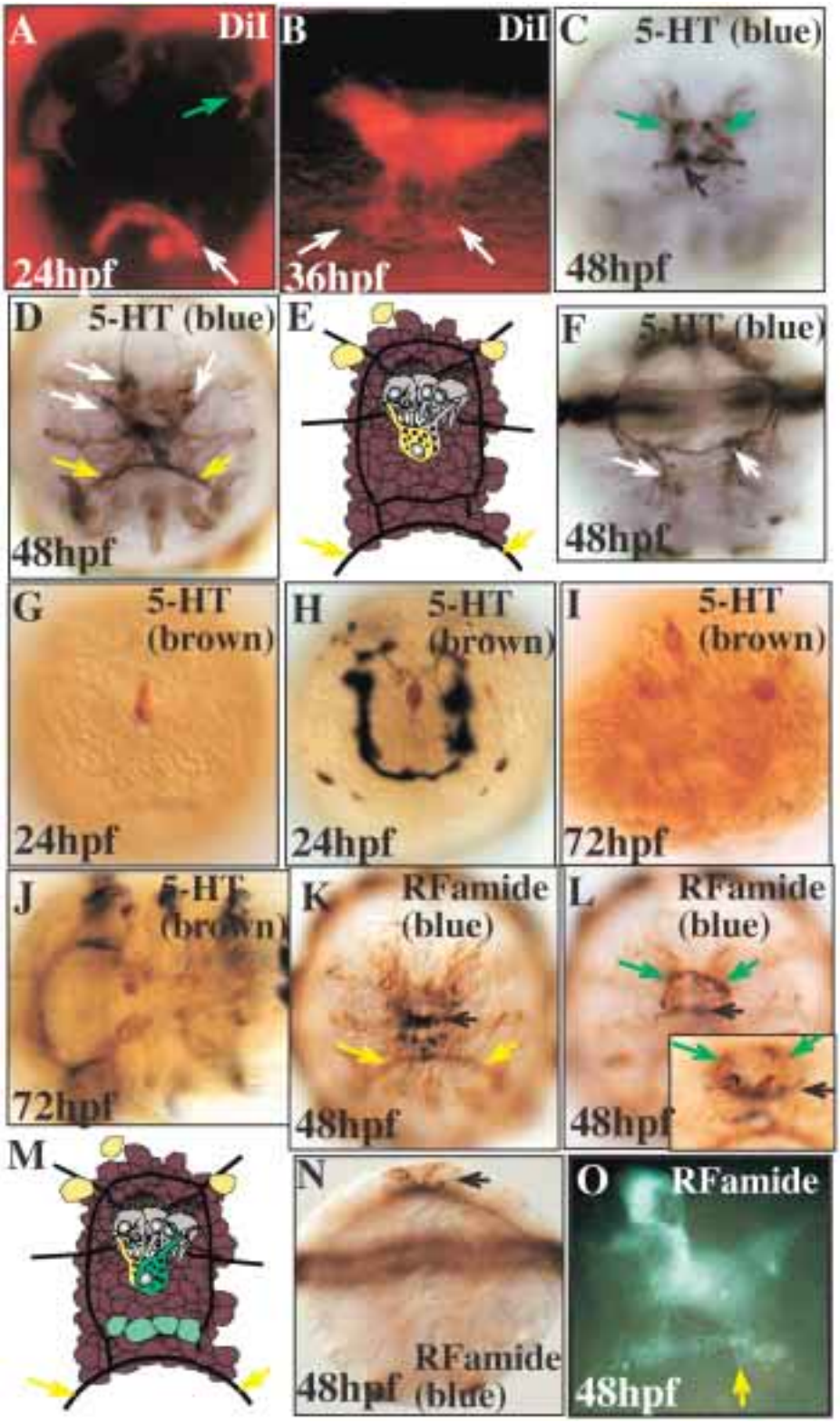


Figure 28. The apical plexus as stained with DiI; and the analyses of RFamide(s) and serotonin in *Platynereis* larvae.

(A, B) Staining of living *Platynereis* larvae with the lipophilic dye DiI (red). A) 24hpf, looking from slightly tilted angle dorso-apically onto the animal. The white arrow marks the 'flask-shaped' crescent cells of the APO (compare to green arrows in C and L, and schematized as grey cells in E and M). Occasionally, 1-2 APO cells encircled by the 'flask-shaped' crescent cells stain as well (red dot below the white arrow). These belong to the group of 'flask-shaped' center cells of the APO (also pointed at by black arrow in L, N). In addition to the cells of the APO, prototrochal cells take up the DiI as well. This visualizes the long extensions they stretch into the episphere (green arrow). B) Ventral view onto an app. 36hpf larva. The two white arrows point to the apical plexus, formed by the neurite extensions of the DiI staining APO cells. **(C-J)** Anti-serotonin antibody stainings of *Platynereis* larvae. Negative controls never showed any staining. C, D,F) 48hpf, blue stainings are serotonergic cells, brown staining is anti-act.tub staining (demarcating ciliated structures and axon tracts). G)-J) Brown stainings are serotonergic cells, blue staining is anti-act.tub staining (demarcating ciliated structures and axon tracts). C) Apical view, green arrows point to the crescent cells of the APO, black arrow point to a serotonin-positive cell that has a 'flask-shaped' appearance and dendritic extensions reaching the surface, defining them as 'flask-shaped' center cells of the APO (also compare to N). D) Slightly deeper focus than C) Black arrow points to the same cells as in C). White arrows point to three additional serotonin positive cells. However, these don't have the extensions to the surface. Yellow arrows for comparison to the schematic shown in E). E) Schematic as introduced in Figure 23. Yellow cells represent serotonergic cells. Yellow arrows pointing to the stomodeal commissure is for comparisons to the real pictures. F) Ventral view. White arrows point to some serotonergic cells occurring in the VNC. G) Apical view (dorsal to the top) at 24hpf. The first cell containing serotonin in the episphere is localised within the APO. The only other cell that stains at this time point is on the opposite site, as visualized in a blastoporal/ posterior view in H). I), J) Detection of serotonergic cells at 72hpf. I) Apical view (dorsal to the top) and J) ventral view (apical to the left). K,L,N,O) Anti-RFamide(s) antibody stainings of *Platynereis* larvae. Negative controls never showed any staining. **(K,L,N)** 48hpf, blue stainings are RFamide(s) positive cells, brown staining is anti-act.tub staining (demarcating ciliated structures and axon tracts). K) Apical view (dorsal to the top). Black arrow point to the 'flasked-shaped' cells (at least four) of the APO positive for RFamide(s). Yellow arrows for an approximate comparison to M. L) Same as in K) with focus being on a slightly upper level. Green arrows point to the crescent cells of the APO. Black arrow has the same position as in K). The larva was tilted slightly to the ventral, allowing an apical –ventral view in the inset, gving a better view onto the 'flasked-shaped' cells of the APO. **(M)** Schematic as introduced in Figure 23. Compare to E) for yellow cells, green cells represent RFamide(s) positive cells. N) Lateral view onto the same larva. Black arrow points to the same cells as in K) and L). Note their extensions (stained by brownish by anti-act.tubulin staining) reaching to the surface. **(O)** The picture shows an anti-RFamide(s) staining of one of these cells. The secondary antibody was fluorescent. Note the long extensions going down and filled with dots (yellow arrow), indicating dense-core vesicles containing RFamide(s).

2.3.3.3 Histaminergic cells are located in the median brain of *Platynereis* larvae and maintained into adulthood

Besides RFamide(s), the other marker highly specific for one group of differentiated cells in the ventral/ median vertebrate forebrain is *histidine decarboxylase* (*hdc*). *hdc* is the specific rate limiting enzyme for histamine biosynthesis (Michal, 1999). This amine is most famous for its role in the mast cells during the inflammatory response (see standard medical textbooks). Histamine however is also present in neurons of the CNS. It is important to note for my median brain cell type comparison, that in all vertebrates, except lamprey, histamine has been found to be restricted to only a few cells within the hypothalamus, i.e. in mammals: tuberomammillary nucleus, in fish: along the whole length of the posterior recess (Eriksson *et al.*, 1998; Haas and

Panula, 2003; Kaslin and Panula, 2001). Its major distribution in lamprey is also in the hypothalamus, however in this animal some histamine positive cells are also present at the ventral base of the mes-/metencephalic boundary, near the reticular nucleus (Brodin *et al*., 1990). Sleep behavior is the only function brain histamine has been associated with, at least indirectly, referring to the direct influence of *orexin/ hypocreatin* on histamine levels in the hypothalamus (Mignot *et al*., 2002). Mice lacking the *orexin/ hypocreatin* gene exhibit a severe narcoleptic phenotype (Chemelli *et al*., 1999). In arthropods, histamine is also present in specific cells of the brain, the functions of which are mostly unclear. The only known function of histamine is in the rhabdomeric PRCs of the arthropod eyes, where it has been shown to serve as transmitter (see above). As mentioned above, I cloned a *Pdu-hdc* N-terminal fragment. However, when I wanted to closer investigate its expression by WMISH, I found that unfortunately, *Pdu-hdc* was repeatedly hardly/ not at all detectable at 24hpf and 48hpf. The reason for this is unresolved, but probably represents an artifact and not a lack of transcript at these timepoints. It is unlikely that no histaminergic cell has differentiated at 48hpf, a stage were almost all analysed differentiated cell type makers (except *Pdu-ci-opsin2*) exhibit expression. Thus only the expression at 72hpf and later stages can be reliably described. Besides its presence in the larval eye area at this stage (Figure 21 C), additional groups of cells stain, the majority of which is located in the median region. Important for this study, a ventral group of histaminergic neurons is located medially within the *Pdu-nk2.1* positive area, a dorsal-median group is at the dorsal border of the *Pdu-nk2.1* expression (Figure 29B). None of the cells belongs to the restricted population APO cells. In addition, a pair of *Pdu-hdc* positive cells is present at the base of the antenna (data not shown). *Pdu-hdc* expression is also present in cells of the peristome and in a pair of cells in the stomodeum two days later. The *Pdu-hdc* staining can be followed long into adulthood (as was also observed for the median epispheral cells containing RFamide(s)). The final position of the median cells is the median adult brain in a region between the two adult eyes (Figure 29J).

As a short synopsis of my investigations on histaminergic cells in *Platynereis* larvae and growing worms, it can be stated that, besides the histaminergic cells in the larvals eye region, two prominent groups of histminergic cells are apparent in the

median brain of *Platynereis*. Cells of these median groups are maintained into adulthood at a very similar position. The median populations of histaminergic brain cells can be considered as further evidence for the validity of the idea that the median brains of *Platynereis* larvae and vertebrates share homologous cell types, given the fact that the only conserved histaminergic population of neurons in the vertebrate brain locates to the hypothalamus.

2.3.3.4 Serotonin is present in highly asymmetric cells in the *Platynereis* larval episphere, including one 'flask-shaped' center cell of the apical organ

Serotonin is the third marker for differentiated cell types used to investigate possible similarities between the ventral/ median forebrain of vertebrates and the median episphere of *Platynereis*. Serotonin or 5–hydroxytryptamine (5-HT) is an ancient biogenic amine that has been found in the brain of different species of all triploblastic phyla investigated (Hay-Schmidt, 2000). In contrast to histamine or RFamide(s), whose presence is very restricted to one or two subpopulations in the vertebrate forebrain, serotonin is present in more regions of the vertebrate brain. However, besides the strong cross-bilaterian conservation of the location of serotonin positive cells (see below), there are two reasons why serotonin is a very useful marker for my comparative analysis. First, despite of the less specific distribution of 5-HT positive cells in the vertebrate brain, they are particularly enriched in the diencephalon of lower vertebrate brains, where many of these are neurons of the developing and adult hypothalamus (in fish: preoptic recess organ, paraventricular organ, posterior recess nucleus; see Kaslin and Panula, 2001; Weigle and Northcutt, 1999). Second, most of these serotonergic cells of the ventral median brain are a special type of neuron, the central spinal fluid contacting neurons (CSF contacting neurons; Vigh and Vigh-Teichmann, 1998). As implicated in the name, CSF contacting neurons are in direct contact with the central spinal fluid (Vigh and Vigh-Teichmann, 1998). Topologically, the ventricular (apical) surface of the neuroepithelium corresponds to the outer surface of a ground-state neural plate like that of the *Platynereis* larva. CSF neurons might therefore derive from ancestral cells that were in contact with the seawater in marine Urbilateria (see discussion) Notably, adult mammals do not possess these hypothalamic 5-HT neurons anymore. Still,

transient expression of 5-HT has been found in substance P positive neurons of the developing mouse hypothalamus (Ni and Jonakait, 1989), supporting the notion that the loss of 5-HT positive neurons in mammals is as a secondary feature, and that the situation observed in lower vertebrates, where these cells persist to adulthood, reflects a more ancestral state.

Apart from the vertebrate ventral median forebrain, serotonergic cells exist as well at other places in the forebrain in all vertebrates, such as the dorsal and ventral thalamus and the pineal gland (Kaslin and Panula, 2001). The other major regions containing 5-HT positive cells are the Raphe nuclei, located at the median base of the midbrain. These nuclei are the major serotonergic nuclei of the mammalian brain (Kandel *et al*., 2000).

Serotonin has also already been investigated in several lophotrochozoan and deuterostomian primary ciliated larvae. In Lophotrochozoa the first cells that can be detected in the trochophora larva belong to the apical organ. In molluscs they have been described as a special type of cells, the type I and II parampullary cells, of which type I are sensory cells (Kempf *et al*., 1997 and see introduction). 5-HT positive cells are as well present in a median position of the apical ganglion (=larval brain) in the larva of echinoderms, and in the apical ganglion of the enteropneust tornaria larva (Beer *et al*., 2001; Hay-Schmidt, 2000). The presence of a group of 5-HT cells in the apical ganglion is conserved across bilaterian primary ciliated larvae (Hay-Schmidt, 2000).

Although apparently many studies have been performed to determine the localization of serotonin in detail in different species, nobody (to my knowledge) has so far analysed these data in conjunction with any expression data available for transcription factors. My comprehensive analysis of transcription factors present in the median brain of *Platynereis* larvae, the knowledge that serotonergic cells are rather specifically present in the median larval brain regions of primary cilated larvae across Bilateria, combined with the notion that serotonergic cells are highly prominent in the hypothalamus of non-mammalian vertebrates, prompted me to complement my data from above with an analysis of serotonergic cells in *Platynereis* larvae.

I investigated the location of 5-HT positive cells in the *Platynereis* larval brain, using an antibody directed against this amine. Moreover, I confirmed the presence of serotonin in the immunoreactive cells by performing WMISH with a riboprobe directed against a fragment of *Platynereis dopa-decaboxylase* (data not shown), one rate limiting enzyme in serotonin biosynthesis (Michal, 1999). No specific 5-HT staining could be detected in the larvae at 15hpf, when compared with a negative control (incubated only with the secondary antibody). The first cells are visible at 24hpf, as one cell staining at the apical and one at the posterior pole (Figure 28G,H). The cell at the apical pole belongs to the APO. More cells are added to this during subsequent development. Their total number is four to five between two and three days of development (see control animals in Table 1). The axons of these cells join the prototrochal nerve and by this encircle the episphere (Figure 38 E, G). Important for my ventral/ median brain comparison, at least one of the serotonegic cells exhibits long dendritic extensions reaching up to the outer surface, identifying this cell as a 'flask-shaped' center cell of the APO (Figure 28 C, D, E). This cell most likely also contains RFamide(s) (compare to Figure 28 K, L, N) and expresses *Pdu-nk2.1* (compare to Figure 14 D, E).

Two additional observations are interesting to note. First, the 5-HT positive cells are one of the rare cases of cells that stay asymmetric beyond 72hpf (Figure 28I). This is interesting, because the origins and molecular mechanisms leading to asymmetries in the brains of different animals are only little understood. Most genes that are asymmetrically expressed in *Platynereis* during earlier developmental stages, become symmetrical between two and three days of development, as this is the case for *Pdu-ptx* (data not shown). Second, serotonergic cells can be found in the VNC as early as at 48hpf (Figure 28F). The most prominent positive cells in the VNC at this time are located above the first post-stomodeal commissure. The amount of the cells in the VNC increases during subsequent stages (Figure 28J).

In conclusion, serotonin occurs first and most prominently in the APO and median brain of *Platynereis* larvae. At least one of the serotonergic cells belongs to the group of 'flask-shaped' center cells of the APO. This cell is highly likely to co-express *Pdu-nk2.1* and produce RFamide(s). The other serotonergic cells in the median episphere are located at the dorsal- median border of *Pdu-nk2.1* expression, in a

region demarcated by the expression of three genes *Pdu-otp*, *Pdu-vax* and *Pdu-rx* (see above). This predominant location of larval serotonergic cells in an epispheral region otherwise demarcated by vertebrate ventral/ median brain markers is highly reminiscent of the vertebrate situation, where serotonerigic cells are prominently present in the vertebrate hypothalamus.

2.3.3.5 The *Platynereis gnrh-receptor* gene is present in the median region of the larval episphere and overlaps with *Pdu-nk2.1* expression

The fourth example of a molecule present in differentiated cells in the vertebrate ventral/ median forebrain, and thus a candidate for the median forebrain comparison between vertebrates and *Platynereis*, is the *gonadotropin-releasing hormone receptor*. *Gonadotropin-releasing hormone* (*gnrh* or *lhrh*) and its receptors (*gnrh-r* or *lhrh-r*) are best known for their role in the hypothalamus-pituitary axis (Norris, 1997). Several vertebrate paralogs of the *gnrh-r* exist (see Figure 30). In the "classical" case, *gnrh* acts as a classical releasing hormone on the *gnrh-r* of the gonadotropic cells of the pituitary leading to the release of *gonadotropins* LH and FSH from the pituitary cells. These molecules have various effects on sexual maturation and development (Norris, 1997). Less known, the vertebrate *gnrh-r* genes have also prominent expression sites within the vertebrate forebrain. Vertebrate specific genome duplications resulted in three *gnrh-r* subgroups (Jodo *et al*., 2003). *gnrh-r* paralogs are prominently present in the median/ ventral forebrain of vertebrates, notably in the ventral hypothalamus (mammals: arcuate, ventromedial nuclei of hypothalamus, fish: nucleus lateralis tuberis) and the preoptic area (*Oncorynches mykiss,* goldfish). In addition expression is present in restricted areas in the optic tectum (*O.mykiss*, goldfish), in the telencephalon (cells in addition to the poa in goldfish and mouse, olfactory bulb (goldfish)), periventricular nucleus of thalamus (mouse) and dorsal cerebellum (goldfish) (for expression in vertebrates see: Jennes and Conn, 1994; Jennes *et al*., 1997; Madigou *et al*., 2000; Peter *et al*., 2003). The expression in the mammalian brain is believed to be also involved in reproductive behavior (Jennes *et al*., 1997). In the context of this thesis it is important to note, that the expression sites of the vertebrate *gnrh-r* genes in the hypothalamic (arcuate nucleus, ventromedial nucleus), as well as the preoptic area

are located within the *nk2.1* expression territory (see above and Kimura *et al*., 1996; Nakamura *et al*., 2001). Moreover, the arcuate nucleus is absent and the ventromedial nucleus strongly effected the in *nk2.1* knockout mouse. The preoptic area also expresses the *vax* gene, and this area is absent in *vax1* knock-out mouse (see above). These facts make the *Platynereis gnrh-r* ortholog a useful differentiated cell type marker for a comparison between the ventral/ median vertebrate brain and the *Platynereis* median episphere. I thus investigated, if a *Pdu-gnrh-r* is present in the *Pdu-nk2.1* and *Pdu-vax* positive area in the larval episphere of *Platynereis*. I cloned and investigated the expression of a fragment of the *Pdu-gnrh-r*. The *Pdu-gnrh-r* fragment I cloned, encodes the segment between the second and the sixth transmembrane helix and is a true *gnrh-r* ortholog (Figure 30). I determined the expression of this fragment by WMISH using a riboprobe directed against this *Pdu-gnrh-r* fragment. Transcripts were first detectable at 48hpf. In the trunk, two cells are visible in the peristomeal region, between the stomodeum and a prominent lateral neurite tract, the latter visualized by an anti-acetylated tubulin antibody. In the episphere, the most prominent cell is located behind the ciliated crescent of the apical organ in the dorso-median region (Figure 29E).

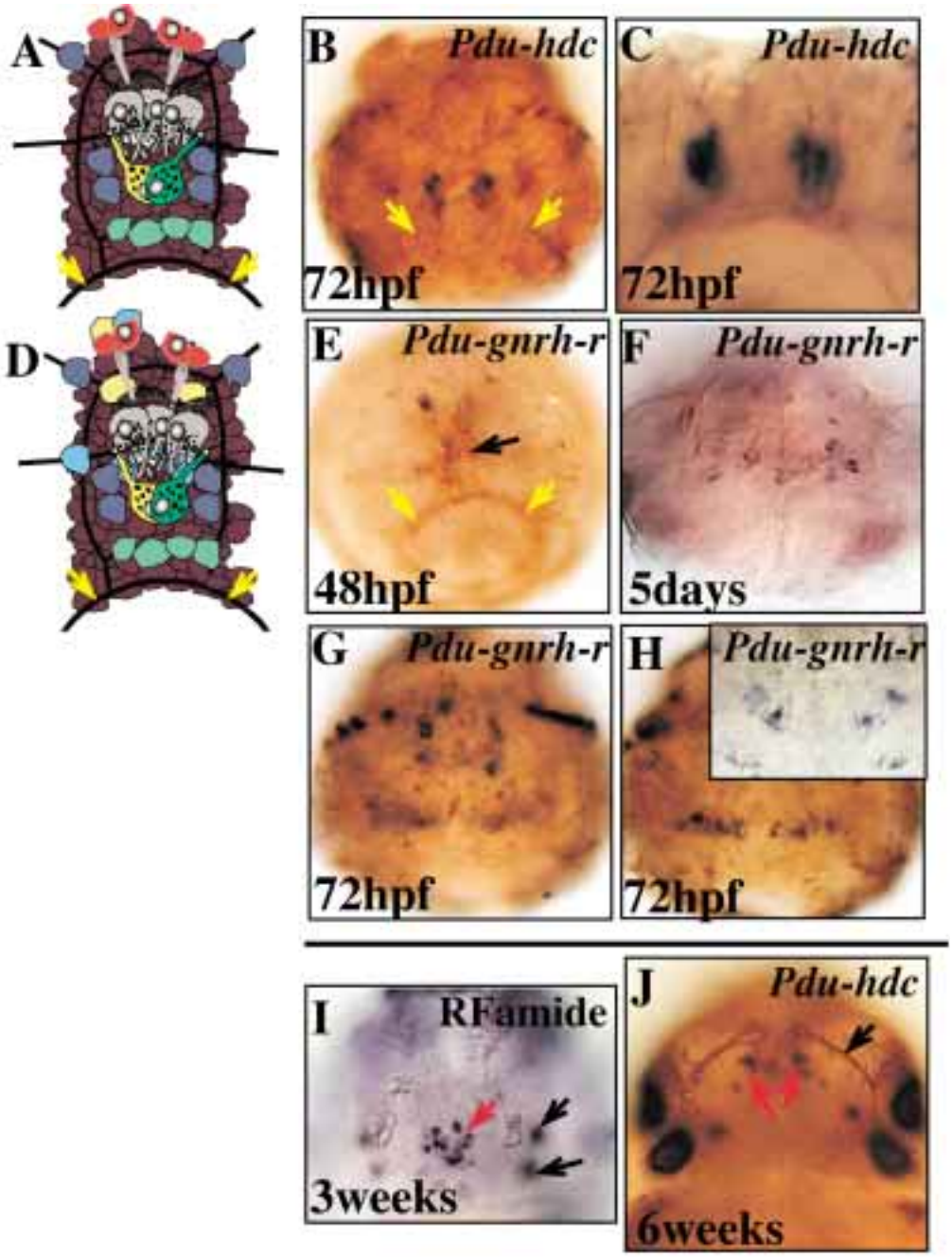


Figure 29.Analysis of *gnrh-r* and *hdc* in *Platynereis*.

Pictures: Blue stainings are cells stained by WMISH, brown staining is anti-act.tub staining (demarcating ciliated structures and axon tracts). For **(A)** and **(D)**: Schematic as introduced in Figure 23 and Figure 28. Yellow-serotonergic cells, green- RFamide(s), red- ciliary PRCs, dark blue- *Pdu-hdc* expression, light blue- *Pdu-gnrh-r* expression. Yellow arrows pointing to the stomodeal commissure is for comparisons to the real pictures. **(B),(C)** Expression of *Pdu-hdc* at 72hpf. B) Apical view (dorsal to the top). Same animal as in Figure 21C, upper focus. Yellow arrows for comparison to A). C) Ventral view at 72hpf, magnifying the median median *Pdu-hdc* positive brain cells. They are located at the very base of the brain. **(E-H)** Expression of *Pdu-gnrh-r*. E) 48hpf, apical view (dorsal to the top). Black arrow points to weak, but real expression in the APO. Yellow arrows for comparison to D). F) Apical view at 5days (dorsal up). G) and H) Apical views at 72hpf (dorsal up). Focus on

more dorsal brain cells in G) and more ventral in H). Note the dotted appearance of the staining in the ventral brain cells (inset in H). **(I)** and **(J)** Maintenance of larval epispheral cells in later development. Dorsal views onto a 3 and a 6 week-old worm. (Note that the spatial relationship between brain and trunk changes during development. The brain extends and gets bend towards the back. Former apical cells will thus now be visible from the dorsal side. Red arrows point to the former median apical brain cells, which are: I) RFamide(s) and J) *Pdu-hdc* positive. Black arrows in I) point to the adult eyes (also visible as big black lateral dots in J) and black arrow in J) points to the adult eye projection.

This cell likely overlaps with the dorsal part of the *Pdu-vax* expression, but is at the dorsal border of the *Pdu-nk2.1* expression domain (compare to Figure 13 C, E, Figure 14 D, Figure 23 E, F). In addition, several cells showing lower transcript levels of *Pdu-gnrh-r* are located directly in the middle epispheral region, in the apical organ (Figure 29E). These cells are clearly located in the *Pdu-nk2.1* expression domain (compare to Figure 13 C, E, Figure 14 D). No cells are visible outside the median region in the episphere. At 72hrs, cells in the ventral–median region stain strongly, in addition cells in the dorso–median region can be detected as well (Figure 29G,H). Again, compared to the *Pdu-nk2.1* and *Pdu-vax* expression domain, the majority of the *Pdu-gnrh-r* cells are located within these domains. The *Pdu-gnrh-r* staining of the ventral–median cells is interesting for an additional reason. It repeatedly exhibits a dotted appearance (Figure 29H). This is unusual for WMISH stainings. Usually the nuclei of the brain cells are very large, leaving only a small stripe of cytoplasm, where the mRNA is located by the WMISH method. The dotted appearance indicated that the cytoplasm is full of granules (a typical feature of secretory cells), and the detected mRNA is located between them, yielding the dotted appearance observed. More cells stain in the dorso-median region three days later (Figure 29F). The *Pdu-gnrh-r* positive cells closely surround the ciliary extensions of the ciliary PRCs (data not shown). The *Pdu-gnrh-r* positive ventral–median cells are still present. Few additional *Pdu-gnrh-r* positive cells can be detected in the lateral region of the episphere at the age of 5 days (data not shown).

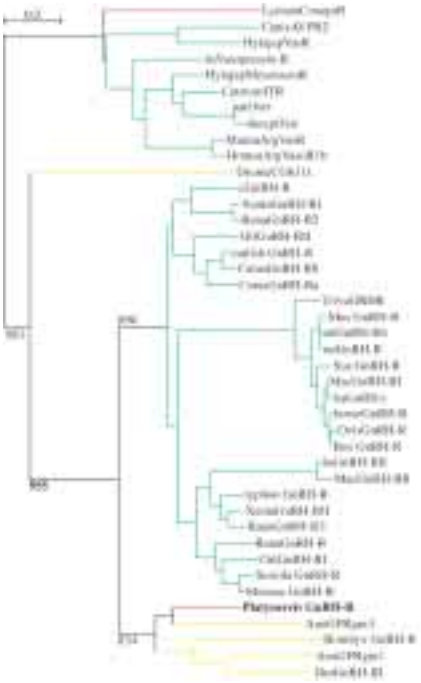


Figure 30. Phylogenetic tree of the *Platynereis* GnRH receptor.

The tree was calculated using AA sequences. Numbers at the branch points represent bootstrap values. Color code: green Deuterostomia, yellow Ecdysozoa, red Lophotrochozoa. The *Platynereis* GnRH receptor gene is represented in bold and clusters together with the ecdysozoan GnRH receptors. Together with the vertebrate GnRH receptors these proteins form a group clearly distinct from the closest related receptors, the Vasopression/ Oxytocin receptors.

The alignment and accession numbers belonging to this phylogenetic tree can be found in the Appendix.

In summary, the *Pdu-gnrh-r* is first and most prominently expressed in cells of the median episphere of larval *Platynereis*. These *Pdu-gnrh-r* positive cells are located in an area positive for *Pdu-vax* and (partly) *Pdu-nk2.1* and therefore are likely to co-express these transcription factors. This situation is reminsicent to the ventral/ median forebrain of different vertebrate species, where *gnrh-r* orthologs are expressed in an area positive for *vax* and *nk2.1* orthologs. The *gnrh-r* is thus the fourth molecule corroborating the idea that the ventral/ median brain of vertebrates and the median brain of *Platynereis* are also highly similar on the level of distinct cell types.

In the two previous chapters I analyzed the expression of seven different transcription factors in the brain of *Platynereis* larvae. These transcription factors were chosen to compare their expression in the *Platynereis* episphere to the expression of their orthologs in the ventral/ median forebrain of vertebrates. The similarities in the expression patterns of these transcription factors between vertebrates and *Platynereis* are very pronounced and thus highly unlikely to be caused by random independent recruitment of these factors in the different groups. This prompted me to speculate that the two median brain regions are in fact

homologous. In this chapter, I further extended this comparative analysis beyond the level of transcription factors by including molecules demarcating differentiated cell types. I first performed a theoretical survey to determine if neuropeptides shown to be downstream targets of some of the analyzed transcription factors in vertebrates are likely to be evolutionarily ancestral. Despite the fact that these neuropeptides are missing from the genomes of the sequenced Ecdysozoa, I found that at least three of them are present in lophotrochozoan animals, arguing for their presence in Urbilateria, and make them thus highly likely to exist also in *Platynereis*. This indicates that the level of comparability probably extends to the level of downstream targets. Second, I investigated the distribution of the four differentiated cell type markers, RFamide(s), *hdc*, serotonin and *gnrh-r*, which are prominently present in the ventral/ median forebrain and partly overlap with some of the transcription factors investigated above in vertebrates. The analysis of the distribution of these factors in the *Platynereis* episphere showed again high similarities with the situation in the ventral/ median forebrain of vertebrates, further corroborating the idea that cell types of the median brains are homologous to a vast extent across Bilateria.

For my comparisons, the apical organ that demarcates the center of the median brain in trochophora-type larvae, was of particular interest. This was due to its central location in the median brain, the peculiar shape of many of its cells, as well as the set of investigated genes expressed in this region. I could distinguish two catagories of cells in the APO, the ‘flask-shaped’ center cells and the ‘flask-shaped’crescent cells. The cells of the apical organ have been suggested to be chemosensory in other species (see introduction). In order to further investigate cellular morphology and possible function of these cells, I employed a method adapted from *C.elegans* to stain sensory cells contacting the outer environment.

2.3.4 *The lipophilic dye DiI stains a distinct set of cells in* Platynereis *larvae, including cells of the apical organ*

In *C.elegans* the lipophilic dyes DiI and DiO can be used to stain ciliated sensory amphid and phasmid neurons. The ability to take up dye depends on sensory ciliary structures, which are exposed to the out environment. Sensory cell exposed to the

outer environment that do not have ciliary structures don't stain with the lipophilic dye (Hedgecock, ; Perkins *et al*., 1986). It should be noted that the exact reason for the dye uptake is unclear, and the two different dyes (DiI and DiO) stain slightly different cells (see in standard *C.elegans* methods e.g. at http://elegans.swmed.edu/). I tested three different lipophilic dyes (DiO, DiI C16 and DiI) on *Platynereis* larvae. Of those only DiI gave useful results. Between 24hpf and 48hpf the prototroch and cells posterior to the telotroch (possibly also the telotroch itself as well) stain, as well as the ciliated crescent of the apical organ (Figure 28A,B and data not shown). The latter are the 'flask-shaped' crescent cells mentioned in the previous sections. Occasionally, but reproducible, few additional cells belonging to the more ventral cell cluster of the apical organ stain (Figure 28A). By position, these cells are likely to belong to the 'flask-shaped' center cells analyzed in the previous sections. The stained cells of the apical organ form (at least part of) the apical plexus (Figure 28B). Interestingly, since the prototrochal and posterior cells are as well highlighted by this dye, one can also observe their extensions (Figure 28A). The posterior cells form a very similar plexus like the apical organ. These cells likewise form long, interdigitated processes (data not shown). It is an interesting notion that by position they very likely include the posterior serotonergic cell (see Figure 28 H). Recently, a posterior sensory organ (PSO) has been described for the polychaete larva of *Phyllodoce maculata*, which exactly also includes (in this case two) posterior serotonergic cells (Nezlin and Voronezhskaya, 2003). The cells of the prototroch likewise extend deep into the episphere, but don't interdigitate (green arrow in Figure 28A). Long basal extensions of the prototrochal cells have already been described for a different polychaete trochophora (Marsden, 1982), however their function is enigmatic. Because three ciliated cell groups stain, I investigated if the dye stains all ciliated cells unspecifically. This is not the case. Several cells that located in the trunk region (e.g. metatrochal cells) or in the episphere do not take up the DiI, although they extend cilia to the surface. Also, the period during which the apical organ, prototrochal and telotrochal cells take up the DiI is restricted. These cells still exist partly at 5days, but don't stain anymore at this timepoint (data not shown). At 5days, a different set of cells starts to stain. These are the cells of the nuchal organs (paired lateral sensory organs below and behind the adult eyes (Ax, 1988)). In few occasions it is possible to observe their projections. The other group

of cells taking up DiI at this timepoint are the sensory cells of the antenna. Their projections end after a relative short distance away from the origin of the antenna in the brain (data not shown).

To resume, distinct sets of cells take up the lipophilic dye DiI at different stages of *Platynereis* development. In analogy to the use of this staining technique in *C. elegans*, it is likely that the uptake of DiI by these cells implies that they act as chemosensory neurons, which are in contact with the environment. Notably, the cells characterized by this property include cells in the APO. Additionally, DiI confirms the morphology of the APO cells as long and 'flask-shaped' and visualizes the apical plexus formed by the cells of the APO. By morphology and their presumed chemosensory function these cells share similarities with the CSF-contacting neurons in the median ventral brains of (lower) vertebrates (see section describing serotonin in *Platynereis* above and discussion below). This provides further and independent evidence for the relatedness of the cell types in median forebrains across Bilateria.

2.4 A whole mount in situ hybridization screen uncovers additional genes involved in bilaterian brain development

In order to investigate the molecular composition of the brain of *Platynereis* larvae and compare its transcription factor expression patterns and cell types to the vertebrate brain, I cloned and analysed candidate genes known from vertebrates in *Platynereis dumerilii*. This approach is a) rather time consuming, b) restricted to known genes, and thus somewhat biased, as well as c) limited to genes with conserved regions long enough to design degenerated primers. The last point is very important considering the peptides secreted by the neurosecretory hypothalamic cells. Several peptide precursors (e.g. the GAP and GnRH common precursor (Mason *et al*., 1986) are too short for degenerated primer design.

In order to circumvent these problems, I participated in a larger project of the lab, involving EST sequencing as well as a whole mount in situ screen. I contributed by constructing a cDNA library from 48hpf whole larvae, and was involved in parts of the WMISH screen, investigating genes of interest for my project.

During the screening, the following criteria helped to identify genes of interest for my study: First, genes represent orthologs of those vertebrate genes, that are known to be present in the ventral/ median forebrain. This deals with the situation a) and c) described in the beginning of this section. Second, genes exhibiting prominent expression in the apical organ/ median brain region are of similar interest, since they address the problem b) described above. Third, because of the similarities of cells of the apical organ, the prototroch, the telotroch and the posterior organ (PSO) between 24hpf and 48hpf mentioned in the previous chapter, I am interested in molecules that might support these similarities on the molecular level by forming a synexpression group in these structures. Synexpression groups were first defined as a set of molecules whose expression sites overlap specifically in distinct tissues or structures Gawantka *et al.*, 1998; Niehrs and Pollet, 1999. Genes belonging to the same synexpression group are commonly co-regulated on the transcriptional level Niehrs and Pollet, 1999. The sharing of members of one synexpression group by different tissues or structures indicates that these tissues or structures exhibt profound molecular resemblances and thus likely also functional similarities.

Among the first 1000 ESTs sequenced and analyzed by WMISH, several fullfill one of the criteria mentioned above. Two vertebrate orthologs present in the ventral/ median forebrain are the genes *orthopedia* (*otp* – see analysis above), and *netrin*. The *netrin* gene of vertebrates is demarcating the ventral midline of the spinal cord, as well as in the forebrain (Strahle *et al.*, 1997). I therefore expected it to be expressed in the median brain region of *Platynereis* trochophora - type larvae as well. This is in fact the case. It is already present in the median episphere at 24hpf (see Figure 31A). Second, the following interesting synexpression groups start to emerge:

i) expression in the apical organ and median brain region (Figure 31C,D),

ii) expression in apical organ region/ median brain, prototroch and PSO and/ or telotroch, (but not in the other ciliary bands in the trunk; Figure 31 E,F),

iii) expression in prototroch and/ or teletroch and ciliary bands in the trunk (Figure 31I, J, K) Of course, these are just preliminary groupings, which will certainly get better defined in the course of the WMISH screen.

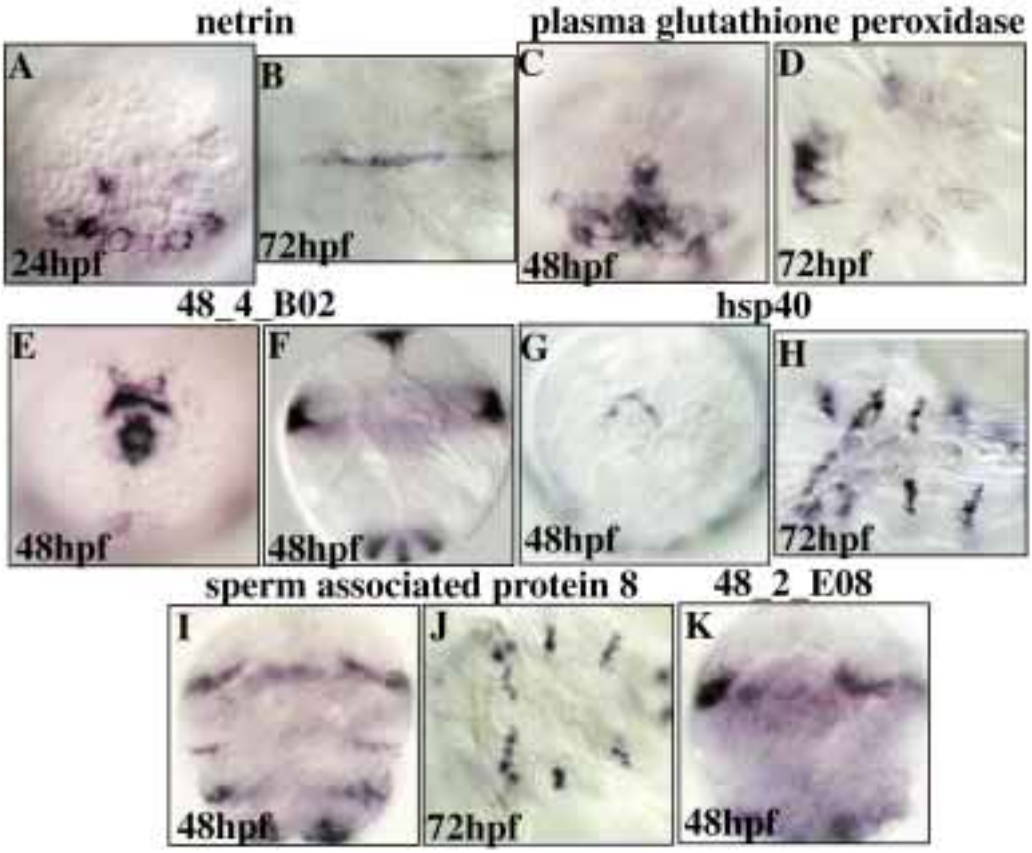


Figure 31. Exemplary expression patterns from a WMISH screen performed with clones of a 48hpf cDNA library.

Sampling of expression patterns of the WMISH screen originating from clones of a 48hpf cDNA library. A gene name was written on top of the pictures, if the sequence of the clones yielded a high Blast score. A number indicated that there has either been no Blast hit or the cDNA has homology to a unknown protein. **(A)** apical view (dorsal up), **(B)** ventral view (apical to the left, right side down), **(C)** apical view (dorsal up), **(D)** ventral view (apical left, right side down), **(E)** apical view (dorsal up), **(F)** ventral view (apical to the top, right side left), **(G)** apical view (dorsal to the top), **(H)** lateral view (apical to the left, ventral down), **(I)** ventral view (apical up, right to the left), **(J)** dorsal view (apical left, left down), **(K)** ventral view (apical top, right side to the left). Pictures taken by: A) P. Steinmetz; B)F.Raible; C,D,E,F, H,J) my own pictures; G,I, K) E. Navikova and N. Bakalenko.

Taken together, the characterization of a 48hpf cDNA library by sequencing and WMISH screening added relevant genes to the comparison of the median forebrains across Bilateria. Additionally, the screen gives first molecular evidence that the APO, prototroch, telotroch and PSO might share more than their stainability by DiI, since these structures jointly express genes, not/ rarely present at other places. The analysis of this library has just started as a collaborative efford involving several labs. More gene expression patterns and sequences are currently being produced, helping with an unbiased analysis and comparison of the investigated structures, such

as the cells of the median brain or the determination of possible synexpression groups.

2.5 The Hedgehog (Hh) pathway is involved in the development of the episphere in the *Platynereis dumerilii* trochophora

The analysis of the orthologous groups of seven transcription factors (*nk2.1*, *rx*, *vax*, *otp*, *sim*, *pou3*, *gsx*), the axon guidance molecule *netrin* and four differentiated cell type markers (RFamide(s), *hdc*, serotonin, *gnrh-r*) revealed that the overlap of their distribution is highly specific for the median brain of the trochophora-type larva, highly reminiscent to the situation in the vertebrate ventral/ median forebrain, with the hypothalamus as its central structure. This would suggest that the regulatory curcuits establishing these patterns might also be similar. Two signalling pathways, the Nodal and Hedgehog pathways have been implicated in vertebrate vental/ median forebrain development (Mathieu *et al*., 2002; Wilson *et al*., 2002; for an overview of the Hh-pathway see Figure 33 K). In one of the first chapters of this thesis I also described my investigations of the light sensitive cells of *Platynereis* and their possible comparison to the vertebrate optic system, including the eyes. The Hh pathway has also been implicated in eye development in various vertebrates, as well as in *Drosophila melanogaster*, where it is early required for the formation of the larval and adult eye primordia (Chang *et al*., 2001) and a wave of Hh triggers the differentiation of the PRCs (Dominguez and Hafen, 1997; Pappu *et al*., 2003).

In order to extend my comparative analyses to a functional level I first started to investigate the role of the Hedgehog signalling pathway in the brain development of *Platynereis dumerilii*. In a second step, I compared the expression pattern and functional data obtained with the situation in other species, especially vertebrates.

Three different *hedgehog* genes (*sonic hedgehog-shh*, *desert hedgehog- dhh*, *indian hedgehog- ihh*) exist classically in vertebrates (teleost fish have at least one additional family member (*tiggle-winkle hedgehog- twhh*) due to the additional genome duplication). In vertebrates, *shh* has been investigated most extensively. Mice lacking *shh* have severe defects in the ventral forebrain, especially they exhibit

a loss of the hypothalamus, and *shh* can promote hypothalamic fates in vitro assays (reviewed in Wilson *et al*., 2002). On the other hand, in fish some hypothalamic development occurs in all known Hh pathways mutants. Most importantly, in mouse loss of *shh* leads to the entire absence of *nk2.1*, whereas in zebrafish a blockage of the Hh pathway has only little effects on the two *Zebrafish nk2.1* genes (*Zfnk2.1a* and *Zfnk2.1b*; Chiang *et al*., 1996; Rohr *et al*., 2001; Rohr and Concha, 2000; Wilson *et al*., 2002). In the vertebrate eye, extraretinal Hh signalling is important for the split of the initially single eye primoridium, optic stalk formation and the initiation of retinal differentiation (Stenkamp and Frey, 2003 and references therein). Similarly to the situation for the *Drosophila* rhabdomeric PRCs, a wave of Hh traveling through the eye is required for the successive differentiation of further retinal ganglion cells (Neumann and Nüsslein-Volhard, 2000; Stenkamp *et al*., 2000). Finally, Hh-signaling from the retinal pigment epithelium has been speculated to be required for the initiation of *rx1* expression in the underlying ciliary PRCs, and thus for their differentiation (Stenkamp and Frey, 2003; Stenkamp *et al*., 2000).

It is important to note that several discrepancies exist in vertebrate, preventing a reliable judgement of possible ancestral roles of this pathway. To examplify some of these discrepancies: a) the dependency of the *nk2.1* expression in mouse vs. fish (see above), b) *pax6* expression in a loss of Hh-pathway situations is expanded in the vertebrate telencephalon, but strongly reduced in the teleost diencephalon. c) The *vax* genes are severely reduced in a *Zebrafish smo* mutant. However, the same genes are hardly affected if the *smo* gene is inhibited by a chemical component (for a, b, c see Perron *et al*., 2003; Rallu *et al*., 2002b; Take-uchi *et al*., 2003; Varga *et al*., 2001).

In the following part I focused my investigations on the possible influence of the Hh-pathway on the development of the optic system, as well as on the transcription factors and markers for differentiated cell types of the median brain to complement the molecular comparison of cell types of the *Platynereis* brain with those of the vertebrate brain. First insight was gained in the developmental roles ancestrally exerted by the Hh-signaling pathway in Bilateria.

2.5.1 *Cloning and expression of members of the Hh-pathway in* Platynereis dumerilii

First, I cloned three components of the pathway in *Platynereis*, *Pdu-hh*, *Pdu-smo* and *Pdu-ptc* (Figure 32, for position in the pathway: Figure 33 K). Unfortunately, the fragments of *Pdu-ptc* and *Pdu-smo* give a staining that is very difficult to judge reliably. For *Pdu-ptc* this is probably due to the relatively short length of the probe. The reason for this problem with *Pdu-smo* is unclear, since the probe is over 2kb long. The presented picture of the apical *Pdu-smo* expression should thus be interpreted with care. It indicates that this gene is broadly expressed in the brain, with some stronger expression in the ventro-median region and the dorsal-lateral region (Figure 33J). This broad expression is reminiscent of the vertebrate situation (Figure 33L,M; Perron *et al.*, 2003; Varga *et al.*, 2001).

Pdu-hh transcripts can be found in the brain and trunk at 19hpf. In the episphere the gene is weakly present in the ventro-median, as well as in the ventral-lateral region (Figure 33A). This staining extends becomes stronger at 24hpf, in addition staining in close proximity to the apical organ, as well as in the anlagen of the nuchal organs at the dorsal episphere is visible at this stage (Figure 33B,C). The staining in these regions becomes broader and more pronounced during subsequent development (48hpf, Figure 33D). It ceases in the ventral domains at 72hpf, but is maintained in the nuchal organs (Figure 33H,I). It is also present in the stomodeum from early stages onwards throughout 72hpf (Figure 33D,E,H and data not shown). This is interesting, because the stomodeum huddles against the ventral side of the brain, and could thus also directly influence epispheral development. In the trunk *Pdu-hh* transcripts are present from 19hpf onwards, its expression forms transversal stripes at 48hpf, probably corresponding to the larval segment boundaries (data not shown and Figure 33E,F).

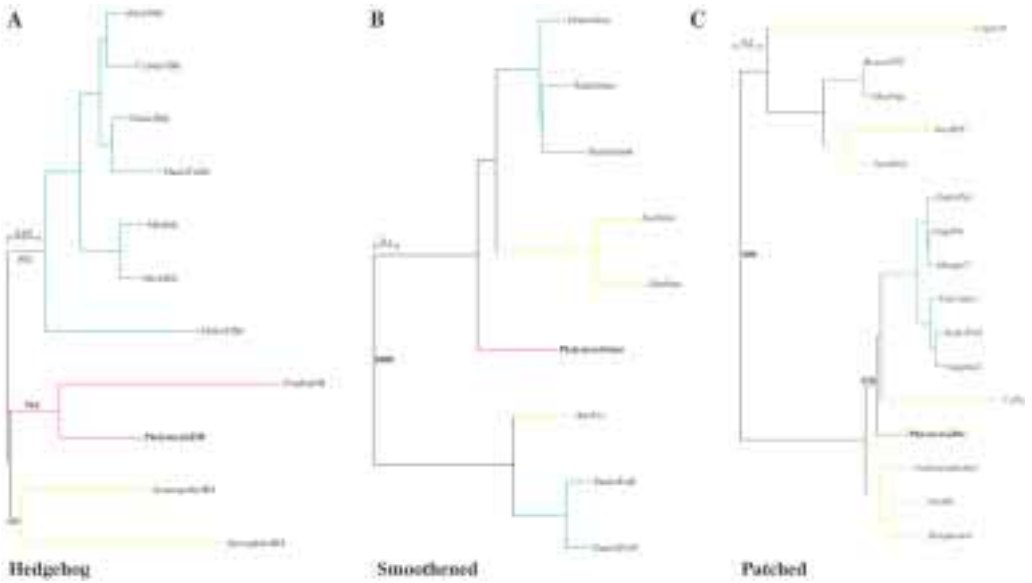


Figure 32.Phylogenetic trees of HH, Smo and Ptc.

Trees were calculared using AA sequences. Color code: Blue- sponge, green- Deuterostomia, yellow- Ecdysozoa, red- Lophotrochozoa. Platynereis protein name is in bold. **(A)** Phylogenetic tree of Hedgehog. Pdu-HH clusters next to Pvu-HH, the other lophotrochozoan HH in the tress. **(B)** Phylogenetic tree of Smoothened. Pdu-Smo clusters together with ecdysozoan and deuterostomian Smo proteins. The Frz proteins are taken as outgroup. **(C)** Phylogenetic tree of Patched. Platynereis Ptc clusters within the Ptc-group of the tree. The Npc proteins are taken as outgroup.

The alignments and accession numbers belonging to these phylogenetic trees can be found in the Appendix.

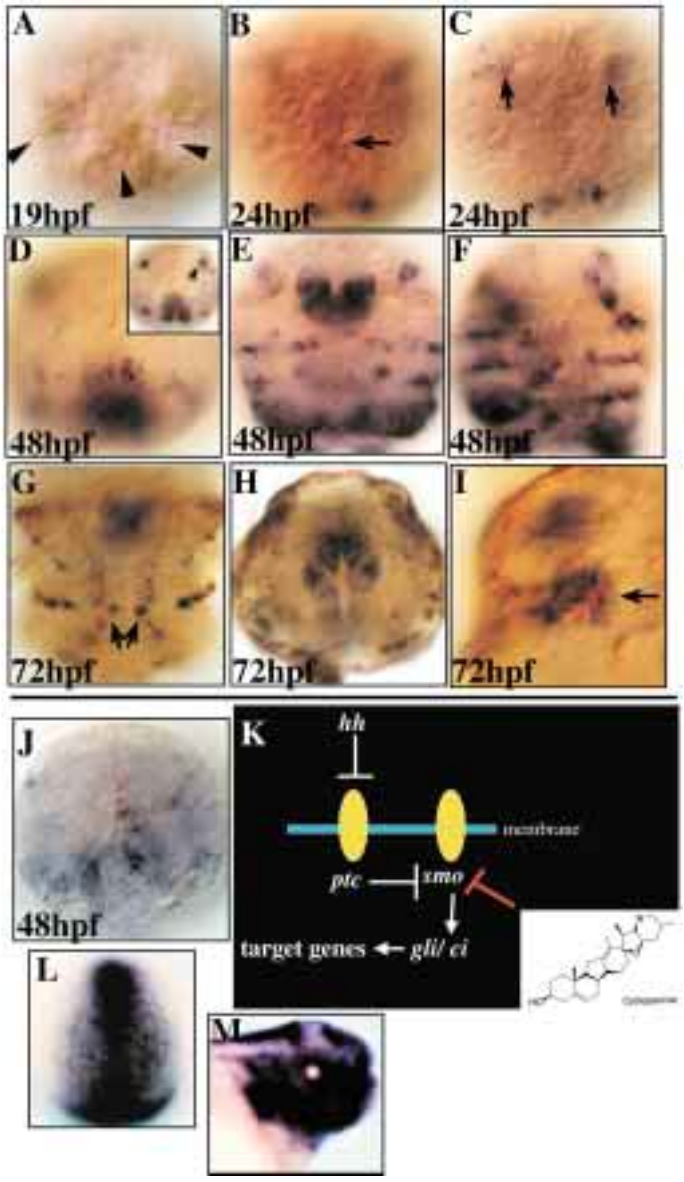


Figure 33. Expression of *hh* and *smo* in *Platynereis* larvae.

Blue stainings are cells stained by WMISH, brown staining is anti-act.tub staining (demarcating ciliated structures and axon tracts). **(A-I)** *Pdu-hh* WMISH. A) 19hpf apical view (dorsal to the top). Arrowheads point to the first cells weakly starting to express *Pdu-hh* in the episphere. B) 24hpf, apical view (dorsal up) Arrow points to the expression in the median region, close to the APO. C) like B) focus slightly deeper. Arrows point to the dorsal brain expression in the presumptive nuchal organ anlagen. D) 48hpf, apical view (dorsal to the top). Expression has broadened in the ventral brain. Inset is slightly deeper plane of focus, arrow points to the continued expression in the presumptive nuchal organ anlagen. E) Ventral and F) lateral views at 48hpf. Expression is strong in the stomodeum and in stripes around the body in the trunk. G) Ventral and H) apical view at the level of the prototroch at 72hpf. Arrow in G) point to *hh* positive cells above the trunk connectives. Stomodeal staining is visible in H). I) Magnification of the staining in the nuchal organs (black arrow) in an apical view at 72hpf. Brown staining surrounded by the blue cells are the cilia of the organ hanging into an open channel.

(J), (L), (M) WMISH stainings of *smo* in different species. J) 48hpf apical view (dorsal up) onto *Platynereis*. The staining is very weak and bad, but was repeatedly obtained in the regions also visible in the picture. It seems to be slightly stronger in the ventral episphere, as well as in the adult eye region. However weak staining seems to be all over. As can be seen from other species L) *Zebrafish* tailbud stage (taken from Varga *et al*., 2001) M) Xenopus stage41 (taken from Perron *et al*., 2003), *smo* would be expected to have a rather broad expression in the CNS. **(K)** Simplified schematic of the Hh signalling pathway taken from standard textbooks. The drug cyclopamine inhibits the pathway by binding to the 7TM molecule *smo*.

Summarizing the expression data obtained from members of the Hh-signalling pathway, I can state that two key members of the pathway, *hh* and *smo*, are present at

timepoints and places, where they can influence brain development. In order to further test, if this is true and if true, to get a first impression which cell types or structures need the Hh- signalling pathway for their normal development, I disrupted the pathway in *Platynereis* embryos and larvae.

2.5.2 *Interfering with the* hh-*pathway using the drug* cyclopamine

I took advantage of the commercially available drug *cyclopamine* to inhibit Hh-signalling. This drug has been shown to bind between the 6th and 7th transmembrane helix of the vertebrate Smo protein, and by this to inhibit the function of the pathway (Chen *et al*., 2002; Cooper *et al*., 1998; Frank-Kamenetsky *et al*., 2002). Additional evidence comes from the fact that the defects induced by the drug in vertebrates are very reminiscent of mutation in the Hh signalling pathway (reviewed in King, 2002). It is interesting to note that no report on cyclopamine effects in *Drosophila melanogaster* or *C.elegans* has yet been published. I applied different concentrations of the drug to mainly two different stages. One stage was 22/24hpf and the other was 15hpf.

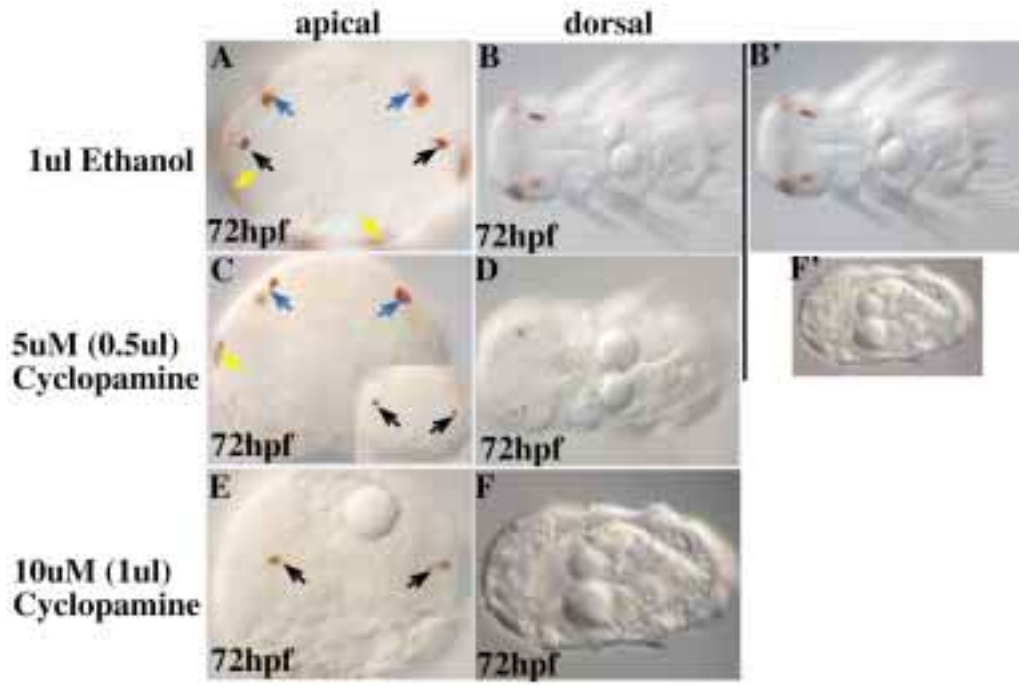


Figure 34. Effects of cyclopamine on the morphology of *Platynereis* embryos.

Different concentrations of cyclopamine have concentration dependent effects in the morphology. Embryos were treated from 22/24hpf onwards. Blue arrows point to the pigment of the adult eyes, black arrows point to the pigment of the larval eyes. Yellow arrows point to additional pigment occasionally visible in the episphere. A) and B) Ethanol is used as control. A) Apical view, note that one of the adult eyes is out of focus and visible as a red shadow below. C) and D) Moderate concentrations of cyclopamine have already effects on the adult eye pigment (C), as well as on the the parapodia (D). E), F) Strong effects of this cyclopamine concentration on eyes , parapodia and antenna. Only the larval eye pigment is visible (E), chaetae and antenna are entirely absent (F). B' and F'- same pictures as B and F, respectively. They reflect the real size ration of non- treated vs. treated embryos. Treated embryos (F') have a reduced size compared to control (B').

Since the application of drugs always comes with the flavour of unspecific effects, four important points should be kept in mind: First, in all the experiments 100 percent (with maybe very few exceptions) of the animals were affected similarly. Second, these effects were strictly concentration dependent. Third, I used only one fifth to one tenth of the concentration that is used in vertebrate experiments, which are considered to show specific effects (see e.g. Perron *et al.*, 2003; Sbrogna *et al.*, 2003). Fourth, a general cytotoxic drug would probably kill the animal pretty soon, since it should effect the survival of all cells.

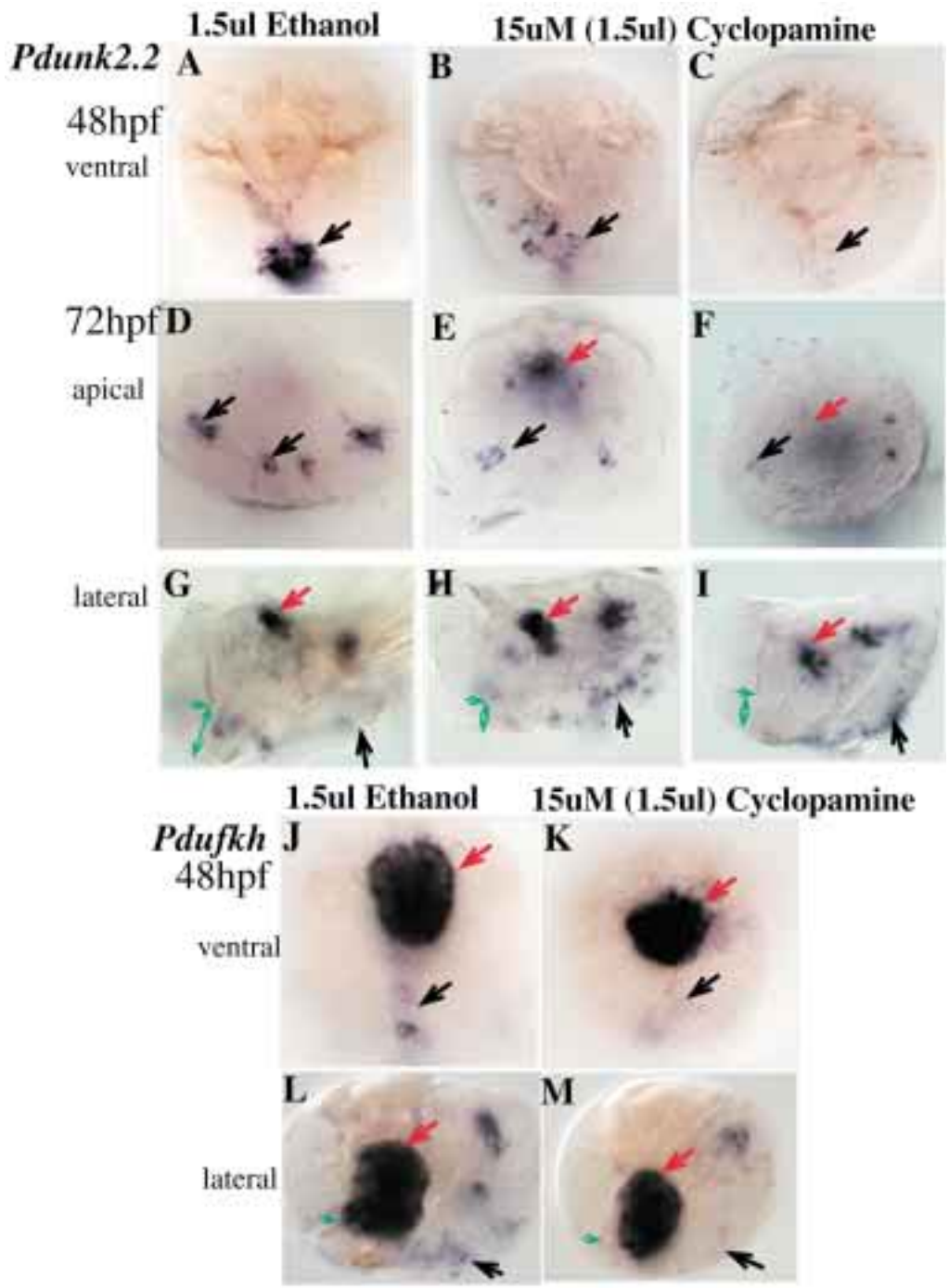


Figure 35. Effects on *Pdu-nk2.2* and *Pdu-fkh* expression in cyclopamine treated embryos.

Embryos were treated from 22/24hpf onwards. Note that only J)- M) reflect the real overall size ratios. A)-I) Effects of cyclopamine on *Pdu-nk2.2* expression. A) Black arrow in ethanol control points to the normal expression in the midline of the VNC. B) and C) show inhibited embryos. Black arrow points to the same position as in A). Note the different strength of the phenotype. In milder affected embryos the staining is reduced and more extended to the lateral (B), whereas the staining is absent in strongly affected embryos. Black arrow in D), E), and F) points to *Pdu-nk2.2* expression in the brain. Note again the different extend of the phenotype in E) and F). Red arrow points to staining from the stomodeum in these embryos. G), H) and I) Lateral views of the same embryos as in D), E) and F).

Red arrow points to the expression in the stomodeum that is unchanged. Now expression in the VNC is visible in the treated embryos (black arrows). Green double arrows indicate the length of the proximal stomodeum that is not surrounded by visceral mesoderm (start of visceral mesoderm indicated by green arrow). This part appears strongly reduced in treated embryos. J)-M) Effects of cyclopamine on *Pdu-fkh* expression. Black arrows point to the expression in the VNC, red arrows in the stomodeum. Green arrow indicates position of the border between proximal and distal stomodeum.

2.5.2.1 Treatment of *Platynereis* embryos with cyclopamine causes specific morphological defects, partly reminiscent to defects seen in vertebrates with Hh- pathway disruptions

In the case of *cyclopamine* inhibited embryos (started at 22/24hpf) overall development appears to be normal, and they survived at least up to five days. They will probably die after this time, because feeding needs to start, and normal stomodeal development appears affected. Stomodeal defects have also been observed in the more derived lophotrochozoan relative, the leech (Kang *et al*., 2003 and see discussion below). Embryos inhibited from 15hpf onwards showed more severe malformations at later stages. This is probably due to more primary and secondary effects that have accumulated. In both versions of the inhibition, similar defects can be perceived on the morphological level. Most apparent is the reduced sizes of the treated animals, in case of the laterinhibition condition to two third or half of the wildtype size (Figure 34B', F'). This is highly reminiscent of the situation in mice, where the Hh- pathway has been implicated in cell proliferation and a reduction of size occurs in the loss-of-function situation (Kawakami *et al*., 2002; Milenkovic *et al*., 1999; Zhang *et al*., 2001). Additionally, the absence of parapodia, chaetae, and antennae can be observed (Figure 34A-F).

2.5.2.2 Treatment of *Platynereis* embryos with cyclopamine reduces *Pdu-nk2.2* and *Pdu-fkh* transcript levels in the VNC

I next investigated, if disruption of the Hh- pathway leads to similar molecular effects as in vertebrates. One of the immediate downstream target genes of this pathway in vertebrates is the *nk2.2* gene, a gene distant but related to the *nk2.1* gene (Barth and Wilson, 1995; Varga *et al*., 2001 and see Figure 22A). I thus investigated the expression of the gene in treated *Platynereis* embryos. In normally developing embryos at 48hpf of development, *Pdu-nk2.2* (cloned by H.Snyman) transcripts are present in the VNC, few cells in the distal stomodeum, as well as some cells within

the trunk of the embryo, which probably correspond to endoderm by position. Additionally, cells in the episphere express *Pdu-nk2.2* at 72hpf (Figure 35A, D, G and data not shown). Treatment with cyclopamine severely reduced *Pdu-nk2.2* transcript levels in the central nervous system. Transcripts are however still present in the stomodeum (Figure 35A-C, G-I). Similary, cyclopamine has been shown to disrupt the dorsal-ventral patterning of the vertebrate neural tube reminiscent of loss-of-function mutations in the Hh- pathway, and thus results in a severe reduction to complete absence of the *forkhead* gene *hnf3-beta/fkh* in the CNS (Chiang *et al.*, 1996; Incardona *et al.*, 1998). Strikingly reminiscent, I find that *Pdu-fkh* (cloned and initially described for wildtype *Platynereis* development by S. Klaus) is strongly reduced in the VNC (Figure 35J-M). Similar to *Pdu-nk2.2* its stomodeal expression domain does not appear severely affected (Figure 35J-M). It should be noted however that the high expression level of *Pdu-fkh* in the stomodeal cells precludes the detection of minor changes in the amount of *Pdu-fkh* in these cells. Therefore, slight effects of cyclopamine treatment on these cells might have been missed. Contrariwise, the transcript levels of *Pdu-nk2.2* are lower and specifically localized to few cells in the distal portion of the stomodeum. Because of this, even milder effects of cyclopamine on these cells should have been detectable.

Both morphological and molecular data suggest that the distal part of the stomodeum (i.e. that is the part where the stomodeum is surrounded by visceral mesoderm) is mainly unaffected. It is however noteworthy, that this looks different for the proximal part at least from the morphological level. (It was not possible to access this molecularly so far.) This part appears to be strongly reduced (Figure 35 green arrows in G,H,I,L,M).

To summarize the previous and this section, the effects of cyclopamine on *Platynereis* are specific. The influences of *cyclopamine* treatments on *Pdu-nk2.2* and *Pdu-fkh* in the CNS resemble the effects caused by disrupted Hh- signalling in vertebrates. This molecular similarity to vertebrates, together with the overall size reduction, which is again reminiscent to the situation in vertebrates, is a strong indication that cyclopamine interferes with the Hh-signalling pathway in *Platynereis dumerilii*. Unfortunately, the *Pdu-smo* fragment so far does not include the 6th and 7th

transmembrane helix. Therefore, the conservation of the cyclopamine binding site could not be determined.

2.5.2.3 Cyclopamine reduces *Pdu-pax6* expression more than *Pdu-nk2.1* expression

As mentioned above, Hh-signalling has been implicated in the proper development of the ventral/ median forebrain in vertebrates. Therefore, I next investigated, which of the transcription factors analyzed in the previous chapters are affected by cyclopamine treatment, and how this might compare to the vertebrate situation.

As explained above, the genes *pax6* and *nk2.1* define lateral vs. median forebrain regions in vertebrates, as well as in *Platynereis*. The treatment of *Platynereis* larvae with cyclopamine caused the *pax6* gene to be much stronger reduced in its epispheral expression domain than *nk2.1* (Figure 36A-H). This can be clearly stated for embryos inhibited from 15hpf onwards (Figure 36A-F), but seems equally true for embryos inhibited from 22hrs onwards (Figure 36G,H). It could not be distinguished whether this reduction in expression was due to a reduced proliferation of pax6 expressing cells, or to a downregulation of the gene. There is no (late inhibition) to some (early inhibition) reduction of the *nk2.1* staining region in both cases (Figure 36A, C-H). Since *nk2.1*, an eminent marker of the median forebrain across Bilateria, was not remarkably influenced, I wondered if a disruption of the Hh signalling pathway influences other transcription factors or differentiated cell type markers of ventral/median forebrain.

2.5.2.4 Cyclopamine treatment reduces *rx* and RFamide(s) levels, disrupts axon tract formation, but has no reducing effects on *vax* or serotonin levels in the episphere

I then tested whether cyclopamine treatment influences *rx* and *vax* expression in the *Platynereis* larval forebrain. Strikingly, the inhibition of embryos from 22hpf onwards leads to an almost entire absence of *Pdu-rx*, with only few cells in the dorsal brain region and in the nuchal organs remaining. However, even these regions are strongly reduced in *Pdu-rx* transcript levels (Figure 37A,B). In contrast, *Pdu-vax* expression is not affected in the very same brain regions in embryos treated under identical conditions. Rather, the staining of *Pdu-vax* seems to be expanded in the

dorsal brain region (Figure 37C,D). However, since some fluctuations of *Pdu-vax* transcript levels were observable in treated animals, they have to be carefully quantified, to see if they increased significantly above background in the cyclopamine treated animals. It should be noted however, that *Pdu-vax* transcript levels were never reduced in cyclopamine treated larvae.

Besides the effects on transcription factors, I investigated the influence of cyclopamine on differentiated cell type markers present in the median forebrain of *Platynereis*. Cyclopamine inhibited embryos exhibit a lowered number of RFamide(s) positive neurons in the episphere, as compared to control embryos. The number was reduced by at least half (see Table 1 and Figure 38A-D). Additionally, axon emerging from the RFamide(s) positive neurons were greatly diminished (Figure 38C,D). This is not only true for the epispheral axon tracts, but also visible in the trunk (data not shown). The serotonergic neurons were not significantly reduced in number in the episphere, however appeared severely misplaced (see Table 1 and Figure 38E-H). Their spatial position was randomized (Figure 38E,F). However in contrast to the episphere, a remarkable reduction of serotonergic neurons can be noted in the ventral nerve cord. The exact difference has not been quantified yet. In contrast to the disruption of axon tract formation of RFamidenergic neurons by cyclopamine, serotonergic neurons developed long axonal extensions similar to control animals. These axons managed to enter the peritrochal nerve ring in many case, as they do in the control case (Figure 38G,H). Nevertheless, their pathfinding is often severly disturbed in cyclopamine treated embryos. It is likely, that this miswiring is at least partly due to the severe misplacement of the neuronal cell bodies.

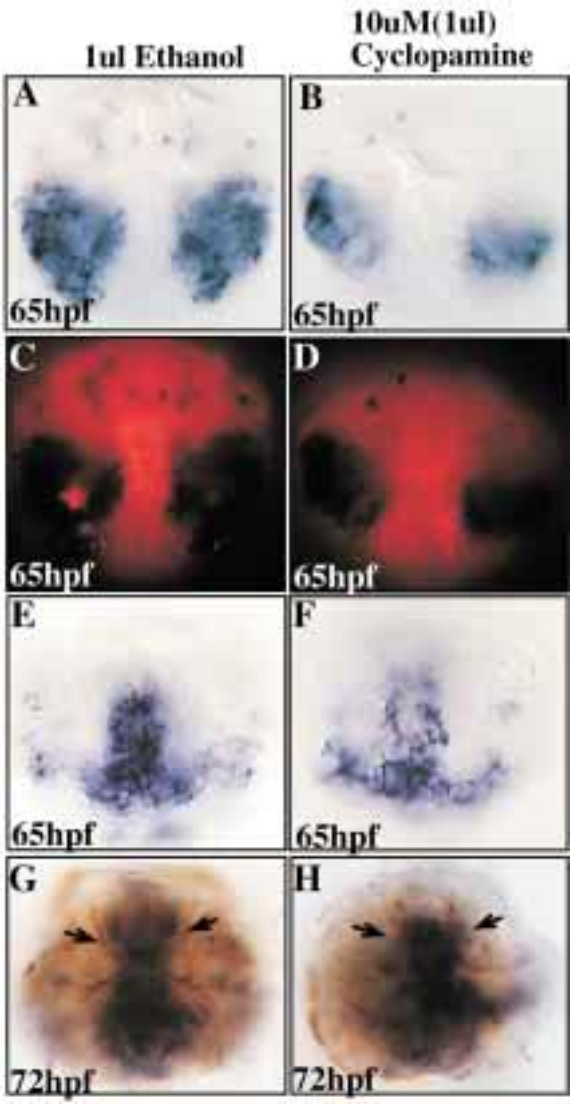


Figure 36.Effects of cyclopamine on *Pdu-pax6*, and *Pdu-nk2.1* expression and the axon schaffold.

Embryos treated from 15hpf **(A-F)** or 22/24hpf **(G,H)** onwards. Note that these pictures were scaled and don't reflect the real overall size ratios of the embryos. A) and B) Apical view (dorsal up) of *Pdu-pax6* stained embryos. C) and D) pictures of the same embryos as in A) and B), respectively, showing *Pdu-nk2.1* under fluorescent light now in red. *Pdu-pax6* appears black. E)-H) Apical view (dorsal up) of embryos stained with *Pdu-nk2.1* (blue). *Pdu-nk2.1* appears slightly weaker in F) compared to E), but almost stronger in H) compared to G). The extension to the dorsal part of the episphere was never as strongly reduced as in *Pdu-pax6* stained cyclopamine treated embryos (A-D). G) and H) were in addition stained with anti act. tubulin (brown) to reveal the axon schaffold. Black arrows point to parts of the schaffold were misguided neurites are apparent in cyclopamine (H) vs. control (G) embryos.

In addition to this, there seems to be a more direct effect of the Hh – signalling on axon scaffold formation. Indirect, as well as direct effects of this pathway on the axon pathfinding and thus scaffold formation have been described for different parts of the vertebrate CNS (Charron *et al.*, 2003; Dakubo *et al.*, 2003; Karlstrom *et al.*, 1999; Salinas, 2003). As I found by staining the entire axonal scaffold with an antibody against actylated tubulin, the entire axon scaffold itself is disrupted in several cases in both episphere and trunk (Figure 36G,H black arrows). This is in line with the observed function of the Hh pathway in axon guidance in vertebrates.

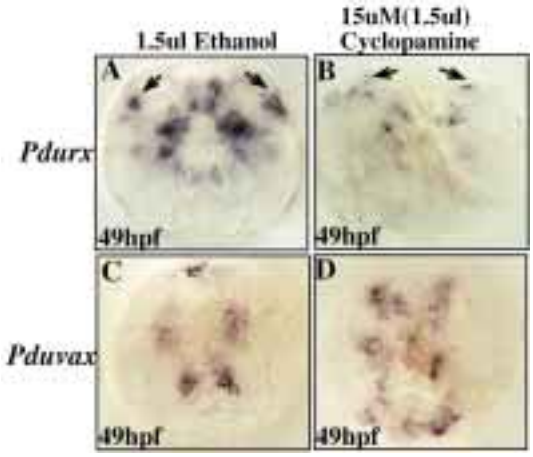


Figure 37.Effects of cyclopamine on *Pdu-rx* and *Pdu-vax* expression.

Apical views (dorsal up) onto control (ethanol, A), C)) vs. inhibited embryos (B,D). Note the strong reduction of *Pdu-rx* in **(B)** vs. **(A)**. *Pdu-vax* expression appears to be slightly expanded to the dorsal in **(D)** vs. **(C)**. Embryos of A)/ C) and B)/D) belonged to the same control and inhibited embryo batches, respectively. The pictures were scaled and don't reflect the real overall size ratios of the embryos.

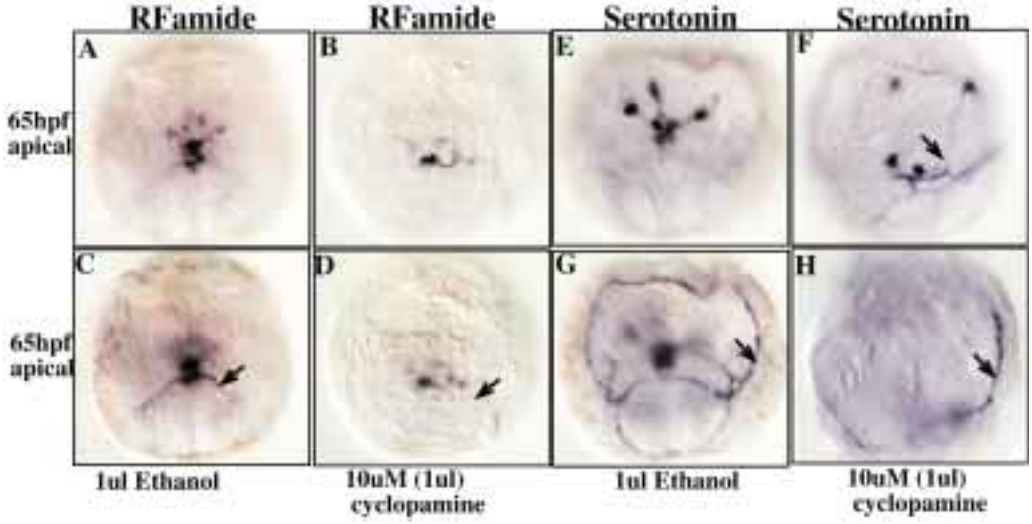


Figure 38. Effect of cyclopamine on RFamide(s) positive and serotonergic cells.

Apical views onto control (ethanol A, C, E, G) vs. cyclopamine inhibited (B,D, F, H)embryos. Note that embryos of A,C, E,G vs. B,D, F, H belonged to the same control vs. inhibited batches, respectively. These pictures were scaled and don't reflect the real overall size ratios of the embryos. Lower row shows the same embryos as upper row in deeper plane of focus. Arrows in C, D, G, H, F point to the axon tracts.

Taken together, the likely disruption of the Hh pathway in *Platynereis* larvae, using the drug cyclopamine has different effects on transcription factors and differentiated cell type markers specifying the median episphere. The level of *Pdu-rx* and RFamide(s) distribution appears strongly reduced, while *Pdu-nk2.1* and serotonin levels are not remarkably reduced, and *Pdu-vax* transcripts is not reduced, but apparently expanded. This argues that as in vertebrates *hh* influences the

development of the median forebrain. The absence of Hh –signalling does not lead to the entire absence of the *nk2.1* positive area, comparable to the situation in zebrafish.

2.5.2.5 Disruption of Hh signalling via cyclopamine reduces adult eye pigment cells and rhabdomeric photoreceptor cells

As mentioned above, Hh signalling is required for rhabdomeric PRC development in *Drosophila* and ciliary and rhabdomeric PRC development in vertebrates. The reduction of *rx* positive cells in the *Platynereis* episphere upon *cyclopamine* treatment indicates, that Hh signalling might also play a role in the development of the ciliary PRCs in *Platynereis*. *Pdu-ci-opsin1* WMISH stainings of *cyclopamine*-treated and control larvae showed a very variable amount of *Pdu-ci-opsin1* transcript in the controls (data not shown). Unfortunatly, this made any clear quantification of the transcript level in treated vs. non-treated larvae difficult.

I next investigated the question, if cyclopamine has an effect on eye pigment cells and rhabdomeric PRCs. I first give details on the effects of cyclopamine on eye pigment cells when the larvae were incubated starting at 22/ 24hpf. Second, I describe the phenotypes caused by cyclopamine on rhabdomeric PRCs. For the latter I tested two different time points, 15hpf and 22-24hpf, which differed in the severity of the produced effects. As mentioned above, larval eyes start to differentiate around 19hpf and adult eyes around 43hpf. An inhibition at 22hpf had no effect on the pigment of the larval eyes, even at highest concentrations (Figure 34A,C,E). Later, adult eye pigment can only occasionally be observed, however always in a very reduced shape (Figure 34A,C,E), indicating defects in the formation of adult eye pigment cells. Effects on eye pigment cells after an inhibition at 15hpf have not been investigated yet.

The normal development of the adult eye rhabdomeric PRC is also disrupted by cyclopamine treatment. Embryos treated from 22/24hpf onwards suffered from the following eye developmental defects: No expression of *Pdu-r-opsin* was detected in the developing adult eyes in contrast to the controls at 48hpf (Figure 39A,B). At 48hpf the differentiation of the adult eye rhabdomeric PRCs, as judged by *Pdu-r-opsin* expression, should have been initiated for more than 5hrs (see above). Later, *Pdu-r-opsin* transcript levels are often severely reduced in the adult eyes, however

some expression is almost always visible. The larval rhabdomeric photoreceptors appear to be occasionally fused to the adult eye anlage. The *Pdu-r-opsin* staining of the larval eye PRCs was undistinguishable from the remaining *Pdu-r-opsin* staining of the adult eye rhabdomeric PRCs in some cases, indicating that the larval eye PRCs got either fused into the remainder of the adult eye anlage or were lost (Figure 39D,E). Embryos inhibited at 15hpf showed more severe defects. 50% (14 out of 27 embryos) had no recognizable *Pdu-r-opsin* staining anymore. The other half (13 out of 27 embryos) showed occasionally some spots of *Pdu-r-opsin* staining in the episphere (Table 1). It was impossible to judge, if these *Pdu-r-opsin* positive cells corresponded to larval or adult eye PRCs, because of the abnormal morphologies and locations of these severely misplaced *Pdu-r-opsin* expressing cells (Figure 39G).

Taken together, disruption of the Hh-signalling pathway in *Platynereis* embryos/ larvae does not only affect the cell types of the median brain region, but also leads to severe disruptions of normal eye development in these animals.

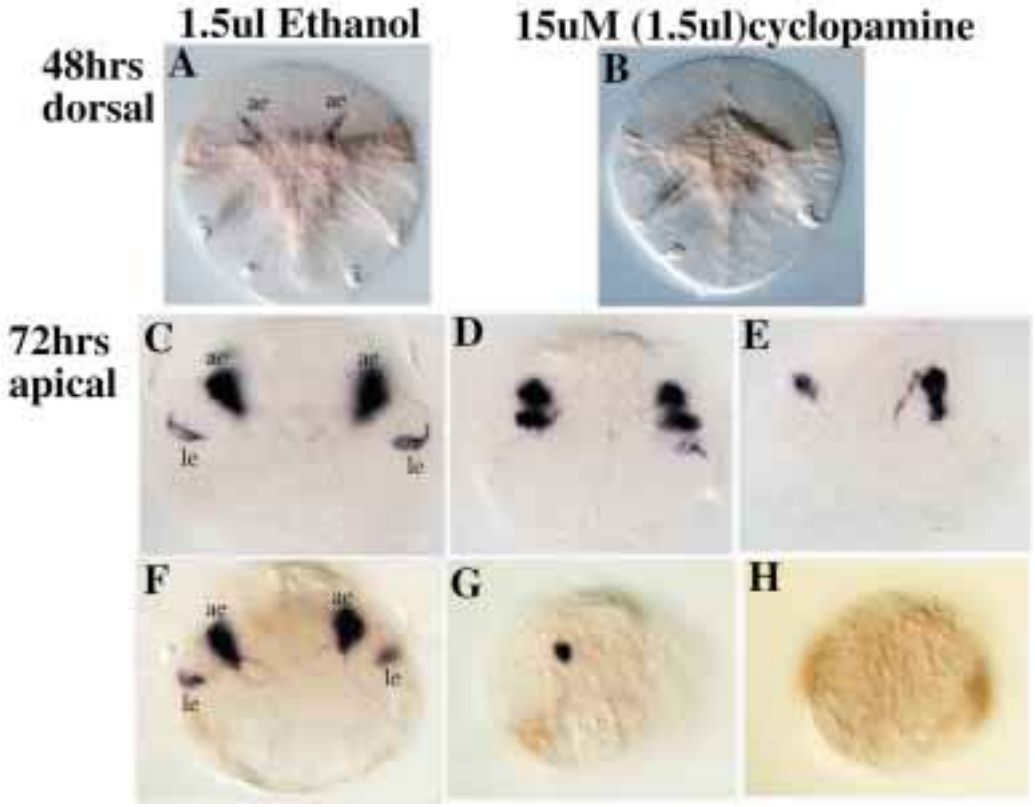


Figure 39.Influence of cyclopamine on *Pdu-r-opsin* expression.

(A-E) Embryos treated with cyclopamine from 22/24hpf onwards, **(F-H)** Embryos treated with cyclopamine from 15hpf onwards. (A, B) Dorsal view (apical to the top) onto control (A) vs. inhibited

embryos (B) at 48hpf stained with a riboprobe directed against *Pdu-r-opsin*. *Pdu-r-opsin* expression in adult eyes at 48hpf. No staining can be detected in the adult eyes (ae) of inhibited embryos. C-H) Apical views (dorsal top) onto embryos stained with *Pdu-r-opsin*. C) and F) control embryos, D,E and G,H represent the different intensities of phenotypes (see also Table 1 for G, H). Note that only C)-H) reflect the real overall size ratios of the embryos. ae- adult eye, le-larval eye

cells stained against:	Ethanol control	Cyclopamine inhib.
RFamide(s)	10 (n=22)	4 (n=29)
Serotonin	5 (n=26)	4 (n=53)
Pdu-r-opsin	4ae;2le (n=10)	0ae; 0le (n=14); and 1,2 or 3 rPRCs (n=13)

Table 1. Quantification of RFamide(s), serotonergic and *Pdu-r-opsin* positive cells in the episphere of cyclopamine treated larvae compared to controls.

Countings were performed on batches that were inhibited from 15hpf onwards; For antibody stainings against RFamide(s) and serotonin (rows 1 and 2), countings were done at 65hpf; for detection of *Pdu-r-opsin* transcripts (row 3), stainings were performed on 72hpf embryos; embryos are identical to those represented in Figure 38 and Figure 39. For all countings, the average number of positive cells per embryo is indicated, along with the number of embryos (n) that were evaluated. In the evaluation of *Pdu-r-opsin*-positive cells, 'ae' indicates adult eye rPRCs, 'le' indicates larval eye rPRCs. In cyclopamine-treated embryos, larval and adult eye rPRCs can not be distinguished; therefore, in the 13 cases where positive cells were observed, they are referred to as 'rPRCs' in this table.

3 DISCUSSION

3.1 Equivalence between bilaterian photosensory and neurosecretory systems on different levels

In the following, I will first provide a short summary of the main results of this thesis, and then evaluate these results from an evolutionary viewpoint. As I laid out in my introduction, my thesis addresses the question to which extent photosensory and neurosecretory/neuroendocrine systems are conserved across Bilateria. I focused my comparisons mainly on individual cell types in the brains of vertebrates and *Platynereis dumerilii*, an important reference organism for lophotrochozoan and ancestral protostomian development. I started my comparison between the forebrains of vertebrates and the larval episphere (brain) of *Platynereis dumerilii* by showing that a major molecular subdivision of the brain applies to both groups, namely the distinction between the lateral vs. median brain territories which are, respectively, marked by *pax6* and. *nk2.1* in an overall *six3/6* positive CNS territory. I continued my comparative analysis by focusing on the distinct cell types. The first differentiated cell types I investigated were the different photoreceptor cell (PRC) types present in the forebrains of both groups, showing that two distinct PRC types are molecularly ancestral and conserved across Bilateria. The ciliary PRCs are likely to originate from the median brain region in both vertebrates and the *Platynereis* trochophora larva, further corroborating the idea that the median brain regions are comparable between both groups. I then focused on the comparison of the median forebrain regions by investigating transcription factors of seven orthology groups and the precursor cell types they specify. Of those, four (*nk2.1*, *rx*, *vax*, *otp*) are expressed highly specifically and have been described to jointly specify the unique molecular identity of the vertebrate ventral/median forebrain. Similarly, the three *Platynereis* orthologs (*Pdu-nk2.1*, *Pdu-vax, Pdu-otp*) are restricted to the median larval brain. The fourth gene (*Pdu-rx*), is also mainly present in the median region (though less specific). Importantly, all four genes are prominently present in cells of the apical organ (APO). I investigated three additional transcription factors orthology groups that have been shown to play an essential role in the formation of distinct neuropeptidergic cell types within the hypothalamus. Individually, these genes are

less specific for the ventral/ median region of the vertebrate brain than the previous four, but their combined expression in the *nk2.1* positive region is specific. I analysed the expression of the *Platynereis* orthologs of these genes and found them to be present combinatorily in the median region of the *Platynereis* brain, including the APO.

Next, I again extended my comparison to differentiated cell types, specific for the median forebrain regions. I included four differentiated cell type markers, as well as cellular morphologies and potential functional characteristics. I first investigated if neuropeptides, such as Vasopressin, Oxytocin or Growth hormone releasing hormone, which are known to be downstream of the analysed transcription factors in vertebrates, are present in Protostomia. From the literature I found evidence that at least three neuropeptide precursors encoding such downstream targets, are likely to exist in *Platynereis*. I continued by closer analysing four differentiated cell type markers, RFamide(s), *histidine decarboxylase* (*hdc*), serotonin and families of the *gnrh-r* genes. These markers all show a very prominent (and partly highly specific) occurence in the vertebrate ventral/ median forebrain. Consistent with the hypothesis that the ventral/ median brain of vertebrates shares a common evolutionary origin with the median episphere of *Platynereis* larvae, all four cell type markers are most prominent (and mostly highly specific) in this region in *Platynereis* larvae. RFamide(s) and serotonin localised to a specific group of cell within the apical organ, the 'flask-shaped' center cells, co-expressing *Pdu-nk2.1*. I further characterized the cells of the APO by their potential to take up DiI. This staining allowed me to visualize the 'flask-shaped' crescent cells, as well as some of the 'flask-shaped' center cells of the APO and the plexus formed by the extensions of these cells. Importantly, the uptake of the dye in other animals has been correlated with the presence of ciliated dendritic nerve endings directly contacting the outside, a hallmark of chemosensory cells. A possible chemosensory function has been suggested for APO cells of primary ciliated larvae from different species. This would infer another, morphological and functional equivalence of cells in the APO and a specialized cell type in the median brain of basal vertebrates, the so-called 'central spinal fluid contacting neurons' (CSF contacting neurons).

All these results support the notion that the ventral/ median brain of vertebrates, with the hypothalamus as its central structure and the median brain of the *Platynereis* median larval brain can be homologenized. This assumption is based on the specific combinatorial code of transcription factor expression, specifying specific precursor cell types, and the differentiated cell types that descend from these precursors (also see below).

Moreover, I reported on my contribution to a large-scale WMISH screen performed in the lab and in collaboration with several other labs. Two of the genes that were found in the pioneer phase of this screen, *Pdu-otp* and *Pdu-netrin*, were of use for my brain comparisons, and their expression in the median forebrain was again consistent with the expression of their vertebrate orthologs.

I finished my comparative analysis by investigating the role of the Hh signalling pathway in the epispheral development of *Platynereis dumerilii*. The Hh pathway has been implicated in the development of ciliary PRCs and retinal ganglion cell (the likely rhabdomeric PRCs of vertebrates), as well as the median forebrain of vertebrates. I therefore analysed if functional aspects of the development of the median forebrain might be conserved as well across Bilateria.

I found two key molecules of the Hh pathway (*Pdu-hh* and *Pdu-smo*) to be expressed at the right time and place to be involved in *Platynereis* brain patterning. The drug cyclopamine, which inhibits the Hh pathway in vertebrates and leech, causes distinct and specific morphological, as well as molecular defects in *Platynereis* embryos. Several of these defects resemble defects of the Hh pathway in vertebrates, indicating that cyclopamine inhibits indeed the Hh pathway (and not other pathways) specifically in *Platynereis dumerilii*. Importantly, in several cases (*nk2.2*, *fkh*) only some domains are affected, whereas others were unaffected. This argues strongly against unspecific effects caused by this treatment. The disruption of the Hh pathway had several effects on the transcription factors and differentiated cell type markers analysed before in conjunction with the PRC and median brain comparison. Cyclopamine treatment reduced the epispheral domain of *Pdu-pax6* stronger than the domain of *Pdu-nk2.1*. It did, however, strongly reduce other markers of the median episphere, in particular *Pdu-rx* and RFamide(s). Other factors characteristic of the median brain, serotonin and *Pdu-vax*, were unaffected in their levels (serotonin) or

even expanded (*Pdu-vax*). In addition, the disruption of the Hh pathway caused a reduction of the pigment cells of the adult eyes, as well as of rhabdomeric PRCs.

The comparison of these results to other protostomia and vertebrates indicates that, ancestrally, the Hh- pathway likely played a role in the development of specific cell types of the median forebrain region, as well as of the eye.

3.2 Common ancestry/homology is the most likely cause for the occurence of equivalent cell types across Bilateria

My results reveal several cell types of the *Platynereis dumerilii* larval episphere that share extensive and specific cell morphological and molecular similarities to vertebrate prosencephalic cell types. Importantly, independent of the evolutionary implications one might draw from these comparisons, these similarities are a useful indicator to define which genes are crucial for the formation and function of a particular cell type, and which were added specifically in the vertebrate or polychaete lineage to modify the cell type. This approach is equivalent to the standard *modus operandi* applied to compare any molecular data from *Drosophila* or *C.elegans* to the vertebrate systems. However, the molecular set-up of the former model organisms is highly derived. In contrast, the sequences of *Platynereis* genes are much closer to the vertebrate sequences (Tessmar-Raible and Arendt, 2003) and are thus highly likely to be much closer to the ancestral and vertebrate biological function. This, combined with the simple brain morphology of *Platynereis*, is a good prerequisite to understand which ancestral gene functions the vertebrate CNS cell types (homologous or non-homologous) are based upon.

However, one major aim of this thesis is to try to establish homologies across Bilateria. I would like to start this discussion with a general consideration of the arguments that permit the formulation of such homologies. I have already introduced the idea that specific cell types are a better unit for evolutionary comparisons than whole organs or structures.

From the evolutionary point of view, the occurrence of any strong similarities between individual cell types of distant phyla must be linked to a common principle,

most likely a common genetic program driving the differentiation of this cell type. Two fundamentally distinct views exist on the question how equivalent genetic programs can appear in different lineages: a) The random switching of a key regulatory/ 'master control' gene establishes a whole cascade of gene regulatory events at unrelated places in different species. This means that the compared cell types are analogous. b) The molecular cascades are maintained in these cell types, however their position might vary due to cell migrations. In addition, duplication of genes may occur, followed by slow modification of the resulting paralogs and, finally, the separation of the resulting sister cell types by cell migrations (see introduction). However, all these cell types in the different compared species trace back to the same precursor cell type in the last common ancestor of the compared groups, implicating homology.

3.2.1 The master control gene concept: a valid alternative explanation?

The first interpretation has gained support by several experiments with 'master control genes'. The definition of a 'master control gene' was originally introduced for the homeotic genes of the *Bithorax Complex*, and perhaps the most impressive demonstration of their role in development has been the genetic construction of four-winged and eight-legged flies (Lewis, 1992; Schneuwly *et al.*, 1987). The discovery of the ability of murine and fruit fly *pax6* orthologs to induce eyes (and thus rhabdomeric PRCs) at endogenous as well as ectopic places in *Drosophila melanogaster* (Halder *et al.*, 1995) led to a somewhat broader use of the term. In this sense, a 'master control gene' is a gene that is capable of activating an entire molecular cascade at ectopic places, leading to the development of a particular structure or cell type at such places. Besides *pax6*, this phenomenon has now been described for many developmental transcription factors, such as *sim*, *myoD, gata1/2*, *myocardin* and *gcm* (Bonini *et al.*, 1997; Cao and Moi, 2002; Egger *et al.*, 2002; Huard *et al.*, 1998; Nambu *et al.*, 1991; Wang *et al.*, 2003; Weintraub *et al.*, 1989). The newly occurring cell type, induced by the ectopic action of a master control gene, e.g. photoreceptor cell type by *pax6*, would now be molecularly indistinguishable from the other, already existing population in the eye. This would,

however, not be because of common origin (homology), but because of the common recruitment of a ‘master control gene’, and thus a secondarily very similar molecular setup. However, two lines of argumentation indicate that this scenario does not describe what commonly occurs during evolution.

3.2.1.1 Spatially separated, but molecularly similar cell types in one species usually share common ancestry in ontogeny

If ‘master control genes’ could be easily switched on/ off, detectable continuity between cell types during ontogeny of one species or during phylogeny of different species should be rare events. However, this is not the case. In contrast, it can regularly be found that molecularly similar cell types of one species that are located at different places in the adult animal derive from one common precursor population and underwent migrations to localise at their final place of function (see introduction for details and examples). Despite many known cases of an initial common anlage for one cell type and subsequent cell migrations (see introduction for more details and examples), there is, to my knowledge, not a single natural case of a ‘master control gene’ switch, as it should be noticable in closely related species.

3.2.1.2 “Closed for reconstruction” does not exist in evolution

Every phenotypic change will be subject of selection in the adult body plan. This implies two issues. A) The phenotypic changes have to occur rather stepwise, because they must not reduce the ability to survive. This argues for rather little changes on the molecular level, and less for a sudden on/off switch of a ‘master control gene’ in unrelated cell types. B) The new cell type/ structure can not take the time of several generations to become functional, e.g. in the case of the PRCs acquiring the correct wiring. A non-functional structure will get abolished rapidly. This argues again against prominent roles of 'master control genes' in the prosses of evolutionary changes. To exemplify: In the case of the ectopic rhabdomeric PRCs induced by *pax6*, the flies had either their antenna replaced with an eye which was connected to the brain, but thus lost a major portion of their important olfactory sense. Alternatively, the eyes where located at other places, but were not wired functionally to the brain, and thus useless or even disadvantageous (Halder *et al.*, 1995).

In summary, although the term 'master control gene' is clearly valid to describe molecular hierarchies, the relevance of ectopic activation experiments for the real evolutionary changes is unclear. As a consequence, molecular, morphological (and spatial) similarities of cell types in different species can thus usually be considered as indication of a close phylogenetic connection, and are conversely useful to trace the relationships of structures and cell types. Therefore, my aquired data can be used for the establishment of true homologous cell types between vertebrates and Lophotrochozoa, and consequently for conclusions about the possible state of the nervous system of Urbilateria.

3.2.2 The molecular fingerprint is a useful criterium for cross-species comparisons

Finally, I want to address one issue that concerns the distribution of some markers used in my analysis. In several cases, the analysed factors, such as *Pdu-nk2.1* or *Pdu-vax*, are quite restricted in their distribution to the region of interest. However, others – such as *Pdu-rx* or *Pdu-hdc* –are not restricted exclusively to this region. Does this broader expression pose a problem to the intended cross-species comparison of cell types? They are useful for cross-species comparisons, because several factors are highly specific in vertebrates, as well as in *Platynereis*, and those that are less specific can be correlated with the region demarcated by the specific markers. The combination of transcription factors and cell type specification markers in the median forebrain region provides a unique fingerprint for this region.

However, it is certainly an important question how these broader expressions have to be interpreted in vertebrates, as well as in *Platynereis*. At the moment it is difficult to decide if the broader expression domains are more ancestral or newly aquired.

3.3 Is there conservation on the level of brain areas across Bilateria?

As I explained in my introduction, the comparison of distinct cell types is a very useful level of analysis, when homology between evolutionary distant organisms should be defined. However, my comparison started off with the investigation of larger brain areas. I hypothesized that the molecular lateral vs. median subdevision of

the anteriormost *six3/6* positive area in *Platynereis* and vertebrates (marked by *pax6* and *nk2.1*, respectively) forms a basic groundplan that appears to be conserved across different groups of Bilateria. This raises several further question that will be discussed below. Are such "regions" valid units for evolutionary comparisons, i.e. can "molecular regions" be homologous? If this were the case, would such a conservation on the level of regionalization be consistent with the approach of comparing cell types? Finally, is a conservation of brain areas likely to be a more general rule, or is it an exception?

To approach these questions, it is useful to consider two arguments. First, the concept of homologous cell types does not necessarily only apply to differentiated cells, but can also apply to precursor cells. In the field of hematopoiesis, for example, clear genealogies of cell types can be drawn that indicate which precursor cell type differentiates into which daughter cell types. In this system, precursor cells can well be addressed – and thus compared – by criteria like individual combinations of transcription factors. In brain development, these cell type genealogies are less established, but it is clear that terminally differentiated cell types have distinct precursors, and that individual sets of transcription factors expressed in these precursors account for the later specific differentiation into distinct terminal cell types. Usually, one precursor cell type gives rise to several terminal differentiated cell types. The population of precursor cells can be large, and thus the transcription factors specifying them will be present many cells. If these cells cluster together in larger areas, rather than in individual patches, these areas of a given precursor cell type can be compared.

Second, the vertebrate ventral/ median forebrain has many different precursor cell types, as evident by the patchwork of combinations of transcription factors in this region. It is possible to compare and localize these precursor cell types in a different animals by analysing the transcription factors they specify, as I did with *nk2.1*, *vax*, *rx, otp*, *sim*, *pou3*, *gsx* expressing cells. The more distinct precursor cell types co-occur together in a distinct area (such as the median forebrain) in the compared species, the more likely can the whole area be considered homologous in the compared species.

In conclusion, these arguments indicate that homologies between precursor cell types can also correlate with homologies between distinct areas of the developing animals. However, as with organs, one should treat larger structures, like areas, with special care, before proposing homology for them. Possible sources of error include dynamic expression patterns and cell mixing, both of which could render a seemingly homogenous area more heterogeneous. Only if many precursor cell types and final differentiated cell types co-localize similarly in the compared animals, then the logic of evolutionary parsimony indicates that this co-localisation in one area was already present in the common ancestor of the compared groups. There is good indication that at least in vertebrates specific precursor cell types of the CNS are indeed highly localized, and not spread throughout the brain. The complex distribution pattern of the resulting differentiated cell types seems to be rather due to secondary migration (see introduction and Rallu *et al.*, 2002a; Wilson and Rubenstein, 2000).

3.3.1 Robustness in molecular specification of regions within phyla as prerequisite for interphyletic comparison

The expression of *six3/6*, *pax6* and *nk2.1* has been analysed in many vertebrate species, and the results are consistent with the notion that the distribution of these three genes belongs to a conserved ancestral groundplan (see above). Even in the ancestral lamprey, the orthologs of these genes display similar expression characteristics (for references see introduction and results). Outside of vertebrates, *six3/6* has been analyzed in the directly developing enteropneust *Saccoglossus kowalevskii*, where it also demarcates the most anterior brain region (Lowe *et al.*, 2003). *pax6* in this animal is expressed very broadly in the anterior region, overlapping with the *six3/6*positive domain, but also extending beyond it (Lowe *et al.*, 2003). However, the regional expression of *nk2.1* is exceptionally well conserved. Its median forebrain expression domain is not only highly specifically present in vertebrates. A specific median brain expression domain within the *six3/6* positive area is also present in the adult body plan of the enteropneust *Saccoglossus kowalevskii*, and, most importantly, in the larvae of the enteropneust *Ptychodera* (Takacs *et al.*, 2002). Moreover, the correlation between the apical organ and the postulated *nk2.1* positive ancestral median brain appears to be conserved. This

correlation has been reported for the primary ciliated larvae of the aforementioned enteropneust, *Ptychodera*, as well as for the sea urchin *Strongylocentrotus purpuratus,* where *nk2.1* expression is present in cells of the apical organ (Takacs *et al.*, 2002). The expression of *nk2.1* in two different enteropneust species establishes a correlation of the apical organ of a primary ciliated hemichordate larvae, and the median brain expression in a *six3/6* positive region in a closely related hemichordate, that skipped its larval stage. This indicates a transformation of the deuterostome larval apical organ region into the ventral brain region of the deuterostome adult stage.

In conclusion, the complementary expression patterns of *nk2.1* and *pax6* in an overall *six3/6* expressing brain have correlates in many other deuterostomian species, supporting the notion that this molecular distinction between two brain areas is also ancestral for the deuterostome branch of evolution

3.3.2 *No conservation of* otx, bf-1, emx *regions?*

Many genes show a broad regionalized expression during earlier stages in vertebrates. These include genes like families of *bf-1*, *otx* (Shimamura *et al.*, 1995), *emx* or *dlx*-family (Redies and Puelles, 2001; Sheng *et al.*, 1997). In contrast, the orthologs of these genes in *Platynereis* show so far only a rather scattered, cell specific expression (see above and data not shown). This is especially remarkable for the *otx* gene, which in both *Drosophila* and vertebrates is broadly expressed in the anterior CNS and has been considered to be a gene with a main function in regionalization (Redies and Puelles, 2001). There are two explanations that can account for these differences. First, it is possible that at earlier embryonic stages of *Platynereis* than those investigated, some of these genes would indeed exhibit a regionalized expression. Second, the regional dimensions of expression patterns might change more easily between different species, than the location of the expression sites themselves in the animal. One mechanism for such changes could be changes in the proliferation rate of precursor cells that would then expand or shrink the dimensions of an ancestral expression domain in one or the other evolutionary lineage.

In summary, the general extent of conservation on the level of bilaterian brain regionalization is, unclear, and awaits further investigations, such as the study of expression patterns at earlier stages in *Platynereis* and in different species. Additionally, further characterizations and comparisons of the enhancer regions of the genes in question will also help to discern between ancestral/conserved and acquired aspects of their regulation. An important problem that remains to be solved is the question why natural selection has maintained regional expression domains like those of the *nk2.1* gene.

3.4 Eye development in Bilateria

It had been hypothesized that Urbilateria already possessed two PRC- types, but evidence for this was lacking on the molecular level (see introduction and results). I isolated two members of an additional *opsin* group from *Platynereis dumerilii*, which by phylogenetic tree analysis and transcript expression confirmed the ancestral presence of two different PRC types on the level of differentiated cell type markers (see results). The phylogenetic grouping was moreover strongly affirmed by further and more detailed sequence comparisons of the amino acids present in the transducin activation and transducin binding domains (D.Arendt pers. comm.). The specific correlation of distinct transcription factors with these two distinct PRC types further completed this analysis (see results). Therefore, my own data, combined with previous analyses, provides evidence on three experimental levels – sequence analysis, developmental gene expression and ultrastructure – that supports the co-existence of two distinct photoreceptor cell types in *Platynereis,* and argues that ciliary photoreceptor cells are not an evolutionary novelty of the vertebrate lineage. Rather, both ciliary and rhabdomeric photoreceptor cells appear to be of ancient origin. This analysis does not only allow conclusions about PRC evolution, but in consequence also about vertebrate eye evolution.

3.4.1 Ancestrally, rhabdomeric PRCs watched the surroundings, ciliary PRCs governed the timepoints of activity

Urbilateria already possessed two distinct photoreceptor cell types, one of which – the rhabdomeric photoreceptor cells – evolved to constitute the eyes of insects or polychaetes, while the ciliary type remained located in the brain in most animals. These two distinct PRC types are likely to derive from one initial PRC type earlier in evolution, since they employ paralogous opsin molecules. Whether this split occurred in urbilaterian or pre-urbilaterian times is currently unclear.

It is very interesting in this regard that the rhopalia (organs containing mechano- and/or light sensory cells) of cnidaria already contain a combination of photoreceptors that express the *pax6* ancestor *paxB* (uniting *pax2/5/8* and *pax6*) and *brn3* (Jacobs and Gates, 2001; Kozmik *et al.*, 2003). It will be very interesting to investigate what kind of *opsin* (or other kind of photosensory molecule) is expressed in these cells. From these indications it is possible that rhabdomeric PRCs and the molecular cascade controlling their formation, as well as their function in vision might be even more ancient than Urbilateria. The other type – the ciliary cell type – was not directly involved in true vision (probably rather in circadian clock regulation) and was only present in the brain, probably under the control of an ancestral *rx* gene. Very similarly, the ciliary photoreceptors still exist in the inner brain of vertebrates. However, a change occurred in the evolution of vertebrate eyes: The ciliary photoreceptor cells were recruited from the brain to form the true photoreceptor cells of the retinal layer, as well as the ciliary photoreceptors of the pineal organ.

3.4.2 Evolution of vertebrate retinal ganglion cells from rhabdomeric PRC precursors

As explained already in the results section, the retinal ganglion cells of vertebrates are the cell type that shares many molecular characteristics with the rhabdomeric PRCs of invertebrate eyes (first proposed by D. Arendt in Arendt *et al.*, 2002). They express a rhabdomeric opsin, *melanopsin*, and require the molecular cascade of *pax6*, *ath5*, *brn3* for their development (Arendt, 2003 and this study). Moreover, retinal

ganglion cells as well as rhabdomeric photoreceptor cells are the cells that directly extend their axons to transmit the visual information into the brain (Arendt *et al.*, 2002). In vertebrates as well as in *Drosophila* they express and use Eph receptor tyrosine kinases to project to the target area and build a topographic map (Dearborn *et al.*, 2002). It is unclear however, to which extent the similarities between invertebrate rhabdomeric PRCs and vertebrate RGCs extend on the level of neurotransmitters. Both cell types use acetylcholine as transmitter (Arendt, 2003), but nothing has been stated about the existence of histamine in RGCs of vertebrates, an amine that has an important role in the neurotransmission of insect, and likely also of *Platynereis* larval eye rhabdomeric PRCs. Nevertheless, the probable phylogenetic relationship of rhabdomeric PRC and RGCs is strongly supported.

3.4.3 Sister cell types in the vertebrate retina

Is the described theory also consistent with the presence of other cell types in the vertebrate eye, such as amacrine or horizontal cells (see above)? Their existence is best explained by the sister cell concept, meaning that the other cell types of the eye are either related to (meaning that they are their descendants) ciliary or to rhabdomeric PRCs (or migrated into the eye from independent places, as probably true for the Mueller Glia cells; Arendt, 2003).

3.4.4 Ciliary PRCs and rhabdomeric PRCs originate from one common precursor, both in ontogeny and phylogeny

As mentioned above, it is plausible that ciliary and rhabdomeric PRCs descent from the same precursor earlier in evolution. Such developmental history is often still reflected in the development of extent species, as formulated in Haeckel's biogenetic law Haeckel, 1874; Haeckel, 1886. Consistent with this idea, it has been shown that RGCs and ciPRCs arise from common developmental precursors in the vertebrate retina, since an early precursor in the retina can give rise to several cell types, including RGCs and ciPRCs (Cepko, 1999; Cepko *et al.*, 1996).

In this context, it is further interesting to note, that at earliest stages of vertebrate ciliary marginal zone development one gene of the top level of the developmental

hierarchy of both distinct PRC-types is present. The ciliary marginal zone is a region in which stem cells proliferate and contribute to the vertebrate eye during later life. Thus, the earliest steps in eye development molecularly employ a combination of genes demarcating the forebrain (in this case represented by the *six3/6* family) and developmental genes of each PRC-type, represented by a member of the *rx*-family (*rx1*) and *pax6* (Perron *et al*., 1998). Strikingly, this is also the case for a 'cocktail' of genes determined to be sufficient to induced ectopic eyes in vertebrates outside the nervous system with high frequency (Zuber *et al*., 2003).

Two challenging questions remain to be answered. First, if *pax6* was ancestrally only required to govern the development of only one, the rhabdomeric type, of the two ancestral photoreceptor cell types, how can it be explained that it is able to ectopically induce complete eyes in vertebrates, eyes that include both ciliary and rhabdomeric PRCs (Chow *et al*., 1999; Zuber *et al*., 2003)? It has been shown, that in these cases *pax6* is able to activate members of the *rx*- and *six3/6*- families (Chow *et al*., 1999). Future experiments will show whether this molecular dependance was newly aquired in the vertebrate lineage, or predated the protostome/ deuterostome split. Also, it is yet unclear, whether the different PRCs share common ontogenetic origin in *Platynereis dumerilii*, and in which hierarchy the genes stand to each other in the genetic cascades in this animal.

To clarify these issues in *Platynereis dumerilii*, it will be necessary a) to investigate earliest stages of eye development, before the differentiation of the first eyes has occur, i.e. 19hpf and earlier; b) to perform gain- and loss-of function studies to unravel the molecular hierarchies involved. It is likely that the different PRCs derive from a common precursor: Both *r*-opsins and *ci-opsins* derive from a common *opsin* gene. After duplication of this precursor during evolution, both successor molecules were initially expressed in the same cells. Over time they diverged more and more, together with their developmental cascade. This initial derivation from the same precursor is possibly still reflected in the developmental phenomena mentioned above.

3.4.5 What makes a larval eye different from an adult eye?

Finally, there is the interesting result (first noticed for *Pdu-pax6* by D. Arendt in Arendt *et al*., 2002) that several members of the rPRC-pathway are only clearly expressed in the larval eye rPRCs, but not adult eye rPRCs. In order to be really certain about the expression of *Pdu-brn3* and *Pdu-hdc* in larval, but not adult PRCs of *Platynereis*, two points still need to be considered. First, a double WMISH staining with *Pdu-r-opsin* is useful for further corroboration of the distinct expression. Second, although no indication of gene or genome duplications exists for *Platynereis dumerilii*, it is still a possibility that *Platynereis* paralogs exist for these genes and are expressed in the adult eyes. Therefore, genomic southern blotting and hybridisation needs to be carried out, to further test this observation.

3.5 Neurosecretory cell types in Bilateria and the origin of the vertebrate hypothalamus

Neurosecretory cells are of pre-eminent importance in all animals, since they are involved in the most basal body functions such as metabolism or ion regulation. Therefore, it is highly likely that a central control and regulation of basal body functions is a feature that evolved already early in the evolution of Bilateria. This section discusses my observations on cell types in the *Platynereis* median brain, and describes a possible set of neurosecretory cell types ancestral for the bilaterian brain.

3.5.1 Nk2.1-positive "flask-shaped crescent cells" and "flasked-shaped center cells" of the APO compare to CSF-contacting neurons of the nk2.1 positive area of the hypothalamus

The vertebrate hypothalamus has distinct cell types, not only on molecular, but also on morphological level. The most apparent are the central spinal fluid contacting neurons (CSF contacting neurons, see Figure 40C,D). These neurons exist in various shapes, are located in the walls of the brain ventricles, and extend their ciliated dendrites into the central spinal fluid. They have been considered to be sensory neurons, based on their electron microscopic morphology and their cilia (Vigh and

Vigh-Teichmann, 1998; Vigh-Teichmann and Vigh, 1983). One subgroup of them is photosensory, as judged by the additional presence of ciliary opsin. This subgroup is also known as (ciliary) deep brain photoreceptor cells (Vigh *et al*., 2002). The second type is of presumptive chemosensory nature. This speculation is based on three points: i) the morphologically sensory appearance of these cells, ii) the non-reactivity with anti-Opsin antibodies and iii) their reaction to chemical changes in the CSF (Vigh and Vigh-Teichmann, 1998; Vigh-Teichmann and Vigh, 1983). Besides eye and pineal, visual and presumptive chemosensory CSF contacting neurons are exclusively localized to the walls of the ventral/ median forebrain, especially in the preoptic area and hypothalamus. Since this is the 'core area' of *nk2.1* expression, it is highly likely that they are *nk2.1* positive (see results part). A third CSF contacting neuron type is of presumptive mechanosensory nature and located in the spinal cord (Vigh and Vigh-Teichmann, 1998), where *nk2.1* is not expressed. The CSF neurons of the forebrain have been found to be often highly neurosecretory, as they contain a large amount of dense core vesicles (Figure 40D).

In *Platynereis dumerilii* the *nk2.1* gene does not only demarcate the median brain region in the larval episphere, importantly, its expression includes special types of cells, the 'flask-shaped' crescent and 'flask-shaped' center cells of the apical organ. These cells exhibit several features, highly reminiscent to the presumptive chemosensory CSF contacting neurons of the vertebrate ventral/ median forebrain (see below).

3.5.2 *Photosensitive and chemosensory CSF contacting neurons in vertebrates and their relationship to cell types in the* Platynereis *episphere*

CSF contacting neurons contain ciliary Opsins and/ or many ancestral neurosecretory substances. Are the cell types present in the trochophora – type larval brain, in particular ciPRCs and cells of the APO, comparable to the CSF contacting vertebrate forebrain neurons on an extended basis, including further molecular details, as well as aspects of cell morphology and function? The CSF neurons containing members of the ciliary opsin group are certainly most reminiscent to the brain ciliary photoreceptor cells of *Platynereis*. It should be noted that the ciliary opsins

expressed in the CSF contacting neurons are encephalopsins/ val *opsins* (Figure 17), those Opsins that repeatedly group together with Pdu-Ci-Opsin. Pdu-Ci-Opsin should cluster outside the vertebrates together with the CI-Opsins of *Anopoheles gambie*, because of their phylogenetic closer distance (see legend of Figure 5 for further explanation). Although artificial, the joined clustering with vertebrate sequences indicates that the Opsins of the deep brain PRCs contain more ancestral sequence features, implying that the vertebrate deep brain ciliary PRC are least derived, i.e. the most ancient ciPRC-type, and best comparable to the *Platynereis* deep brain ciliary PRCs. Another notion can be interpreted as further support for this idea. Several genes that are involved in the development of the vertebrate eye (such as *rx* or *vax*) have as well a broader, yet highly specific expression domain in the ventral/ median forebrain of vertebrates. This observation probably reflects the fact that at least some of the cell types of the vertebrate eyes are evolutionary descendants of this ventral/median area. Therefore, the photosensitive CSF contacting neurons are likely to correspond to the *Platynereis* ciliary PRCs.

The CSF contacting neurons that do not express ciliary *opsins*, but are of presumptive chemosensory nature and contain neuropeptides and serotonin exhibt a remarkable similarity to other cells in the *Platynereis* episphere, the *Pdu-nk2.1* expressing 'flask-shaped' crescent and center apical organ cells. First, the DiI staining of the apical organ cells is interesting in this context. DiI was not only used to visualize the apical organ neurosecretory plexus as it had been described for different trochophora type larvae, but it also indicates that several cells of the APO have direct contact to the outer environment. The uptake of DiI by few and distinct neurons is reminiscent to the situation in the nematode *C.elegans*, where DiI is exclusively taken up in a subset of sensory neurons that contact the outside via ciliated endings (see results for citations and more details). Therefore, the DiI fillings indicate that the stained APO neurons directly contact the outside via their ciliated dendrites.

Consitent with this, staining with an anti-acetylated tubulin antibody revealed that some of the ventral apical organ cells extend long fathom-like structures from their dendrites (referred to as 'flask-shaped' center cells). At least a subset of them is likely to correspond to the ventrally located DiI positive apical organ cells by position. These cells are also reminiscent of the sensory ampullary and parampullary apical

organ cells existing in mollusc trochophora – type larvae by morphology, spatial location and molecular composition (see introduction and results and below). Several of the sensory parampullary cells of molluscs have been described to be positive for RFamide(s), as well as for serotonin (Dickinson and Croll, 2003). Similarly, 'flask-shaped' center cells of the *Platynereis* trochophora type larvae are positive for RFamide(s) and (one cell) for serotonin (Figure 28C,D,E,K,L,M,N, see below for further discussion on RFamide(s) and serotonin).

In striking resemblance, two of the preoptic area and hypothalamic neurosecretory substances contained by several CSF contacting neurons are RFamide(s) and serotonin (Oksche and Vollrath, 1980; Vigh and Vigh-Teichmann, 1998). This comparison indicates that the APO cells of trochophore-type larvae are homologenizable to (at least subgroups) of the presumptive chemosensory CSF contacting neurons of the vertebrate forebrain. Thus, the ventral/ median forebrains of vertebrates and lophotrochozoan trochophora - type larvae are not only similar in their combination of transcription factors and cell type specifying molecules (see above results and below). They also share cells with a similar morphological characteristics and putative functions.

In the following sections I will further discuss on cell types, focusing now mainly on their molecular composition. This will include precursor cell types, at the moment only characterized by a limited set of transcription factors, as well as some differentiated cell types, that (where possible) I tried to link to precursor cell types by expression of the described transcription factors.

3.5.3 Nk2.1-*positive serotonergic and RF-amidergic cells*

Two genes are highly specific in their expression in the brain of *Platynereis* larvae. These are serotonin and RFamide(s). Their first site of expression in the brain is the apical organ. In both cases, the staining later extends to more cells in the median brain. Similar to RFamide(s), serotonin has an astonishingly high spatial specificity for the median brain of *Platynereis*, and especially for the apical organ. Reminiscent of *nk2.1* expression, the presence of serotonin in the apical organ is highly stereotypical across Bilateria, since it has been reported not only from protostomian

larvae, but also from deuterostomian larvae, like the primary ciliated larvae of enteropneusts, as well as from echinoderms (Hay-Schmidt, 2000). Since deuterostomian and protostomian primary ciliated larvae can be considered homologous (Arendt *et al.*, 2001), this strongly argues that already Urbilateria possessed serotonergic cells in a *nk2.1* positive region. In contrast, RFamidetergic cells positive cells have so far only been shown to be associated with the apical organ of protostomia, such as different polychaetes and molluscs (including the basal polyplacophoran *Mopalia muscosa*; Dickinson *et al.*, 1999; Friedrich *et al.*, 2002; Hay-Schmidt, 1995).

To resume, the best-conserved markers that – in combination –characterize cells of the APO across Bilateria are serotonin and *nk2.1*. Consequently, if a *nk2.1* positive apical organ region with serotonergic cells already existed in Urbilateria, can remnants of this ancestral equipment still be traced in vertebrates, where primary ciliated larvae with apical organs can not be found any more? Serotonergic cells in invertebrates have often been compared them to the serotonergic cell in the raphe nucleus of vertebrates (Hay-Schmidt, 2000). This is in fact the major nucleus containing serotonergic neurons in mammals (Kandel *et al.*, 2000). However, outside mammals, all other vertebrates have a major serotonergic region in the hypothalamus (Sano *et al.*, 1983 and results part above), and consequently co-expresses *nk2.1* (because, as laid out above, *nk2.1* demarcates the entire hypothalamus and preoptic area also in lower vertebrates). Therefore, the co-presence of serotonin and *nk2.1* in the hypothalamus of lower vertebrates is likely to be the direct reflection of an ancestral developmental program involved in the specification of serotonergic apical-organ-like cells.

Interestingly, a similar link emerges from the analysis of RFamide(s) positve neurons in vertebrates. As mentioned above, RFamide(s) positive cells have only two places of origin in the vertebrate forebrain (see results part). Of these two, one is located in the tuberal hypothalamus, including the arcuate nucleus, or its resprective counterparts in non-mammals. Exactly this region is affected in the *nk2.1* knock-out mice, where this nucleus in particular is completely absent (Kimura *et al.*, 1996). Notably as serotonin, RFamide(s) localizes into the CSF contacting neuron type in the *nk2.1* positive area in vertebrates, very reminiscent of the RFamide(s) containing

and *nk2.1* expressing 'flask-shaped' center cells of the the apical organ. This provides strong evidence for a shared ancestry of this cell type in vertebrates and annelids, and hence also for the ancestral co-appearance of *nk2.1*, serotonin and RFamide(s) in cell types of the median bilaterian forebrain (see Figure 40C,D,E,F and discussion about the central spinal fluid contacting neurons above).

3.5.4 Nk2.1-*positive* gnrh-r *cells*

In addition to serotonin and RFamide(s), two additional likely ancestral differentiated cell types are shared between the apical organ region and the vertebrate ventral/ median forebrain (especially the hypothalamus). One gene, *gonadotropin releasing hormone receptor* (*gnrh-r*), is far less specifically expressed in the vertebrate brain than the previous markers. For example, *gnrh-r* is expressed in the adenohypophysis, but one of its major expression sites in the brain is the tuberal hypothalamus, the central region of *nk2.1* expression. Consistent with the equivalence of this region with the apical organ, I found that the *Platynereis* ortholog *Pdu-gnrh-r* is present in *Pdu-nk2.1* positive cells in the apical organ region of *Platynereis* larvae as well (see see results and Figure 40E,F).

3.5.5 Possible nk2.1- *positive histaminergic neurons*

The expression of the enzyme *histidine decarboxylase* marks sites of histamine synthesis. Although it is widely occurring in different tissues of many species, its expression in the vertebrate brain is highly specific for the hypothalamus. In all vertebrates, including fish, it is expressed in one particular nucleus, which in mammals is called the tuberomammillary nucleus (Haas and Panula, 2003; Kaslin and Panula, 2001). In good accordance with the equivalence of these brain regions, an ortholog of this enzyme is also expressed in the median region of the *Platynereis* larval brain, close to the apical organ. However, it should be noted that, despite the clear restriction of *histidine decarboxylase* expression in vertebrates, the distribution of this enzyme in other animals is less restricted. First, in lamprey (a very basal vertebrate) it appears, in addition to the hypothalamic expression, also to be expressed at the mid-/ hindbrain boundary (Brodin *et al*., 1990). Second, as mentioned in the section about the photosensory systems, *histamine* is also a marker

for the photoreceptor cells of insects. A conserved role in protostomian PRCs could be reflected by its likely presence in the larval eye photoreceptor cells of *Platynereis*. (I could, however, not detect it in the adult eye photoreceptors.) In conclusion, *histamine* might have had two or even three ancestral expression sites in Urbilateria. As a consequence, the single vertebrate histaminergic nucleus in the hypothalamus could theoretically also relate to one of the potential additional ancestral presence sites of *histamine*, even if this appears to be less likely, based on all the circumstitial evidence that I have presented here. A clearer answer could be gained if the histaminenergic nuclei of the vertebrate hypothalamus co-express *nk2.1*, but this has not been investigated (S.Kimura, pers. comm.).

In summary, at least three differentiated cell type markers (RFamide(s), serotonin and *gnrh-r*) exist in the *nk2.1* positive area in the median brain of vertebrates as well as in the primary ciliated larva of *Platynereis*. The fourth cell type gene (*hdc*) has not been investigated in respect to its expression in the vertebrate *nk2.1* area, but its expression is consistent with a homology of many cell types of the median forebrain across Bilateria (Figure 40E,F).

3.5.6 Otp-*positive Vasopressin/Oxytocin, Somatostatin secreting cells*

Similar to the *nk2.1* expression, the aspect of *otp* expression in the APO is well conserved, since it is also present in apical organs of organisms outside the polychaetes. As mentioned above, the exclusive expression site of the *otp* counterpart in the larval brain of the mollusc, *Patella vulgata* locates to cells of the apical organ (Nederbragt *et al.*, 2002). What effector genes are downstream of the apparently conserved *otp* expression? In vertebrates, *otp* has been shown to be essential for a neuropeptidergic subtype of cells in the hypothalamus (Acampora *et al.*, 1999; Wang and Lufkin, 2000). These peptides (such as AV/O or SS) are extremely well conserved between vertebrates and Lophotrochozoa in those cases where they have been identified, indicating a strong selective pressure on their function (see results), They thus represent likely candidates for ancestral effector genes downstream of *otp*.

Interestingly, a sea urchin (*Paracentrotus lividus*) *otp* gene has been cloned, but has so far not been described to be expressed in the apical organ region of the primary ciliated larvae (pluteus) of this species (Di Bernardo *et al*., 1999). This finding is surprising, since the primary ciliated larvae of sea urchin and polychaetes are considered to share homology (Arendt *et al*., 2001). The most likely explanation for this controversial finding would therefore be the degree of evolutionary 'derivedness' characteristic for echinoderms in general, as illustrated by their highly derived morphology (Storch and Welsch, 1993; Westheide and Rieger, 1996). The observed discrepancy for *otp* expression in sea urchin probably reflects a functional aspect. The peptides regulated by *otp* in vertebrates (such as AV/O or SS), despite their apparent cross-bilaterian conservation, have not been described nor can be found in available EST collections for echinoderms. Therefore, if *otp* is indeed an ancestral regulator of these neuropeptides, its absence from the apical organ in sea urchin pluteus larvae would be expected to correlate with a loss of the corresponding neuropeptidergic cells. This hypothesis remains to be tested.

Three additional transcription factor orthology groups (*pou3*, *sim1/2* and *gsx*) that play a role in neuropeptidergic cell development of the hypothalamus of the vertebrate median forebrain were tested and found to be expressed in the median region of the *Platynereis* brain, and in particular in the apical organ. Two of those (*sim1/2* and *brn2*- one of the vertebrate *pou3* genes-) are known to act in the same molecular cascade in a subset of (*nk2.1* positive) neuropeptidergic hypothalamic neurons (CRH and AV/O positive neurons) in parallel to *otp*. Strikingly, both genes can also be found co-expressed in the *Platynereis nk2.1* positive median epispheral region, further corroborating the ventral/median brain equivalence across Bilateria (Figure 40A,B and results).

Finally, the CSF-contacting neurons of the vertebrate hypothalamus have been shown to contain several of the preoptic area and hypothalamic neurosecretory substances, e.g. members of the AV/O peptide family, SS, PROLACTIN, substance P, VIP and NPY (Vigh and Vigh-Teichmann, 1998; Vigh-Teichmann and Vigh, 1983 and see above). As mentioned above, SS and AV/O are direct downstream targets of *otp*, *sim* or *pou3* orthologs in vertebrates. The finding of VIP in these neurons is interesting, because it is a member of the bilaterian-wide conserved

superfamily PACAP (see results) and thus highly related to GHRH, the peptide under the control of *gsx* orthologs in vertebrates. These are important correlations, because as layed out above, *otp*, *sim*, *pou3* and *gsx* orthologs are present in cells of the APO of primary ciliated larvae, of which at least subgroups are highly reminiscent to the CSF contacting neurons of the vertebrate forebrain (see above).

In conclusion, the following scenario emerges for ancestral cell types in a *six3*/6 positive bilaterians forebrain. The cell types contained neuropeptides, like AV/O or PACAP, and belonged at least partly to a presumptive chemosensory neuron type contacting the outside. Transcription factors ancestrally specifying these cell types was in all cases *nk2.1*. This *nk2.1* precursor cell type was then further subdivided by *otp*, *sim* and *pou3* orthologs to specify AV/O containing cells and *gsx* orthologs, which specified the PACAP-family positive subtype.

3.5.7 Vax-*positive Netrin chemoattractor cells*

Another transcription factor subfamily that is involved in the presumptive ancestral area of the median forebrain is the vax subfamily. *Pdu-vax* expression shows two features remarkably similar to the two vertebrate *vax* genes. First, it is highly specific for the median region of the *Platynereis* episphere overlapping with *Pdu-nk2.1*. Second, in this region it demarcates the growing axon tracts (Figure 40A,B; first noted byD.Arendt). This could be indicative of a conserved function in axon guidance, as *vax* positive cells are closely associated with distinct median axon tracts in mice. *Vax* knock-outs in mouse display, among others, defects in axon guidance of the retinal ganglion cells and of the median aspects of several other major axon tracts in this area (Barbieri *et al.*, 1999; Bertuzzi *et al.*, 1999; Hallonet *et al.*, 1999). These defects are ar least partly caused by a loss of the chemoattractent *netrin* in the axon assiociated cells in *vax1-/-* mice (Bertuzzi *et al.*, 1999). The latter detail is highly remarkable, given the fact that also in *Platynereis* larvae *Pdu-netrin* is highly reminiscent and thus likely overlaps with *Pdu-vax* in the ventro-median episphere as early as 24hpf (compare Figure 23 E and Figure 31 A). *Vax* is unlikely to play a more general role in axon guidance, since for this, it would on the one hand have to be present at more sites, and, on the other hand, in this case, the gene should not be dispensable from any species. *Drosophila* apparently, however, seemed to have lost

this gene, although it is present in another derived protostomian, *C. elegans*. In accordance with additional functions of *vax,* a mouse knock-out of *vax1* abolishes the preoptic area (Hallonet *et al*., 1998[Hallonet, 1999 #2792).

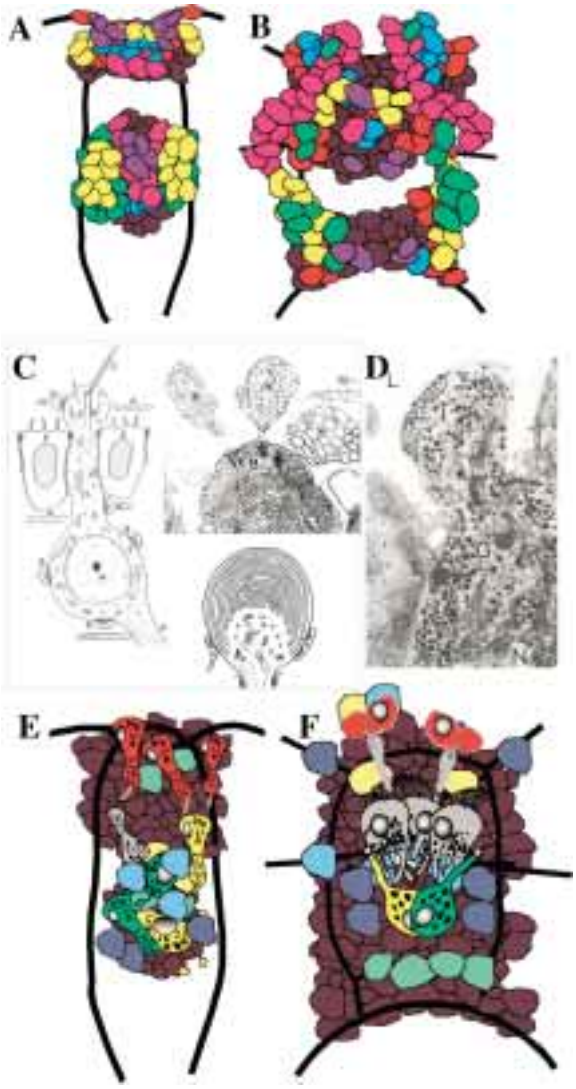


Figure 40. Vertebrate central spinal fluid contacting neurons and schematic comparisons of the vertebrate median forebrain and the *Platynereis* apical organ regions.

(A,B,E,F) As in the previous figures, simplified and schematized views of the vertebrate median/ ventral forebrain (A,E) and the median region of the *Platynereis* episphere (B,F); later major axon tracts are given in black as reference. **Color code:** (A,B) dark red: *nk2.1*; light red: *vax*; blue: *otp*; pink: *rx*; yellow: *sim*; green: *pou3*; lilac: *gsx*. (E,F) dark red: *nk2.1*; light red: ciliary PRCs; yellow: serotonergic cells; green: RFamide(s) positive cells; light blue: *gnrh-r* positive cells; dark blue: *hdc* positive cells. Cells with special shapes in E represent CSF-contacting neurons, in F ciPRCs and cells of the APO (see also Figure 8). For more explanations and comparisons of the schematics see Figure 23 for A, B and Figure 29 for E, F.

(C) Different types of CSF-contacting neurons in the vertebrate forebrain. **(D)** Dendritic terminal of a CSF contacting neuron reaching into the fluid. The whole cell is filled with many dense core vesicles. This indicates high levels of neurosecretion. Pictures in panels C and D taken from Oksche and Vollrath, 1980.

In conclusion, one likely ancestral function of *vax* is the specification of a median brain specific cell type, (at least partly) positive for *netrin* and required for the correct axon tract formation of a restricted set of median forebrain axon tracts. The specification of additional cell types is likely, given the broader phenotype observed in mouse mutants. *Platynereis* could be useful to identify those additional candidate cell types that could be controlled by *vax* and thus provide a guide for a more refined analysis of the role of this gene also in vertebrate development.

3.5.8 Rx *positive precursor cells*

Rx is another gene that supports the equivalence of median brain regions. *Rx* is highly specific for the median brain and its derivatives in vertebrates, and *Pdu-rx* consistently shows clear expression in the APO and median brain region in *Platynereis* (see results and Figure 40A,B). Besides its very median expression (in the apical organ and the ciliary PRCs), later expression of *Pdu-rx* includes two additional lateral wing-shaped expression domains that might represent a developmental specialty of *Platynereis* (see above). One likely role for *rx* in the median brain region is the specification of ciliary PRCs which are comparable to the photosensitive CSF contacting neurons of the ventral/ median vertebrate forebrain and it derivatives, as I have discussed in greater detail above. Beyond that, and consistent with the broader expression of *rx* in median/ventral brain of Bilateria, evidence from a medaka fish mutant of the *rx3* gene indicates that this gene might have a more prominent function also for other cell types in this area, since the hypothalamus in the mutant fish appears to be disorganized (S. Winkler, personal communication). To date, however, no detailed studies have been performed that would answer the question which additional hypothalamic cell types depend on the function of *rx*.

3.5.9 *Evolutionary origin of the nuchal organ, a possible polychaete synapomorphy*

The analysis of RFamide(s) positive cells yielded an interesting side aspect. Besides the prominent presence in the apical organ region, RFamide(s) is observed in only one additional population of cells in the episphere. These are the cells of the nuchal organs (presumed chemosensory organs, see in Ax, 1988). How has this population to be interpreted in respect to the cross-bilaterian median forebrain comparison? From the present data in *Platynereis,* this question is difficult to answer, but notably, the nuchal organs in other polychaete larvae (e.g. *Polygordius*) have been described to derive from much more medially located primordia, in closest vincinity to the apical organ (Hatschek, 1878). This is an interesting notion, since these presumptive chemosensory organs express as well other median brain markers, such as *Pdu-hh* or *Pdu-rx*, and so the nuchal organ and the apical organ might be linked by a common

ancestor organ. The determination of the very early origin of the nuchal organs in *Platynereis dumerilii* larvae by cell labelling and tracing experiments will be one possible way to address this issue.

3.5.10 Evolutionary origin of CSF-contacting neurons

In the following part, I want to discuss the existing theories on the origin of the vertebrate CSF contacting neurons. CSF contacting neurons have been described as such from lamprey onwards, but get progressively reduced in higher vertebrates and are not present anymore in mammals (Oksche and Vollrath, 1980). Two major hypothesis of their origin have been put forward. The first theory suggests, that CSF contacting neurons represent a common type of 'protoneuron' from which all other neurons derive (Vigh-Teichmann and Vigh, 1983). It might be true, that neurons of more primitive animals are less protected and thus more often exposed to the outside. However, I find it doubtful that neurons with very specific morphological and molecular characteristics should serve as precursor of all other neurons. The CSF contacting neurons of the vertebrate forebrain are a mixed population of molecularly clearly distinct cell types. Thus, it seems to be more probable to compare them to similar distinct cell types in other invertebrate brains. The second theory proposes, based on morphological criteria, that apical organ cells where taken over into the vertebrate brain. T. Lacalli proposed that these apical organ cells formed the evolutionary core of the later vertebrate eye complex, which involves CSF-contacting neurons (Lacalli, 1994). This latter theory is highly consistent with the outcome of the comparative molecular studies of this thesis.

3.5.11 Evolutionary conservation of the neuroendocrine aspect of the hypothalamus?

One major aspect of the vertebrate hypothalamus is not only its neurosecretory, but also its neuroendocrine function, meaning the release of neurosecretory molecules into the body fluid. Has this feature evolved in the deuterostome lineage, or could it reflect an ancestral state as well? A definitive answer to this question can not be given at the moment, and will require further studies. Three arguments should however be considered in this context. First, a subgroup of the neurosecretory CSF

neurons forms part of the vertebrate hypothalamic neuroendocrine system and thus is directly functional in this process (Oksche and Vollrath, 1980; Vigh and Vigh-Teichmann, 1998). Since I have stressed the likely homology between CSF-contacting neurons with cells in the polychaete apical organ, this finding is at least consistent with the hypothesis that the endocrine function is ancestral. Second, many adult polychaetes possess a well – elaborated vasculatory system that is contacted by a neurohaemal organ, most commonly called the infracerebral gland, which acts as a neuroendocrine system. Third, circumstantial evidence exists that cells of the apical organ could, in the course of development, contribute to this infracerebral gland. Generally, the fate of the apical organ cells in later developmental stages is strongly debated in different organisms. They have been stated to vanish completely or, alternatively, to become more and more integrated into the developing brain and to be maintained at least partly into adulthood (Chia and Rice, 1978; Dickinson and Croll, 2003; Kempf *et al.*, 1997; Lacalli, 1981; Lacalli, 1984). In this study, I found that the median RFamide(s) positive cells of the *Platynereis* larva, as well as probably the *Pdu-hdc* positive cells are maintained long into adulthood. In consequence, this argues (at least partly) rather for the latter possibility. Moreover, the final position of these cells in *Platynereis* is a location in the middle of the brain between the two adult eyes. Interestingly, this is very reminiscent of the RFamide(s) positive cells described for adult worms of the close relative *Nereis*, where these cells are located in the middle of the brain directly above the infracerebral gland (Thorndyke and Goldsworthy, 1988). Whether or not these cells project their axons into the infracerebral gland of *Nereis*, has not been determined. This is, however, likely, because in young *Platynereis* larvae, I observed projections of these cells that clearly extend to the base of the brain, where later the infracerebral gland is located. Moreover, in a different annelid, the leech *Theromyzon tessulatum*, RFamide(s) brain neurons have been shown to project into its neurohaemal organ (Lefebvre and Salzet, 2003).

Therefore, one interesting hypothesis would be that not only the neurosecretory cell types in the median brain of Bilateria might be homologous and thus ancestral, but that these cells might also have contributed to an ancestral neuroendocrine system, which became modified in the different evolutionary lineages, giving rise to the

infracerebral gland of polychaetes as well as the neuroendocrine system of the vertebrate hypothalamus. As pointed out, this hypothesis requires additional validation. As part of this, a more detailed time series in *Platynereis* could serve as a good basis for the analysis to which extent the apical organ is indeed continuous with the later infracerebral gland.

3.5.12 Possible influences on the evolution of the hypothalamus during phylogeny

As I have discussed above, many data are highly consistent with the notion that cell types of the ventral/ median forebrains of vertebrates and lophotrochozoan trochophora–type larvae are homologous, i.e. share common ancestry. Clearly, this does not imply that all the cell types in this area have to have direct counterparts. There are many possible reasons for this. First, cell types can expand or become lost in one lineage, but not in the other. Secondly, one should consider that the vertebrate ventral – median brain, including the hypothalamus, may well include cell types of different origins. By this, I mean that cell types that ancestrally belonged to other areas of the urbilaterian nervous system might have mixed with the ancestral set of median brain cells during the course of phylogeny. One likely source for such mixing could be the rearangements associated with the process of dorsal-ventral body inversion during phylogeny, during which cells of the ancestral stomatogastric nervous system might have been included in this region (Dohrn, 1875). In protostomia, these cells would be expected in the nervous system of the stomodeum.

3.6 An ancestral role of the Hedgehog–signalling pathway in median forebrain development

Two signaling pathways have mainly been implicated in early ventral – median forebrain, and especially hypothalamic development in vertebrates. These are the Nodal and the Hedgehog/Hh signaling pathways (Mathieu *et al*., 2002). For practical reasons, I focused my analysis on the Hh signalling pathway to investigate if the Hh pathway plays a role in the specification of cells types in the *Platynereis* episphere. Indeed, my analysis showed that Hedgehog signalling has an impact on the proper

specification of presumably ancestral cell types in the *Platynereis* median episphere, and is thus likely to represent an ancestral signal that influenced the median forebrain region of Urbilateria.

3.6.1 Platynereis *Hedgehog pathway signalling components are present at the right time and place to act in median brain patterning*

In order to investigate if a function of the Hh pathway in the *Platynereis* episphere is likely, I cloned several members of this signaling pathway and confirmed their orthology to the respective vertebrate and invertebrate counterparts with the help of phylogenetic trees. These genes were the two transmembrane 'receptors' *patched*/*Pdu-ptc* and *smoothened*/*Pdu-smo*, as well as *hedgehog*/*Pdu-hh* itself. All of these were found as single orthologs, consistent with the previous notion that cases of gene duplications only rarely occur in *Platynereis dumerilii*. Their cloning from a larval cDNA library indicated that all three genes are transcribed in embryogenesis. However, the expression analysis by WMISH yielded only a strong signal for *Pdu-hh* and a very faint signal for *Pdu-smo*. For technical reasons, I was only able to clone fragments of these genes, which – in the case of *Pdu-ptc* – might not suffice to yield a strong hybridisation signal.

In the case of *Pdu-smo*, the cloned fragment is long enough to yield a confident signal in WMISH (2kb). The observed expression is only weak, indicating the fact that *Pdu-smo* is expressed at low levels. Consistent with the broad expression of *smoothened* orthologs in fish and frog (Figure 33L,M), *Pdu-smo* is present in broad regions in the *Platynereis* episphere, with a slightly stronger expression in the median region and eye area. This indicates that the majority of epispheral cells are competent to receive Hh signalling, and are thus possibly affected by the inhibition of the Hh pathway. From early brain developmental stages onwards, *Pdu-hh* itself is expressed at two sites that have the potential to influence median brain development: the brain itself, and the stomodeum. Hh has been found to be a diffusible molecule, thus brain cells lying above the stomodeum or adjacent to *Pdu-hh*- expressing epispheral cells could be influenced by it. The major brain developmental events take place between 19hpf and 72hpf, when most brain structures have started to

differentiate, as judged by DIC microscopy and the analysis of molecular markers. Notably, *Pdu-hh* is expressed over this period. Thus, main components of the signalling pathway are expressed at the right time and place to strongly influence *Platynereis* median forebrain development.

3.6.2 *Cyclopamine specifically inhibits the Hedgehog signalling pathway in* Platynereis dumerilii

My results showed that the drug cyclopamine is a very useful tool to investigate the influence of the Hh pathway on the development of the *Platynereis* larva. One important consideration for the use of small molecules like cyclopamine is the question whether their effects can be considered to be specific. In summary, seven reasons argue in favour of the specificity of cyclopamine for the interruption of the Hedgehog singalling pathway in *Platynereis*:

i) the drug has been used successfully in several experimental systems of both the deuterostomes (vertebrates) and the protostomians (leech) for specific interference with the Hh signalling pathway, thus providing a good basis for its specific activity in *Platynereis;*

ii) the final concentrations of cyclopamine that I used in my experiments were around 10 times lower than those concentrations regularly used for vertebrate experiments, reducing the likelihood of unspecific effects;

iii) the cytotoxicity of cyclopamine was apparently low; consistently, the animals could survive in cyclopamine solution for days (at least 5d when inhibitions were started 22-24hpf); in addition, even higher concentrations of ethanol did not display any phenotypes similar to the drug;

iv) moreover, the observed phenotypes were strictly dependent on concentration and timing of the application, with 100 percent penetrance and without visible defects in controls;

v) different cellular markers were affected to different extents, further arguing against a general effect on cellular viability; for example, *rx* and RFamide(s) positive cells are strongly reduced in number, whereas *vax* and serotonin positive cells were not reduced;

vi) several effects of the drug on *Platynereis* morphogenesis were reminiscient of the effects observed in vertebrates; e.g the small body size of treated embryos compared to controls. All mouse knock-outs affecting major players of the Hh signalling pathway exhibit misregulations of the overall body size;

vii) lastly, molecular similarities exist in the affected downstream genes; *nk2.2* and *fkh*, two downstream target gene in vertebrates (see above), are also strongly downregulated in cyclopamine-treated *Platynereis dumerilii* embryos, indicating that the effects caused by cyclopamine are due to a specific disruption of the Hh signalling pathway.

3.6.3 *Cyclopamine affects ventral/ median brain parts in* Platynereis

Cyclopamine treatment of *Platynereis* embryos reduces the lateral *Pdu-pax6* expression much stronger than the median *Pdu-nk2.1* expression. At first glance, this appears to be contradictory to the situation in mammals, since loss of the Hh pathway in the mouse forebrain has so far been interpreted to reduce first the ventral (median) fates (including *nk2.1* positive cells of the forebrain) and not the dorsal (lateral) *pax6* positive cells (Chiang *et al*., 1996; Rallu *et al*., 2002b). In contrast, the observed effects of cyclopamine in *Platynereis dumerilii* are more reminiscent of the situation in the zebrafish thalamus. In the zebrafish telencephalon, like in the mouse, reduction of the Hh pathway leads to an increase of *pax6*. But in the zebrafish diencephalon, the *pax6* expression domain is strongly reduced, whereas the two zebrafish *nk2.1* genes are only little affected (Rohr *et al*., 2001; Varga *et al*., 2001; Wilson *et al*., 2002). The molecular hierarchy in the vertebrate thalamus of teleosts might thus reflect the more ancestral genetic programme present in the urbilaterian median brain. Several lines of evidence point towards the fact that Hh signaling is required for normal hypothalamic development. The most conclusive work on this results from zebrafish. As mentioned, the two zebrafish *nk2*.1 genes, *nk2.1a* and *nk2.1b* are not primarily affected by the reduction of Hh signalling. Nevertheless, Hh signalling promotes early anterior-dorsal hypothalamic development, and is, together with the secreted signaling molecule Nodal, required to maintain hypothalamic fate (Mathieu *et al*., 2002). In line with this, the treatment of *Platynereis dumerilii*

embryos with cyclopamine has profound effects on median brain cell type specification molecules. In particular, *rx* and RFamide(s) positive cells are strongly reduced upon cyclopamine treatment. While these specific defects have so far not been discovered in the vertebrate hypothalamus, the underlying functional connection is at least in the case of *rx* reminiscent of other experimental evidence. As mentioned in the results section, it has been speculated that vertebrate *rx1* expression in the (ciliary) photoreceptor cells of the eye depends on Hh signaling. The dependence of hypothalamic *rx* expression in vertebrates on Hh signalling has not been described.

While cyclopamine treatment affects *rx*- and RFamide(s) positive cells in the median brain, other cell types appear to occur in normal number, indicating that the presence of Hedgehog might only be required for particular cell types. As an example, the amount of *vax* expression is not reduced. Moreover, serotonergic cells are not significantly reduced or enhanced in number, however they are abnormally placed in the brain. In contrast to the brain, where a reduction in cell number is not apparent, the cell number of serotonergic cells in the VNC seems vastly reduced. The latter is very reminiscent of the situation in the vertebrate neural tube, where serotonergic neurons are also strongly reduced in numbers upon disruption of the Hh signaling pathway (Matise *et al.*, 1998). Also, these results jointly support that Hedgehog signalling in the brain has different functions than Hedgehog signalling in other regions of the embryo. I have not yet addressed the question, which of these effects are primary defects, and which are only caused as a secondary consequence. Similar uncertainties, however, exist in the vertebrate studies. In conclusion, the Hh pathway has an ancestral role for the formation of ventral/ median forebrain (in particular the hypothalamus). In consistency to the situation in a lower vertebrate, the zebrafish, my experiments argue that the ancestral function of Hh in the brain was rather connected to the level of distinct cell types (such as RFamide(s) or *rx* positive cells) than to the specification of the entire median forebrain area.

3.6.4 *Hedgehog is required for axon wiring and both larval and adult eye development in* Platynereis

Besides the distinct effects of Hh on cell specification, early Hh signalling is also

required for proper cell placement and wiring. The effect of Hh signalling on cell placement is evident from the misplacement of serotonergic and rhabdomeric photoreceptor cells in embryos treated with cyclopamine as early as 15hpf. Miswiring was observed when embryos were treated from 15 hpf or 22-24hpf onwards, the latter being the time point when the first axon tracts become visible. Notably, miswiring is apparent in both the brain and the neurons of the VNC, indicating a general effect of Hh on axonal pathfinding. This is likely to reflect a primary effect, because Hh signalling in vertebrates has recently been shown to be directly necessary for correct axon guidance (Charron *et al*., 2003; Dakubo *et al*., 2003; Karlstrom *et al*., 1999; Salinas, 2003).

As in vertebrates, eye and PRC development are affected in several ways by the loss of Hh signalling. The earliest treatment was performed at 15hpf, at a stage before the larval eye has developed, which occurs around 19hpf (Arendt *et al*., 2002). This treatment completely abolished eye formation in roughly half of the animals, strongly suggesting a role of Hh in larval eye formation. In the other half of the cases, single cells positive for *Pdu-r-opsin* were detected at various mislocated places in the episphere. The decision if these were larval or adult eyes or both was impossible. This misplacement was strongly reminiscent of the phenotype observed for serotonergic cells, corroborating the requirement of Hh signalling for correct cell specification and / or positioning in the polychaete episphere. If the underlying mechanisms are the same for the different cell types, still remains to be elucidated.

Besides the effect on early eye specification, I also noted a distinct, albeit more subtle, requirement of Hh signalling on later eye development. When animals were treated with cyclopamine at a stage when the larval eyes should be fully differentiated, while the adult eyes are still undevelopped, eye-like structures at roughly correct positions are found in all animals. However, adult eye differentiation is severely abnormal. Normally, adult eye differentiation starts around 43hpf of development (Arendt *et al*., 2002), but treated embryos do not show signs of this differantiation even at 48hpf. Subsequent development overcomes this initial failure, and adult eye PRCs differentiate. However, these stay much smaller and are more disorganized than in control animals. In addition to this, larval and adult eyes tend to be fused together. Either a single larval eye PRCs is still separately visible, but in

contact with the adult eyes, or it is completely integrated into the adult eye anlage. This is an interesting observation because it suggests that larval and adult rhabdomeric PRCs develop from one initially common anlage, which – in the absence of Hh signaling – does not separate. As an alternative explanation, the larval eye PRCs might fuse with the adult eye PRCs secondarily. This, however, appears less likely, because it would require the disassembly, movement and fusion of a previously correctly established structure (the larval eye) with the adult eye PRCs. To finally clarify this point, it will be necessary to perform labelings of the early eye anlage and investigate the fate of the labeled cells. If a common primordium indeed exists, labeled cells of the early eye primordium should give rise to both larval and adult eye PRCs.

There is one alternative explanation for the later development of the adult eye PRCs. It cannot be entirely ruled out that the reason is not a developmental compensation of an initial developmental defect, but rather that the development of the treated animals is delayed. Two points argue against a developmental delay. First, there is no indication that the treated animals are severely delayed in their development, when compared to controls (at least when the drug is added from 22/24hpf onwards, see e.g. Figure 34 or Figure 39). Although treated animals are generally smaller, prominent developmental events like the extension of the body axis occur approximately at the right time point. Second, the phenomenon of developmental compensation does not necessarily indicate delay. Rather, this phenomenon is known from many studies in other organisms, even where loss- or gain of function experiments lead to severe initial defects (J. Wittbrodt, personal communication).

Finally, there is one additional interesting aspect of the role of Hh in eye development. Although some adult eye PRCs can be detected by WMISH with a *Pdu-r-opsin* riboprobe after cyclopamine treatment at 22/24hpf (see above), the adult eye pigment is hardly visible at all stages examined. This indicates that the adult eye pigment is independently affected by interference with Hh- signalling, as it is the case in vertebrates (Perron *et al.*, 2003). This is an interesting parallel, because it is so far completely unclear if the eye pigment cells of vertebrates are comparable to any other eye pigment cells outside vertebrates. Enzymes synthesizing the pigments and transcription factors involved in the formation of retinal pigment cells will be

further worthy of investigation in this respect.

3.6.5 Both stomodeum- and brain-derived Hedgehog could influence median brain development

In addition to the conclusion that interference with Hh signalling affects several aspects of brain development, including cells of the photosensory and presumptive neurosecretory system, the question remains which endogenous center produces the Hh signal that is responsible for proper development under normal conditions. As described above, *Pdu-hh* transcripts are present at two places, the episphere itself and the stomodeum. Both have, in their respective position, the potential to influence epispheral cells. For the brain-derived Hh signal, this is self-explanatory. However, evidence grows that the stomodeum could act as an important signalling center that hence could indirectly influence the development of the episphere. Laser ablation of the stomodeum leads to a severe reduction in epispheral *rx* expression, indicating that stomodeal signals are indeed at least partly involved in epispheral development. (S. Klaus, personal communication). Of course, the signals eminating from the stomodeum and influencing brain development are likely to include more than the Hh pathway. The Nodal signalling pathway is another good candidate for a signalling pathway originating in the stomodeum and influencing brain development. If cyclopamine treatment abolishes stomodeal development, and thus all signals eminating from this structure, the observed epispheral defects could be caused very indirectly. What is the evidence that the epispheral defects observed are linked to the Hh pathway and not purely indirect effects caused by the lack of all signalling pathways eminating from the stomodeum? This question is important, since in the leech – like *Platynereis* a lophotrochozoan species – a lack of foregut structures has been described as the major result of cyclopamine treatment (Kang *et al.*, 2003). In *Platynereis,* my results are consistent with the idea that the observed epispheral defects are rather directly linked to the Hh pathway, since the part of the stomodeum, which underlays most of the brain, appears almost normal by morphological and molecular criteria. Even in the presence of severe developmental brain defects, I did not observe a complete absence of the foregut/ stomodeum in the treated specimens (also see below). The stomodeum of *Platynereis* consists of at least two

morphologically distinct regions. One proximal region (close to the mouth opening) that is not surrounded by visceral muscle cells, as well as one more distal region, which is surrounded by the visceral muscle. The most proximal region seems to be absent after a treatment started at 22/24 hpf, the more distal part is always present as can be observed with DIC-optics (Figure 34). Moreover, two molecular markers also indicate that most of the stomodeum is unaffected by cyclopamine treatment. Molecularly, *Pdu-fkh* and *Pdu-nk2.2* expression is absent in other regions of the animal after treatment, but is not visibly reduced (or misplaced in the case of *Pdu-nk2.2*) in the stomodeum of the same animals (Figure 35), under conditions that cause severe defects in the brain. In summary, it appears unlikely that the effects of cyclopamine on the *Platynereis* episphere are solely a secondary consequence of a disrupted stomodeum. However, the exact influence of stomodeal signaling on the median forebrain, and the influence of cyclopamine on the stomodeal signalling remains to be resolved. It will be necessary to e.g. investigate if cyclopamine treatment abolishes *nodal* expression in stomodeal cells to further address this question.

In conclusion, at presence, a dual influence of both potential Hedgehog signalling centers on median brain development is likely. But additional investigations are necessary to separate between the effects of these centers, as well as between the respective contributions of different pathways, like the Hedgehog- and Nodal-signalling pathways.

3.6.6 *Possible reasons for differences in the effect of cyclopamine on leech and* Platynereis *development*

The results discussed above leave the question why cyclopamine treatments display different effects in two lophotrochozoan species, a leech and the polychaete, *Platynereis*. Why do cyclopamine- treated *Platynereis* embryos not display a similar degree of foregut defects as observed in treated leech?

In this context, it is noteworthy that Hh signalling also plays a role in the development of parts of the foregut of *Drosophila melanogaster*. In this species, mutant analyses show that Hh is required for the final inward movements of cells

building the most inward part of this organ in *Drosophila*, the proventriculus (Hoch and Pankratz, 1996; Pankratz and Hoch, 1995). So, the correlation between Hh signalling and correct foregut development is supported more widely in Protostomia. Several reasons could explain why the foregut in leech is much more severely affected (or even completely absent) at cyclopamine concentrations that in *Platynereis* only disrupt proximal stomodeal development (see above). On the one hand, it is possible that higher concentrations of cyclopamine or earlier treatment of *Platynereis* embryos will result in much more severe and thus comparable stomodeal defects. Likewise, *Drosophila* embryos in which both maternal and zygotic Hh is absent, might also display more severe abnormalities than zygotic mutants, where only a part of the foregut is affected (see above). On the other hand, it should be considered that the description of the leech foregut defects were only based on morphological and not molecular level. Therefore, another possibility is that the leech foregut tissue is disordered, but still molecularly correctly specified. Third, it has to be stated that the exact degree of homology between the foregut structures of the three systems in question – fruitfly, leech, and polychaete – is not clear. In this light, it could also be possible that e.g. the proximal stomodeum of *Platynereis dumerilii* equals to the total foregut of leech- making the effects than very well comparable.

Besides the effects on the foregut, another difference is apparent between the two lophotrochozoans. The only additional effect of cyclopamine that has been described is the influence on mesodermal development in leech. No defects have been reported for the CNS development of this species, different to my observations in *Platynereis*. (I did not investigate the effects of cyclopamine on mesoderm, however.) To explain these differences, it should be noted, that the expression described for the *hh* gene in leech has many features that are not comparable to insects or vertebrates (Kang *et al*., 2003), whereas the *Pdu-hh* expression shows several features much more reminiscent of *Drosophila* or vertebrates. These are e.g. the segmental expression at 48hpf reminiscent of *Drosophila* or the median/ ventral brain expression comparable to vertebrates. This is consistent with the presumed more ancestral position of *Platynereis dumerilii*. The failure to detect similar defects in leech as compared to *Platynereis* upon disrupted Hh signaling might thus be at least partly a hirudinean

specific feature.

3.7 How could Urbilateria have looked like? How might it have changed on the way to vertebrates?

3.7.1 Conservation …

In the end of this study, I would like to resume what picture emerges for the presumed structure of the Urbilaterian brain. By necessity, this picture will remain largely fragmentary. Nevertheless, a clear outcome of my analysis is that this ancestral blueprint, in its complexity, goes much beyond what has been assumed to be the case, based on traditional protostomian model species. First, on the molecular as well on the cell morphological level, the analysis of the basal protostomain, *Platynereis dumerilii*, showed that there are many cells types that resemble vertebrate brain cell types much closer than this was ever assumed from comparisons of the classical protostomian model organisms *Dosophila melanogaster* or *C.elegans*. This argues convincingly for a complex last common ancestor of polychaetes and vertebrates and, in turn, supports the notion that organisms like insects or nematodes possess an evolutionarily derived mode of CNS development.

Second, some assumptions can be made about the regional and/or functional subdivision of the urbilaterian nervous system. Most likely, this nervous system contained a core region demarcated by the expression of a *six3/6* ortholog. This region was further subdivided into a lateral *pax6* and a median *nk2.1* region.

Third, considering the differentiated cell types arising from these regionally distributed precursors, the more lateral regions of the brain presumably contained rhabdomeric PRCs, which, together with a pigment cell, probably formed the primitive eyes of this animal. The median brain, in contrast, contained a different type of PRCs, the ciliary PRCs, which were located in close neighborhood to neurosecretory cells best comparable to the cells present in the today's vertebrate hypothalamus (and partly pineal organ). These neurosecretory cells contained serotonin, RFamide(s), histamine and *gnrh-r*, and presumably also produced other

'hypothalamic' neuropeptides, such as the AV/O or members of the PACAP superfamily like GHRH, whose presence in Urbilateria can be inferred from the wide occurence of their highly conserved precursors across Bilateria. Several of these median forebrain cells might have had contact to the outside, probably for sensory functions. The core of this presumed sensory-neurosecretory complex was probably formed by a structure equivalent to the apical organ of extant primary ciliated larvae.

3.7.2 ...Changes...

As far as the modification of this ancestral blueprint over the following millions of years is concerned, certain trends can be assumed. The ancestral cell types and their respective transcription factors were maintained or even multiplied on the evolutionary lineage leading to the modern representatives of protostomians (like *Platynereis*) and deuterostomians (like the vertebrates). In some cases, different cell types joined together to form new functional units, such as the eyes of vertebrates (consisting of rhabdomeric and ciliary PRCs) or the different nuclei of the hypothalamus (units of various neurosecretory cells). In addition, the vertebrate brain undergoes the process of neurulation leading to additional rearrangements in the cell placement. Neurulation is a process in which a flat structure rolls up into a tube. Topologically, the inside of this tube corresponds to the former outer surface of the flat brain. This explains the continuity between the presumed chemosensory cells in the primitive apical organ and the central spinal fluid contacting neurons in the forebrain of lower vertebrates. Other sensory cells and organs that require to be in direct contact with the outside, e.g. for vision, needed to escape the enclosure of the neural tube. This might be a reason why the vertebrate eyes have to 'bud out' from the brain.

It is further apparent that on the way towards *Drosophila melanogaster* and *C.elegans* many of the cell types got lost or highly modified. The remaining cell types underwent as well duplications, diversifications, migrations and aggregations into new functional units in these organisms. Therefore, even though a relevant core set of original cell types still persists in these species, this set might be hardly recognizable as such due to many modifications that prevent clear correlations with the cell types present in other extant species.

3.7.3 ...And many questions left

Many questions about the complexity of the urbilaterian brain remain. First of all, what were the ancestral functions of the elements we now consider to be conserved? Second, as discussed above, to which extent might a true endocrine system have existed as well? Which of the chemosensory senses existed already, in particular can widely distributed senses like olfaction be traced to a common origin in Urbilateria? Distinct olfactory and gustatory senses exist in *Drosophila melanogaster* (3rd antennal segments and maxillary palps), as well as in vertebrates (nose). So far molecular comparisons have been impossible, mainly because of the fast evolving receptor molecules, but a more comparative view might help to identify conserved components, if they indeed exist. Another question concerns the origin of the vertebrate adenohypophysis, a structure in close functional association with the hypothalamus and a composite of chemosensensory and neurosecretory components. Finally, the identification of core components of the urbilaterian brain also serves to address a retrospective question. One exciting challenge will be to relate this setup to the data that start to emerge from the diploblastic animals, like cnidaria or sponges. Apical concentration of particular neurons and sensory cells is already present in diploblastic animals such as the cnidarians and ctenophores. So where did the urbilaterian brain itself come from initially?

Finally, beyond the specific questions of ancestral brain development addressed in this thesis, this study has also stressed the importance of *Platynereis dumerilii* as a reference organism for comparative developmental analyses. On the one hand, in comparison to other protostomian model organisms like *Drosophila melanogaster* or *C.elegans*, *Platynereis dumerilii* contains a more complete set of ancestral urbilaterian genes and cell types, as example *ciliary opsin* and ciliary photoreceptor cells. On the other hand, in comparison to vertebrates, *Platynereis dumerilii* exhibits a far lower complexity of genes and cell types, and thus a less complex development and morphology that can be more easily studied. In synthesis, this makes *Platynereis dumerilii* a highly valuable invertebrate model organism. Research on it can answer questions left open by *Drosophila melanogaster* and *C.elegans*, and will thus produce further valuable predictions for vertebrate development.

4 MATERIALS AND METHODS

4.1 Technical equipment

Eppendorf centrifuge 5417C, rotor F45-3011

Zeiss Stemi 2000

Sorvall rotor H4000

Mikroskop Zeiss Axiophot

Objective: Zeiss 20x or 40x air objectives

Camera :Zeiss AxioCam HRc, software: AxioVision 3.1

Gaze net for *Platynereis* embryos: nylon-Siebgewebe NITEX, Maschenweite 100um, Typ 03-100/44 ca. 102cm breit and 3m lang, Gebr.Stallmann, Suentelstrasse 82,25462 Rellingen b. Hamburg

4.2 Strains

The bacterial strains used for vector amplification were *E.coli* (*XL1 blue* or *DH10B*). The constructed cDNA library was transformed into *E.coli DH10B*. *E.coli TOP10* was used for direct subclonings of PCR products with the help of the TopoTA kit (Invitrogen Life Science). LB medium and LB agar were used. For selection of Ampicillin-resistant clones,, a final concentration of 50μg/ml Ampicillin was used.

The *Platynereis dumerilii* material (embryos or D/RNA) stemmed from an inbred culture at EMBL. This culture has its origin in the *Platynereis dumerilii* population bred in Mainz since the 1960ies. The first worms came from the Golf of Neaples, but got two additional incrosses from worms of very different regions during the last 20 years. The worms were grown at 18°C and according to standard protocol (see http://www.uni-giessen.de/%7Egf1307/breeding.htm and Dorresteijn *et al*., 1993)

4.3 Chemicals and solutions

The following overview comprises general buffers and solutions. Special solutions and reagents are mentioned together with the methods. All chemicals, if not noted otherwise, were purchased from the companies Applichem, Merck, Roth and Sigma.

Agarose was purchased from Pharmacia, Bromophenolblue and Xylenecyanol from Serva

10x loading buffer:	50% Glycerol
	100mM EDTA (pH 7.5)
	1.5 mM Bromophenolblue
	1.9 mM Xylenecyanol
TE:	10 mM Tris/HCl pH 7.4
	1mM EDTA pH 8.0
10xTBE:	890 mM Tris
	890 mM boric acid
	20 mM EDTA pH 8.0
50xTAE	242 gm Tris base
	57.1 ml Acetic acid
	100ml 0.5M EDTA
	Add ddH2O to 1 liter and adjust pH to 8.5.
1xTBST	0.14M NaCl, 2.5mM KCl, 25mM Tris pH7.5, 0.1% Tween 20
1xTNT	0.15M NaCl, 0.1M Tris pH7.5, 0.1% Tween 20
1xTNB	TNT containing 1%NEN TSA blocking reagent, when preparing, heat mixture to app. 55°C and stir to dissolve the blocking reagent more quickly, cool down before use!
MEMPFA-T	0.1M MOPS pH 7.4; 2mM EGTA; 1mM MgSO4; 4% PFA; 0.1% Tween
35%formaide hybmix (10ml) for readioactive hybridisations	Denhardts (50X) 1ml; calfthymus (10mg/ml) 100ul; Tris Hcl 1M pH 7.5 500ul; SDS 20% 500ul; SSC (20x) 2.5ml; H_2O 1.7ml; formamide 3.5ml
20xSSC	3M NaCL (175.32g/l) and 0.3M Trinatrium citrate (88.23g/l)
MaNaT	100mM Maleic acid pH7.5; 150mM NaCl; 0.1% Tween
10xPBS	70g NaCL; 62.4g Na2HPO4.2h2O; 3.4g KH2PO4
	pH7.4

4.4 Antibodies

A monoclonal anti-mouse anti-acetylated tubulin antibody was purchased from Sigma {clone no. 6-11B-1(SigmaT6793)} and used in a 1:500 dilution in PBS. A polyclonal anti-rabbit anti-serotonin antibody was purchased from Immunostar/ DiaSorin (#20080), and used at an 1:1000 dilution in PTW. The anti- rabbit anti-RFamide(s) antibody was a generous gift from N.Rebscher and used in a final dilution of 1;50. All three primary antibodies detect interphyletically conserved epitopes.

The secondary anti-mouse antibody used was either coupled with alkaline phophatase (anti-mouseAP, purchased from Zymed) or biotin (anti-mousebiotinylated, purchased from Vector laboratories, Inc. (#BA-9200)). The secondary anti-rabbit antibody used was either coupled with alkaline phophatase (anti-rabbitAP, purchased from Zymed #62-1822) or biotin (anti-rabbit biotinylated, purchased from Zymed laboratories #81-6140). All secondary antibodies were used in a 1:250 dilution in PTW. In few cases an anti-rabbit FITC secondary antibody (Sigma-Aldrich) was used 1:100.

4.5 Whole mount in situ hybridization (WMISH)

Embryos were fixed in 4% paraformaldehyde/2´ phosphate-buffered saline (PBS)-Tween (PFA/PTw) for 2 hours. An established in situ hybridisation protocol (Loosli *et al*., 1998) was followed with a modification of ProteinaseK treatment in 100 mg/ml for 30sec (15-24hpf), 1min (24 hpf-72hpf), 2min (3d-5d), 3min (older than 5d). After staining, embryos were refixed in (PFA/PTw) for 20min, washed and cleared in 87% glycerol. Embryos were mounted in glycerol and pictures taken under Nomarski optics using a Zeiss Axiophot.

4.5.1 WMISH for young embryos (15hrs and younger)

Hybridization Buffer

750mM NaCl	5M stock – 15ml in 100ml
75mM NaCitrate pH7	1M = 294.10 – 2.206g in 100ml
50% Formamide	50ml of a 100% stock

Torula RNA	100ug/ml –10mg in 100ml
1.5% Blocking Reagent	1.5g in 100ml
5mM EDTA	500mM stock – 1ml in 100ml
0.1% Tween	100ul of 100% in 100ml
	500ul of 20% in 100ml

APB

100mM Tris pH 9.5	2M stock- 5ml in 100ml
50mM MgCl	1M stock- 5ml in 100ml
100mM Nacl	5M stock- 5ml in 100ml

EMBRYO FIXATION AND STORAGE

-The normal fixation for insitus works as well but this is the better fixation for this method

-Make acidified sea water by adding about 13 drops of 32% HCl to a pH of 3.9

-Remove jelly by washing embryos in acidified sea water – pour embryos onto mesh, add acidified water and then rinse well with sterile natural sea water

-Fix in eppendorf tubes for 1hr in MEMPFA-T with shaking

-Dehydrate progressively with 5 min washes in a series of MeOH / MEMPFA-T (25/75, 50/50, 75/25, 100/0)

-Wash for 15min in 100% MeOH

-Change MeOH and store at –20

WMISH

DAY 1

-Rehydrate in MeOH / TBST (75/25, 50/50, 25/75, 0/100)

-Digest in Proteinase K (50ng /ml) in TBST for 20 mins at 37C

-Wash in 0.1M TEA with 2.5ul acetic anhydride/ml

-Wash in 0.1M TEA with 5ul acetic anhydride/ml

-Wash 2x5 mins in TBST

-Refix for 20mins in MEMPFA-T

-Wash 5x5 min in TBST

-Wash 10 mins in 50/50 TBST / hybridization buffer

-Prehyb in 400ul HB for 6hrs @ 68C in the waterbath

-Prepare probe in 100ul HB

-Denature probe for 10 mins a@ 80C and add to prehyb

-Hybridize overnight at 68C

DAY2

-Wash in 50/50 HB/2xSSC for 20mins @68C

-Wash in 25/75 HB/2xSCC for 20mins @68C

-Wash in 2xSCC for 30mins @68C

-Wash 2x30mins in 0.2xSSC with 0.3%CHAPS @68C

-Wash 2x10mins in MaNaT @ RT

-Block for 1Hr in BB @RT

-Dilute anti-Dig 1:5000 in BB

-Remove preBB and incubate in antibody for 1Hr @RT

-Wash 2x5 mins and 6x15mins in MaNaT

-Wash in APB 5 mins (or overnight @4C)

-Stain in APB containing 10%PVA (heat to dissolve) with 2.63ul -100mg/ml NBT and 3.5ul -50mg/ml BCIP per ml solution required

-Stop staining by washing 2x in TBST

-Refix in MEMPFA-T for 20mins

-Wash 2x in TBST

-Store in 87% glycerol @ 4C

4.5.2 WMISH for two colors

This protocol is a mixture of the standard WMISH protocol for *Platynereis* embryos (see above and attachment) and a protocol adapted from Ciruna and Rossant, 2001, using the TSA Fluorescent Systems (PerkinElmer) as second staining method. So far we tried the cy3 conjugated tyramid from NEN (PerkinElmer#704A). DIG- or fluorescein labeled riboprobes for WMISH were produced from 1μg of linearized DNA templates using correspondingly modified dNTP solutions (Roche, see protocol in attachment). Both probes were mixed together, embryos were hybridized, washed and the first staining detected with the standard WMISH conditions. After the detection the embryos are NOT postfixed in 4%PFA, but washed 3x 5min in PTW and then 2x short and 2x 5min in TBST. After this, the embryos were washed once 5min in TNT and blocked for at least 1hr at RT in TNB. Replace the TNB by a 1:50 dilution of the peroxidase conjugated anti-fluorescein (Roche #1426346) antibody in TNB, and incubate for 2hrs at RT or for O/N at 4°C. Rinse afterwards with TNT and wash 6x with increasing time intervals (app. from 5-50min) in TNT, rinse in NEN amplification diluent. Color reaction is initiated by adding tyramide working solution (1:25 dilution of reconstituted the cy3 conjugated tyramid in NEN amplification diluent). The color reaction develops at RT in the dark, depending on the signal strength the staining process will last at least 2.5hrs. If the reaction needs to be stopped, rinse 3x in TNT, 2x for 30min in TNT. Mount embryos in 87% glycerol (dapco may be used).

4.6 Antibody staining procedure

Embryos and larvae were fixed as above, dehydrated in methanol, rehydrated in methanol/PTw,or taken from the postfixation solution after the in situ hybridization procedure, and blocked for 2 hours in 1 ml 5% sheep serum/PTW. Blocking solution was replaced by 50- 200ul diluted primary antibody solution, and incubated at room temperature for 1-2 hrs or overnight at 4°C. Control larvae were further incubated in blocking solution, but otherwise treated identical to the batches incubated in primary antibody. Larvae were washed 5 times with increasing duration (5 to 25min) in PTW, and incubated for 1-2hrs at room temperature or at 4°C O/N in diluted secondary antibody. The embryos were washed again afterwards 5 times with

increasing duration (5 to 25min) in PTW. Embryos incubated with a secondary antibody coupled with biotin were processed using the Vectastain ABC Kit (Vector Laboratories) according to manufacturers instructions. Embryos incubated with a secondary antibody coupled with AP were washed twice in WMISH staining buffer and stained in a NBT/BCIP solution (dilution according to WMISH protocol). Post-staining treatment was done as described in all cases as described in the WMISH protocol.

If the anti-rabbit FITC was used as secondary antibody, the embryos were finally mounted in 1,4-diazabicyclo([2.2.2])octane (DABCO)/Glycerol (25mg dabco (Sigma) in 1ml PBS and 9ml glycerol).

4.7 Total RNA and polyA$^+$ RNA isolation from embryos

Two to three well-grown batches of the wanted stage were collected into a cut-open 50ml Falcon tube, where the opening was covered by a small net. They were washed several times with sterilized natural seawater, and it was taken care that no big pieces of dirt or algae were left on the net between the embryos. Afterwards they were transferred with a pipette into a cut-open 15ml Falcon tube, where the opening was covered by a small net. The net was transferred into a 2ml Eppendorf tube and shock-frozen in liquid N_2. Each net was covered with 1ml of TRIZOL reagent (Gibco). Embryos were homogenized by pipetting several times up and down at RT. (The gaze will be dissolved as well, but this does not affect the procedure.) TRIZOL volumes and subsequent handling were according to the manufacturer's recommendation. Resulting total RNA was quantified using photo-spectrometry, and samples were saved for comparative quantification of subsequent steps on agarose gels. For poly-A$^+$-RNA isolation, the Dynabeads® mRNA Purification Kit (Dynal Biotech) together with a Magnet (Dynal MPC®-S) was used according to the manufacturer's protocol. For each purification, the total RNA outcome of two gazes was pooled together. Two purification steps were done. The first elution was done in 300ul. 1ml of binding buffer was added and the mixture subject to the second purification step. The final elution was done in 2 times 15ul 10mM Tris/HCl (pH7.5) at 80°C. Each eluate was kept separate, because the second eluate had usually only

half of the concentration of the first. The amount of purified polyARNA was estimated by gel electrophoresis (take 3-5ul of the eluate), using total RNA as quantification standard.

4.8 cDNA synthesis

cDNA first strand synthesis (for degenerated PCR or 3' extensions race) was performed with SuperScriptII Reverse transcriptase (Gibco) according to the manufacturer´s protocol. The sequence of the following Oligo(dT) was used: raceT17 5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT3'

In order to have more complete 5' cDNA ends and to not only need to rely on be real cDNAlibraries for 5'end cDNA extension races, I used the SMART RACE cDNA amplification kit (Clontech #K1811-1) according to manufacturers instructions.

4.9 48hpf cDNA library construction

App. 4μg of purified polyA RNA (see protocols above) were used for the Invitrogen Superscript Plasmid system (Invitrogen Cat.# 18248). The protocol was followed in all points, except that for the estimation of the first strand synthesis yield, only an aliquot was labeled with radioactive (P^{32}) dCTP, in order to protect the DNA. The second strand reaction was labeled completely. PolyT NotI primer adapter was used to make the first RT reaction. SalI adapters were added, fragments were NotI digested. By this, the fragments were subcloned directionally into the NotI/SalI restriction site of the pCMVSport6 vector (NotI sits 3', thus T7 sequencing give antisense and Sp6 sense sequence). The column fraction that was choosen for subcloning had its major smear between 700/800bp and up to 12kb. The length of 220 of these clones was ascertained by gel electrophoresis. 55% were longer than 1.4kb, but most (app.90%) are not bigger than 2-3kb.

4.10 General gene cloning procedure

New *Platynereis dumerilii* genes were cloned according to the following procedure (for detailed method descriptions see below).

4.10.1 cloning of novel fragments

1.) Degenerated primers (Buck and Axel, 1991) were designed in conserved regions of the protein, using an AA sequence alignment, a standard codon-usage table and the computer program Oligo6.44 for Macintosh. 2.) The primers were used in a degenerated PCR on (usually) first strand library. 10ul of the product were analyzed on an agarose gel. 3a.) If usable fragments of the gene were available from other organisms, the gels were southern blotted and hybridized under low stringent conditions. Bands yielding a positive signal were gel eluted and directly subcloned, using the TopoTA kit (Invitrogen). 3b.)If no usable fragments were available for hybridisations, bands of the approximated size were gel eluted and directly subcloned, using the TopoTA kit (Invitrogen). 4a.) If a usable fragment was available for hybridization, the resulting colonies were filter lifted and hybridized again under low stringent conditions. Positive were treated according to 4b) 4b) Colonies were picked, mini-prepped. The resulting DNA was subject to an EcoRI and a HinfI single digest. EcoRI was used to estimate the insert size, HinfI, which has a 4bp recognition sequence, and is thus a polycutter, was used to analyze which clones are likely to be identical. 5.) Each clone of each HinfI pattern, that had the approximately correct size (as judge by the AA sequence alignment), was send for sequencing. 5.) The resulting sequence was analyzed using BLAST and alignment programs.

4.10.2 Extension of existing fragments

1.) Regions for ‘race’ primers (i.e. extension primers) were chosen by eye. 2.) Race PCR was performed, using one specific primer and: first strand cDNA(with RaceAda primer) or the Lambda-Zap libraries (with T7 primer) for 3’ extensions and Lambda-Zap libraries (with T3 primer) or cDNA created with the SmartRace kit (Clontech #K1811-1) (with the UPM primer). 3.) The products were subject to gel electrophoresis. The gels were southern blotted and hybridized with high stringent conditions with the existing fragment of the gene. Bands yielding a positive signal were gel eluted and directly subcloned, using the TopoTA kit (Invitrogen). 4.) The procedure following is the same as above, except that always high stringent hybridisation conditions were used.

4.11 Polymerase chain reaction (PCR)

Programs and PCR mixtures used, depended on the aim of amplification and primers. If not noted otherwise, 1000bps/ min were used as standard to calculate the extension time.

4.11.1 mixtures

cDNA extensions with λ-Zap cDNA library (gift from C.Heimann/A.Dorresteijn)

3-4ul λ-Zap library (pBS+ vector backbone); 0.5ul T3 primer (for 5' race extension, 100uM) or T7 primer (for 3' race extension, 100uM); 0.5ul specific primer (100uM); 2.5ul 5mM dNTPs; 5ul PCR buffer (Qiagen 10x); 0.2ul Taq polymerase (Qiagen); 37.3ul ddH_2O

SMART RACE cDNA amplification kit (Clontech #K1811-1)

3ul library, 5ulUPM (universal primer mix, see kit), 2.5ul (5uM) specific primer, 5ul PCR buffer (Qiagen 10x); 0.2ul Taq polymerase (Qiagen); 31.8ul ddH_2O

cDNA extensions with RaceAda primer

2-3ul library, 1ul specific primer (100uM); 1ul RaceAda primer (100uM); 2.5ul 5mM dNTPs; 5ul PCR buffer (Qiagen 10x); 0.2ul Taq polymerase (Qiagen); 37.3ul ddH_2O

4.11.2 programs

degenerated PCR for *Pdu-ath*:

1.) 1 min 94°C, 2.) 2min 42°C, 3.) 4min 72°C, 4.) repeat 1.-3. 5 times, 5.) 1min 94°C, 6.) 2min 47°C, 7.) 4min 72°C, 8.) repeat 5.-7. 35 times, 9.) 10min 72°C, 10.) 4°C ∞

Kridegen (all other degenerated PCRs):

1.) 1 min 94°C, 2.) 2min X°C-5°C, 3.) 4min 72°C, 4.) repeat 1.-3. 5 times, 5.) 1min 94°C, 6.) 2min X°C, 7.) 4min 72°C, 8.) repeat 5.-7. 35 times, 9.) 10min 72°C, 10.)

4°C ∞

X= recommended temperature of the primer analysis software (Oligo 6.0 for Macintosh)

KriRace (program for cDNA extension):

1.) 1 min 95°C, 2.) 2min 55°C (with raceAda, Sp6, T3 or T7 primer 48°C), 3.) 4min 72°C, 4.) repeat 1.-3. 5 times, 5.) 1min 95°C, 6.) 2min 58°C (with raceAda, Sp6, T3 or T7 primer 50-52°C), 7.) 4min 72°C, 8.) repeat 5.-7. 35 times, 9.) 10min 72°C, 10.) 4°C ∞

4.11.3 primers

target	forward primer(s)	reverse primer(s)
Pdu-ath:	ACNAAYGTNGTNCARAARCA	GCRTTDATRTANRTYTGNGCCATYTG
	nested: CARMGAMGNYTNGCNGCNAAYGC	nested: GCCATYTGGARNGTYTCRTAYTT
Pdu-brn3	NWSNCARWSIACIATHTGYMGITTYGAR	NYKYTGICKIYKRTTRCARAACCAIAC
Pdu-brn1/2/4	NWSNCARWSIACIATHTGYMGITTYGAR	NYKYTGICKIYKRTTRCARAACCAIAC
Pdu-barH1	MGNACNGCNTTYACNGAYCAYCAR	CNSHRTARTTNCCNGCYTCNGC
	nested: WSNTTYGARMGNCARAARTAYYT	nested: CCNACNGCNGTYTGNCKYTTCCAYTT
	nested: WSNGTNCARGAYMGNATGGANY	
Pdu-ci-opsins	for Pduci-opsin1:	NGC NGC ICK RAA YTG IKT RTT CAT
	WSN TGY WSI GTI AAY TGG	nested:NCK RAA YTG IKT RTT CAT IMM IAC RTA DAT
	nested: WSN TAY ATH ATH TTY YTI TTY RTI TTY	nested: TTC ATN MMI ACR TAD ATD ATI GGR TTR TA
	for Pduci-opsin2:	
	TTYATGGTAGCNTGGACNCCNTAYGC	
Pdu-emx	YTNMGNYTNGARCAYGCNTTYGA	TRYTTNGTNCKNCKRTTYTGRAACCA NAC
	nested: CAYTAYGTNGTNGGNCARGARMGNAAR CA	
Pdu-hh	ACNCCNYTXGTXTTYAARCARMAY	CCARTCRAAXCCXGCYTCXACXGC
	nested: GARGARGGXACXGGXGCXGAY	nested: RTCNACNGCXCKXCCYTCRTARTG
Pdu-smo	TGYTGGGCXGYXATHCARCC	NGTCCANACCCAXGTXSWCAT
	nested: GARGGNTGYGGXRTXCARTGY	nested: RTANCCNACRAARCADATXCC
Pdu-ptc	ACNCCNYTNGAYTGYTTYTGGGARGG	GGDATNGCNSWNARYTTDATNCC
	nested: AARTAYATGCAYTGGCMNGARGAR	nested: ACYTGNGTNGTNGCNGCRTTRAA
Pdu-gnrh-r	GCNAARATGARRACIYTIAARATGAC	ARNARRTARTANGGNGTCCARCA
Pdu-hdc and Pdu-DopaDC	DopaDC: GGNAARGARATGGTXGAYTAYAT	DopaDC: RCARTCRAARTGXACCATXARCCA

target	forward primer(s)	reverse primer(s)
	HDC: GGNAARGARATGGTXGAYTAYAT nested: CCNGAYGTXCARCCXGGXTAY	nested: YTCNARYTCXGTRCAXGCXGG HDC: NARNGGDATYTGCCARTGCAT nested: RCARTCRAARTGXACCATXARCCA
Pdu-ptx	GARGARATHGCIRTITGGACIAA nested: AARAAYGGITTYGGICCICART nested: DSNTAYHSITAYAAYAAYTGGGCI	NSWNSHRTGYTGYTTIGCYTT nested: RAAYTGIGGICCRAAICCRTTYTT

Table 2. Sequences of degenerated primers used in this study.

Target genes are listed in the first column; sequences of the primers used to clone these genes are given accordingly; letters follow the IUPAC standards for nucleic acids. All gene fragments were cloned from cDNA, except for *Pdu-gnrh-r,* which was only cloned from genomic male DNA.

target	3'RACE primer(s)	5'RACE primer(s)
Pdu-ath:	atg aac agc ctg aac ggg g	ccc cgt tca ggc tgt tca t
	nested: gta tga aag ggc aaa gac a	nested: tgt ctt tgc cct ttc ata c
		nested: gga agg ttt cgt act tgg aca
Pdu-brn3:	gagccataacaacatgatagcc	ttgaggtccaacttttcagcg
	nested: cagctgctagggagagcaaggc	nested: cccgaggggcgtggctgcacg
Pdu-pit1	acaggaggaggaagaggcgcacc	ccttgtccaggtgcagtgcccctg
	nested: gtgtcctggccaaagatgccc	nested: tcctgcagatgtcgtgggagg
Pdu-brn1/2/4:	gaacatgtgcaaactgaagcc	gagatctcctgggcagctggc
	nested: agactccacttccggctcccc	nested: aagtgctgttccagggctccc
Pdu-barH1:	gtc cag gat aga atg gaa tta gc	gtt ctt cga ttc tgg tac cac g
	nested: cag aca ccc aag tca aaa cgt gg	nested: ttg act tgg gtg tct gtc aag ttc
Pdu-ci-ops1:	gtt tcg tat ggt ctc ata tat gac	agc tgg aat cac cgt tgc aac agg
	nested: act ggt gga agt gtg gcc aag	nested: tcc gaa aga tgc aag cat gca g
Pduci-ops2:	tcc gaa aga tgc aag cat gca g	gct ctt ggc aaa gag gga agg
	nested: ggc ctg ccc atc tac gct gag g	nested: gta gat ggg cag gcc att agg
Pdu-hh:	acacagcgctgcaaagacaag	gtcttctgtgtgatgtaaatc
	nested: acgttcgcaattgcggtcatg	nested: ctctgtgactctgagtttgac
Pdu-smo	agacggcactgtgaggacgcaag	ctgcagacaatttcatctcttgc
	nested: tctggggaatctatatcttgcac	nested:taaactgagcagcccaaccaattg
Pdu-ptc	aac agc gac aat gtc ctt cct ttc	gaa agc tct ttg cca ggc att gag
	nested: gac aga tgc cgt cca gtc cca gag	nested: ctt cag ctt tga caa tgt tgc cg
Pdu-emx	cac tac gtt gta ggc cag gag	gac ctg tgt ttc tgt tag gcc
	nested: gat ctg gct gct aat tta ggc	nested: agc agc cag atc ttt cct ctc c
Pdu-DopaDC	ccggtggtagctccggttcaacc	gaaataggcgtggaagtttgg
	nested: ctgcacaagctgattccggaatc	nested: tggcataatgaccctctcgat

target	3'RACE primer(s)	5'RACE primer(s)
Pdu-ptx	gagcgtatgttggcgggagag	gcgcaaacgcgagcgaaccatgg
	nested: cacgcagcttggctgctgacc	nested: ttatgccccctacaacaactgg

Table 3. Sequences of RACE primers used in this study.

Target genes are listed in the first column along with the primers used for the extension of the initial gene fragments by RACE. The sequence of the general raceAda primer used was `ACTGCAGTCGACATCG.`

Pdu-nk2.2, *Pdu-gsx* were cloned by Heidi Snyman. In addition, Heidi Snyman helped in cloning *Pdu-barH1* and with the initial degenerated PCR for cloning the *pou-box* genes. *Pdu-six3*/6, *Pdu-r-opsin, Pdu-pax6*, *Pdu-rx, Pdu-vax* were cloned by Detlev Arendt *Pdu-fkh* was cloned by Sebastian Klaus.

Pdu-nk2.1 was a gift from Guillaume Balavoine.

4.12 Southern blots and filterlifts from bacterial plates:

I followed the protocol of (Sambrook *et al.*, 1989). For filter lifts of bacterial plates I used the method 2, for filter lifting and method 1 for lysis.

4.13 Radioactive hybridizations of DNA blots

4.13.1 probe preparation

The probe was produced by gel eluting a digested DNA fragment. This fragment was labeled with the "Megaprime DNA labeling Kit (Amersham Life Sciene #RPN 1604/5) according to manufacturer's instructions. The radioactive nucleotide used was P^{32}dCTP.

4.13.2 high stringency hybridization

The filters were rinsed with 1xSSC, prehybridized in RapidHyb Buffer (Amersham Life Science) for 10-30min at 65°C. The buffer was replaced by fresh buffer when colony lifts were hybridized, in case of southern blots the buffer was not renewed. Half of the total amount of probe was added (denatured shortly before for 5min at

95°C), and hybridized for 2hrs at 65°C. Afterwards the blots were rinsed once shortly,and washed twice 20min with 0.1xSSC/0.1%SDS at 65°C. The blots were exposed with an intensifier screen at -80°C.

4.13.3 low stringency hybridization

The filters were rinsed with 1xSSC, prehybridized in 35% FA-Hybmix for 1-2hrs at 42°C. The buffer was replaced by fresh buffer when colony lifts were hybridized, in case of southern blots the buffer was not renewed. Half of the total amount of probe was added (denatured shortly before for 5min at 95°C), and hybridized for O/N at 42°C. Afterwards the blots were rinsed shortly twice, and washed twice 20min with 5xSSC/0.1%SDS at 42°C. The blots were exposed with an intensifier screen at -80°C.

4.14 Sequencing

DNA sequencing was performed by the EMBL intern service unit of Vladimir Benes.

4.15 Attribution of library clone identities after sequencing

Sequences were first tested via BLASTX and BLASTN (http://www4.ncbi.nlm.nih.gov/BLAST/) to the closest likely ortholog in other species, and to exclude that the gene was cloned from an organism not *Platynereis dumerilii*. Afterwards the protein sequence was analyzed with other protein sequences in ClustalX and a phylogenetic tree generated. Sequences designated as *Platynereis* orthologs of other genes usually clustered with high bootstrap value into the respective orthology group.

4.16 Sequence alignments and phylogenetic tree constructions

Protein sequences of a selected number of species were obtained from the database and aligned using CLUSTALX (Thompson et al., 1997). These alignments spanning

the conserved domains such as HD (homeodomain), SD (Six domain), or bHLH (basic Helix-Loop-Helix) domain, were used to calculate a 1000-fold bootstrapped phylogenetic tree using the neighbour-joining method, excluding all positions with gaps in the alignment, and correcting for multiple substitutions, using the programme CLUSTALX (Thompson et al., 1997).

4.17 Cyclopamine treatments

Most of the cyclopamine used in the experiments was a gift ofCarl Neumann and Alena Shkumatava. Cyclopamine was also purchased from Toronto Research Chemicals (#C988400). One ore two batch(es) of well developed embryos of 15hpf or between 22-24hpf were washed with NSW in a 50ml Falcon tube with a gaze covering the one side. Batches were mixed at this time. The embryos were transferred into a Nunc petridish and subsequently aliquoted in equal amount into the single vials of a 24-well Nunc dish. The number of vials filled depended on the amount of different concentrations tested or embryos needed. All vials were filled with either 1ml or 2ml of NSW (two are better for the suvival of the larvae, but of course more cyclopamine is needed). The volume of NSW was always the same in cyclopamine and control embryos. It is really important to use only well-developed embryos, and not to have a to high density of embryos in each vial! Initially, different amounts of cyclopamine were tested, ranging from 1uM to 100uM. This equals a volume from 0.1 to 10 ul of cyclopamine solution in 1ml NSW. Since the cyclopamine is solved in 95% EtOH (in ddH_2O), 95% EtOH in ddH_2O in the respective amounts was used as control. A concentration range of 5-15uM, was found to be suitable, showing penetrant phenotypes, but letting the embryos survive for several days. Embryos were either photographed alive, using 20x or 40x magnification on the objective. Alternatively, they were fixed and processed as described in the other protocols.

4.18 DiI labellings

Embryos of the stages interesting where collected in a 50ml Falon tube covered by gaze and washed with natural seawater. 1ml of a solution highly enriched with embryos was transferred to an Eppendorf tube. 100ul (1/100 of the volume of the

tube) of a DiI or DiO (Molecular Probes) at a concentration of 1mg/ml in DMSO (centrifuged 5min at 10000rpm before use to avoid crumbles) was added, mixed with care and shook at very low speed for at least 2-4hrs, depending on the age of the embryos. For microscopy, embryos were rinsed 3x with natural seawater, and put onto a slide in a mixture of 50%NSW and 50% 7.5%$MgCl_2$. The $MgCl_2$ was added to inhibit movements (it does not inhibit cilia movements, D. Arendt, personal communication) . DiI can be observed with the TRITC filter, DiO with the FITC filter of a microscope. It is certainly worth for each batch to test slightly different DiI concentrations and incubation times in parallel to get best results.

4.19 Further molecular standard techniques

DNA restriction digests, DNA ligations, bacterial mini preparations, electrotransformation of *E.coli*, agarose gel electrophoresis (0.6%–3% agarose, TAE buffer) were performed according to standard protocols (Sambrook *et al.*, 1989). Gels were stained for 5-10 minutes in a 1:10000 dilution of EtBr in water and used for gel extraction or photography on a gel documentation station.

Bacterial maxi preparations were done using the "Qiagen Maxiprep Kit" according to manufacturers instructions. DNA was eluted from agarose gels by cutting the respective piece out of gel under long-wave UV conditions (λ=366nm). The piece was centrifuged for 5min at maximum speed in a 0.5ml Eppendorf tube half filled with glass wool and a hole at the bottom (agarose piece on top). The 0.5ml Eppendorf tube had been placed into a 1.5ml Eppendorf tube to collect the liquid. The eluted DNS solution was cleaned by using the "PRC nucleotide removal kit" from Qiagen, and eluted in a final volume of 30ul ddH_2O.

4.20 Additional genes cloned by myself, but not mentioned in this study

Pdu-ptx (entire ORF cloned), *Pdu-emx* (entire ORF cloned), *Pdu-lim3* (entire ORF cloned)

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6 APPENDIX

6.1 Sequence alignments of novel *Platynereis* genes and indices of accession numbers

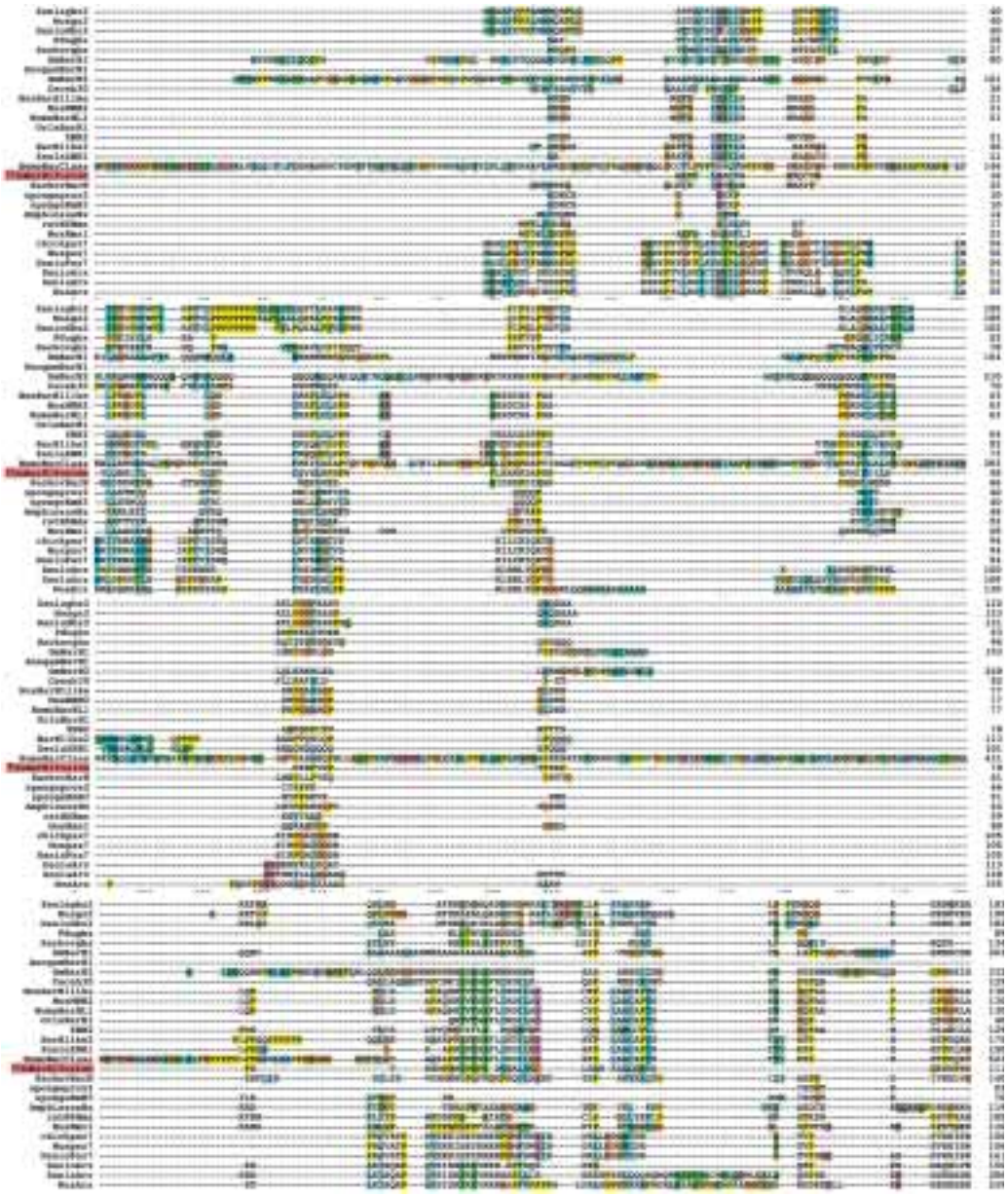
In the following sections, I include the sequence alignments that I used to construct the phylogenetic trees shown in this thesis. Note that for the large Opsin analysis, the alignment is already included in the result section of my thesis.

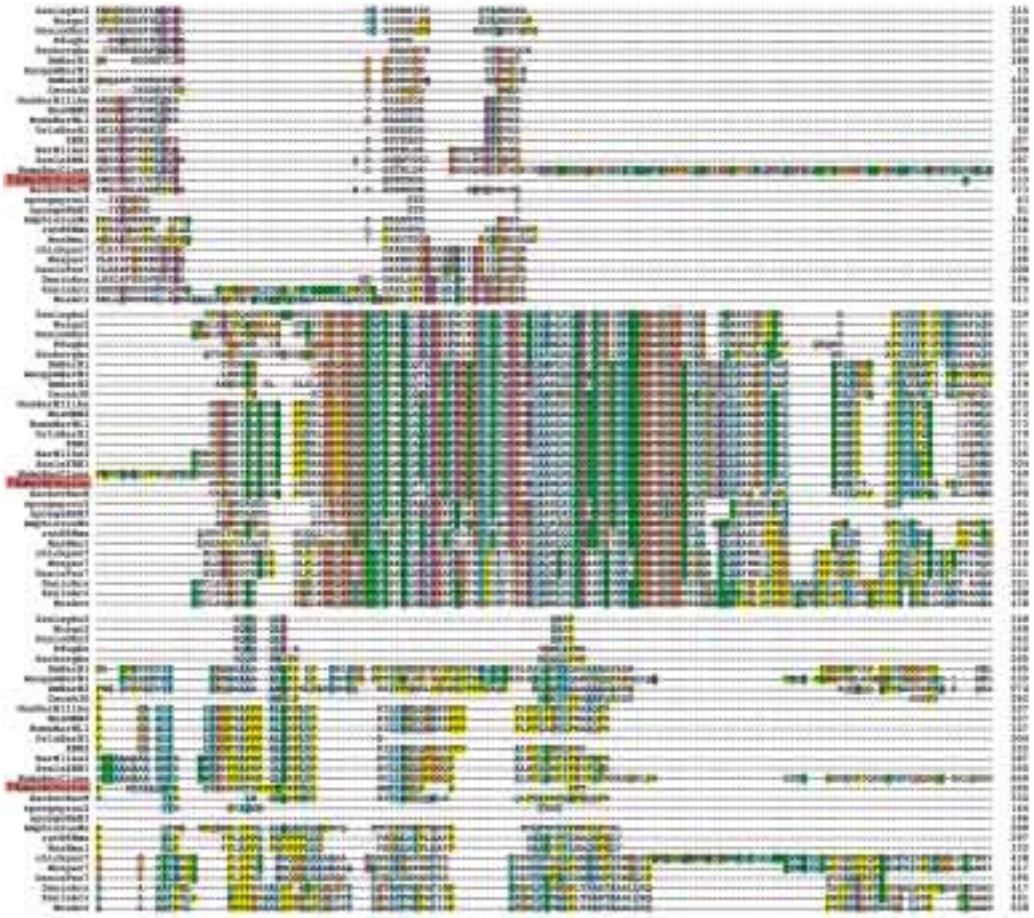
All alignments were produced with the CLUSTAL algorithm as described in the material and methods section. For convenience, *Platynereis dumerilii* proteins are always highlighted in red.

Under each alignment, I provide an index that contains the public accession numbers/names of the corresponding proteins, following the nomenclature of the NCBI database (http://www.ncbi.nlm.nih.gov). Except otherwise noted, all of the shown *Platynereis* sequences were first identified and reported by myself in this study. Exceptions include Pdu- Nk2.1 and Pdu-Vax. Their sequence and assignment to the respective orthology groups was provided by G. Balavoine and D. Arendt, respectively, but they were included in my alignment analyses (Vax served as an outgroup for the Emx alignments).

I used additional unpublished *Platynereis dumerilii* markers that were identified by others and hence are not listed in this index. These are Pdu-Rx (sequence and annotation by D. Arendt); Pdu-Nk2.2 and Pdu-Sim (sequence and annotation by H.Snyman and D. Arendt); Pdu-Fkh (S.Klaus, Diploma thesis, University of Heidelberg, 2003); as well as Pdu-Gsx (sequence and annotation by D. Ferrier).

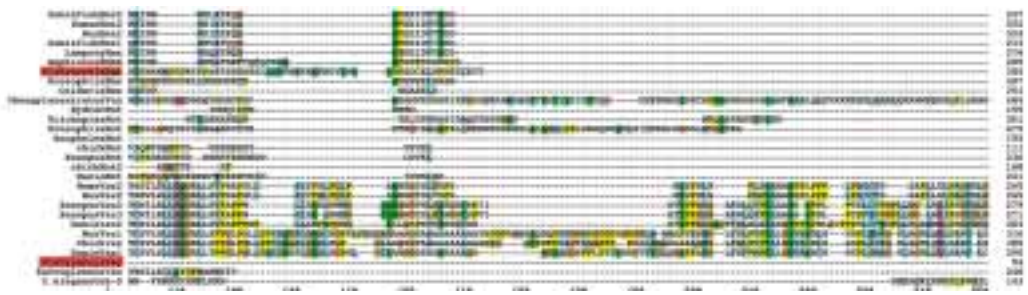
6.1.1 *Platynereis* BarH1





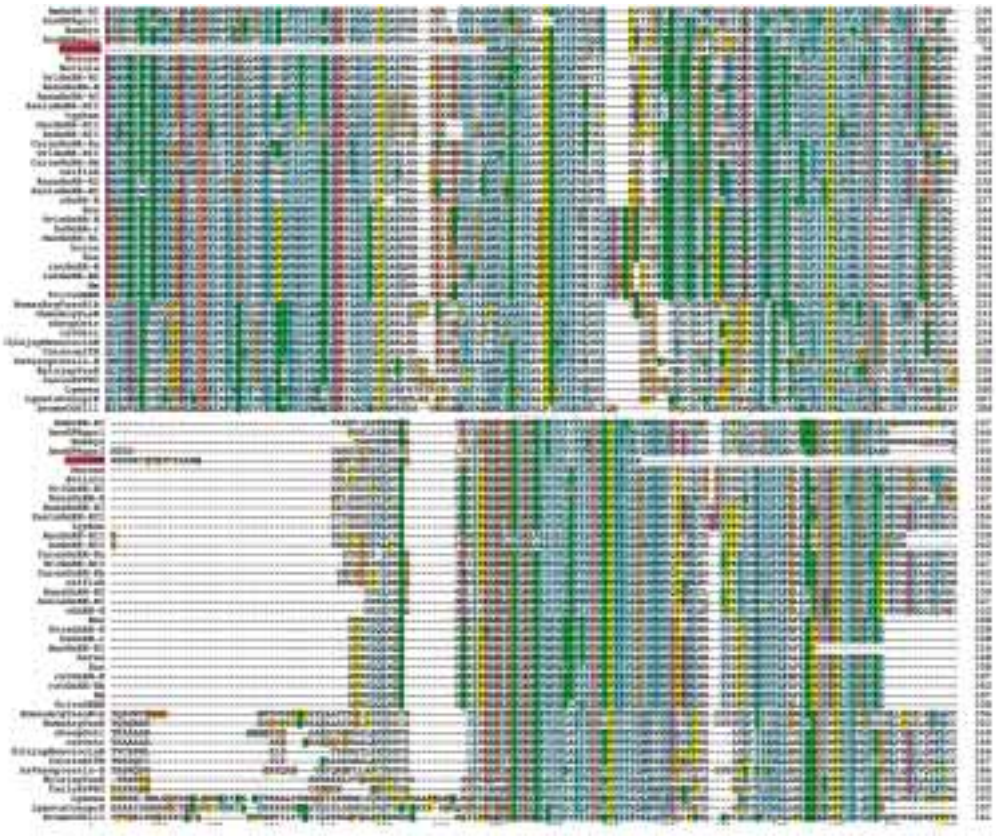
Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
DmBarH1	ref|NP_523387.1|	BarH1 CG5529-PA [Drosophila melanogaster]
AnogamBarH1	ref|XP_314421.1|	ENSANGP00000013333 [Anopheles gambiae]
DmBarH2	ref|NP_523386.1|	BarH2 CG5488-PA [Drosophila melanogaster]
OrlaBarH1	emb|CAD19778.1|	Bar protein [Oryzias latipes]
MusBarH1like	ref|NP_062319.1|	BarH-like 1 [Mus musculus]
HomoBarHL1	ref|NP_064448.1|	BarH-like 1 [Homo sapiens]
BarHlike2	ref|NP_064447.1|	BarH-like 2 [Homo sapiens]
XenlaXBH1	gb|AAG14450.1|	Bar homeobox protein XBH1 [Xenopus laevis]
XBH2	gb|AAG14451.1|	Bar homeobox protein XBH2 [Xenopus laevis]
SackovBarH	gb|AAP79299.1|	barH [Saccoglossus kowalevskii]
MusMBH2	ref|NP_062319.1|	BarH-like 1 [Mus musculus]
Ceceh30	ref|NP_508524.1|	C.Elegans Homeobox (ceh-30)
HomoBarClass	ref|NP_064447.1|	BarH-like 2 [Homo sapiens]
DanioGbx2	gb|AAN76664.1|	Gbx2 [Danio rerio]
Pdugbx	emb|CAD43609.1|	homeobox protein [Platynereis dumerilii]
chickpax7	dbj|BAA23005.1|	PAX7 protein [Gallus gallus]
DanioArx	ref|NP_571459.1|	paired-like (aristaless) homeobox 2a [Danio rerio]
DanioPax7	ref|NP_571401.1|	paired box gene 7a [Danio rerio]
XenlaArx	gb|AAN05413.1|	aristaless-related homeobox [Xenopus laevis]
AmphioxusHx	gb|AAL09323.1|	homeobox Hx [Branchiostoma floridae]
Muspax7	ref|NP_035169.1|	PAX7 [Mus musculus]
MusArx	ref|NP_031518.2|	aristaless related homeobox gene [Mus musculus]
spongeEmH3	gb|AAC18965.1|	EmH-3 [Ephydatia muelleri]
ratH6Hmx	ref|XP_341239.1|	similar to Hmx1 [Rattus norvegicus]
MusHmx1	ref|NP_034575.1|	H6 homeo box 1 [Mus musculus]
DmHmx	ref|NP_524951.1|	H6-like-homeobox CG5832-PA [Drosophila melanogaster]

6.1.2 Platynereis *Emx and Vax*



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
C.elegansCeh-5	ref|NP_492586.1	C.Elegans Homeobox (ceh-5)
DanioNot	ref|NP_571130.1	floating head [Danio rerio]
AmphioxusEMXA	gb|AAF76327.1|	homeoprotein [Branchiostoma floridae]
chickNot	pir||I50622	Cnot protein - [Gallus gallus]
chickNot2	emb|CAA66662.1|	homeobox protein [Gallus gallus]
DrosophilaEms	sp|P18488|HMES_DROME	Empty spiracles homeotic protein [Drosophila melanogaster]
HomoVax2	gb|AAP36469.1|	Homo sapiens ventral anterior homeobox 2 [synthetic construct]
HydraCNot	emb|CAB88387.1|	homeoprotein [Hydra vulgaris]
MusVax1	ref|NP_033527.1|	ventral anterior homeobox containing gene 1 [Mus musculus]
MusVax2	ref|NP_036042.1|	ventral anterior homeobox containing gene 2 [Mus musculus]
MedakaVax1	emb|CAB88699.1|	Vax1 transcription factor [Oryzias latipes]
StrongylocentrotusVax	gb|AAD20328.1|	homeodomain protein Not [Strongylocentrotus purpuratus]
XenopusVax2	emb|CAB58181.1|	Vax2 protein [Xenopus laevis]
XenopusVax3	gb|AAF25692.1|	ventral anterior homeobox 3 [Xenopus laevis]
XenopusNot	pir||A46305	Xnot protein [Xenopus laevis]
AnophelesNot	ref|XP_321115.1|	ENSANGP00000018309 [Anopheles gambiae] gb|EAA01471.1|
DrosophilaEms	sp|P18488|HMES_DROME	Empty spiracles homeotic protein [Drosophila melanogaster]
CnidariaEmx	emb|CAA72534.1|	Cn-ems protein [Hydractinia symbiolongicarpus]
ZebrafishEmx1	ref|NP_571354.1|	empty spiracles homeobox 3; empty spiracles homeobox 1 [Danio rerio]
ZebrafishEmx2	ref|NP_571355.1|	empty spiracles homeobox 2 [Danio rerio]
LampreyEmx	dbj|BAB13506.1|	LjEMX [Lethenteron japonicum]
MusEmx2	ref| NP_034262|	empty spiracles homolog 2 [Mus musculus]
HumanEmx2	ref|NP_004089.1|	EMX2 [Homo sapiens]
Chickvax	dbj|BAA84282.1|	homeodomain protein [Gallus gallus]
Saccoglossusvax	gb|AAP79280.1|	ventral anterior homeobox [Saccoglossus kowalevskii]
Daniovax2	ref|NP_919390.1|	Vax2 [Danio rerio]
Musvax1	ref|NP_033527.1|	ventral anterior homeobox containing gene 1 [Mus musculus]
DanioVax1	ref|NP_919391.1|	Vax1 [Danio rerio]
StrongylocentrotusNot	gb|AAD20328.1|	homeodomain protein Not [Strongylocentrotus purpuratus]
DrosophilaNot	ref|NP_650701.1|	CG18599-PA [Drosophila melanogaster]
TrichoplaxNot	gb|AAQ82694.1|	Not [Trichoplax adhaerens]

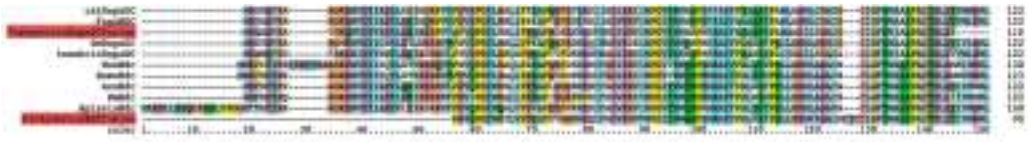
6.1.3 Platynereis *GnRH-Receptor*



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Anguilla GnRH-r	dbj|BAB11961.1|	gonadotropin-releasing hormone receptor [Anguilla japonica]
Astal GnRH-R	gb|AAK29745.1|	gonadotropin-releasing hormone receptor [Astatotilapia burtoni]
Bos GnRh- R	ref|NP_803480.1|	gonadotropin-releasing hormone receptor; [Bos taurus]
Bombyx AkH-R	gb|AAL95712.1|	AKH receptor [Bombyx mori]
CarasGnRH-Rb	gb|AAD20002.1|	gonadotropin releasing hormone receptor type B [Carassius auratus]
Catastomus AVT-R	sp|Q90352|AVT_CATCO	[Arg8]vasostocin receptor (AVT) [Catostomus commersoni]
CarasGnRH-Ra	gb|AAD20001.1|	gonadotropin releasing hormone receptor type A [Carassius auratus]
catfish	sp|O42329|GRR2_CLAGA	Gonadotropin-releasing hormone II receptor (Type II GnRH receptor) [Clarias gariepinus]
Cercopithecus GnRH-RII	sp|Q95MH6|GRR2_CERAE	Gonadotropin-releasing hormone II receptor (Type II GnRH receptor) [Cercopithecus aethiops]
cGnRH-R	emb|CAC18674.1|	chicken gonadotropin-releasing hormone receptor [Gallus gallus]
DmGnRH-RI	gb|AAN10047.1|	putative AKH receptor [Drosophila melanogaster]
DmGnRH-RII	ref|NP_648571.1|	CG10698-PA [Drosophila melanogaster]
hsGnRH-r	ref|NP_000397.1|	gonadotropin-releasing hormone receptor; hormone receptor; GnRH receptor; GnRHR [Homo sapiens]
hsGnRH-RII	ref|NP_476504.1|	gonadotropin-releasing hormone (type 2) receptor 2 [Homo sapiens] gb|AAL89821.1|
hsVasopressin-R	ref|NP_000697.1|	V1-vascular vasopressin receptor AVPR1A; SCCL vasopressin subtype 1a receptor [Homo sapiens]

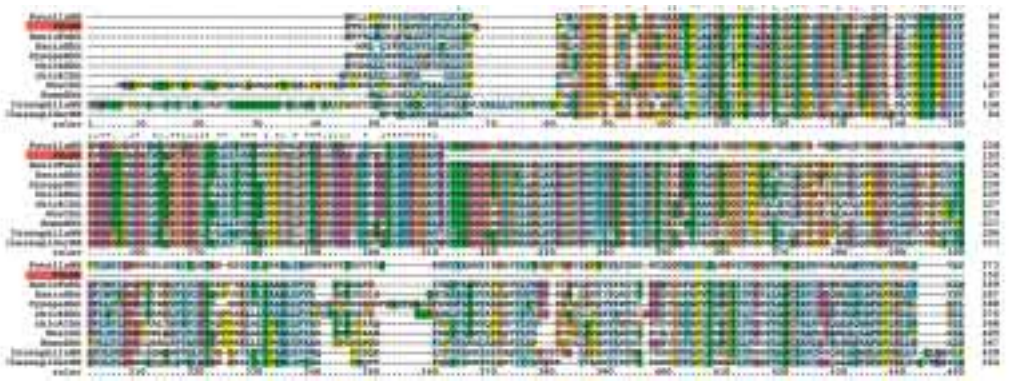
Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
horse GnRH-R	sp|O18821|GRHR_HORSE	Gonadotropin-releasing hormone receptor (GnRH receptor) [Equus caballus]
Lymnea Conopressin R2	gb|AAC46987.1|	conopressin receptor 2 prf||2208362A Lys-conopressin receptor 2
MacGnRH-RII	sp|Q95JG1|GRR2_MACMU	(GnRH-II-R) gb|AAK52745.1| GnRH receptor II [Macaca mulatta]
MacGnRH-RI	gb|AAG43378.1|	gonadotropin releasing hormone receptor [Macaca radiata]
Mm GnRH-R	gb|AAA37716.1|	gonadotropin releasing hormone receptor
Morone GnRH-R	gb|AAF28464.1|	pituitary gonadotropin releasing hormone receptor [Morone saxatilis]
Onkym GnRH	emb|CAB93351.1|	gonadotropin-releasing hormone receptor [Oncorhynchus mykiss]
OrlGnRH-RI	dbj|BAB70504.1|	gonadotropin-releasing hormone receptor 1 [Oryzias latipes]
OrlGnRH-RII	dbj|BAB70503.1|	gonadotropin-releasing hormone receptor 2 [Oryzias latipes]
OvisGnRH-R	emb|CAA50978.1|	GnRH receptor [Ovis aries]
RanaGnRH-R	gb|AAG42575.1|	GnRH receptor-1 [Rana catesbeiana]
RanaGnRH-R2	gb|AAG42949.1|	GnRH receptor-2 [Rana catesbeiana]
RanaGnRH-R3	gb|AAG42574.1|	GnRH receptor-3 [Rana catesbeiana] gb|AAL11631.1| [Rana catesbeiana]
ratGnRH-R	emb|CAA48776.1|	gonadotropin-releasing hormone receptor; GnRH-R [Rattus sp.]
ratGnRH-Rb	ref|NP_112300.1|	[Rattus norvegicus] gonadotropin-releasing hormone receptor
Seriola GnRH-R	emb|CAB65407.1|	gonadotropin-releasing hormone receptor [Seriola dumerili]
Sus GnRH-R	gb|AAA31067.1|	luteinizing hormone-releasing hormone receptor
typhon GnRH-R	gb|AAD49750.1|	gonadotropin-releasing hormone receptor [Typhlonectes natans]
TrivuGRHR	sp|Q9TTI8|GRHR_TRIVU	Gonadotropin-releasing hormone receptor (GnRH receptor) (GnRH-R) gb|AAF21641.1| [Trichosurus vulpecula]
XenlaGnRH-RI	gb|AAF89754.1|	gonadotropin releasing hormone receptor type I [Xenopus laevis]
XenlaGnRH-RII	gb|AAK49334.1|	gonadotropin-releasing hormone receptor type II [Xenopus laevis]
AnoGPRgnr1	ref|XP_308034.1|	ENSANGP00000019362 [Anopheles gambiae] gb|EAA03704.1|
GPRgnr2	ref|XP_321555.1|	ENSANGP00000018165 [Anopheles gambiae] emb|CAD27924.1
AnoGPRgnr3	ref|XP_321591.1|	ENSANGP00000011679 [Anopheles gambiae] gb|EAA00865.1|
Dromecorazin	gb|AAN10045.1|	putative corazonin receptor [Drosophila melanogaster]
HomsaArgVasoR1b	ref|NP_000698.1|	arginine vasopressin receptor 1B; arginine vasopressin receptor 3; [Homo sapiens]
DromeGH21228p	gb|AAN10047.1|	putative AKH receptor [Drosophila melanogaster]
SussroOT-R	sp|P32306|OXYR_PIG	OXYTOCIN RECEPTOR (OT-R) [Sus scrofa]
MumuOxtr	ref|XP_144956.2|	oxytocin receptor [Mus musculus]
sheepOxtr	gb|AAK28287.1|	endometrial oxytocin receptor [Ovis aries]
MumuArgVasR	ref|NP_036054.1|	V3/V1b vasopressin receptor, V1b arginine vasopressin receptor [Mus musculus]
ratOxtr	ref|NP_037003.2|	oxytocin receptor [Rattus norvegicus]
HylajapMesotocinR	dbj|BAC23056.1|	mesotocin receptor [Hyla japonica]
CatstomITR	sp|Q90334|ITR_CATCO	Isotocin receptor (ITR) -white sucker [Catostomus commersoni]
HylajapVasR	dbj|BAC23055.1|	V2 type arginine vasotocin receptor [Hyla japonica]
CanisAVPR2	sp|O77808|V2R_CANFA	vasopressin receptor type II [Canis familiaris]
DromeCG6111	ref|NP_651449.2|	CG6111-PA [Drosophila melanogaster]
LymstaConoprR	gb|AAA91998.1|	conopressin receptor
LymstaLSCPR2	gb|AAC46987.1|	conopressin receptor 2 prf||2208362A Lys-conopressin receptor 2
spiderLeucotR	gb|AAF72891.1|	leucokinin-like peptide receptor [Boophilus microplus]
LymstagGRL106	gb|AAB92258.1|	cardioexcitatory receptor [Lymnaea stagnalis]

6.1.4 Platynereis *Dopa Decarboxylase and Histidine Decraboxylase*



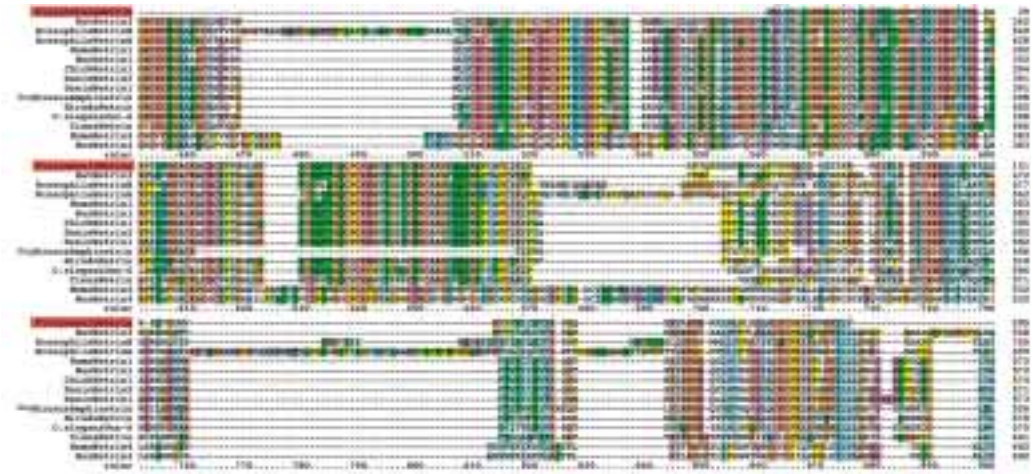
Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
MusHdc	gb|AAH52833.1|	Histidine decarboxylase [Mus musculus]
HomoHdc	sp|P19113|DCHS_HUMAN	Histidine decarboxylase (HDC) pir||A49882 histidine decarboxylase (EC 4.1.1.22) - human
AplysiaHdc	gb|AAP34326.1|	histidine decarboxylase [Aplysia californica]
AnoHdc	ref|XP_319749.1|	ENSANGP00000017218 [Anopheles gambiae]
DmHdc	ref|NP_523679.2|	Histidine decarboxylase CG3454-PA [Drosophila melanogaster]
ratDopaDC	ref|NP_036677.1|	Dopa decarboxylase (aromatic L-amino acid decarboxylase) [Rattus norvegicus]
tenebrioDopaDC	dbj|BAA95568.1|	dopa decarboxylase [Tenebrio molitor]
DmDopaDC	ref|NP_724489.1|	CG30446-PA [Drosophila melanogaster]
FuguHDC	SINFRUP00000065269	SINFRUP00000065269 Fugu genome

6.1.5 *Platynereis Hedgehog*



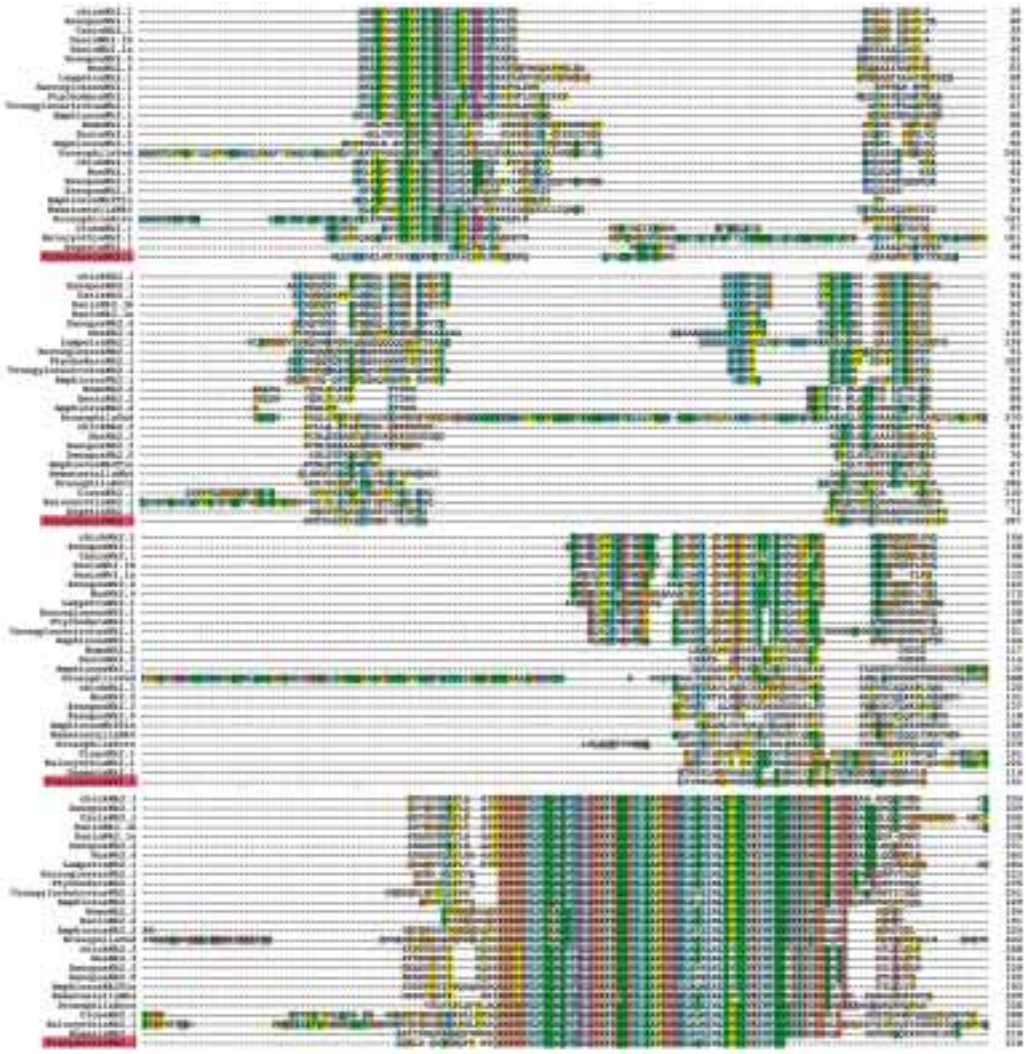
Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
DrosophilaHH	ref|NP_524459.2|	hedgehog CG4637-PA [Drosophila melanogaster]
DanioTwhh	ref|NP_571274.1|	tiggy winkle hedgehog; [Danio rerio]
housespiderHH	dbj|BAD01490.1|	hedgehog [Achaearanea tepidariorum]
CynopsShh	sp|Q90385|SHH_CYNPY	Sonic hedgehog protein precursor [Cynops pyrrhogaster]
HomoDhh	ref|NP_066382.1|	desert hedgehog preproprotein [Homo sapiens]
chickShh	sp|Q91035|SHH_CHICK	sonic hedgehog [Gallus gallus]
DanioShh	ref|NP_571138.1|	sonic hedgehog; [Danio rerio]
PatellaHH	gb|AAM60752.1|	hedgehog [Patella vulgata]
chickIhh	sp|Q98938|IHH_CHICK	Indian hedgehog protein precursor (IHH)
MusIhh	ref|NP_034674.1|	Indian hedgehog [Mus musculus]

6.1.6 Platynereis Netrin



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
HomoNetrin1	ref|NP_004813.1|	netrin 1 [Homo sapiens]
MusNetrin1	sp|O09118|NET1_MOUSE	Netrin-1 precursor [Mus musculus]
DanioNetrin2	ref|NP_571104.1|	netrin 1a; netrin 2 [Danio rerio]
RatNetrin3	ref|XP_343868.1|	Netrin 3 [Rattus norvegicus]
ChickNetrin1	sp|Q90922|NET1_CHICK	Netrin-1 precursor - chicken
C.elegansUnc-6	ref|NP_509165.1|	UNCoordinated locomotion UNC-6 [Caenorhabditis elegans]
DanioNetrin1	ref|NP_571073.1|	netrin 1 [Danio rerio]
DrosophilaNetrinB	ref|NP_511155.1|	Netrin-B CG10521-PA [Drosophila melanogaster]
DrosophilaNetrinA	sp|Q24567|NETA_DROME	Netrin-A precursor [Drosophila melanogaster]
HirudoNetrin	gb|AAC83376.1|	netrin precursor [Hirudo medicinalis]
CionaNetrin	dbj|BAB68352.1|	netrin [Ciona savignyi]
AmphioxusAmphinetrin	emb|CAB72422.1|	amphinetrin [Branchiostoma floridae]
MusNetrin4	ref|NP_067295.1|	netrin 4; beta-netrin [Mus musculus]
HomoHetrin	gb|AAF69670.2|	hepar-derived netrin-like protein [Homo sapiens]
HomoNetrin4	gb|AAG53651.1|	netrin 4 precursor [Homo sapiens]

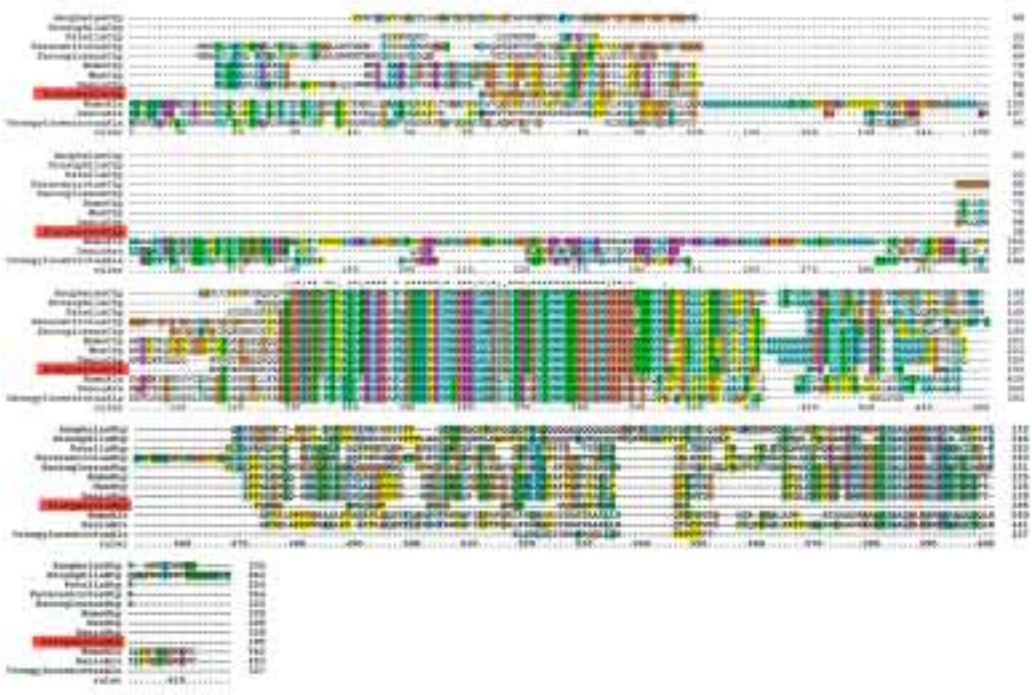
6.1.7 Platynereis Nk2.1



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
CionaNk2.1	dbj|BAB68343.1|	Cs-TTF1 [Ciona savignyi]
CanisNk2.1	X77910	C.canis Thyroid Transcription Factor-1
AmphioxusNk2.1	gb|AAC35350.1|	homeobox protein Nkx2-1 [Branchiostoma floridae]
DrosophilaScarecrow	gb|AAF26436.1|	homeobox protein Scarecrow [Drosophila melanogaster]
chickNk2.1	emb|CAA11493.1|	Homeodomain protein NKx2.1 [Gallus gallus]
DugesiaNk2.1	sp|Q00401|HMH2_DUGTI	Homeobox protein DTH-2 (Dugesia tigrina)
DanioNk2.1a	ref|NP_571664.1|	thyroid transcription factor 1a [Danio rerio]
SaccoglossusNk2.1	gb|AAP79291.1|	nkx2-1 [Saccoglossus kowalevskii]
StrongylocentrotusNk2.1	gb|AAM94862.1|	homeodomain protein NK2.1 [Strongylocentrotus purpuratus]
PtychoderaNk2.1	gb|AAM93268.1|	NK2.1 homeodomain protein [Ptychodera flava]
HalocynthiaNk2.1	dbj|BAA33413.1|	Thyroid Transcription Factor-1 [Halocynthia roretzi]

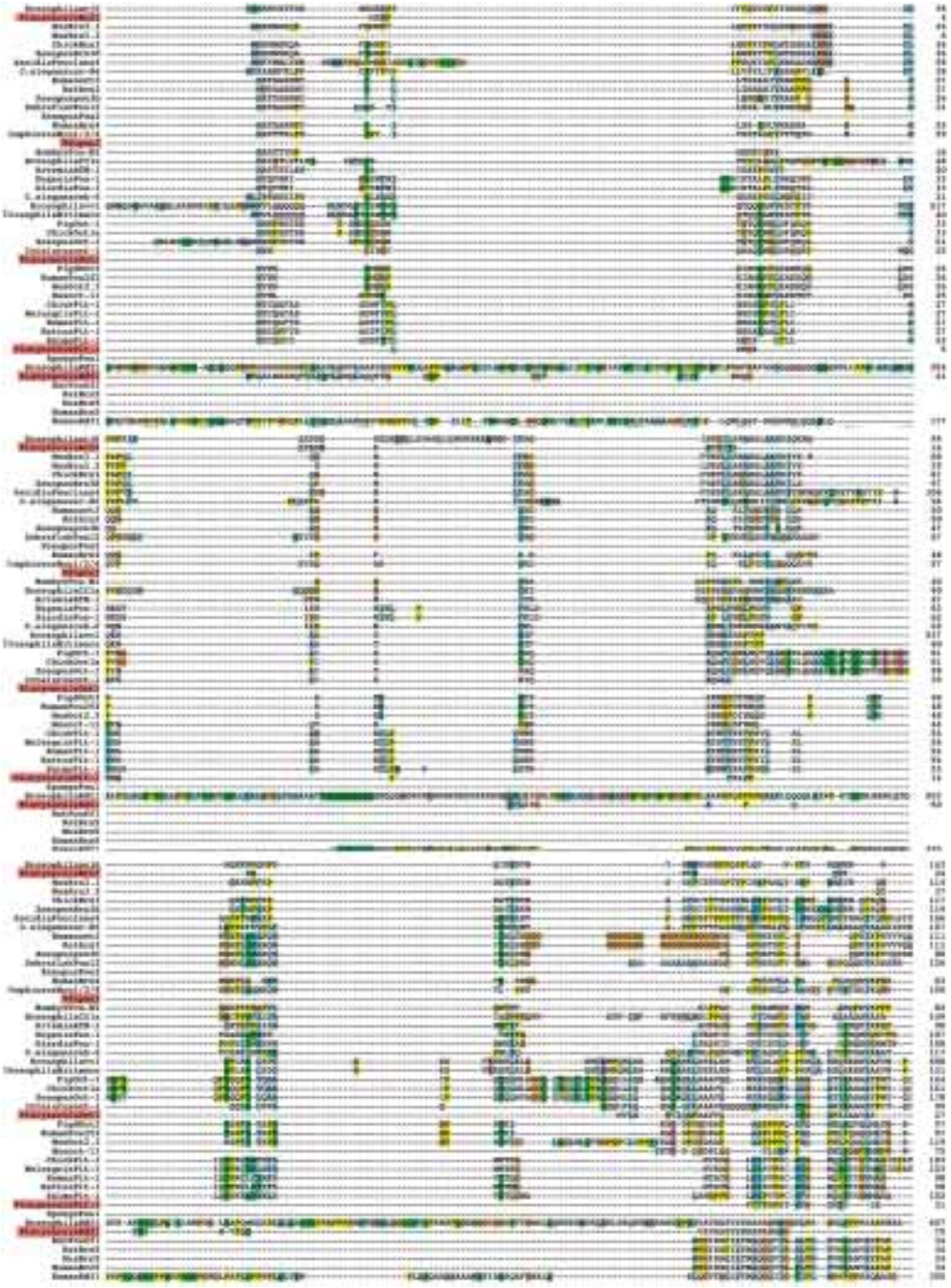
Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
DanioNk2.1b	ref|NP_571851.1|	thyroid transcription factor 1b [Danio rerio]
XenopusNk2.4	gb|AAG17404.1|	homeobox protein Nkx2-4 [Xenopus laevis]
MusNk2.4	ref|NP_075993.1|	NK2 transcription factor related, locus 4 [Mus musculus]
XenopusNk2.1	gb|AAG17405.1|	homeobox transcription factor Nkx2-1 [Xenopus laevis]
LampetraNk2.1	dbj|BAB32434.1|	thyroid transcription factor-1 [Lethenteron japonicum]
C.elegansCeh-24	ref|NP_506419.2|	C.Elegans Homeobox, NK-2 class, (ceh-24)
AmphioxusNk2.2	gb|AAD01958.1|	homeodomain protein [Branchiostoma floridae]
HomoNk2.2	ref|NP_002500.1|	NK2 transcription factor related, locus 2 [Homo sapiens]
DanioNk2.2	ref|NP_571497.1|	nk2.2 protein [Danio rerio]
DrosophilaScro	gb|EAA45970.1|	CG17594-PA.3 [Drosophila melanogaster]
chickNk2.3	emb|CAA66257.1|	cNKx-2.3 [Gallus gallus]
DrosophilaVnd	ref|NP_476786.2|	CG6172-PA [Drosophila melanogaster]
XenopusNk2.5	sp|P42583|NK25_XENLA	(XNkx-2.5) pir||I51442 homeobox protein - African clawed frog
MusNk2.3	gb|AAD38415.1|	homeobox protein NKX2-3 [Mus musculus]
NematostellaNk4	gb|AAP88432.1|	NK-4 homeobox protein [Nematostella vectensis]
AmphioxusNk2Tin	gb|AAM90855.1|	Amphink2-tin [Branchiostoma floridae]
chickNk2.8	emb|CAA71665.1|	NKX2.8 [Gallus gallus]
XenopusNk2.2	sp|P42587|HNK2_XENLA	Homeobox protein XENK-2 [Xenopus laevis]
XenopusNk2.3	gb|AAA99181.1|	putative [Xenopus laevis]

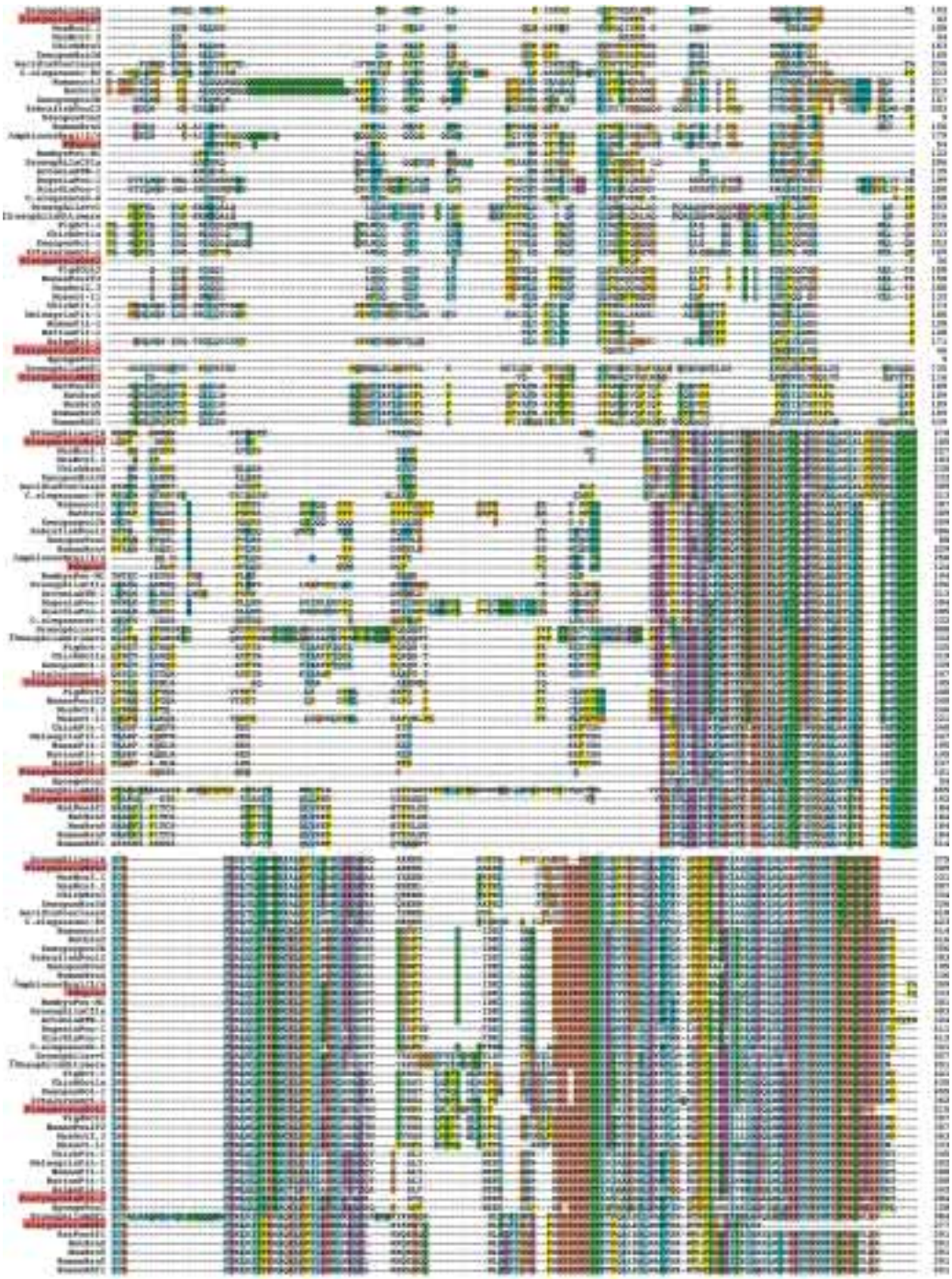
6.1.8 *Platynereis Orthopedia*



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
PatellaOtp	gb|AAM33145.1|	orthopedia [Patella vulgata]
ParacentrotusOtp	sp|O76971|OTP_PARLI	Homeobox protein orthopedia-related (PLOTP) [Paracentrotus lividus]
SaccoglossusOtp	gb|AAP79292.1|	orthopedia [Saccoglossus kowalevskii]
HomoOtp	ref| NP_115485.1|	orthopedia [Homo sapiens]
AnophelesOtp	ref|XP_313975.1|	ENSANGP00000009907 [Anopheles gambiae]
DanioOtp	ref|NP_571175.1|	orthopedia protein [Danio rerio] gb|AAD42021.1|
DrosophilaOtp	ref|NP_523799.3|	orthopedia CG10036-PA [Drosophila melanogaster]
MusOtp	ref|NP_035151.1|	orthopedia homolog; [Mus musculus]
HomoAlx	ref|NP_620689.1|	aristaless related homeobox [Homo sapiens]
DanioArx	ref|NP_571459.1|	aristaless related homeobox; paired-like (aristaless) homeobox 2a [Danio rerio]
DrosophilaAlx	ref|NP_722629.1|	aristaless CG3935-PA [Drosophila melanogaster]
StrongylocentrotusAlx	sp|Q26657|ALX_STRPU	Aristaless homeobox protein (ALX) (SpPrx-1) [Strongylocentrotus purpuratus]
AnophelesAlx	ref|XP_317481.1|	ENSANGP00000011877 [Anopheles gambiae]

6.1.9 Platynereis *Pou proteins*

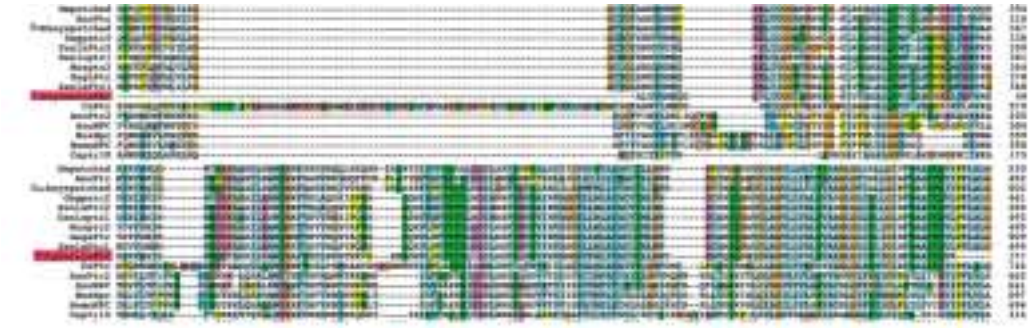




Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Drosophilaacj6	sp|P24350|POU_DROME	Inhibitory POU protein (I-POU)
Drosophilavvl	ref|NP_476659.1|	nubbin CG6246-PA [Drosophila melanogaster]
MusBrn3.1	pir||B49642	POU-domain protein Brn-3.1 - mouse
ChickBrn3	sp|Q91998|BRN3_CHICK	Brn-3 [Gallus gallus]
HumanBrn3b	emb|CAA50589.1|	Brn-3b [Homo sapiens]
HumBrn3a	gb|AAA57161.1|	Brn-3a
XenopusBrn3d	gb|AAG17008.1|	class IV POU-homeodomain protein [Xenopus laevis]
DrosophilaCf1a	prf||1709357B	Cf1-a protein
AscidiaPouclass4	gb|AAB62538.1|	class IV POU protein [Herdmania curvata]
HumanPou4f3	gb|AAC06203.1|	transcription factor POU4F3 [Homo sapiens]
AmphioxusBrn1/2/4	gb|AAL85498.1|	AmphiBrn1/2/4 [Branchiostoma floridae]
C.elegansunc-86	ref|NP_498796.1|	UNCoordinated locomotion UNC-86 [Caenorhabditis elegans]
PlecoglossusPit-1	gb|AAM00354.1|	pituitary-specific transcription factor [Plecoglossus altivelis]
SparusPit-1	pir||JC6186	Pit-1 - [Sparus aurata]
SalmoPit-1	emb|CAA67433.1|	Pit-1 protein [Salmo salar]
ChickPit-1	sp|Q9YGL7|PIT1_CHICK	Pit-1 [Gallus gallus]

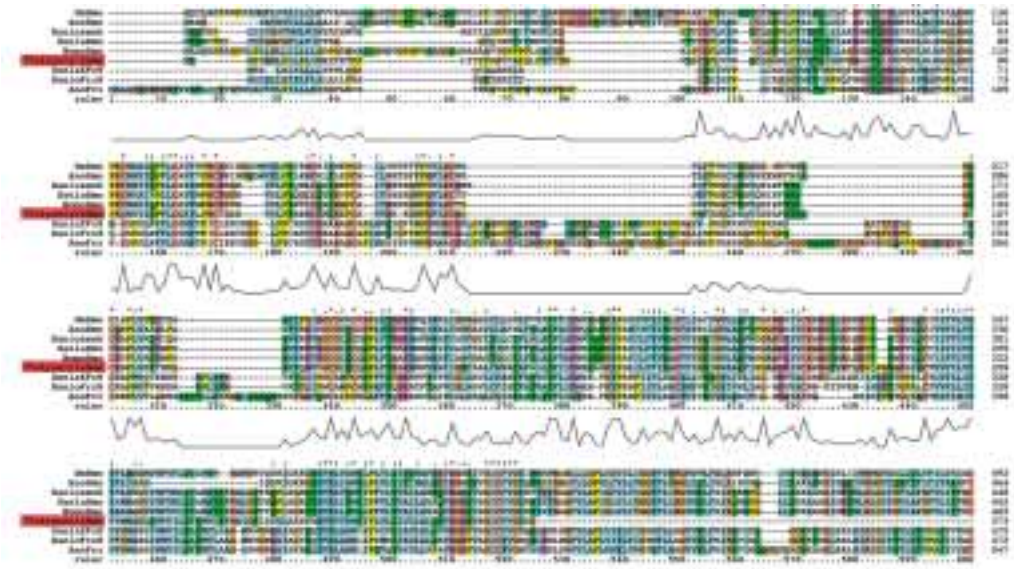
Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
MeleagrisPit-1	sp|Q05749|PIT1_MELGA	Pit-1beta* [Meleagris gallopavo]
HumanPit-1	dbj|BAA02291.1|	pituitary-specific POU-domain DNA binding factor [Homo sapiens]
MacacaPit-1	gb|AAB51352.1|	pit-1/GHF-1
RattusPit-1	gb|AAH61564.1|	Pou1f1 protein [Rattus norvegicus]
PigPit-1	gb|AAB65789.2|	POU-domain transcriptional regulator [Sus scrofa]
DugesiaPou-1	sp|P31370|POU1_DUGJA	POU domain protein 1 (DJPOU1) Dugesia japonica
AnolisBrain-1	dbj|BAA28665.1|	Brain-1 gene [Anolis carolinensis]
GiardiaPou-1	emb|CAA49383.1|	transcription factor [Girardia tigrina]
DrosophilaNubbin	ref|NP_476659.1|	nubbin CG6246-PA [Drosophila melanogaster]
DrosophilaMitimere	gb|AAA28481.1|	dPOU-28
PigOct-1	sp|Q29076|PO21_PIG	(Oct-1)
MusOct2.3	pir||S22543	Oct2.3 [Mus musculus]
XenopusOct-1	emb|CAA40454.1|	maternal transcription factor [Xenopus laevis]
ChickOct1a	sp|P15143|PO21_CHICK	Oct-1, - chicken
PigdOct2	sp|Q29013|PO22_PIG	Oct-2 - pig
C.elegansceh-6	ref|NP_492304.1|	(ceh-6) [Caenorhabditis elegans]
Ictalurusoct-1	emb|CAA03984.1|	Oct1 transcription factor [Ictalurus punctatus]
HumanPou2f2	sp|P09086|PO22_HUMAN	Octamer-binding transcription factor 2
Musoct-11	ref|NP_035269.1|	octamer binding protein-11 [Mus musculus]
BombyxSgf-3	sp|Q17237|SGF3_BOMMO	Silk gland factor 3 (SGF-3) silkworm
ArtemiaATH-1	emb|CAA75353.1|	APH-1 [Artemia franciscana]
XenopusPou2	gb|AAA92684.1|	transcription factor POU2
ZebrafishPou12	sp|P56224|ZP12_BRARE	ZP-12 [Danio rerio]
Xenopuspou3b	sp|P70030|PO3B_XENLA	POU3b [Xenopus laevis]
Humanoct3	sp|P20265|PO32_HUMAN	POU domain, class 3, transcription factor 2
Ratbrn2	ref|XP_345511.1|	POU domain, class 3, transcription factor 2 [Rattus norvegicus]
Zebrafishzp23	sp|P79745|ZP23_BRARE	POU domain protein ZP-23 [Danio rerio]
Zebrafishpou1	ref|NP_571177.1|	POU domain gene 1 [Danio rerio]
HumanBrn4	pir||A55557	transcription factor Brn-4 - human
ZebrafishZp-23	emb|CAA69214.1|	Pou1 protein [Danio rerio]
AnolisBrn4	dbj|BAA28667.1|	Brain-4 gene [Anolis carolinensis]
SpongePou1	gb|AAD01888.1|	Pit-1/GHF-1 [Gallus gallus]
DrosophilaRdf1	ref|NP_610377.1|	CG11641-PA [Drosophila melanogaster]
HumanRdf1	gb|AAC83404.2|	unknown [Homo sapiens]
XenopusOct60	gb|AAA49997.1|	XOCT-60
MusBrn5	ref|NP_034257.1|	POU domain, class 6, transcription factor 1 [Mus musculus]
HumanBrn5	gb|AAH51326.1|	POU6F1 protein [Homo sapiens]
RatBrn5	sp|P56223|PO61_RAT	Brn-5 - rat
DmAT16994p	ref|NP_610377.1|	CG11641-PA [Drosophila melanogaster]

6.1.10 Platynereis Patched



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Dmpatched	ref|NP_724694.1|	patched CG2411-PB [Drosophila melanogaster]
buckeye patched	gb|AAD31595.1|	putative hedgehog receptor [Junonia coenia]
AnoPtc	ref|XP_317925.1|	ENSANGP00000011383 [Anopheles gambiae]
CePtc	ref|NP_495662.1|	patched family member (ptc-1) [Caenorhabditis elegans]
AnoPtc2	ref|XP_317322.1|	ENSANGP00000010415 [Anopheles gambiae]
Gagpatc2	gb|AAK97655.1|	patched 2 [Gallus gallus]
XenlaPtc1	gb|AAK15463.1|	patched-1 [Xenopus laevis]
GagaPtc	sp|Q90693|PTC1_CHICK	Patched protein homolog 1 (PTC1) - chicken
XenlaPtc2	dbj|BAB18575.1|	patched-2 [Xenopus laevis]
Danioptc1	ref|NP_571063.1|	patched1 [Danio rerio]
MusPtc2	ref|NP_032984.1|	patched homolog 2 [Mus musculus]
MusPtc1	ref|NP_032983.1|	patched; Patched 1; [Mus musculus]
MusNpc	ref|NP_032746.1|	Niemann Pick type C1; sphingomyelinosis [Mus musculus]
HomoNPC	ref|NP_000262.1|	Niemann-Pick disease, type C1 [Homo sapiens] [Homo sapiens]
Ceptr10	ref|NP_491658.1|	PaTched Related (ptr-10) [Caenorhabditis elegans]
AnoNPC	ref|XP_310493.1|	ENSANGP00000007352 [Anopheles gambiae]

6.1.11 Platynereis Smoothened



Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Daniosmoh	ref|NP_571102.1|	smoothened homolog [Danio rerio]
XenlaSmo	gb|AAK15464.1|	smoothened [Xenopus laevis]
HomoSmo	ref|NP_005622.1|	smoothened [Homo sapiens]
DmSmo	ref|NP_523443.1|	smoothened CG11561-PA [Drosophila melanogaster]
AnoSmo	ref|XP_314720.1|	ENSANGP00000013048 [Anopheles gambiae]
XenlaXFz8	sp|O93274|FZD8_XENLA	Frizzled 8 precursor [Xenopus laevis]
DanioFrz8	ref|NP_571629.1|	frizzled homolog 8a; frizzled homolog 5; frizzled homolog C [Danio rerio]
AnoFrz	ref|XP_311505.1|	ENSANGP00000024916 [Anopheles gambiae]

6.1.12 Accession numbers of Vasopression/ Oxytocin-like neuropeptides

These accession numbers relate to the proteins I used to construct the alignment of Figure 27 B.

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
CatostomIT	sp|P15210|NEU1_CATCO	Isoticin-neurophysin IT 1 [Catostomus commersoni]
CatostomVTN	sp|P17668|NEU3_CATCO	Vasotocin-neurophysin VT 1 - white sucker
FuguIsot	sp|O42493|NEUI_FUGRU	Isoticin-neurophysin IT 1 precursor [Takifugu rubripes]
FuguVTN	sp|O42499|NEUV_FUGRU	Vasotocin-neurophysin VT 1 precursor [Takifugu rubripes]
sheepOTNPI	sp|P13389|NEU1_SHEEP	Oxytocin-neurophysin 1 precursor [Ovis aries]
SeaurchinOTC	sp|P24787|NEUV_CHICK	Vasotocin-neurophysin VT precursor [Gallus gallus]
TorpedoIsotocin	gb|AAA74284.1|	isotocin/neurophysin
MasusalmonIsotocin	dbj|BAA01738.1|	prepro-isotocin-I [Oncorhynchus masou]
TyphlonecMesotocin	gb|AAF76848.1|	mesotocin preprohormone [Typhlonectes natans]
DromeMST98Ca	ref|NP_524899.1|	CG11719-PA [Drosophila melanogaster]
BufojapMT	sp|P08162|NEUM_BUFJA	MESOTOCIN-NEUROPHYSIN MT PRECURSOR (MT) - Japanese toad
Musoct	ref|NP_035155.1|	oxytocin [Mus musculus
BosAVNII	ref|NP_789824.1|	arginine vasopressin; neurophysin II [Bos taurus]
BosONI	emb|CAA23448.1|	[Bos taurus] oxytocin neurophysin I precursor
OncorynchAVNII	sp|P16042|NEU4_ONCKE	Vasotocin-neurophysin VT 2 precursor [Oncorhynchus keta]
Danioisotocin	ref|NP_840076.1|	isotocin neurophysin [Danio rerio]
dipnoiAVNII	dbj|BAA24026.1|	prepro-vasotocin [Neoceratodus forsteri]
chickAVNII	sp|P24787|NEUV_CHICK	Vasotocin-neurophysin VT precursor – [Gallus gallus]
LampetraAVNII	dbj|BAA06669.1|	vasotocin precursor [Lethenteron japonicum]
hagfish	dbj|BAA06668.1|	vasotocin precursor [Eptatretus burgeri]
AplysiakAVNII	dbj|BAB40371.1|	Lys-conopressin preprohormone [Aplysia kurodai]
octvulcephaloticin	dbj|BAC82436.1|	cephalotocin [Octopus vulgaris]
EiseniaAVNII	dbj|BAA36458.1|	Annetocin precursor [Eisenia fetida]
Octopvuloctopressin	dbj|BAC82435.1|	octopressin [Octopus vulgaris]
Lymnea	sp|Q00945|CONO_LYMST	Conopressin/neurophysin [Lymnaea stagnalis]
MusAVP	ref|NP_033862.1|	arginine vasopressin [Mus musculus]
PlatichAVNII	dbj|BAA98140.1|	vasotocin precursor [Platichthys flesus]
DanioAVNII	ref|NP_840078.1|	vasotocin neurophysin [Danio rerio]

6.1.13 Accession numbers of Vasopression/ Oxytocin like receptors

These accession numbers relate to the proteins I used to construct the alignment of Figure 27 A.

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
MusVP1A	ref|NP_058543.2|	arginine vasopressin receptor 1A; [Mus musculus]]
HomoVP1A	ref|NP_000697.1|	arginine vasopressin receptor 1A; [Homo sapiens]
OvisVP1	gb|AAC41627.1|	vasopressin V1 receptor
flounderVPR	gb|AAF00506.1|	arginine vasotocin receptor [Platichthys flesus]
HomoVPRV3	ref|NP_000698.1|	arginine vasopressin receptor 1B; arginine vasopressin receptor 3; [Homo sapiens]
SusOR	sp|P32306|OXYR_PIG	OXYTOCIN RECEPTOR (OT-R) [Sus scrofa]
RatVPR1b	gb|AAC52235.1|	arginine-vasopressin V1b receptor
MusOR	ref|XP_144956.2|	oxytocin receptor [Mus musculus]
BosOR	ref|NP_776559.1|	oxytocin receptor [Bos taurus]
CatostomIR	sp|Q90334|ITR_CATCO	Isotocin receptor (ITR) [Catostomus commersoni]
AstatoVPR	gb|AAM70493.1|	arginine vasotocin receptor [Astatotilapia burtoni]
CatostomVPR	sp|Q90352|AVT_CATCO	[Arg8]vasostocin receptor (AVT) [Catostomus commersoni]
OctopusOCCER	dbj|BAC81147.1|	OT/VP superfamily peptide receptor-1 [Octopus vulgaris]
GallusVR	gb|AAG17937.2|	pituitary vasotocin receptor [Gallus gallus]
HylaVMRV2	dbj|BAC23055.1|	V2 type arginine vasotocin receptor [Hyla japonica]
DmCG6111	ref|NP_651449.2|	CG6111-PA [Drosophila melanogaster]
Ano	ref|XP_308034.1|	ENSANGP00000019362 [Anopheles gambiae]
OrylaGnRHR3	dbj|BAC97832.1|	gonadotropin-releasing hormone receptor 3 [Oryzias latipes]
BombyxAKHR	gb|AAL95712.1|	AKH receptor [Bombyx mori]
RnnaGNRHR1	gb|AAP15162.1|	GnRH receptor 1 [Rana ridibunda]
CionaGnRHR2	dbj|BAC57060.1|	Gonadotropin-releasing hormone receptor [Ciona intestinalis]
DmGnRHR	gb|AAN10047.1|	putative AKH receptor [Drosophila melanogaster]
CanisV2R	sp|O77808|V2R_CANFA	Vasopressin V2 receptor [Canis familiaris]
DmGnRGR2	pir||JE0278	GnHR receptor homolog (Drosophila melanogaster)
BufoMR	sp|Q90252|MTR_BUFMA	Mesotocin receptor (MTR) [Bufo marinus]
LymneaCPR2	gb|AAC46987.1|	Lys-conopressin receptor 2
LymneaCPR	gb|AAA91998.1|	conopressin receptor

6.1.14 Accession numbers of Opsins

These accession numbers relate to the proteins I used to construct the alignment of Figure 16 and phylogenetic trees of Figure 17 and Figure 18.

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Drome-rh5	ref|NP_477096.1|	Rhodopsin 5 CG5279-PA [Drosophila melanogaster
Drome-rh4	ref|NP_476701.1|	Rhodopsin 4 CG9668-PA [Drosophila melanogaster]
Drome-rh3	ref|NP_524411.1|	Rhodopsin 3 CG10888-PA [Drosophila melanogaster]
Drome-rh2	ref|NP_524398.1|	Rhodopsin 2 CG16740-PA [Drosophila melanogaster]
Drome-rh6	ref|NP_524368.3|	Rhodopsin 6 CG5192-PB [Drosophila melanogaster]
Drome-rh1	ref|NP_524407.1|	CG4550-PA [Drosophila melanogaster]
Manse-opsin	gb|AAD11964.1|	opsin [Manduca sexta]
Apime-LW-rhodopsin	sp|Q17053|OPSD_APIME	Rhodopsin, long-wavelength - honeybee
Papgl-rhodopsin	gb|AAD34220.1|	rhodopsin [Papilio glaucus]
Hemsa-bcrh1	sp|Q25157|OPS1_HEMSA	BcRh1 [Hemigrapsus sanguineus]
Schmidtea-opsin	gb|AAD28720.1|	opsin [Schmidtea mediterranea]
Xenla-melanopsin	gb|AAC41235.1|	melanopsin [Xenopus laevis]
Xenla-rhodopsin	sp|P29403|OPSD_XENLA	Rhodopsin [Xenopus laevis]
Todpa-retinochrome	sp|P23820|REIS_TODPA	Retinochrome (Retinal photoisomerase) [Todarodes pacificus]
Girardia-opsin	emb|CAB89516.1|	opsin [Girardia tigrina]
Todpa-rhodopsin	sp|P31356|OPSD_TODPA	Rhodopsin [Todarodes pacificus]
Sepof-rhodopsin	sp|O16005|OPSD_SEPOF	Rhodopsin [Sepia officinalis]
Schgr-rhodopsin	sp|Q94741|OPS1_SCHGR	Opsin 1 [Schistocerca gregaria]
salmon-ancient-opsin	sp|O13018|OPSO_SALSA	Vertebrate ancient opsin
red-swamp-crayfish-opsin	sp|P35356|OPSD_PROCL	Rhodopsin [Procambarus clarkii]
Petma-rhodopsin	sp|Q98980|OPSD_PETMA	Rhodopsin gb|AAB62981.1| rhodopsin
VAL-opsin	ref|NP_571661.1|	vertebrate ancient long opsin; (VAL)-opsin [Danio rerio]
Petma-pineal opsin	sp|O42490|OPSP_PETMA	Pineal opsin (P-opsin) [Petromyzon marinus]
Homsa-rhodopsin	ref|NP_000530.1|	rhodopsin; human
Patye-Gq-Rhodopsin	sp|O15973|OPS1_PATYE	Rhodopsin, GQ-coupled (GQ-rhodopsin) [Mizuhopecten yessoensis]
Homsa-encephalopsin	ref|NP_055137.1|	opsin 3 (encephalopsin, panopsin); [Homo sapiens]
Patye-Go-rhodopsin	sp|O15974|OPS2_PATYE	Rhodopsin, G0-coupled (G0-rhodopsin) [Mizuhopecten yessoensis]
Musmu-peropsin	ref|NP_033128.1|	retinal pigment epithelium derived rhodopsin homolog [Mus musculus]
Musmu-RGR	ref|NP_067315.1|	RGR opsin [Mus musculus]
Octdo-rhodopsin	sp|P09241|OPSD_OCTDO	Rhodopsin [Octopus dofleini]
Galga-rhodopsin	sp|P22328|OPSD_CHICK	Rhodopsin [Gallus gallus]
Musmu-melanopsin	ref|NP_038915.1|	opsin 4 (melanopsin) [Mus musculus]
Lolsu-rhodopsin	sp|Q17094|OPSD_LOLSU	Rhodopsin [Loligo subulata]
Lolfo-rhodopsin	sp|P24603|OPSD_LOLFO	Rhodopsin [Loligo forbesi]

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Homsa-melanopsin	ref|NP_150598.1|	opsin 4 melanopsin [Homo sapiens]
Hemsa-rhodopsin	sp|Q25158|OPS2_HEMSA	Compound eye opsin BCRH2 [Hemigrapsus sanguineus]
Bosta-RGR	ref|NP_786969.1|	opsin homolog [Bos taurus]
Limulus-ocellus	sp|P35361|OPS2_LIMPO	opsin, ocellar - Atlantic horseshoe crab
Lamja-rhodopsin	sp|P22671|OPSD_LAMJA	Rhodopsin - Japanese lamprey
Homsa-RGR	sp|P47804|RGR_HUMAN	RPE-retinal G protein-coupled receptor [Homo sapiens]
Homsa-peropsin	ref|NP_006574.1|	peropsin [Homo sapiens]
Serinus-SWS-opsin	emb|CAB91994.1|	SWS opsin [Serinus canaria]
Schistosoma-opsin	gb|AAF73286.1|	RHO G-protein coupled receptor [Schistosoma mansoni]
Columba-SWS2-opsin	gb|AAD38035.1|	SWS2 opsin [Columba livia]
Pladu-opsin	emb|CAC86665.1|	rhabdomeric opsin [Platynereis dumerilii]
Rat-RGR	ref|XP_224673.1|	similar to RGR opsin [Rattus norvegicus]
Xenla-RGR	BX704587	Xenopus leavis EST (translated)
Galga-RGR	gi|25468016|gb|BU228798.1| BU228798	Gallus gallus cDNA clone ChEST766p11 (translated)
Bos-RGR	ref|NP_786969.1|	retinal G protein coupled receptor [Bos taurus]
Oryla-RGR	gi|22181724|dbj|BJ522912.1| BJ522912	cDNA Oryzias latipes cDNA clone MF01SSB026E22 (translated)
Danre-RGR	gi|40318893|gb|CK353006.1| CK353006	Danio rerio cDNA clone IMAGE:7071698 (translated)
Homsa-RGR	sp|P47804|RGR_HUMAN	RPE-retinal G protein coupled receptor [Homo sapiens]
Musmu-encephalopsin	ref|NP_034228.1|	opsin (encephalopsin) [Mus musculus]
Anolis_carolinensis	gb|AAD32622.1|	P opsin [Anolis carolinensis]
Takifugu_TNT_opsin	gb|AAL83430.1|	TMT opsin [Takifugu rubripes]
Ciona_int_opsin	dbj|BAB68391.1|	opsin [Ciona intestinalis]
Anopheles_GPRop11	ref|XP_312503.1|	ENSANGP00000001159 [Anopheles gambiae] gb|EAA07662.2|
Anopheles_GPRop12	ref|XP_312502.1|	ENSANGP00000014846 [Anopheles gambiae] gb|EAA08098.1|
Amphiop6	dbj|BAC76024.1|	opsin [Branchiostoma belcheri]
Amphiop5	dbj|BAC76022.1|	opsin [Branchiostoma belcheri]
Amphiop4	dbj|BAC76021.1|	opsin [Branchiostoma belcheri]
Amphiop1	dbj|BAC76019.1|	opsin [Branchiostoma belcheri]
Amphiop2	dbj|BAC76020.1|	opsin [Branchiostoma belcheri]
Amphiop3	dbj|BAC76023.1|	opsin [Branchiostoma belcheri]

6.1.15 Accession numbers of Otx/Gsc proteins

These accession numbers relate to the proteins I used to construct the phylogenetic tree of Figure 5B.

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Dugesiaotx	dbj|BAA90698.1|	homeobox [Dugesia japonica]
Girardiaotx	gb|AAD55327.1|	homeobox protein [Girardia tigrina]
Pf-Otx	dbj|BAA89013.1|	Pf-Otx [Ptychodera flava]
Saccoglossusorthodenticle	gb|AAP79293.1|	orthodenticle [Saccoglossus kowalevskii]
LjOtxA	dbj|BAA33409.1|	LjOtxA [Lethenteron japonicum]
Leucopsarionotx2	dbj|BAB62172.1|	transcriptional factor [Leucopsarion petersii]
Daniootx3	ref|NP_571290.1|	orthodenticle homolog 3 [Danio rerio]
Leucopsarionotx	dbj|BAB62171.1|	transcriptional factor [Leucopsarion petersii]
HomoOTX1	ref|NP_055377.1|	orthodenticle 1; homeobox protein OTX1 [Homo sapiens]
HMOC_DROME	sp|P22810|HMOC_DROME	Homeotic protein orthodenticle (Ocelliless protein) [Drosophila melanogaster]
LjOtxB	dbj|BAA33410.1|	LjOtxB [Lethenteron japonicum]
Danioconerodhox	emb|CAB63872.1|	OTX5b protein [Xenopus laevis]
DanioOtx1	sp|Q91994|OTX1_BRARE	Homeobox protein OTX1 [Danio rerio]
Musotx1	gb|AAL24809.1|	Otx1 [Mus musculus]
PetromyzonOtx	gb|AAC82470.1|	Otx [Petromyzon marinus]
Pdotx	emb|CAC19028.1|	homeobox transcription factor [Platynereis dumerilii]
XenlaOTX5	dbj|BAA86260.1|	XOTX5 [Xenopus laevis]
XenlaOTX5b protein	emb|CAB63872.1|	OTX5b protein [Xenopus laevis]
ScyliorhinusOtx5	gb|AAK85128.1|	homeobox protein Otx5 [Scyliorhinus canicula]
OlaOtx2	emb|CAA04396.1|	Otx2 [Oryzias latipes]
Pristinaotxlike	gb|AAK64220.1|	orthodenticle-like homeobox protein 1 [Pristina leidyi]
CynopsOTX2	dbj|BAC53612.1|	OTX2 [Cynops pyrrhogaster]
Cs-OTX	dbj|BAB68341.1|	Cs-OTX [Ciona savignyi]
Homootx2	pir||S39407	homeotic protein otx2 - human
XenlaOtx1	gb|AAK31735.1|	transcription factor Otx1 [Xenopus laevis]
AAB63527.1orthodenticle 2	gb|AAB63527.1|	orthodenticle 2
PleurodelesOtx2	gb|AAM09955.1|	transcription factor Otx2 [Pleurodeles waltl]
PodocoryneOtx	gb|AAF04002.1|	homeodomain protein Otx [Podocoryne carnea]
GallusOTX2	emb|CAD32962.1|	homeobox protein OTX2 [Gallus gallus]
Daniootx2	ref|NP_571326.1|	orthodenticle homolog 2 [Danio rerio]
CionaintOtx	gb|AAG59802.1|	Otx [Ciona intestinalis]
HelobdellatrisLox22-otx	gb|AAB61443.1|	Lox22-otx [Helobdella triserialis]
LabidochromisOtx2	dbj|BAC02578.1|	Otx2 [Labidochromis caeruleus]
Lethenteron Pax6	dbj|BAB62531.1|	Pax6 [Lethenteron japonicum]

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Branchiostomagoosecoid	gb|AAF97935.1|	goosecoid [Branchiostoma floridae]
amphioxusOtx	gb|AAC00193.1|	amphioxus Otx transcription factor [Branchiostoma floridae]
PleurodelesOtx5	gb|AAN17797.1|	transcription factor Otx5 [Pleurodeles waltl]
Daniogsc	ref|NP_571092.1|	goosecoid; [Danio rerio]
Pdgsc	emb|CAC19336.1|	homeobox transcription factor [Platynereis dumerilii]
Dmgsc	sp|P54366|GSC_DROME	goosecoid [Drosophila melanogaster]

6.1.16 Accession numbers of Six proteins

These accession numbers relate to the proteins I used to construct the phylogenetic tree of Figure 5A.

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
Saccoglossus Six3	gb|AAP79281.1|	six3 [Saccoglossus kowalevskii]
XenlaSix6	gb|AAH42277.1|	Six6-A protein [Xenopus laevis]
GiardiaSix3	gb|AAN77127.1|	six3 [Girardia tigrina]
Dmoptix	ref|NP_524695.2|	Optix CG18455-PA [Drosophila melanogaster]
DanioSix7	ref|NP_571429.1|	sine oculis homeobox homolog 7 [Danio rerio]
MusSix6	ref|NP_035514.1|	SIX6 [Mus musculus]
XenlaOptx2	gb|AAD47356.1|	homeobox protein Optx2 [Xenopus laevis]
HomoSix3	ref|NP_005404.1|	SIX3 protein [Homo sapiens]
MusSix9	emb|CAA09775.1|	Six9 protein [Mus musculus]
HomoSix9	ref|NP_031400.1|	OPTX2 [Homo sapiens]
GallusOptx2	sp|O93307|SIX6_CHICK	SIX6 [Gallus gallus]
DanioSix3	ref|NP_571438.1|	Six3 [Danio rerio]
GagaSix3	sp|O42406|SIX3_CHICK	SIX3 [Gallus gallus]
DanioSix6	ref|NP_571438.1|	Six6 [Danio rerio]
AnogamSix4	ref|XP_309580.1|	ENSANGP00000022269 [Anopheles gambiae]
DugesiaSix12	emb|CAD89530.1|	six1-2 protein [Dugesia japonica]
DmSix4	ref|NP_649256.1|	CG3871-PA [Drosophila melanogaster]
PduSix2	emb|CAC86663.1|	Six2 protein [Platynereis dumerilii]
ceh-34	ref|NP_504419.1|	ceh-34 [Caenorhabditis elegans]
ceh-33	ref|NP_504420.1|	ceh-33 [Caenorhabditis elegans]
CEH-32	ref|NP_505958.1|	ceh-32 [Caenorhabditis elegans]
GirardiaSix1	emb|CAB89515.1|	homeodomain transcription factor [Girardia tigrina]
CionaSix2	AK112392	Ciona intestinalis cDNA, clone:ciad046p13 (translated)
AnogamSix2	ref|XP_320814.1|	ENSANGP00000004790 [Anopheles gambiae]
Dugesiasix12	emb|CAD89530.1|	six1-2 protein [Dugesia japonica]
MusSix1	AAH23304.1	Sine oculis homeobox homolog 1 [Mus musculus]

Name in phylogenetic tree/ AA alignments	Accession number	Name as it occurs in the NCBI database
DanioSix2.1	ref|NP_571858.1|	sine oculis homeobox homolog 2.1 [Danio rerio]
XenlaSix2	gb|AAD39895.2|	homeobox protein SIX2 [Xenopus laevis]
HomoSix2	sp|Q9NPC8|SIX2_HUMAN	SIX2 [Homo sapiens]
GirardiaSix2	emb|CAB89515.1|	homeodomain transcription factor [Girardia tigrina]
PetromyzonSix1	gb|AAD39897.1|	homeobox protein SIX1 [Petromyzon marinus]
DmSineoc	ref|NP_476733.1|	sine oculis CG11121-PA [Drosophila melanogaster]

6.2 Abbreviations

SI units (système international d'unités) and symbols of standard multiples (m, μ, etc.) are not listed below. Additional abbreviations are introduced in the text at the site of their first appearance.

AA	amino acid
AV/O	Argenine-Vasopression/ Oxytocin
AP-conjugated	alkaline phosphatase-conjugated
APO	apical (sensory) organ
ATP	Adenosine-5´triphosphate
bHLH	basic helix-loop- helix (domain)
BSA	bovine serum albumine
cAMP	cyclic Adenosine-5´monophosphate
°C	degree Celsius
CNS	Central nervous system
CSF	Central spinal fluid
C-terminus	Carboxy-terminus (of a peptide)
CTP	Cytosine-5´triphosphate
Da	Dalton
DAB	3'-3'-diaminobenzidine
DAG	diacylglycerol
DCV	dense core vesicle
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-A/C/G/T-trisphosphate
DTT	dithiothreitol
DV	dorso-ventral
EST	expressed sequence tag
Fgf (FgfR)	Fibroblast growth factor (receptor)
g	gravital force
GTP	Guanosine-5´-triphosphate
h	hour(s)
hh	hedgehog (signalling molecule)
HH	Hamburger-Hamilton stage (chick embryonic staging)

hpf	hours post fertilization
IC_{50}	Inhibitory concentration resulting in 50% inhibition
ISH (WMISH)	(whole-mount) *in situ* hybridisation
M	mol/l
min	minute(s)
mRNA	messenger RNA
NPY	Neuropeptide Y
NSW	natural sea water
N-terminus	Amino-terminus (of a peptide)
OD	optcial density
Pd	Platynereis dumerilii
PCR	polymerase chain reaction
poa	preoptic area
PRC	photoreceptor cell
RNA	ribonucleic acid
RCF	relative centrifugal force (x g)
rpm	rotations per minute
RT	room temperature
SC	spinal cord
SDS (SDS-PAGE)	Sodium-dodekylsulfate (polyacrylamide gel electrophoresis)
SS	Somatostatin
TTP	Thymidine-5´triphosphate
UTP	Uridine-5´triphosphate
UTR	untranslated region
UV	ultraviolet
VNC	ventral nerve cord
wt	wild-type
'	min
''	s

Short Summary

This thesis deals with the origin of the photosensory and neurosecretory cell types in the bilaterian brain. As the main experimental system, I used the annelid *Platynereis dumerilii*. *Platynereis* is an emerging protostomian model organism that is ideally suited for comparisons with vertebrates because it has retained many ancestral cell types, yet has a relatively simple morphology and mode of development.

In the first section, I describe my contribution to the reconstruction of the urbilaterian photosensory system. By cloning and analyzing a novel *opsin* gene from *Platynereis*, I was able to provide molecular support for the hypothesis that Urbilateria (the last common ancestors of all Bilateria) already possessed two photoreceptor cell types (PRCs) – rhabdomeric and ciliary PRCs. I further corroborated this by the comparative analysis of different upstream regulators for each PRC type. Presumably only the rhabdomeric type was ancestrally involved in vision, while the ciliary type was a light detector in the inner brain (like the ciliary PRCs of the vertebrate pineal organ). Both types, however, were recruited into the vertebrate eye, which is thus a compound structure. This finding provides a novel basis for understanding both the molecular similarities and differences between the vertebrate and invertebrate eyes.

I extended these analyses in the second section by investigating the molecular and morphological set-up of the median brain of the *Platynereis* larva and in particular of the apical organ (APO). The APO is a specialized, highly neurosecretory structure. From a detailed analysis of molecular markers and cellular morphologies I concluded that the median brain of trochophora type larvae with the APO as its core structure and the ventral/ median prosencephalon of vertebrates with the hypothalamus as its center share common heritage from their urbilaterian ancestor.

Both vertebrate eyes and hypothalamus have been shown to require proper Hedgehog (Hh) signalling for their correct development. From the expression studies and functional experiments in the third part of my thesis, I obtained evidence that Hh signalling might have played an ancestral role on median forebrain development. Moreover, the analysis permits a clear prediction for specific cell types in vertebrates that are likely under the control of the Hh- signaling pathway. Thereby, this study also supports the use of *Platynereis* as a model organism for Bilaterian-wide comparisons.

Zusammenfassung

Diese Dissertation befaßt sich mit der vergleichenden Analyse der Entwicklung lichtsensorischer und neurosekretorischer Systeme in der Evolution der Bilateria. Die Hauptfragestellung der Arbeit ist, ob die lichtsensorischen und neurosekretorischen Systeme, die in den verschiedenen Zweigen des evolutionären Stammbaums existieren, auf gemeinsame Anlagen in den Vorfahren aller Bilateria (Urbilateria) zurückführbar sind, also homolog sind, oder ob diese Systeme mehrfach unabhängig voneinander entstanden, und daher als konvergent zu betrachten sind.

Die Arbeit knüpft, vor allem im Bezug auf die lichtsensorischen Systeme, an einen langen wissenschaftlichen Disput an, in dem wahlweise die partiellen morphologischen und molekularen Gemeinsamkeiten oder die offenkundigen Unterschiede zwischen den verschiedenen natürlich vorkommenden Augentypen als Evidenz für oder wider die Homologie dieser Strukturen ins Feld geführt wurden. Die vorliegende Arbeit wählt im wesentlichen zwei Lösungsansätze, um diese Fragestellung neu zu untersuchen:

Erstens sucht sie Entsprechungen vornehmlich nicht auf der Ebene von Organen (z.B. dem Auge), sondern auf der Ebene von einzelnen Zelltypen. Diese Betrachtungsweise wird der Möglichkeit gerecht, daß von der Vielzahl der Zelltypen, aus denen sich komplette Organe zusammensetzen, nur ein Teil (z.B. die Photorezeptorzellen des Auges) in dem Vorläufer-Organ beteiligt war, während andere später in der Evolution hinzutraten und für substantielle Unterschiede zwischen den entsprechenden Organen in den heutigen Lebewesen verantwortlich sind.

Zweitens wählt diese Arbeit auf Seiten der Protostomia einen Organismus zum Haupt-Studienobjekt, der sich für den Bilateria-weiten Vergleich sehr viel besser eignet als die herkömmlichen Modellorganismen *Drosophila melanogaster* und *Caenorhabditis elegans*. Dies ist der Polychaet *Platynereis dumerilii* (Annelida, Nereididae). Neuere Erkenntnisse belegen, daß verschiedene Tiergruppen unterschiedlich schnell molekular and morphologisch evolvieren. Diese Tatsache spiegelt sich im jeweiligen Repertoire von Genfamilien und Zelltypen wider, die in den verschiedenen Organismen gefunden werden können. Während das

Genrepertoire von *Drosophila melanogaster* und *C.elegans* im Lauf der Evolution stark reduziert wurde und mit einem hoch abgeleiteten Entwicklungsmodus korreliert, weist *Platynereis dumerilii* neben seiner ursprünglicheren Lebensform auch ein breiteres Repertoire an Genen und Zelltypen als die oben erwähnten Organismen auf. Die bisherigen Vergleiche der Gene und Zelltypen von *Platynereis dumerilii*, anderer Lophotrochozoa und Cnidaria mit Deuterostomia (insbesondere Wirbeltieren) legt den Schluß nahe, daß viele dieser Zelltypen und Gene ursprünglich sind, in der Evolution von *Drosophila melanogaster* oder *C.elegans* jedoch verloren gingen. Wie auch die vorliegende Studie zeigt, eignet sich *Platynereis dumerilii* daher außerordentlich gut, um in Fragen der Homologie und Konvergenz insbesondere auf der Zelltyp-Ebene Klarheit zu schaffen, und damit auch aufzudecken, in welchem Grade Urbilateria bereits lichtsensorische und neurosekretorische Systeme besaßen. Der erste Teil der Arbeit beschreibt die Rekonstruktion der Bestandteile des lichtsensorischen Systems von Urbilateria. Um die großen Unterschiede in den Augen von Protostomia (v.a. Insekten) und Deuterostomia (v.a. Wirbeliteren) zu erklären, war in jüngerer Vergangenheit die Hypothese aufgestellt worden, daß Urbilateria nicht nur einen, sondern bereits zwei verschiedene Photorezeptor-Zelltypen besaßen, die aber im Verlauf der Evolution in verschiedener Weise in die Augen von Protostomia und Deuterostomia integriert wurden. Diese zwei Zelltypen korrelieren nach dieser Theorie mit den heutigen rhabdomeren und ciliären Photorezeptorzellen, die sich ultrastrukturell und molekular unterscheiden lassen. Die rhabdomeren Typen bilden die Photorezeptorzellen der larvalen und adulten *Drosophila*-Augen, während die ciliären Photorezeptorzellen als Stäbchen und Zapfen des Wirbeltierauges fungieren.

Sollten beide Zelltypen in der Tat ursprünglich sein, dann sollte man erwarten, daß sie sich auch heute noch gemeinsam in den verschiedenen Bereichen des Stammbaums der Lebewesen nachweisen lassen, insbesondere in den beiden großen Bereichen der Protostomia und der Deuterostomia. In der Tat existieren molekulare und morphologische Hinweise, daß Wirbeltiere beide Zelltypen besitzen. Außerdem sind bei *Platynereis* – nicht aber bei *Drosophila* – morphologisch neben den rhabdomeren auch ciliäre Photorezeptorzellen beschrieben. Der wichtige Nachweis aber, daß insbesondere die ciliären Zellen von *Platynereis dumerilii* auch molekular

mit den ciliären Photorezeptorzellen der Deuterostomia homolog sind, konnte bislang nicht erbracht werden.

Die vorliegende Arbeit beschreibt daher in ihrem ersten Teil eine weitergehende molekulare Charakterisierung der ciliären und rhabdomeren Photorezeptorzellen von *Platynereis dumerilii* und ihren Vergleich zu den Photorezeptorzellen der Wirbeltiere. Die Klonierung eines neuen *Platynereis opsin* Gens spielt in dieser Analyse eine Schlüsselrolle. Einerseits erlaubt die Expressionsanalyse dieses Gens die Schlußfolgerung, daß die morphologisch charakterisierten ciliären Photorezeptor-Zellen in *Platynereis* tatsächlich photosensitiv sind. Andererseits erweist die Analyse der Sequenz des zugehörigen Opsin Proteins, daß es (zusammen mit zwei Opsinen aus *Anopheles gambiae*) molekular deutlich näher an den Opsinen der ciliären Wirbeltier-Photorezeptorzellen steht als an den Opsinen der (rhabdomeren) Protostomia-Photorezeptorzellen. Das bestätigt auch auf molekularer Ebene, daß in Urbilateria ursprünglich zwei Typen von Photorezeptorzellen existierten. Diese Daten deuten darauf hin, daß die ciliären Photorezeptorzellen des Wirbeltiergehirns und –auges (neben den Zapfen und Stäbchen des Auges auch die lichtsensitiven Neurone des Hypothalamus und Pinealorgans) und die ciliären Photorezeptorzellen im Gehirn von *Platynereis dumerilii* homologe Zelltypen sind. Im Gegensatz dazu ist das bereits bekannte und charakterisierte *Platynereis opsin* klar mit den restlichen wirbellosen *opsinen* verwandt und im Einklang mit der Theorie nur in den Augen von *Platynereis* exprimiert, welche aus dem zweiten, rhabdomeren Photorezeptor-Typ bestehen.

Neben diesem wichtigen Befund erlaubte die weitergehende Expressionsanalyse, den ciliären und rhabdomeren Rezeptorzellen jeweils auch Transkriptionsfaktoren zuzuordnen, deren Orthologe sowohl in Wirbeltieren wie auch in *Platynereis dumerilii* spezifisch mit entweder dem einen oder dem anderen Photorezeptor-Zelltyp korrelieren (*rx* mit dem ciliären Typ; *ath*, *brn3* mit dem rhabdomeren Typ). Dies lieferte weitere Bestätigung für die These, daß beide Zelltypen molekular stark unterschiedlich und ursprünglich für Bilateria sind.

Diese Theorie erlaubt einige Annahmen über die mögliche ursprüngliche Funktion sowie die Evolution der beiden ursprünglichen Zelltypen: Der rhabdomere Photorezeptor-Zelltyp spielte wahrscheinlich in einem ursprünglichen primitiven

Auge eine Rolle für das Sehen (wie das im Auge der rezenten Insekten der Fall ist), während die im inneren Gehirn liegenden ciliären Photorezeptorzellen ursprünglich nur der Lichtwahrnehmung dienten, ähnlich den Photorezeptorzellen im Pinealorgan und Hypothalamus der heutigen Wirbeltiere. Der ciliäre Zelltyp wurde jedoch zusätzlich ins heutige Wirbeltierauge rekrutiert und übernahm dort schließlich die Funktion der visuellen Wahrnehmung, während der rhabdomere Zelltyp als heutige retinale Ganglienzellen persistieren. Somit stellt das heutige Wirbeltierauge eine Kompositionsstruktur dar, die auf Ebene der einzelnen Zelltypen jedoch mit einzelnen Bestandteilen des Protostomia-Gehirnes homologisierbar ist. Diese Erklärung liefert eine neue Basis für das Verständnis der molekularen Ähnlichkeiten und Unterschiede, die zwischen den Wirbeltier- und Insektenaugen gefunden werden können.

In Fortsetzung dieser Analyse untersuchte der zweite Teil der Arbeit den gesamten medianen Hirnbereich der *Platynereis*-Trochophoralarve und seine Zelltypen und vergleicht diese Ergebnisse mit dem medianen Vorderhirnbereich der Wirbeltiere. Das Expressionsstudium von sieben verschiedenen Transkriptionsfaktoren in *Platynereis*, und der Vergleich zu den Expressionsorten der Orthologen in Wirbeltieren, ergibt eine bemerkenswerte Übereinstimmung zwischen ventral/ medianen Vorderbereich des Wirbeltiergehirns und dem medianen Bereich der Episphere der Trochophoralarve. Die molekulare Ähnlichkeit zwischen *Platynereis* und Wirbeltieren in diesem Bereich ist insbesondere bemerkenswert, da vier der Orthologen (*nk2.1*, *vax*, *otp*) hochspezifisch für das ventral/ mediane Vorderhirn der Wirbeltiere einerseits und das mediane Vorderhirn von *Platynereis* andererseits sind. Die Ausdehnung des Vergleichs auf molekulare 'Marker' für differenzierte Zelltypen (Serotonin, RFamid(e), *gnrh-r* und *hdc*) und auf spezielle Zellmorphologie führt zu der These, daß die mediane Episphere der primären ciliären Larven mit ihrem zentralständigen stark neurosekretorischen Apikalorgan und das ventrale/ mediane Vorderhirn der Wirbeltiere mit dem zentral-liegenden Hypothalamus auf einen gemeinsamen Vorläufer in den Urbilateria zurückführbar sind. Das bedeutet, daß viele der Zelltypen des Hypothalamus der Wirbeltiere einen sehr alten Ursprung haben, und wirft die spannende Frage auf, was die ursprüngliche Funktion dieser

Zellen gewesen sein könnte, und welche Rolle sie zudem in wesentlich einfacheren Organismen spielen, wie die *Platynereis*-Trochophoralarve sie darstellt.

Sowohl die Augen als auch der Hyphothalamus sind in ihrer Entwicklung in Wirbeltieren von einem funktionierendem Hedgehog- Signaltransduktionsweg abhängig. Es stellt sich daher die Frage, ob sich die durch Vergleiche von Genexpression und Zellmorphologie postulierte Homologie von Photorezeptor-Zelltypen und medianen Vorderhirn-Zelltypen bei Wirbeltieren und *Platynereis dumerilii* auch in den Mechanismen widerspiegelt, die bei der Entwicklung dieser Zelltypen und Bereiche ein Rolle spielen. In Erweiterung der vorangegangenen vergleichend-entwicklungsbiologischen Arbeit um diesen funktionalen Aspekt, befaßt sich der dritte Teil dieser Studie mit der Frage, ob der Hedgehog (Hh)-Signaltransduktionsweg eine Rolle in der Hirnentwicklung in *Platynereis dumerilii* spielt. Eine Expressionanalyse von *hh* und *smoothened*, zwei Bestandteilen des Signaltransduktionsweges, legt nahe, dass dies ähnlich wie bei Wirbeltieren der Fall sein könnte. Das Molekül Cyclopamin hat in Wirbeltieren einen spezifisch inhibitorischen Effekt auf den Hh- Signalweg. Die Arbeit zeigt, daß bereits ein Fünftel bis ein Zehntel der in Wirbeltieren wirksamen Konzentrationen zu morphologisch und molekular distinkten Defekten im larvalen Zentralnervensystem von *Platynereis dumerilii* führen. Es ist wichtig darauf hinzuweisen, dass mehrere Faktoren nur in Teilen ihrer Expressionsdomänen betroffen sind, an anderen Orten aber nicht beeinträchtigt sind. Das macht eine allgemeine Entwicklungsverzögerung als Begründung für die erwähnten Defekte unwahrscheinlich. Morphologische und molekule Defekte, die durch Cyclopamin-Inkubation in *Platynereis* hervorgerufen werden können, ähneln Defekten, die man in Wirbeltieren bei Störungen des Hh-Signalweges beobachten kann. Das deutet stark darauf hin, daß Cyclopamin auch in *Platynereis* den Hh- Signalweg beeinträchtigt.

Diese Analyse führt, neben einem besseren Verständnis der Entwicklung des Zentralnervensystems bei *Platynereis dumerilii*, auch zu einem besseren Verständnis von Gemeinsamkeiten und Unterschieden bei der Gehirnspezifizierung in Bilateria. So deutet die Studie darauf hin, welche ursprünglichen Rollen der Hh-Signaltransduktionsweg in Bilateria hat. Ein Funktionsverlust führt in *Platynereis,* ähnlich zu der Situation in Wirbeltieren, zu einer stärkeren Reduktion der lateralen

Vorderhirnexpression von *pax6* als der medianen *nk2.1* Expression (vergleichbar mit der Situation im Diencephalon des Zebrafisches), außerdem zu einer Reduktion der Pigmentzellen und Photorezeptorzellen des Auges, und zu einer Fehlsteuerung der axonalen Verbindungen. Desweiteren deutet diese Analyse auf Faktoren hin, die auch im Wirbeltier-Hypothalamus unter der Kontrolle vom Hh- Signalweg stehen könnten, unter diesem Aspekt dort jedoch noch nicht analysiert worden sind (z.B. *rx* oder RFamid (e).

In ihrer Gesamtheit unterstreicht die vorliegende Arbeit letztlich auch die herausragende Eignung von *Platynereis dumerilii* für die Studie von Entwicklungsprozessen im Bilateria-weiten Vergleich. Neben der Klärung evolutiver Fragestellungen beinhaltet diese Studie einen weiteren Punkt, der zukünftig von weitergehender Nützlichkeit auch für die Erforschung von Wirbeltieren sein könnte. Denn sie beschreibt eine initiale Charakterisierung und Lokalisierung von Zelltypen im Vorderhirn von *Platynereis*, die wahrscheinlich hohe Verwandschaft, zumindest aber starke molekulare Ähnlichkeiten zu den neurosekretorischen Zelltypen des ventral/ medianen Vorderhirns, besonders des Hypothalamus haben. Dieses Bezugssystem kann in Zukunft genutzt werden, um einerseits bisher unbekannten, aber auch in Wirbeltieren konservierten Genen erste mögliche Funktionen zuzuordnen (beispielsweise über die, in dieser Arbeit bereits erwähnte systematische Expressionsmusteranalysen von cDNA Banken). Desweiteren stellt die Trochophora-Larve von *Platynereis* mit ihrer relativ einfachen, aber wahrscheinlich ursprünglichereren Morphologie ein attraktives Modellsystem dar, in dem sich die Funktion ursprünglicher Gene in manchen Aspekten besser analysieren läßt als in den molekular reduzierteren und morphologisch stärker abgeleiteten klassischen Modellorganismen *Drosophila melanogaster* oder *Caenorhabditis elegans*.

Erklärung

Ich versichere, daß ich meine Dissertation

"*The evolution of sensory and neurosecretory cell types in bilaterian brains*"

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 9. März 2004

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Curriculum Vitae

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11/2001 to present	PhD student of Dr. Monika Hassel at the University of Marburg.
7/2002– 8/2003 *Heidelberg, Germany*	visiting research student in Dr. Detlev Arendt's lab at the European Molecular Biology Laboratory (EMBL), working on the sensory and neurosecretory system of the annelid worm *Platynereis dumerilii*
12/2001 – 4/2002 *San Francisco, USA*	visiting research student in Dr. Graeme Davis's lab at the University of California, San Francisco, working on the development of the neuromuscular junction in the fruit fly *Drosophila melanogaster*

University of Heidelberg, Heidelberg, Germany; 1995 to 2001

2/2001 – 11/2001 *Heidelberg*	visiting research student in Dr. Jochen Wittbrodt's lab at the EMBL, Heidelberg, working on vertebrate eye and brain development
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12/1999 – 4/2000	final exams in biochemistry, cell biology and zoology (mark 1.0)
11/1997 – 9/1998 *Boston, USA*	visiting research fellow in Dr. Gary Ruvkun's lab at the Mass. General Hospital/ Harvard Medical School, Boston, USA, working on neuronal development of the nematode *Caenorhabditis elegans*
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Christian– Weise– Gymnasium, Zittau, Germany, 1991 to 1995

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5/1995 passed the final exam in piano with A grade level after 11 years playing

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11/2001-12/2003 PhD scholarship from the Boehrinher Ingelheim Foundation

10/1996-3/2001 scholarship from the German National Merit Foundation (Studienstiftung des deutschen Volkes)

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Arendt D, **Tessmar-Raible K**, Snyman H, Dorresteijn AW, Wittbrodt J: *Conservation of a simple photosensitive system in Bilateria, and evolution of vertebrate eyes*; in revision

Del Bene F, **Tessmar-Raible K**, Wittbrodt J. (2004): *Direct interaction of geminin and Six3 in eye development*. Nature 427(6976):745-749.

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Hobert O, **Tessmar** K and Ruvkun G. (1999); *The Caenorhabditis elegans lim-6 LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons*. Development 126 (7), 1547- 1562.

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