Development of pigment-cup eyes in the polychaete *Platynereis dumerilii* and evolutionary conservation of larval eyes in Bilateria

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SUMMARY

The role of *Pax6* in eye development in insects and vertebrates supports the view that their eyes evolved from simple pigment-cup ocelli present in their last common ancestors (Urbilateria). The cerebral eyes in errant polychaetes represent prototype invertebrate pigment-cup ocelli and thus resemble the presumed ancestral eyes. We have analysed expression of conserved eye specification genes in the early development of larval and adult pigment-cup eyes in *Platynereis dumerilii* (Polychaeta, Annelida, Lophotrochozoa). Both larval and adult eyes form in close vicinity of the optic anlagen on both sides of the developing brain ganglia. While *pax6* is expressed in the larval, but not in the developing, adult eyes, expression of *six1/2* from trochophora stages onwards specifically outlines the optic anlagen and thus covers both the developing larval and adult eyes. Using *Platynereis rhabdemic opsin* as differentiation marker, we show that the first pair of adult eye photoreceptor cells is detected within bilateral clusters that transitorily express *ath*, the *Platynereis atonal* orthologue, thus resembling proneural sensory clusters. Our data indicate that – similar to insects, but different from the vertebrates – polychaete *six1/2* expression outlines the entire visual system from early developmental stages onwards and *ath*-positive clusters generate the first photoreceptor cells to appear. We propose that *pax6*-*, six1/2*- and *ath*-positive larval eyes, as found in today’s trochophora, were present already in Urbilateria.

Key words: *Platynereis*, Eye, Evolution, Larval eyes, Adult eyes, *six*, *pax6*, Lophotrochozoa, Annelids

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INTRODUCTION

The finding that *eyeless/pax6* and *sine oculis/six* transcription factors play important roles in eye development in insects and vertebrates (Cheyette et al., 1994; Chow et al., 1999; Halder et al., 1995α; Hill et al., 1991; Loosli et al., 1999; Quiring et al., 1994; Zubler et al., 1999) has initiated a heated debate about homology of eyes in Bilateria (Arendt and Wittbrodt, 2001; Gehring and Ikeo, 1999; Pichaud et al., 2001). All bilaterian eyes should trace back to a common, *pax6*-dependent precursor resembling the ‘prototype eye’ of Charles Darwin (Gehring and Ikeo, 1999). Still, the notion of eye homology across the Protostomia and Deuterostomia split is disputed from the morphological viewpoint (Nilsson, 1996; Salvini-Plawen and Mayr, 1977). To advance on the controversial issue of eye homology, molecular studies on diverse eye types have been initiated in Bilateria other than insects and vertebrates. In Protostomia, these involve the lens eyes in cephalopods (Tomarev et al., 1997), and the pigment-cup-eyes in nemertines (Loosli et al., 1996) and in planarians (Callaerts et al., 1999). In Deuterostomia, the simple pigment-cup eyes in amphioxus (Glardon et al., 1998) and in ascidians (Glardon et al., 1997) have been studied. A caveat for comparison, however, is that all eyes investigated so far in Protostomia are either highly derived (squid lens eyes and insect compound eyes) or developmental stages are not easily accessible (nemertines and planarians). We have therefore investigated eye development in the marine polychaete *Platynereis dumerilii*, which has been chosen for its ancestrality and central position in Lophotrochozoa, and is easy to keep in breeding culture (Arendt et al., 2001; Dorresteijn et al., 1993). This species forms one pair of larval, and two pairs of adult pigment-cup eyes, and all stages of embryonic and larval development can be easily studied.

The paired larval eyespots of the *Platynereis* trochophora larva are composed of only one pigment cell and one photoreceptor cell (Fig. 1A) (Rhode, 1992), and thus match the bilaterian prototype two-celled eye (Gehring and Ikeo, 1999). They are referred to as inverse, because the photoreceptor, the rhabdome, is oriented towards the concavity of the pigment cell. Similar larval eyes are found in the primary ciliary larvae of sipunculan worms, flatworms, molluscs and acon worms. Although structurally divergent to some extent, all larval eyes form at comparable positions left and right of the apical organ, and their widespread distribution in Bilateria makes them good candidates for interphyletic homology (Arendt and Wittbrodt,
The pairs of Platynereis adult pigment-cup eyes show a very characteristic structure with photoreceptorial cell processes traversing the pigment cell layer (Fig. 1B,C), shared with adult eyes in other carnivorous polychaetes, various molluscs, sipunculans, and onychophorans (Eakin and Westfall, 1965; Hermans and Eakin, 1974; Salvini-Plawen and Mayr, 1977). Deviating from the larval eyes, they are referred to as averse, because the rhabdomeric photoreceptors are oriented away from the concavity of pigment. Platynereis adult eyes represent a second separate type of eye that is distinct from the larval eyes that might equally be phylogenetically conserved, at least among Protostomia (Arendt and Wittbrodt, 2001).

We have studied four eye specification genes conserved in evolution with respect to the development of Platynereis larval and adult eyes. *pax6* orthologues are essential for eye formation in vertebrates (Chow et al., 1999; Hill et al., 1991; Walther and Gruss, 1991) and *Drosophila* (Halder et al., 1995a; Quiring et al., 1994). This has led to the hypothesis that *pax6* has an evolutionary conserved function in eye development (master control gene hypothesis) (Gehring and Ikeo, 1999; Halder et al., 1995a; Quiring et al., 1994). *pax6* orthologues are expressed in the developing lens eye of the squid (Tomarev et al., 1997), in the ocelli of regenerating nemertines (Loosli et al., 1996; Tarpin et al., 1999), in the frontal organ of amphioxus (Glardon et al., 1998), and in the ascidian ocellus (Glardon et al., 1997). Genes belonging to the *six1/six2* family of transcription factors are equally involved in eye development across phyletic boundaries. In *Drosophila*, *sine oculis* is expressed in, and required for, the formation of the entire visual system, comprising optic lobes, larval eyes (Bolwig organ), and developing lateral compound eyes and ocelli (Chayette et al., 1994; Seimiya and Gehring, 2000). In planarians, *six1/2/sine oculis* is essential for the regenerating eyes (Pinedo et al., 2000). The vertebrate *sine oculis* orthologues *six1* and *six2* are detected in differentiated ganglion cells of the retina in mouse (Kawakami et al., 1996; Oliver et al., 1995), and in Xenopus (Ghanbari et al., 2001). Orthologues of the bHLH transcription factor *atonal* are involved in cell type specification in the differentiating insect and vertebrate eyes. In the *Drosophila* eye disc, selection of R8 photoreceptor cells requires transient expression of *atonal* in a cluster of competent, ‘proneural’ cells (Jarman et al., 1994). Remarkably, *atonal* is not expressed in the R1-R7 photoreceptor precursors. It is required for their formation, however, because R1-R7 are induced by R8 (Jarman et al., 1994). *Atonal* is also indispensable for the formation of the larval Bolwig organ, where it is again active only in the first, but not in the secondary photoreceptor precursors (Daniel et al., 1999). Formation of the *Drosophila* ocelli also requires *atonal* function (Jarman et al., 1994). In mouse, frog, and fish, transitory expression of *ath5* precedes, and is required for, the determination of ganglion cells (Brown et al., 1998; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Wang et al., 2001). Finally, the expression of *opsin* related genes is initiated in the differentiating photoreceptor cells. The opsin promoter is a direct downstream target of *pax6* in *Drosophila* (Papatsenko et al., 2001; Sheng et al., 1997). In Bilateria two distinct opsins can be distinguished: rhabdomeric opsin (*r-opsin*) that is expressed in invertebrate rhabdomeric receptor cells, and ciliary opsin (*c-opsin*) that is expressed in the vertebrate ciliary photoreceptor cells (Arendt and Wittbrodt, 2001).

We have isolated *pax6*, *six1/2*, *ath* and *r-opsin* orthologues from *Platynereis dumerilii* and investigated their expression in the developing eyes. Our study reveals distinct molecular identities for larval and adult eyes. While the developing larval eyes co-express *six1/2* and *pax6*, the developing adult eyes express *six1/2* only. Both the larval photoreceptors and the first differentiating photoreceptors of adult eyes emerge from cell clusters positive for *ath*. Our data reveal that the early, and common, expression of *pax6*, *six1/2* and *ath* transcription factors, as found in the Platynereis larval eye anlage is a shared feature across Protostomia, and this corroborates the notion that two-celled larval eyes, as found in the polychaete trophochorda, were the evolutionary precursors for at least a subset of cerebral eyes in Bilateria (Arendt and Wittbrodt, 2001; Callaerts et al., 1997; Gehring and Ikeo, 1999; Halder et al., 1995b).

### MATERIALS AND METHODS

#### Worm breeding culture

Embryonic, larval and developing adult stages were obtained from an established *Platynereis* breeding culture at the EMBL Heidelberg, following the protocol of (Dorrestein et al., 1993).

#### Cloning of partial and full-length cDNAs

We isolated fragments of *Platynereis pax6, six1/2, ath* and *r-opsin* genes by nested PCR after reverse transcription of embryonic mRNA (48 hours). Degenerate primers for *pax6* (forward, GIGGIGTITTTYGTAAYGG; nested forward, TIGGIGMTATYAY-GARACIGG; reverse, GCRAANACRTNGGRTARTG; nested reverse, NGCYTNGNARRCTDATYTYT) were used for PCR: 5x(1 minute at 94°C, 2 minutes at 43°C, 4 minutes 72°C) then 35x(1 minute at 94°C, 2 minutes at 48°C, 4 minutes 72°C) followed by 10 minutes at 72°C. Additional degenerate primers to detect potential paralogues (forward, GGCAYWISGNGTIAAYCA; nested forward, GGIGGIGTITTTYGTAAYGG; reverse, ARNARCKT-TCNCKDATYTTTCCA; nested reverse, TCNCKDATYTTCC-ANGCRAA) were used under similar PCR conditions. Degenerate primers for *six1/2* (forward, CCNWSITTYGNTYYACNACG; nested forward, ARGTNCGNTTGYITTTYGARGRT; reverse KNGNSWNGGRTANOARTRTG) were used for nested reverse AARCARTAISWIGTYCTYCICCCRTC) were used for PCR: 5x(1 minute at 94°C, 2 minutes at 42°C, 4 minutes at 72°C) then 35x(1 minute at 94°C, 2 minutes at 47°C, 4 minutes at 72°C) followed by 10 minutes at 72°C. Degenerate primers for *ath* (forward, ACNAAYGTTCTCATYACR; reverse, ATNCAYCTI-KRW A YTITGSTG) were used at 5

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CACCTGCAAAAGAAGCTCGGTCC; nested forward, GTCC- 
TAAAAGCCAAGGTCTGTAG; reverse, CCCCC- 
GTCAGGCTCGGTCTA; nested reverse, TGGTTGCTCCCTT- 
CATAC), r-opsin: forward, TCCCTGAAGGA TTCCAGACA TCTTG; 
nested forward, AACTACACCTACGTCCTCGGCA TGT; reverse, 
TCCCAAGGAC- 
GGCATAGGTGT) and plasmid specific primers (T7, T3) for 30 
seconds at 94°C, 1 minute at 60°C and 4 minutes at 72°C. Identity of 
the clones was confirmed by sequencing [EMBL Nucleotide Sequence 
Database (Accession Numbers: 
pax6, AJ316541; six1/2, AJ316542; 
ath, AJ316543; r-opsin, AJ316544)].

Alignment and construction of phylogenetic trees
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), PD (paired domain) and 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).

Whole-mount in situ hybridisation
Embryos were fixed in 4% paraformaldehyde/2× phosphate-buffered 
saline (PBS)-Tween (PFA/PTw) for 1 to 4 hours. An established in 
situ hybridisation protocol (Loosli et al., 1998) was followed with the 
modification of ProteinaseK treatment in 100 µg/ml for 4 minutes (24 hour 
larvae), or 10 minutes (72 hour young 
worm). After staining, embryos were 
refixed in (PFA/PTw), washed and 
cleared in 80% glycerol. Embryos 
were mounted in glycerol and pictures taken under Nomarski optics 
using a Zeiss Axiophot.

Immunostaining for acetylated tubulin
A commercially available MoAb to acetylated tubulin, clone no. 6- 
11B-1(SigmaT6793), was used that detects an interphyletically 
conserved epitope present in cilia and axons. Embryos and larvae were 
fixed as above, dehydrated in methanol, rehydrated in methanol/PTw, 
or taken from the postfixation solution after the in situ hybridisation 
procedure, and blocked for 2 hours in 1 ml 5% serum/PTw. Blocking 
solution was replaced by 150 µl monoclonal antibody (MoAb) to 
acetylated tubulin diluted 1:500 in serum/PTw, and incubated overnight at 4°C. Larvae were washed 6×10 minutes in PTW, and 
cubated for 2 hours at room temperature in sheep biotinylated Anti- 
Mouse IgG secondary Ab. After additional washes 6×10 minutes in 
PTw staining was performed using the Vectastain ABC Kit (Vector 
Laboratories). Post-staining treatment was done as described above.

RESULTS
Larval and adult eyes develop from the lateral optic 
anlagen
Larval eyes (Fig. 1A) are first visible at 22 hours of 
development as two faint spots of orange pigment in the larval 
episphere (Fig. 1D). Twenty-four hour trochophora larvae were 
immunostained with an anti-acetylated tubulin antibody (Fig. 
1F) to visualise connections of larval photoreceptor cell axons 
to the larval central nervous system [for a general description
of polychaete larval nervous systems see Heimler (Heimler, 1988)). From the larval eyes (white arrows in Fig. 1F), traceable axons extend in opposite directions, medially towards the apical organ to contribute later to the optic commissure, and peripherally to connect to the prototroch ring nerve (Fig. 1F). Most likely these are collaterals of a single photoreceptor cell axon. Larval photoreceptor axons thus contribute to two bilateral nerves interconnecting prototroch ring nerve, larval eyes and apical organ (larval eye nerve, len, in Fig. 1F). Note that in the 2-day-old metatrochophora larva the episphere larval nervous system has been skewed medially, owing to the disproportionate growth of the ventral-peripheral anlagen of the palpae (plp in Fig. 1G).

The developing adult eyes (Fig. 1B) are morphologically visible at 53 hours of development, by the orange shading pigment in the first photoreceptor cell to form (not shown). Slightly later, at 60 hours, adult eyes consist of two
photoreceptor cells and two pigment cells (Fig. 1E) (Rhode, 1992). Notably, adult eye photoreceptor cells form only about three cell diameters dorsal from, and thus in close vicinity to, the larval eyes. This suggests that larval and adult eyes could trace back to common eye precursors (see below). Additional adult photoreceptor cells, pigment cells, and support cells are added continuously, and at 72 hours, the adult eye anlagen on each side have split into two (Rhode, 1992). The visible pigment in *Platynereis* has been isolated and characterised as a mixture of three pterin dimers with autofluorescent activity (Viscontini et al., 1970). Axons that emerge from adult eye photoreceptor cells connect to the axonal scaffold at the level of the dorsal brain commissure, the optic commissure (oc in Fig. 1G,H).

Episphere serial sections of the 72 hour episphere show that both larval and adult eyes form part of lateral cell masses that separate from the medial developing brain by layers of connective tissue (data not shown, and Fig. 6D), and that connect to the brain via the optic nerves (Fig. 1H). Given the continuous growth and the later large size of the adult eyes, it is likely that most cells of these masses will contribute to the developing adult eyes. We refer to the lateral masses as optic anlagen. They do not include the anlagen of the antennae or of the palpae.

Between 72 hours and 96 hours of development, the larval eyes and the adjacent ventral palpae move ventrally and medially, while the two pairs of developing adult eyes remain situated dorsolaterally (data not shown).

**Cloning of pax6, six1/2, ath and r-opsin orthologues in Platynereis**

A *Platynereis* pax6 fragment of about 600 bp length spanning the paired box and the homeobox was isolated by low-stringency RT-PCR using degenerate primers derived from conserved regions in the paired domain and in the homeodomain. This was used to screen a *Platynereis dumerilii* 24 hour cDNA library (C. Heimann, unpublished), yielding seven pax6 clones. Two library clones of 4.3 kb (pax6-17) and of 4.1 kb span the entire pax6 ORF. An extensive PCR search for possible additional pax6 genes involved four pairs of degenerate upper and lower primers in all possible combinations. Eighty clones were picked after low-stringency colony hybridisation and 28 were sequenced, all of them representing the same, single *Platynereis* pax6 gene. In line with this, Southern blot hybridisation of genomic DNA with a *Platynereis* pax6 fragment yielded single bands under moderate stringency conditions (data not shown). An alignment with other bilaterian Pax6 proteins (Fig. 2A), as well as the construction of a phylogenetic tree involving bootstrap analysis (Fig. 3A) reveals that the *Platynereis* pax6 gene obtained clearly clusters within the Pax6 family. It is most closely related to the pax6 genes of nemertine and squid, two other representatives of the Lophotrochozoa.

Several overlapping *Platynereis* six1/2 fragments in the range from 300 bp to 450 bp, encompassing parts of the Six-domain and of the homeodomain, were obtained by low-stringency PCR with degenerate primers. A full-length clone of 1.4 kb was then obtained by vector-anchored PCR from a 48 hour cDNA library (C. Heimann, unpublished). Southern blot hybridisation indicated the presence of a single six1/2 gene in the *Platynereis* genome (data not shown). According to the alignment (Fig. 2B) and the phylogenetic tree (Fig. 3B), *Platynereis* six1/2 is a firm member of the six1/six2/sine oculis subfamily of Six transcription factors. Notably, it is less diverged in sequence from *Drosophila* sine oculis and mouse six1 and six2 than *Dugesia* sine oculis, another Lophotrochozoan member of the group.

![Fig. 4. Platynereis pax6, Pd-six1/2 and Pd-atonal in larval eye development. Apical views of whole-mount in situ stained embryos showing the pattern pax6 (A,D,G,K), six1/2 (B,E,H,L) and atonal (ath) (C,F,I,M) expression at 15 hours (A-C), 19 hours (D-F), 24 hours (G-I) and 36 hours of development (K-M). In 36-hour-old larvae (K-M), the axonal scaffold was counterstained with anti-acetylated tubulin antibody. Developing or mature larval eyes are indicated by white arrows. Black arrowheads point at isolated dorsal cells constantly expressing pax6.](image-url)
A Platynereis ath fragment of about 150 bp spanning the basic helix-loop-helix domain was isolated using low-stringency PCR with degenerated primers. Two longer fragments of 765 bp, including the entire N-terminus and basic helix-loop-helix domain were obtained by vector-anchored PCR from a cDNA library. Both clones have identical protein sequences. Alignment (Fig. 2C) and bootstrap analysis (Fig. 3C) revealed that Platynereis ath is an atonal/ath1/5 orthologue.

Low-stringency PCR with degenerate primers yielded a 450 bp r-opsin fragment. A full-length clone of 1.4 kb was then obtained by vector-anchored PCR from a cDNA library. It contains an open reading frame of 1149 bp, starting from the first ATG and encoding 383 amino acids. In Fig. 2D, the deduced amino acid sequence is aligned with other invertebrate r-opsins. Bootstrap analysis reveals that the Platynereis r-opsin belongs to the subfamily of invertebrate r-opsins (Fig. 3D). Molecules belonging to this subfamily are active in rhabdomeric photoreceptors. The conserved series of amino acid residues between transmembrane segments V and VI which is required for binding and activation of G-protein (Fig. 2D) equals its counterparts in other Lophotrochozoan and Arthropod opsins, but not in vertebrate c-opsins (Arendt and Wittbrodt, 2001). This indicates that Platynereis opsin interacts with the Gq-α subunit canonical for invertebrate r-opsins.

**Larval eye precursors form at the intersect of the pax6 and six1/2 territories from ath-positive precursors**

Expression of pax6, six1/2 and atonal was analysed by whole-mount in situ hybridisation (Fig. 4). The Platynereis pax6 and six1/2 genes are expressed in the developing episphere, in bilateral patches of cells that laterally abut the protorhoch. These patches are detected already at the late embryonic stage (15 hours, Fig. 4A,B), and persist in the early larval stage (19 hours, Fig. 4D,E), in the mature trochophora larva (24 hours, Fig. 4G,H), in the late trophophora (36 hours, Fig. 4K,L; 43 hours, Fig. 5A,B; 48 hours, Fig. 5A,B), and well into the three-segmented young worm (data not shown). The pax6 staining is located in the ventral half of the episphere, and the six1/2 staining in the dorsal half of the episphere. In all stages examined, there is an overlap of expression in the lateral episphere. Two-colour co-staining of both pax6 and six1/2 transcripts with acetylated tubulin (which labels larval photoreceptor axons, see above) reveal that this overlap of expression covers the two-celled larval eyes at 24 hours (data not shown) and at 36 hours (arrows in Fig. 4K,L).

From embryonic (15 hours) to mature larval stages (24 hours), the patches of pax6 expression extend more dorsally than the larval eyes (white arrows in Fig. 4D). These more dorsally located pax6-expressing cells, by position, might correspond to adult eye precursors. At 36 hours of development, pax6 expression is lost in the more dorsal cells, so that the larval eyes now demarcate the dorsalmost extent of the pax6 expression domain (Fig. 4K). However, pax6 expression is maintained in a pair of isolated dorsal cells (arrowheads in Fig. 4A,D,G,K, and see below). Co-staining with acetylated tubulin revealed that these cells send out axons towards the optic commissure (Fig. 4K), identifying them as neurones. pax6-positive dorsal cells can also be detected at 43 hours (Fig. 5B), at 48 hours (Fig. 5A,D), and in the 72 hour developing young worm, where a group of pax6-positive cells is found at the very base of the differentiating adult eyes (data not shown). The Platynereis pax6 gene is also expressed along the developing central nervous system of the body segments, similar to insect and vertebrate pax6 genes (data not shown). Like pax6, also the six1/2 gene is expressed at additional sites in the segmented body regions, starting in the mature larva (out of focus in Fig. 4L, and data not shown).

Expression of the Platynereis ath gene in the developing episphere is very dynamic. This is in line with the proneural function assigned to the insect and vertebrate orthologues. In the 15 hour embryonic episphere, a single cell is stained that, by position, represents a precursor cell of the apical organ (Fig. 4C). By 19 hours of development, two dorsomedial cells, as well as lines of three cells along the apical organ, are ath-positive (Fig. 4F). In addition, there was strong staining of ath matching the pax6/six1/2 overlap at 19 hours of development (arrows in Fig. 4D-F). By the timepoint of their appearance and by position, these large cells transitorily positive for ath represent larval eye precursors. At 24 hours, when the larval eyes are fully differentiated, expression in the larval eye precursors, and in other cells, had disappeared (Fig. 4I). Starting at late larval stages (36 hours), another prominent site...
of ath expression emerged in the episphere, medial to the larval eyes along the larval eye axons (Fig. 4M). This staining demarcated bilateral clusters of cells that slightly later give rise to the first adult eye photoreceptor cells. Expression of the r-opsin gene was undetectable at embryonic and early larval stages. It was present in the larval eye photoreceptors in the mature larvae, though at low levels (data not shown).

A cluster of atonal-positive cells generates the first photoreceptor cell in the adult eye anlage

The next focus was on the molecular characterisation of adult eye development, to find out whether the same combination of pax6, six1/2 and ath expression would also define the adult eye anlagen. As adult eye photoreceptor cells are morphologically visible at 53 hours only, we used Platynereis r-opsin expression as a molecular marker to identify differentiating adult eye photoreceptors. The first, r-opsin-positive adult eye photoreceptor cells were detected at 43 hours of development. These two cells form three cell diameters right and left of the apical organ (Fig. 5D). This position matched the bilateral clusters of atonal-expressing cells that were detected at the same time point in larvae from the same batch (Fig. 5C), and that were already present along the larval eye axons at 36 hours of development (Fig. 4M and see above). This match in position was revealed by a comparison of cellular patterns in the medial episphere (compare Fig. 5C with 5D). For this, we took advantage of the fact that cellular outlines are visible under Nomarski optics, and that the 2-day-old brain anlage in the Platynereis episphere represents a clear, transparent epithelium with clear morphological landmarks such as the apical organ. A plausible explanation for the overlap in expression, which takes into account the functional data from other systems (Brown et al., 1998; Jarman et al., 1994; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Wang et al., 2001), is that the bilateral clusters of ath expression represent proneural clusters that generate the first photoreceptor cells of the adult eye. Remarkably, these clusters of ath-positive cells had already disappeared slightly later on, at 48 hours (data not shown), indicating that ath expression in photoreceptor precursors is very transient and precedes differentiation. As the cell lineage of adult eye photoreceptors is not yet known, we cannot exclude the possibility that additional photoreceptor precursors added later to the developing adult eyes also trace back to initially ath-positive precursors.

Developing adult eyes express six1/2, but not pax6

Interestingly, the ath-positive clusters were enclosed in the six1/2, but not in the pax6 expression territory (compare Fig. 5A,B,C). In addition, the comparison of cellular patterns revealed that the atonal-positive clusters did not match the isolated groups of cells that constantly express pax6 (arrowheads in Fig. 5B and Fig. 6A; detected as early as 15 hours); instead, these pax6-positive cells were located dorsally adjacent to them. Therefore, at 43 hours of development, adult eye photoreceptor precursor cells, and the first differentiating photoreceptors, express six1/2, but are devoid of pax6. However, it is possible that pax6 is expressed in adult eye precursor cells at earlier stages, given the more dorsal extension of the patches of pax6 expression at embryonic and larval stages (Fig. 4A,D,G, see above).

In the 2-day-old metatrochophora, four adult eye photoreceptor cells were detected using r-opsin as a marker (Fig. 6C). These cells are now located in a more peripheral, dorsolateral position, almost abutting the prototroch. They are enclosed within the six1/2 territory (Fig. 6B), but still do not express pax6 (Fig. 6A). This is also true for the latest stage examined, the 3-day-old developing young worm (data not shown).

Platynereis pax6 in larval eyes, chemosensory palps and antennae

It is evident that the two-celled larval eyes represent only a small subset of cells within the ventrolateral patches of pax6 expression. To determine the fate of the remainder of pax6-positive cells, we analysed pax6 expression in 72 hour developing young worms. At that stage, differentiation is well under way and facilitates identification of structures. In a ventroanterior view, three pax6-positive sensory organs and organ precursors can be identified (Fig. 7A). First, the larval eyes are still present and express pax6 (arrows in Fig. 7A; also visible in the optical cross section in Fig. 7B). Second, the majority of pax6-expressing cells ventral to the larval eyes and adjacent to the ventromedial gland field constitute the anlagen of the palps: two fields of mechano- and chemosensory receptor cells located right and left of the mouth that control food uptake (Hauenschild and Fischer, 1969). Third, the two medial cells on both sides of the apical organ represent the tip of the developing antennae (ant in Fig. 7A). The Platynereis antennae likewise host mechano- and chemosensory receptor cells (Hauenschild and Fischer, 1969). Expression of pax6 in head chemosensory organs has been described for nemertines (Loosli et al., 1996), for cephalopods (Tomarev et al., 1997) and for vertebrates (Grindley et al., 1995; Walther and Gruss, 1991), thus representing another recurrent theme in Bilateria.

DISCUSSION

Developing larval and adult polychaete eyes differ in the expression of pax6

In accordance with the well-established role of pax6 in eye development in Bilateria, our expression study also implicates pax6 in Platynereis eye development. In the two distinct types of eyes present in Platynereis, namely larval and adult eyes, pax6 is expressed in larval eye precursors and in the differentiated larval eyes, and possibly also in early adult eye precursors, although this has not been determined with certainty. However, we found that larval and adult eyes differed with respect to pax6 deployment at differentiation stages. While the gene was expressed in the larval eyes at all stages examined, it was not detected in the developing adult eyes from the onset of photoreceptor differentiation onwards, well into the three-segmented young worm stage. This is all the more remarkable, as the Platynereis adult eyes exhibit life-long growth (Hauenschild and Fischer, 1969), with hundreds of cells added to the initial few-celled primordia (Fischer and Brökelmann, 1966; Rhode, 1992). Our data preclude a direct role of pax6 in this process. More precisely, pax6 appears not to be involved in the transcriptional control of Platynereis adult eye differentiation, such as the activation of r-opsin or of any other photoreceptor or pigment cell-specific downstream
Eye development and evolution

We cannot completely rule out the existence of another pax6 paralogue that might exert this function, but we consider this rather unlikely given our extensive searches. pax6 is expressed, however, in a group of dorsal cells, located at the base of the developing adult eyes. By morphology and position, at least a subset of these cells might represent first order sensory interneurones of the visual system [where pax6 is also expressed in mouse (Stoykova et al., 1996)]. It will be interesting to determine whether, at earlier developmental stages, these pax6-positive cells exert an influence on the formation of the adjacent adult eyes.

Adult cerebral eyes that differentiate in the absence of pax6 also exist in Lophotrochozoans other than polychaetes. In the squid (Cephalopoda, Mollusca), pax6 expression covers the early eye anlagen, but is not detected in the differentiating retina (Tomarev et al., 1997). This would suggest an evolutionary relationship between polychaete and cephalopod adult eyes that also exhibit a very similar ultrastructure. Homology of the distinct eye types found in molluscs and polychaetes, however, is an unresolved issue (Arendt and Wittbrodt, 2001; Bartolomaeus, 1992).

From a comparative point of view, the question arises of whether the co-existence of distinct eye types that differentiate with and without pax6 also applies for other groups. Among Lophotrochozoans, separate types of cerebral eyes are present for example in Sipunculans (Salvini-Plawen and Mayr, 1977) that could well correspond to larval and adult eyes in polychaetes but have not yet been investigated at the molecular level. In Ecdysozoan insects, however, two distinct conserved eye types co-exist (Paulus, 1972; Paulus, 1979), the medial ocelli, and the lateral compound eyes [of which the Bolwig organs in Dipteran larvae are evolutionary derivatives (Daniel et al., 1999)]. Remarkably, neither of the Drosophila pax6 orthologues (eyeless and twin of eyeless) are expressed in the differentiating lateral compound eye or Bolwig organ photoreceptors (Czerny et al., 1999; Quiring et al., 1994). However, evolutionary relationships of insect and polychaete eye types are obscure (Arendt and Wittbrodt, 2001; Salvini-Plawen and Mayr, 1977) and more molecular comparative data (also for Drosophila ocelli) will be needed to advance on this issue.

**Fig. 6.** Adult eye development in 2-day-old metatrochophora larvae. Apical views at 48 hours of development. Expression of pax6 (A) six1/2 (B) and r-opsin (C) in the growing adult eye. Black arrowheads indicate isolated dorsal cells constantly expressing pax6. (D) Schematic drawing of gene expression patterns at 48 hours of development. Natural pigment (brown spots) serves as morphological landmark. Abbreviations: ae, adult eyes; le, larval eyes; oa, optic anlagen; oc, optic commissure; ant, antennae; plp, palpae anlagen.

**Fig. 7.** pax6 expression in the 72 hour episphere. Figure shows pax6 expression in 72 hour young worms in a series of optical sections from a ventroanterior view. (A) Surface view showing pax6 expression in larval eyes (white arrows), antennae (ant) and palpae (plp). (B) Deeper optical section with pax6 expression in larval eyes (le, white arrows) and anlagen of the palpae. (C) More dorsal optical section showing pax6 expression in antennae (ant), besides prominent expression in palpae.
It has been proposed that a direct role in photoreceptor cell differentiation should be ancestral for pax6 genes (Gehring and Ikeo, 1999; Pichaud et al., 2001; Sheng et al., 1997). Clearly, this role does not apply for Platynereis adult eyes, but it does apply for the larval eyes that prominently express pax6 at all stages examined. This supports ancestrality of polychaete larval eyes, and corroborates our notion that larval eyes are ancestral for Bilaterians. It will be important to analyse whether pax6 is also active in the development of larval eyes in other ciliated larvae, such as the mollusc, echiurid and sipunculan trochophora-type larvae, or the enteropneust tornaria.

**six1/2 expression defines the entire visual system – but only in Protostomia**

The survey of six1/2 expression in the developing Platynereis episphere from larval to adult stages (Figs 4-6) reveals a very specific and continuous expression that encompasses the developing larval and adult eyes, and that outlines the optic anlagen (Fig. 6D). Medially, six1/2 expression also extends into the optic commissure. The polychaete optic commissure is considered an optic associative neuropil with many synaptic endings, rather than a simple fibre tract (Bullock and Horridge, 1965). Accordingly, six1/2 expression defines the entire Platynereis visual system from early developmental stages onwards (Fig. 6D). In this respect Platynereis eye development resembles Drosophila eye development, where sine oculis/six1/2 is required from early embryonic stages onwards for the formation of the entire visual system (Chayette et al., 1994; Seimiya and Gehring, 2000). In planarians, sine oculis/six1/2 is also expressed early on in the regenerating eye (Pineda et al., 2000). These findings indicate that the role of six1/2 orthologues in early visual system specification could be evolutionarily ancient. The vertebrate six1/2 expression data, however, are at variance with this notion. Deviating from the situation in insects and polychaetes, the vertebrate six1 and six2 genes are not involved in early eye development, but are detected only in the late differentiating retina (Ghanbari et al., 2001; Kawakami et al., 1996). Therefore, early development of visual systems differs across Bilateria, in that six1/2 is active in Protostomia but not in the vertebrates. This is not the first case of ovet non-conservation in bilaterian early eye development, given that the *rx* (retina homeobox) gene has proven crucial for eye formation in the vertebrates (Loosli et al., 2001; Mathers et al., 1997; Mathers and Jamrich, 2000; Zhang et al., 2000) but not in insects (Egert et al., 1998) or in polychaetes (D. A. and J. W., unpublished). If it is not the early role of six1/2 in eye specification that is conserved across Bilateria, what about the later (shared) expression of six1/2 genes in differentiating cells of the developing eye?

**The ganglion cells of the vertebrate retina: evolutionary counterparts to invertebrate rhabdomeric photoreceptors?**

A common feature of six1/2 involvement in vertebrate and in invertebrate eye development is the cell type-specific expression in the developing eye at differentiation stages. In Drosophila (Serikaku and O’Tousa, 1994), planarians (Pineda et al., 2000) and Platynereis (this study), the six1/2-positive cell types are rhabdomeric photoreceptor cells and pigment cells, while in the vertebrates, six2 shows a conserved expression in pigment cells and ganglion cells in the late differentiating eyecup – not, however, in the ciliary photoreceptor cells (Ghanbari et al., 2001; Kawakami et al., 1996). Apart from the possible conservation of expression in pigment cells, this comparison indicates that invertebrate rhabdomeric receptor cells and vertebrate ganglion cells may be evolutionarily related. In line with this, there are additional characteristics that are specifically shared between the two cell types, and that might indicate common descent. First, at the morphological level, both send out their axons towards the optic centres of the brain. Second, invertebrate rhabdomeric photoreceptor cells emerge from atonal/ath-positive precursors, in insects (Daniel et al., 1999; Jarman et al., 1994) and in polychaetes (this study), and vertebrate ganglion cells emerge from ath5-positive precursors in mouse, frog and fish (Brown et al., 1998; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Wang et al., 2001). Notably, vertebrate ciliary photoreceptors do not express ath5 (Marquardt et al., 2001). A third similarity of rhabdomeric photoreceptor cells and ganglion cells is that they express orthologous r-opsin molecules (Arendt and Wittbrodt, 2001): All invertebrate photoreceptors so far examined (including those of Platynereis, this study), employ r-opsin molecules for photodetection. A vertebrate r-opsin orthologue has recently been identified, called melanopsin. It shows restricted expression in retinal ganglion cells but not in the ciliary photoreceptors (Provenco et al., 1998; Provenco et al., 2000).

Is there a plausible explanation for these resemblances in terms of eye evolution? We have recently hypothesised that primary ciliary larvae with ‘rhabdomeric’ eyespots (as found, for example, in today’s trochophora and tornaria larvae) were present also in chordate ancestors (Arendt and Wittbrodt, 2001). Chordate descendants then lost the primary larvae, but might have inherited the larval eyes, in a way that today’s ganglion cells are remnants of the ancestral rhabdomeric photoreceptors. They were then complemented by a population of ciliary photoreceptor cells.

**Reconstructing the eyes in Urbilateria**

Gehring and Ikeo (Gehring and Ikeo, 1999) have recently proposed that bilaterian eyes trace back to rather simple two-celled precursors. Based on a comparative survey of eye positions and morphologies, and of phototransductive cascades, we have further outlined that these precursors might have been a pair of larval eyes present in the ciliated larvae of Urbilateria, with rhabdomeric photoreceptors employing r-opsin (Arendt and Wittbrodt, 2001). This study implies that, besides pax6, six1/2 and ath were also involved in the development of such larval eyes – as observed in the larval eyes of today’s trochophora.

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