The exorhodopsin and melanopsin systems in the pineal complex and brain at early developmental stages of Atlantic halibut (*Hippoglossus hippoglossus*)

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Abstract

The complexity of the non-visual photoreception systems in teleosts has just started to emerge with co-localization of multiple photoreceptor types with unresolved functions. Here we describe an intricate expression pattern of melanopsins in early life stages of the marine flat fish Atlantic halibut (*Hippoglossus hippoglossus*), a period when the un-pigmented brain is directly exposed to environmental photons. We show a refined and extensive expression of melanopsins in the halibut brain already at the time of hatching, long before the eyes are functional. We detect melanopsin in the habenula, suprachiasmatic nucleus, dorsal thalamus and lateral tubular nucleus of first feeding larvae, suggesting conserved functions of the melanopsins in marine teleosts. The complex expression of melanopsins already at larval stages indicates the importance of non-visual photoreception early in development. Most striking, we detect expression of both exorhodopsin and melanopsin in the pineal complex of halibut larvae. Double fluorescent labeling showed that two clusters of melanopsin positive cells are located lateral to the central rosette of exorhodopsin positive cells. The localization of different photopigments in the pineal complex suggests that two parallel photoreceptor systems may be active. Furthermore, the dispersed melanopsin positive cells in the spinal cord of halibut larvae at the time of hatching may be primary sensory cells or interneurons representing the first example of dispersed high-order photoreceptor cells. The appearance of non-visual opsins early the in development of halibut provides an alternative model to study the evolution and functional significance of non-visual opsins.

Introduction

Light has an important impact on life. Almost all animals are dependent on light to modulate their behavior and physiology and as the intensity of light alters with the solar cycle the animals have to adapt to the photic changes. The vertebrate eye is responsible for the image-forming vision and the retina can detect spatial and spectral differences of the light. In addition non-visual photoreception supplies animals with measurements of irradiance and non-directional photoreception. While mammals are dependent on ocular photoreception to adapt to changes in the ambient light, photoreceptors in non-mammalian vertebrates have been detected in a wide range of tissues including dermal melanophores, pineal organ and deep brain cells (reviewed in Peirson et al., 2009; Davies et al., 2010). In contrast to mammals that are dependent on photic information from the retinal ganglion cells (Berson et al., 2002), photoreceptors are present in the pineal organ of non-mammalian vertebrates, which regulate the melatonin synthesis important for photoentrainment of the circadian rhythm directly (reviewed in Korf, 1994). The existence of additional photoreceptive capacity in the teleost brain was early indicated in studies of blinded and pinealectomized European minnows, where skin pigmentation altered in response to light stimulation of the head (Frisch 1911). These deep brain photoreceptors have received little attention in the literature, although a few studies in teleosts have indicated an involvement in behavioral responses to light (van Veen et al., 1976; Tabata et al., 1989; Fernandes et al., 2012) and in birds where hypothalamic photoreceptor cells are thought to be responsible for photoperiodic responses (Halford et al., 2009; Davies et al., 2012). Numerous photoreceptive cell types and their photosensitive pigments have been identified over the last decades, but how these cells contribute to the behavioral and physiological responses to light is just emerging. In the Atlantic halibut the hatching mechanism is known to be regulated by light (Helvik and Walther, 1992). This physiological process takes place long before the retina is differentiated (Kvenseth et al., 1996). Hence, in this study we take the advantage of Atlantic halibut in order to study non-visual photoreception at early stages of teleost development.

The non-visual opsin melanopsin was originally identified in dermal melanophores of *Xenopus laevis* (Provencio et al., 1998) and since been shown to have a significant role in photoentrainment of the circadian rhythm in mammals (Provencio et al., 2000; Gooley et al.,

2001; Panda et al., 2002; Hattar et al., 2003). In non-mammalian vertebrates two different melanopsin classes have been identified, the mammalian-like (*opn4m*) and the *Xenopus*-like (*opn4x*) (Bellingham et al., 2006). In teleosts melanopsins are expressed both in the eye and the brain. In zebrafish expression of melanopsin has been detected in all retinal layers of adult eyes (Davies et al., 2011) and melanopsin is detected in discrete regions of the zebrafish brain already prior to retinogenesis (Matos-Cruz et al., 2011). In the brain of juvenile Atlantic cod expression of melanopsin is detected in the habenula and suprachiasmatic nucleus (Drivenes et al., 2003). Current studies in Atlantic salmon demonstrate expression of different melanopsin genes in multiple regions of the brain and cell populations that are located in regions associated with different neuroendocrine systems (Sandbakken et al., 2012). Studies in teleosts have located different melanopsins in the same retinal layers (Davies et al., 2011) and melanopsin is also suggested to be co-localized with vertebrate ancient opsin in the horizontal-cell layer (Jenkins et al., 2003; Cheng et al., 2009) and in cells resembling amacrine cells of the inner nuclear layer (Sandbakken et al., 2012). In the Atlantic salmon brain co-localization of melanopsin and vertebrate ancient opsin has been observed in the left habenula and in the dorsal thalamus (Sandbakken et al., 2012). The non-visual opsin exorhodopsin has been shown be a pineal-specific opsin expressed in the pineal organ from early in development of teleosts, and has been indicated to have a role in the circadian rhythm (Pierce et al., 2008). By using fluorescent double labeling techniques, the present study reveals how melanopsin is expressed in close connection to the pineal-specific exorhodopsin at the early developmental stages of Atlantic halibut.

Halibut eggs hatch at an early developmental stage and the hatched larvae have a big yolk sac and a primitive larval body (Lönning et al., 1982; Haug, 1990). In halibut the pineal has been indicated to be important in perceiving and mediating photic information in the dark-dependent hatching mechanism. It has been suggested that the pineal may influence the time of hatching, as the pineal contain molecules involved in the phototransduction cascade already before hatching (Forsell et al., 1997). Recent studies in zebrafish have however indicated other regions of the brain to be important for light-dependent physiological processes. In unhatched pre-retinal zebrafish, photoreceptors in the hindbrain were shown to be responsible for a “photomotor response” after exposure to intense light (Kokel et al., 2013). In addition, zebrafish larvae lacking eyes and pineal organs demonstrate a light-seeking behavior triggered by loss of illumination,

and melanopsin expressing cells in the preoptic area were found to regulate this behavior (Fernandes et al., 2012).

To further investigate the early embryonic and pre-retinal light responses in fish we have characterized melanopsin and exorhodopsin non-visual systems in Atlantic halibut. Halibut have a more than a month-long early life history prior to a functional retina and during this period a complex expression pattern of the melanopsins is detected, coincident with the light-regulated hatching mechanism. We find expression of melanopsin in various brain regions and an extensive expression of exorhodopsin in the pineal organ. By fluorescent double labeling techniques the relative distribution of a mammalian-like melanopsin and exorhodopsin is shown in the pineal region. In addition this study evaluates the ontogeny of the melanopsin expressing photoreceptor cells at early stages (pre-retinal) in relation to the melanopsin expression in the brain and retina of halibut larvae with functional eyes.

Materials and methods

Animals

Eggs, larvae and juvenile fishes of Atlantic halibut (*Hippoglossus hippoglossus*) were obtained from the Institute of Marine Research, Austevoll Aquaculture Station, Norway. All experiments described follow the local animal care guidelines and were given ethical approval by the Norwegian Veterinary Authorities.

Molecular cloning

Total RNA was isolated from the retina and brain of juvenile Atlantic halibut (*Hippoglossus hippoglossus*) by Trizol reagent (Life Technologies, Bethesda, MD). Purification of Poly A^+ mRNA was performed with Oligotex Resin (Qiagen, Germany) and preparation of double stranded cDNA and adaptor-ligated cDNA were done using Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA).

Isolation of the halibut mammalian-like melanopsins (*opn4m1* and *opn4m3*) was performed with a nested approach with degenerative primers described in Sandbakken et al., (2012). For the first

round of PCR the annealing temperature was 52°C and 30 cycles were used. PCR product from first round of PCR served as a template for the second round of PCR with annealing temperature 50°C and 30 cycles. The halibut *Xenopus*-like melanopsin (*opn4x2*) was identified by using opsin specific degenerative primers described in Helvik et al., (2001) and then melanopsin specific degenerative primers with a nested approach. Annealing temperature for the first round of PCR was 46° C and 30 cycles were used and the nested PCR had an annealing temperature of 46°C and 35 cycles. Generation of full-length sequence for *opn4m1*, *opn4m3* and *opnx2* were obtained by 5' and 3'RACE (Rapid Amplification of cDNA Ends) nested PCR. The reactions were performed according to the recommendations (Clon-Tech, Palo Alto, CA) by touchdown PCR (Primers are listed in Table 1). To verify the assembly of the RACE products, a PCR with primer binding sites located in the predicted the 5' and 3' UTRs was done. All PCR products were extracted from agarose gel using QIAEX II Gel Extraction Kit (Qiagen, Germany) or MinElute®Gel Extraction Kit (Qiagen, Germany) before cloning into StrataClone PCR Cloning vector pSC-A-amp/kan (Agilent Technologies, LA Jolla, CA) or pGEMT®-Easy Vector (Promega, Madison, WI) and sequencing at the University of Bergen Sequencing Facility.

Recently, the halibut genome was sequenced on an Illumina HiSeq200 (Illumina, San Diego, CA) (Pair End, 100bp reads) to 100x coverage and a contig assembly was made with the CLC software (CLC bio, Denmark). O*pn4m1*, *opn4m3* and *opn4x2*, identified by degenerative PCR and RACE PCR, were verified by searching the genome with BLASTN (NCBI, Bethesda, MD, RRID:nlx_153932) and TBLASTN (NCBI, Bethesda, MD, RRID:OMICS_00999). In addition the genome was searched by TBLASTN using available teleost protein opsin sequences as query in order to obtain more halibut opsin genes, and a second *Xenopus*-like melanopsin (*opn4x1*), a remnant of a melanopsin gene and exorhodopsin (*exorh*) were found. Putative opsin genes were predicted based on the BLAST alignments and GENSCAN (Burge and Karlin, 1997, RRID: nif-0000-30609), and the annotation was based on BLASTX (NCBI, Bethesda, MD, RRID: nlx_153933) against GenBank (NCBI, Bethesda, MD, RRID: nif-0000-02873) and phylogenetic analysis. Verification of the predicted opsins were done by PCR using primers with binding sites in the 5' and 3' UTRs and cloning and sequencing were done as described for the other melanopsins.

Sequence and phylogenetic analyses

Analysis and assembly of the sequences were done using the Vector NTI9 software (Invitrogen, Carlsbad, CA) and primer design was performed by ApE-A plasmid Editor v2.0.36. ClustalX 2.1 (Larkin et al., 2007) was used to align the amino acid sequences of melanopsins. Phylogenetic analysis was carried out by constructing a maximum likelihood tree using MEGA version 5 (Tamura et al., 2011, RRID: nlx_156838) and 1000 bootstrap replicates were applied to ensure the statistical robustness of each node. The four melanopsins identified in Atlantic halibut were named according to the nomenclature in Bellingham et al., (2006) and Davies et al., (2011).

Riboprobes

Digoxigenin (DIG)-labeled and/or fluorescein-labeled riboprobes for the four halibut melanopsins and exorhodopsin were made following the manufacturer's instructions (Roche Diagnostics, Germany). In the synthesis of the riboprobes PCR product was used as template for the reaction as described in Thisse and Thisse, (2008) and the synthesized probes were precipitated by LiCl and EtOH together with tRNA (Roche Diagnostics, Germany). Sequence alignment shows that the similarity between the sequence targets of the melanopsin probes does not exceed 75%.

***In situ* hybridization on whole embryos and larvae**

Embryos and larvae of different developmental stages of Atlantic halibut were fixated in 4% paraformaldehyde-buffred PBS (pH 7.4) for 48 h at 4°C. After a brief wash in 1xPBS the whole embryos and larvae were dehydrated in methanol and stored at -20°C in 100% methanol until use.

Whole mount *in situ* hybridization was started by rehydrating the embryos and larvae in methanol (75-25%) and then rinsing them for 2x5 min in 1xPBS pH 7.4. Embryos were dechorinated and the yolk was removed. Larvae with pigmentation were bleached in 3% H_2O_2/0,5% KOH as described in Thisse and Thisse, (2008) and the bleaching was stopped by first washing 5 min in 1xPBS and then 4x5 min in 1xPBST (0,1% Tween20 (Sigma, St. Louis, MO), in 1xPBS). The tissue was permeabilised with proteinase K (Promega, Madison, WI) treatment (10 µl/ml in 0.1 M Tris-HCl pH 8.0 and 50 mM EDTA) and the time of treatment was optimalized for the size of

the embryos and larvae. After a rinsing step with 1xPBST the embryos and larvae were fixated in 4% paraformaldehyde-buffred PBS (pH 7.4) and then washed 4x5 min in 1xPBST. Pre-hybridization was carried out at 65°C for 2 h in hybridization solution without probe before incubation with hybridization solution with probe overnight at 65°C. The hybridization solution was composed of of: 10 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 mM EDTA, 0.2% Tween20, 1% Blocking reagent (Roche Diagnostics, Germany), 10% dextransulphate (Sigma, St. Louis, MO) and 50% formamide (Sigma-Aldrich, St. Louis, MO). After hybridization washing series of 2x15 min in 50% formamide (VWR, West Chester, PA) in 2xSSCT (300 mM NaCl, 30 mM $C_6H_5Na_3O_7 \times 2H_2O$, 0,2% Tween (Sigma, St. Louis, MO)), 1x30 min in 2xSSCT, 2x15 min in 2xSSCT and 2x15 min in 0.2xSSCT were performed at 65°C. To remove unhybridized probe the embryos and larvae were treated with RNase A (0.02 mg/ml) (Sigma, St. Louis, MO) for 20 min at 37°C before washing with RNase buffer (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA) for 20 min at 65°C. The embryos and larvae were incubated in 2xSSC, 0.05% TritonX-100 (Sigma, St. Louis, MO) and 2% Blocking reagent for 2-3 h before overnight incubation with Anti-Digoxigenin-Alkaline phosphatase, Fab fragments (1:2000) (Cat. No 11093274910, Roche Diagnostics, Germany, RRID:AB_514497) in 2xSSC, 1% Blocking reagent and 0.3% TritonX-100. To remove redundant antibody the embryos and larvae were washed 4x20 min in 1xPBST and 2x10 min in visualization buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Visualization was done by incubation in darkness with freshly made chromogen substrate 45 µl 4-Nitro blue tetrazolium chloride (Roche Diagnostics, Germany) and 35 µl 5-Bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics, Germany) in 10 ml visualization buffer. Sense probes were used as a control for unspecific DIG probe labelling. Visualization was stopped by washing in stop solution (10 mM Tris-HCl pH 7.5, 1 mM EDTA and 150 mM NaCl) before mounting in 70% glycerol (Sigma, St. Louis, MO) in stop buffer.

***In situ* hybridization on sectioned embryos and larvae**

Embryos (14 days post fertilization (dpf)) and larvae (47 days post hatching (dph)) of Atlantic halibut were fixated in 4% paraformaldehyde-buffred PBS (pH 7.4) for 48 h at 4°C. After a brief wash in 1xPBS the embryos and larvae were incubated in a solution of 25% sucrose, 25% Tissue Tek (Sakura Fintek, Netherlands) and 50% 1xPBS overnight at 4°C. They were mounted in a mould of Tissue Tek and rapidly frozen on an iron block pre-cooled in liquid nitrogen. Parallel

sectioning (10 µm) was done in a Leica CM 3050S cryostat (Leica Microsystems, Germany) and before storage at -20°C the tissue was air dried for 1 h in room temperature and for 10 min in 65°C. One parallel of the 47 dph sectioned larvae was Nissl-stained with 0.5% Cresyl Fast Violet (Chroma-Gesellschaft, Germany) and the other parallel was stained by *in situ* hybridization. *In situ* hybridization was carried out as described in Sandbakken et al., (2012).

***In situ* hybridization on whole embryos using fluorescent double labeling techniques**

Fluorescent double labeling *in situ* hybridization was done to identify expression of two genes in the same embryo. Fluorescein-labeled riboprobe for exorhodopsin (*exorh*) and digoxigenin-labeled riboprobe for the mammalian-like melanopsin (*opn4m1*) were used. Preparation and *in situ* hybridization was done as described above, with the following modifications. At the hybridization step, both probes were applied. The fluorescein-labeled probe was first visualized by using the antibody Anti-Fluorescein-horse radish peroxidase (POD), Fab fragments (1:400) (Cat. No 11426346910, Roche Diagnostics, Germany, RRID:AB_840257) and the TSA^{TM}Plus Fluorescein System (Cat. No NEL741001KT, Perkin Elmer, Waltham, MA) according to the producer's protocol. Before applying Anti-Digoxigenin-Alkaline phosphatase, Fab fragments (1:2000) (Cat. No 11093274910, Roche Diagnostics, Germany, RRID:AB_514497) the embryos were blocked for 4 h in 2% Blocking reagent (Roche Diagnostics, Germany) and 2xSCC, and the digoxigenin probe was visualized by use of Fast Red tablets as recommended by the manufacturer (Cat. No 11496549001, Roche Diagnostics, Germany). The stained embryos were mounted in DABCO anti-fading medium (Triethylenediamine, Sigma, St. Louis, MO) and stored in darkness.

Immunohistochemistry

Immunohistochemistry with antibody against serotonin (5HT) to mark the serotonergic system was performed. Whole embryos at 14 dpf were washed in 2%PBST (2% TritonX-100 (Sigma, St. Louis, MO) in 1xPBS) for 2 d at 4°C before washing 2x30 min with 1.5% PBST (1.5% TritonX-100 in 1xPBS). Incubation with primary antibody for 3 d at 4°C was done with 1.5% PBST/1% bovine serum albumin (BSA) (Sigma, St. Louis, MO). The primary antibody was polyclonal anti-serotonin antibody produced in rabbit (Cat. No 20080, DiaSorin, Italy, RRID:AB_572263) with a concentration of 1:1000. Embryos were washed 2x30 min in 1xPBS prior to incubation overnight

at 4°C with the second antibody Anti-Rabbit IgG (H+L), CF™ 555 antibody produced in goat 2 mg/ml (Cat. No SAB4600068, Sigma-Aldrich, St. Louis, MO, RRID:AB_2336059). A concentration of 1:100 was used for the secondary antibody in 1xPBS/1% BSA (Sigma, St. Louis, MO). The incubation was ended by washing in 2x30 min in 1xPBS before mounting in DABCO anti-fading medium (Triethylenediamine, Sigma, St. Louis, MO) and storage in darkness.

Fluorescent *in situ* hybridization and immunohistochemistry

Fluorescent *in situ* hybridization together with immunohistochemistry was done to evaluate the expression pattern in the region of the pineal. *In situ* hybridization on embryos was performed using the TSA^{TM}Plus Fluorescein System together with fluorescein-labeled exorhodopsin riboprobe as described above. Before immunohistochemistry was done on the fluorescent stained embryos they were incubated for 4 h in 2% Blocking reagent (Roche Diagnostics, Germany). Monoclonal Anti-Acetylated Tubulin antibody produced in mouse (Clone 6-11 B-1) (1:1000) (Cat. No T7451, Sigma-Aldrich, St. Louis, MO, RRID:AB_609894) was incubated overnight in room temperature with 1% Blocking reagent in 2xSSC. Prior to incubation with secondary antibody the embryos were washed with 1xPBS 3x10 min. The secondary antibody used was Anti-Mouse IgG (H+L), CF™ 555 antibody produced in goat 2 mg/ml (1:100) (Cat. No SAB4600066, Sigma-Aldrich, St. Louis, MO, RRID: AB_2336060) in 1xPBS/1% BSA. The incubation took place overnight at room temperature before washing 2x30 min in 1xPBS and mounting in DABCO anti-fading medium (Triethylenediamine, Sigma, St. Louis, MO) and storage in darkness.

Antibody characterization

Rabbit anti-serotonin (Cat. No. 20080, DiaSorin, RRID:AB_572263) is a polyclonal antibody against serotonin, and has been showed to have similar staining in classical serotonergic neuronal populations in vertebrates (Ekström and Ebbesson, 1988; Ebbesson et al., 1992; Sandbakken et al., 2012). Mouse anti-acetylated tubulin (Cat. No. T7451, Sigma-Aldrich, St. Louis, MO, RRID:AB_609894) is a monoclonal antibody that recognizes an epitope located on the α3 isoform of *Chlamydomonas axonemal* α-tubulin. The antibody has been shown to recognize a single 55 kDa band (the predicted molecular weight of acetylated tubulin) on western blots of

teleost brain extracts (Liu and Lessman, 2007). In addition, the antibody has also been shown to specifically label axons in the developing CNS of zebrafish (Chitnis and Kuwada, 1990). The antibody has been used to label axons in many organisms including teleosts (Ledizet and Piperno, 1991; Hunter et al., 2011; Verpy et al., 2011). See Table 2 for details.

Microscopy and photographic manipulation

All bright field photos were taken using a digital camera (Leica DFC 320) attached to a Leica DM 6000B microscope (Leica Microsystems, Germany). For fluorescent microscopy a ebx75mc-L90 lamp (Leistrungselektronik Jena GmbH, Germany) was used together with the same microscope, a digital camera (Leica DFC 350) (Leica Microsystems, Germany) and the filter cubes GFP and Y3 (Leica Microsystems, Germany). Adobe Photoshop CS5 (San Jose, CA) was used for adjustments of brightness, contrast, color levels and to sharpen the pictures.

Results

Identification and characterization of opsins in Atlantic halibut

Three full-length cDNAs of melanopsins in Atlantic halibut were identified by PCR and later verified by searching the halibut genome. In addition one melanopsin, a remnant of a melanopsin gene and exorhodopsin were found by searching the halibut genome using a BLAST algorithm. Two of the melanopsins, *opn4m1* and *opn4m3*, are comparable to the mammalian-like melanopsins (*opn4m*) and two, *opn4x1* and *opn4x2*, are similar to the *Xenopus*-like type (*opn4x*). The PCR ran with primer binding sites located in the predicted the 5' and 3' UTRs revealed that the mammalian-like melanopsin (*opn4m3*) has two splice variants (long and short) differing in the cytoplasmatic tail. The long isoform has a stretch of 22 amino acids not seen in the short isoform and by searching the halibut genome we identified an exon coding for 22 amino acids between the two last exons of the short isoform. In zebrafish an additional intronless mammalian-like melanopsin has been identified, thought to have arisen from retrotransposition (Bellingham et al., 2006). In the degenerative PCR screen for halibut melanopsins no orthologue for the intronless zebrafish gene was detected. However, by search in the halibut genome using a BLAST algorithm we identified a remnant of a potential intronless gene. The remnant is a pseudogene that does not span the seven transmembrane region and it has deletions and

frameshifts (data not shown). The other four melanopsin genes detected have an exon-intron structure. GenBank accession number, cDNA length, open reading frame (ORF) and amino acid sequence are listed in Table 3 for the four functional melanopsins and exorhodopsin.

An alignment (Fig. 1) of the four functional melanopsins in Atlantic halibut together with published melanopsin amino acid sequences of zebrafish (Opn4m) shows that they all contain the seven transmembrane α-helical domains (TM1-7) characteristic for the opsins, and they have the lysine (K) in the TM7 that serves as a site for Schiff base linkage of the chromophore 11-*cis* retinal (reviewed in Terakita, 2005). In the third transmembrane domain the glutamic acid (E), a counterion typical for visual opsins, is replaced with an aromatic tyrosine (Y) residue (Provencio et al., 1998) common with the glutamic acid (E) in the extracellular loop between the TM4 and TM5 being the putative displaced counterion of the Schiff base (Terakita et al., 2000). The tripeptide DRY in the interface between TM3 and the second intracellular loop and an asparagine (N) in TM2 thought to be critical for G-protein activation are conserved in the halibut melanopsins (Bockaert and Pin, 1999). To stabilize the tertiary structure a disulfide bridge between cysteins (C) of the first and second extracellular loop is formed (Karnik and Khorana, 1990), these cysteins are also present in the halibut melanopsins.

Phylogenetic analysis

The maximum likelihood tree (Fig. 2) based on amino acid sequences shows the putative evolutionary relationship between the halibut melanopsins and various melanopsins of different species. In addition the visual opsins and exorhodopsin of halibut and zebrafish are included. As shown in Bellingham et al. (2006) the melanopsins divide into two branches, the mammalian-like (*opn4m*-like) and the *Xenopus*-like (*opn4x*-like) melanopsins. The halibut melanopsins position into these two branches and in addition the teleost duplication of melanopsins is present in halibut. Exorhodopsin, the pineal-specific opsin (Mano et al., 1999; Philp et al., 2000), branches together with rod opsin as they arose from an ancient duplication in teleosts (Bellingham et al., 2003).

Expression of melanopsin at the stage of hatching

In situ hybridization on whole embryos (Fig. 3) showed that the two mammalian-like melanopsins (*opn4m1* and *opn4m3*) and one of the *Xenopus*-like melanopsins (*opn4x2*) are expressed in the brain and spinal cord at the time of hatching. No expression of *opn4x1* was detected at the stage of hatching. Sense probes were included as controls for unspecific DIG probe labelling for all the melanopsins and showed no staining (data not shown).

Expression of *opn4m1* (Fig. 3A-H) was detected in several regions of the brain and in the spinal cord. Dorsal in diencephalon *opn4m1* is expressed close to the pineal, and a dorsal view of the expression pattern (Fig. 3A, C) shows that the expression is located a few cells lateral to the midline on both sides. Ventral in diencephalon two clusters of cells of *opn4m1* were detected in the presumptive future preoptic area (Fig. 3B, F). In the midbrain an *opn4m1* positive cell was seen in the optic tectum and a cluster of cells is situated just ventral to the optic tectum (Fig. 3B, G). In the tegmentum a broad cluster of *opn4m1* expression was detected (Fig. 3B, G) and seen from a dorsal view the expression is just medial to the developing eye (Fig. 3A, D). In the hindbrain a small cluster of *opn4m1* cells was detected ventral to the neuromast cell on both sides (Fig. 3B). In the spinal cord distinct cells expressing *opn4m1* were seen just dorsal to the developing notochord (Fig. 3A, B, E, H).

Expression of *opn4m3* was also detected in several regions of the brain and in the spinal cord (Fig. 3I-O). In the brain two regions of the ventral diencephalon have *opn4m3* expression (Fig. 3I, J, K, M). The most rostroventral cluster is located in the presumptive future preoptic area (Fig. 3K), while the other cluster is situated more dorsally (Fig. 3J, M). In the hindbrain a bilateral ball of cells with *opn4m3* negative cells in the middle was seen. The clusters are just caudal to the midbrain-hindbrain boundary and at the level of the lateral neuromasts (Fig. 3I, J, L, N). Expression of *opn4m3* was also detected medial to the ear (Fig. 3I, J, L). In the spinal cord distinct cells expressing *opn4m3* were seen just dorsal to the developing notochord but the *opn4m3* positive cells start more caudally than the *opn4m1* cells (Fig. 3I, J, O). Expression of *opn4x2* (Fig. 3P-U) is located medial to the ear (Fig. 3P, Q, R, T) and in the spinal cord in a similar pattern as *opn4m3* (Fig. 3P, Q, S, U). Staining seen in the ear (Fig. 3P) is not consistent and may be artificial and due to trapping of probe in the ear.

Expression of exorhodopsin and melanopsin in the region of the pineal organ

In situ hybridization revealed expression of exorhodopsin in the halibut pineal at the stage of hatching (Fig. 4A) and the mammalian-like melanopsin (*opn4m1*) is also expressed in the same region (Fig 4B). Cryosections on embryos (14 dpf) showed *exorh* in the pineal (Fig. 4C) and *opn4m1* positive cells surround the pineal, only a few cell rows lateral to the midline (Fig. 4E). The cryosection also pointed out that the melanopsin expression is not in the epithelia cells covering the pineal (Fig. 4E). Fluorescent double labeling on whole embryos further compared the expression of the *exorh* (Fig. 4D) and *opn4m1* (Fig. 4F) in the region of the pineal, and revealed that the mammalian-like melanopsin is expressed in cells just adjacent to the exorhodopsin expressing cells of the pineal (Fig. 4G). The two opsins are not expressed in the same cells (Fig. 4 G). A combination of *in situ* hybridization (*exorh*) and immunohistochemistry (anti-acetylated tubulin) demonstrated an axonal connection between the exorhodopsin expressing pineal and deep brain cells (Fig. 4H-M) at the stage of hatching. A lateral view (Fig. 4H, J, K) shows an axon from the exorhodopsin expressing pineal descending deep in the diencephalon anterior to the posterior commissure. This corresponds to previous studies in halibut suggesting the presence of neural signaling pathways between the pineal and the brain at the time of hatching (Forsell et al., 2001). From a dorsal view it is evident that pineal neuronal cell bodies are located medially where no exorhodopsin expression is seen and the axons from these cell bodies descend laterally in the developing brain (Fig. 4I, M). Detection of antibody against serotonin (5HT) showed serotonin positive cells in the pineal (Fig. 4N, O) and in the ventral part of diencephalon and midbrain (Fig. 4N, P) at the stage of hatching. (See supplementary Figure 1 for magenta/green copy.)

The mammalian-like melanopsin (*opn4m1*) is extensively expressed in the brain of halibut larvae with functional eyes

The spatial expression pattern of *opn4m1* in the brain of halibut larvae with functional eyes (47 dph) was compared with adjacent serial Nissl-stained sections (Fig. 5). Positive cells of *opn4m1* are apparent in the pineal region (Fig. 5A3) and in addition expression of *opn4m1* was detected in the left habenula (Fig. 5B3). Expression in the habenula was also detected at an earlier stage (27 dpf) and a rostral view of a larva with *opn4m1* expression shows the distinct cells around the pineal and in the left habenula (Fig. 5B4-B5). Expression was also detected in the anterior

preoptic area (Fig. 5C3), in the suprachiasmatic nucleus, ventral thalamus and presumably in nucleus pretectalis superficialis magnocellularis (Fig. 5D3). In addition, expression was seen in the optic tectum and dorsal in tegmentum (Fig. 5D5). In ventral diencephalon expression was observed in cells that are most likely the nucleus lateralis tuberis (Fig. 5E3). In tegmentum *opn4m1* positive cells were detected ventrally and they are most likely located in the raphe nuclei (Fig. 5F3). Expression was also detected in the hindbrain presumably in the nucleus medialis octavolateralis and in the descending octaval nucleus (Fig. 5G3).

Melanopsin and exorhodopsin in the brain of halibut larvae with functional eyes

Expression of the other halibut melanopsins (*opn4m3*, *opn4x1* and *opn4x2*) and exorhodopsin (*exorh*) was also investigated at 47 dph. Three of them (*opn4m3*, *opn4x2* and *exorh*) had expression in the brain and the spatial expression pattern was compared with adjacent serial Nissl-stained sections (Fig. 6). The pineal-specific exorhodopsin is extensively expressed in the pineal at 47 dph (Fig. 6A3). The mammalian-like melanopsin (*opn4m3*) is expressed in the suprachiasmatic nucleus (Fig. 6B3), in ventral and dorsal thalamus (Fig. 6C3), in ventral tegmentum most likely in raphe nuclei (Fig. 6D3) and dorsal in the tegmentum and in the optic tectum (Fig. 6E3). The only *Xenopus*-like melanopsin (*opn4x2*) positive cells detected in the brain were seen medially, rostral in the hindbrain (Fig. 6F3).

Melanopsin expression in a functional retina

At the stage of hatching no melanopsin positive cells were found in the neuroblastic cells of retina. In contrast, at first feeding when the larvae have functional eyes, expression of three of the melanopsins (*opn4m1*, *opn4x1* and *opn4x2*) was detected (Fig. 7). No transcripts of *opn4m3* were found. The mammalian-like melanopsin (*opn4m1*) is expressed in the ganglion cell layer and in the inner nuclear layer. In the inner nuclear layer *opn4m1* was found in cells resembling amacrine cells and in addition distinct cells probably similar to horizontal cells were seen close to the outer plexiform layer (Fig. 7A). The *Xenopus*-like melanopsin (o*pn4x1*) was located in the ganglion cell layer and in cells resembling amacrine cells of the inner nuclear layer (Fig. 7B). Expression of *opn4x2* was just seen in the inner nuclear layer. Close to the outer plexiform layer distinct cells were observed and they are most likely horizontal cells. In addition a more diffuse expression

was seen in cells similar to bipolar cells (Fig. 7C). A schematic drawing of the retina illustrates the distribution of the melanopsin positive cells (Fig. 7D).

Discussion

Appearance of non-visual opsins early in development of halibut provides an alternative model to study the evolution and functional significance of non-visual opsins. The present study describes non-visual photoreception during early life stages of teleosts, when the brain is uncovered and transparent and directly exposed to environmental photons. We show a refine and extensive expression of melanopsin in Atlantic halibut larvae illustrating the importance of non-visual photoreception from early stages of development. In the pineal complex (Ekström and Meissl, 1997), we detect expression of both melanopsin and exorhodopsin in adjacent cells suggesting that two different photoreceptor systems may be active in the pineal complex.

The transparent halibut brain has extensive melanopsin expression

The literature has defined two distinct groups of melanopsins in non-mammalian vertebrates, the mammalian-like melanopsins (*Opn4m*) that encode melanopsin proteins more similar to human and mouse and the *Xenopus*-like melanopsins (*Opn4x*) resembling *Xenopus laevis* melanopsin (Bellingham et al., 2006). In agreement with other teleosts (Davies et al., 2011; Sandbakken et al., 2012) and as a result of a whole-genome duplication event early in the evolution of ray-finned fish (Christoffels et al., 2004; Jaillon et al., 2004; Naruse et al., 2004), this article identifies two halibut melanopsins in each group and a pseudogene of a potential intronless melanopsin. The halibut melanopsins are expressed already at an early developmental stage when the light regulated hatching takes place. Illustrated by Figure 8 the transparent unpigmented halibut embryo (Fig. 8A) has at the stage of hatching differential expression of the melanopsins in several distinct regions of the brain and in the spinal cord (Fig. 8C). After retinogenesis when the eyes are functional the melanopsins are expressed in several retinal layers (Fig. 8D). However the extensive melanopsin expression in the brain at a pre-retinal stage persists in the transparent brain of halibut larvae after retinogenesis (Fig. 8B, D), indicating that photoreception directly through the brain is still vital even though the eyes are functional. An apparent exception is the *opn4m3* expression in the hindbrain. At hatching, ball-shaped aggregated clusters of cells are seen medial

to the neuromasts, but this expression pattern is not detected when the eyes become functional, indicating that the clusters may be involved in an early light receptive function e.g. hatching.

Interaction of exorhodopsin and melanopsin in the pineal complex

As illustrated in this article, melanopsin (*opn4m1*) is flanking the pineal-specific exorhodopsin expression and the cells are positioned next to each other (Fig. 4). This expression pattern is apparent both at the time of hatching and at first feeding (Fig. 4, Fig. 5 and Fig. 6). Expression of two different opsins in the pineal complex indicates that two parallel photoreceptor systems may be active in the pineal complex. The complex may be able to detect light with different wavelengths and thus expand the spectral information it can obtain from the environment. In zebrafish melanopsins are shown to have a maximal sensitivity to blue light at 470 and 484 nm (Davies et al., 2011), while exorhodopsin has a maximal sensitivity towards the green light at 498 nm (Tarttelin et al., 2011). The flanking melanopsin expressing cells may also function as a photoisomerase for the exorhodopsin expressing pineal cells. Recent evidence show that only two of the zebrafish melanopsins display invertebrate bistability, while the others are monostable and function more like classical vertebrate-like photopigments (Davies et al., 2011), known to be dependent on dark-isomerization to regenerate back to the *11-cis* configuration (reviewed in Lamb, 2009). The same study has suggested that bistable melanopsins in the zebrafish retina may provide 11-*cis* retinal to the monostable melanopsins in the same retinal layer. Phylogenetic analysis branches the halibut melanopsin expressed around the pineal together with the bistable melanopsins of zebrafish (Fig. 2), while exorhodopsin branch together with the monostable visual opsins (Davies et al., 2010). This may indicate that the halibut melanopsin could provide 11-*cis* retinal to the presumably monostable exorhodopsin expressing cell of the pineal, in addition to its role in photoreception. Consistent with previous studies in halibut embryos we show axonal projections from the pineal to the deep brain regions, indicating presence of neural signaling pathways between the two structures (Forsell et al., 2001). In addition, we demonstrate that the projections descend from exorhodopsin negative neurons medial in the pineal complex and that serotonin positive cells exist in the pineal complex at the same developmental stage (Fig. 4).

Differential melanopsin expression in the halibut brain

Prior to retinogenesis the halibut melanopsins are differentially expressed in the brain and spinal cord (Fig. 3). Mammalian-like melanopsins are expressed dorsal and ventral in diencephalon, presumably around the pineal (*opn4m1*) and in the prospective preoptic region (*opn4m1*/*opn4m3*). Expression of o*pn4m1* is also detected in the tegmentum and optic tectum, while both mammalian-like (*opn4m1*/*opn4m3*) and *Xenopus*-like (*opn4x2*) melanopsins are detected in the hindbrain and spinal cord. From the literature early expression of melanopsin has received little attention, but in zebrafish pre-retinal expression of melanopsin has also been reported (Matos-Cruz et al., 2011). Evaluating the early melanopsin expression between the two teleosts, the only consistent expression is around the pineal. But, in contrast to the mammalian-like melanopsin expressed around the pineal in halibut, a monostable *Xenopus*-like melanopsin is expressed in zebrafish. Although speculative, the potential bistable property of *opn4m1* in halibut opens for melanopsin driven photoisomerase activity in the pineal complex, while regeneration back to *11-cis* configuration in zebrafish has to be dependent on other systems.

This article contributes further information on the complexity of melanopsin expression in the brain of marine species and gives a possibility to compare the distribution of mammalian-like and *Xenopus*-like melanopsins in different species. In agreement with our previous findings in juvenile Atlantic cod (Drivenes et al., 2003) and juvenile Atlantic salmon (Sandbakken et al., 2012), melanopsin was detected in the habenula (*opn4m1*) and suprachiasmatic nucleus (*opn4m1*/*opn4m3*) of Atlantic halibut larvae (Fig. 5 and Fig. 6). But in contrast to cod and salmon that express *Xenopus*-like melanopsins in these diencephalic brain structures, the mammalian-like melanopsins are expressed in the same structures in halibut. Moreover, the left asymmetry of melanopsin expression in the halibut habenula is consistent with findings in salmon, where the asymmetric photoreceptive habenula was indicated to be linked to the photoreceptive function of the parapineal organ (Sandbakken et al., 2012). In accordance with salmon, mammalian-like melanopsins are expressed in thalamus (*opn4m1*/ *opn4m3*) and in the nucleus lateralis tuberis of the hypothalamus (*opn4m1*) (Sandbakken et al., 2012). The melanopsin expression detected in halibut larvae is highly comparable to that of juvenile cod and salmon, however we also found new brain regions expressing melanopsin in halibut. We discovered melanopsin positive cells in several regions of the midbrain of halibut larvae. We found melanopsin expression presumably in

nucleus pretectalis superficialis magnocellularis, in the dorsal tegmentum, in the optic tectum and most likely cells of the raphe nuclei. Melanopsin positive cells were also detected in the hindbrain. We indicate that *opn4m1* is expressed in the nucleus medialis octavolateralis and in the descending octaval nucleus while *opn4x2* was detected medially in the rostral hindbrain.

Melanopsin positive cells in the brain may have a role in modulation

Recently it has been demonstrated that non-visual opsins are expressed in interneurons of medaka and zebrafish (Fischer et al., 2013). According to the classification of photoreceptor cells based on distribution and neural identity (Ramirez et al., 2011), photosensitive interneurons represent high-order photoreceptor cells that are able to both receive and send electrical signals. With the refined and extensive halibut melanopsin expression in mind, one can speculate about the identity of the melanopsin expressing cells. They may represent primary sensory neurons transducing external stimuli into electrical signals, or they may be photosensitive interneurons that modulate the incoming electrical signal before it is transmitted. Several of the brain regions expressing melanopsins, such as the preoptic area, habenula, dorsal thalamus and tegmentum, are shown to have retinal and pineal innervations (Ekström and Meissl, 1997). One can further speculate if these melanopsin positive cells are photosensitive interneurons having a role in the modulation of the signals from the retina and pineal. Interestingly, high-order photoreceptor cells have so far just been identified in aggregated cluster of cells (Ramirez et al., 2011). This article shows dispersed photoreceptor cells in the spinal cord of halibut larvae at the time of hatching and one can speculate that these melanopsin positive cells are primary sensory cells or interneurons representing the first example of dispersed high-order photoreceptor cells.

Melanopsin expression in the halibut retina

In halibut larvae three of the melanopsins (*opn4m1*, *opn4x1* and *opn4x2*) are differentially expressed in the retina at the time of first feeding, showing that melanopsins are extensively expressed in the pure cone retina of halibut prior to rod-development and dim-light vision. In accordance to juvenile cod (Drivenes et al., 2003) and salmon (Sandbakken et al., 2012), the *opn4x1* is expressed in ganglion cells and in the inner nuclear layer in cells resembling amacrine cells. Expression of *opn4x2* is also consistent with previous findings in cod and salmon, showing melanopsin positive horizontal cells and a diffuse expression pattern in bipolar cells. The

mammalian-like melanopsin *opn4m1* is detected in the inner nuclear layer presumably in amacrine cells as seen in salmon, but in addition *opn4m1* is identified in horizontal cells of the inner nuclear layer and in the ganglion cell layer. Comparing our findings in brain and retina of marine teleosts it is apparent that the expression of *Xenopus*-like and mammalian-like melanopsins is more conserved in the retina than in the brain. In zebrafish retina the melanopsins are detected in all cell layers including the photoreceptor cell layer with expression of the intronless *opn4m2* in cones (Davies et al., 2011). Halibut have no melanopsin expression in the photoreceptor cell layer, and this may be due to lack of a functional orthologue of the intronless zebrafish *opn4m2*. Based on the expression of melanopsins in all cell layers of the adult zebrafish retina, it has been suggested that the melanopsins confer global photosensitivity to the teleost retina and may permit direct fine-tuning of the retinal circuitry (Davies et al., 2011). Our study on halibut suggests that light influences fine-tuning of retinal and brain circuitry early in development, expanding opsin´s modulatory role in lower vertebrates.

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Conflict of interest statement

The authors certify that there are no conflicts of interest.

Role of authors

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: ME, JVH. Acquisition of data: ME, JVH, ØD, CAB, RBE. Analysis and interpretation of data: ME, JVH, LOEE. Drafting

the manuscript: ME, JVH. Critical revision of the manuscript for important intellectual content: ME, JVH, LOEE. Obtained funding: JVH. Study supervision: JVH, LOEE, ØD.

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Table 1 Primers

Primer name	Sequence (5'–3')	Use
Opn4mFw2	GGGCATCACMGGCATGSTGGGAAACYT	Degenerative primer for *opn4m1* and *opn4m3*
Opn4mFw2-N	ATCTGCTCSATGATCACRCTSAYRTKAT	Nested degenerative primer for *opn4m1* and *opn4m3*
Opn4mRv2	GATGWGTKATGGCRTADATGATGGGGTTGT	Degenerative primer for *opn4m1 and opn4m3*
Opn4mRv2-N	CCARGAGATGACAWAMADCAGTAGSACWAT	Nested degenerative primer for *opn4m1 and opn4m3*
OpsinFw	AAGAAGYTCMGTCMACCTCTYAAYT	Degenerative primer for *opn4x2*
OpsinRv	GTTCATGAAGACRTAGATDAYAGGGTTRTA	Degenerative primer for *opn4x2*
MopsF	GCTKTSTTCGGMATMACGTCMATG	Nested degenerative primer for *opn4x2*
MopsR	GMAGCAGCASAGCAKCAWSGTGTA	Nested degenerative primer for *opn4x2*
HhMelF1	GGGTCTGCTGACTTCCTGTTCCT	3'RACE *opn4m1*
HhMelF2	GGGGAAGTTTAACGGCAGCACTC	Nested 3'RACE *opn4m1*
HhMelR1	CGCACCGACGGCGTAAAGGTCAT	5'RACE *opn4m1*
HhMelR2	GTCCCAGGAACAGGAAGTCAGCA	Nested 5'RACE *opn4m1*
HhMelF3	CGCTCCTACACGATGCTGCTCTT	3'RACE *opn4m3*
HhMelF7	TGTGGCCCTCACTGCATTCG	Nested 3'RACE *opn4m3*
HhMelR3	GTAGACCCAGGCAACAGCCAAGA	5'RACE *opn4m3*
HhMelR4	GCTAAGGGCTTTCCTGCGAGACA	Nested 5'RACE *opn4m3*
HMops1F3	GAGGGGCTGATGACGTCTTGT	3'RACE *opn4x2*
HMops1F2	ACGTCTTGTACGTGGGATTACGTC	Nested 3'RACE *opn4x2*
HMops1R3	GATATAAGAGCTCCAGCCGACGA	5'RACE *opn4x2*
HMops1R2	TGATAACCACGTAGCGGTCGAT	Nested 5'RACE *opn4x2*

Table 2 Antibodies

Antibody	Immunogen	Manufacturer, host species, mono- vs. polyclonal, catalog number, RRIDs	Dilution used
Anti-serotonin	Serotonin (5HT)	DiaSorin (Italy), Rabbit polyclonal, #20080, RRID:AB_572263	1:1,000
Anti-acetylated tubulin	*Chlamydomonas axonemal* α-tubulin within four residudes of acetylated Lys-40	Sigma-Aldrich (St. Louis, MO), Mouse monoclonal, IgG2b, #T7451, RRID:AB_609894	1:1,000
Anti-digoxigenin-alkaline phosphatase, Fab fragments	Digoxigenin (DIG)	Roche Diagnostics (Germany), Sheep polyclonal, #11093274910, RRID:AB_514497	1:2,000
Anti-Fluorescein-horse radish peroxidase, Fab fragments	Fluorescein	Roche Diagnostics (Germany), Sheep polyclonal, #11426346910, RRID:AB_840257	1:400
Anti-Rabbit IgG (H+L), CF™ 555	Rabbit IgG (H+L)	Sigma-Aldrich (St. Louis, MO), Goat polyclonal, #SAB4600068, RRID:AB_2336059	1:100
Anti-Mouse IgG (H+L), CF™ 555	Mouse IgG (H+L)	Sigma-Aldrich (St. Louis, MO) Goat polyclonal, #SAB4600066, RRID: AB_2336060	1:100

Table 3 Sequence information

Name	GenBank no.	cDNA length	predicted ORF	Predicted aa length	Binding site for the *in situ* probe (5'-3')	Probe length
opn4m1	KF941289	2138 bp	1527 bp	508 aa	159 - 1392 bp	1172 bp
opn4m3short	KF941290	1780 bp	1638 bp	545 aa	1 - 1089 bp	1089 bp
opn4m3long	KF941291	1846 bp	1704 bp	567 aa	1 - 1089 bp	1089 bp
opn4x1 (partial)	KF941292	1321 bp	1302 bp	434 aa	1 - 569 bp	569 bp
opn4x2	KF941293	2328 bp	1671 bp	556 aa	657 - 1859 bp	1203 bp
exorh	KF941294	1274 bp	1059 bp	352 aa	1-1274 bp	1274 bp

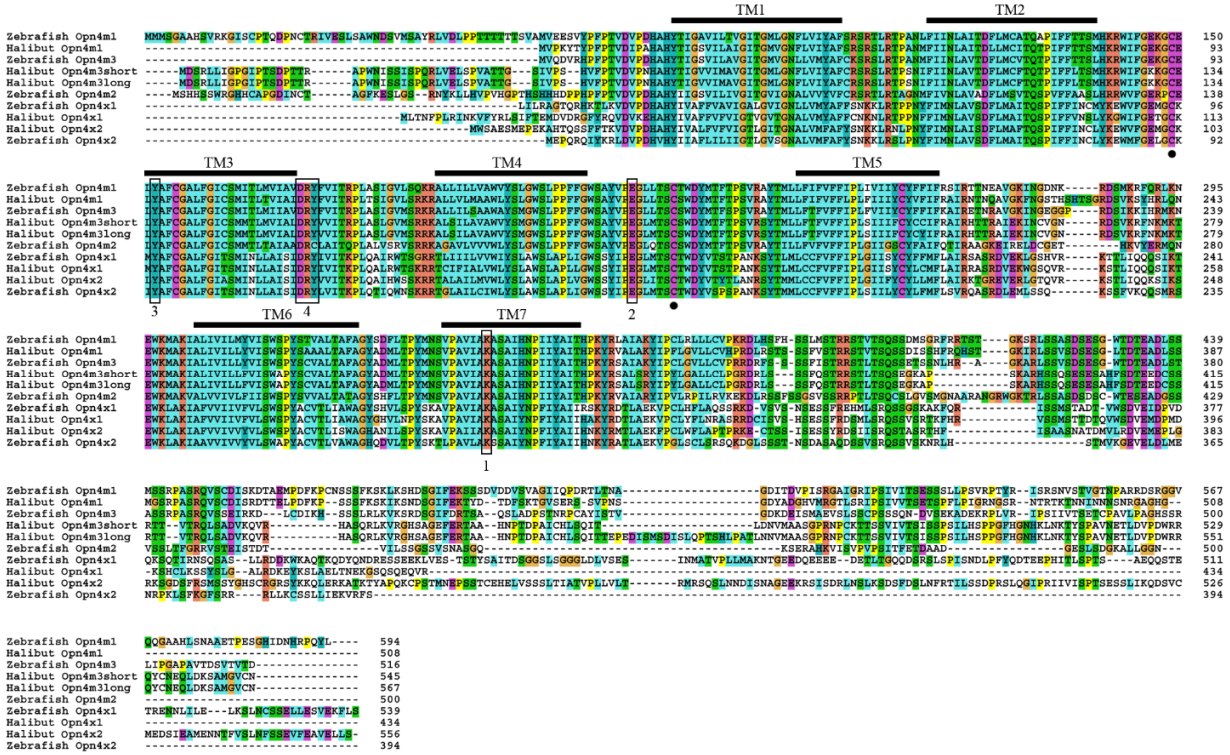

Figure 1 Alignment

Alignment of the deduced amino acid sequence of Atlantic halibut melanopsins against published melanopsin sequences of zebrafish. The seven transmembrane (TM) domains are indicated by TM1-7 (in accordance with Matos-Cruz et al. (2010)) and the alignment shows that all the halibut melanopsins span the domains. The retinal attachment site (K) in TM7 (1), the potential Schiff base counterions E and Y (2,3) and the DRY tripeptide (4) are outlined. Cysteins involved in the disulfide bridge formation are indicated with solid circles.

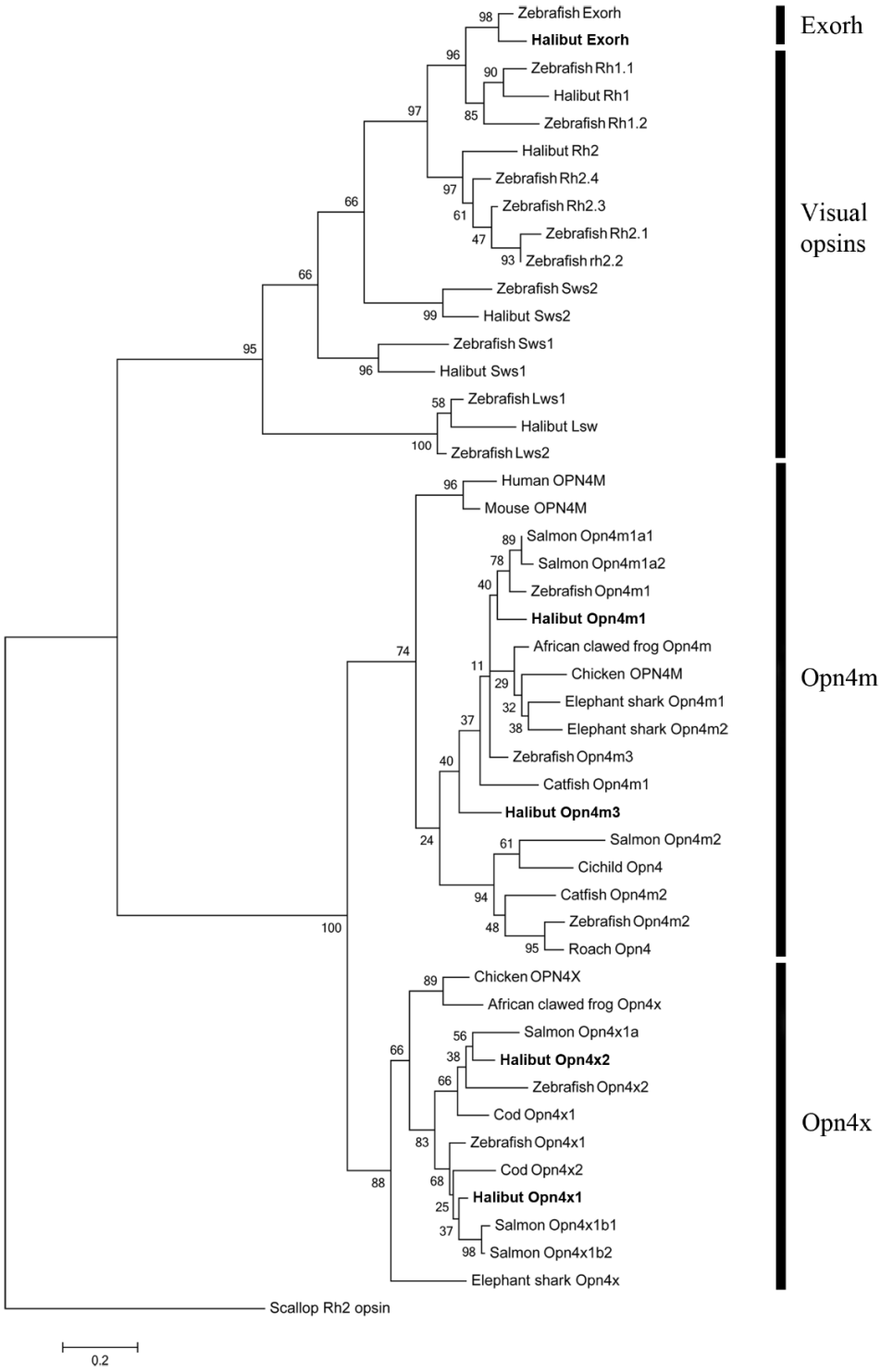

Figure 2 Phylogenetic tree

The maximum likelihood tree shows the phylogenetic relationship between the halibut melanopsins and melanopsins of other species. In addition the visual opsins and exorhodopsin of

halibut and zebrafish are included. Scallop (*Mizuhopecten yessoensis*) BAA22218 (Rh2 opsin) has been used as an out-group. A bootstrap value of 1000 has been applied. The opsin sequences used for generating the tree are: i) Exorhodopsin: zebrafish (*Danio rerio*) NP571287 (Exorh); halibut (*Hippoglossus hippoglossus*) KF941294 (Exorh) ii) Visual opsins: zebrafish (*Danio rerio*) NP571159 (Rh1.1); halibut (*Hippoglossus hippoglossus*) AAM17918.1 (Rh1), zebrafish (*Danio rerio*) ADK38855; halibut (*Hippoglossus hippoglossus*) AAM17916.1 (Rh2); zebrafish (*Danio rerio*) NP571328 (Rh2.1), NP878311 (Rh2.2), NP878312 (Rh2.3), NP571329 (Rh2.4); zebrafish (*Danio rerio*) NP571267 (Sws2); halibut (*Hippoglossus hippoglossus*) AAM17920.1 (Sws2); zebrafish (*Danio rerio*) NP571394 (Sws1); halibut (*Hippoglossus hippoglossus*) AAM17917.1 (Sws1); zebrafish (*Danio rerio*) NP571250 (Lws1); halibut (*Hippoglossus hippoglossus*) AAM1792.1 (Lws1); zebrafish (*Danio rerio*) NP001002443 (Lws2) iii) Mammalian-like melanopsin: human (*Homo sapiens*) ENSP00000361141 (OPN4M); mouse (Mus musculus) ENSMUSP00000022331 (OPN4M); salmon (*Salmo salar*) AFI61534.1 (Opn4m1a1), AFI61536.1 (Opn4m1a2); zebrafish (*Danio rerio*) ENSDARP00000109133 (Opn4m1); halibut (*Hippoglossus hippoglossus*) KF941289 (Opn4m1); African clawed frog (*Xenouus laevis*) ABD37674.1 (Opn4m); chicken (*Gallus gallus*) ABX10832.1 (OPN4M); elephant shark (*Callorhinchus milii*) AFU50495.1 (Opn4m1), AFU50496.1(Opn4m2); zebrafish (*Danio rerio*) ENSDARP00000070530 (Opn4m3); catfish (*Ictalurus punctatus*) ACP43590.1 (Opn4m1); halibut (*Hippoglossus hippoglossus*) KF941291 (Opn4m3); salmon (*Salmo salar*) JN210550.1 (Opn4m2); Cichild (*Astatotilapia burtoni*) ACB29678.1 (Opn4); catfish (*Ictalurus punctatus*) ACP43591.1 (Opn4m2); zebrafish (*Danio rerio*) ENSDARP00000070530 (Opn4m2); Roach (*Rutilus rutilus*) AAO38857.1 (Opn4) iv) *Xenopus*-like melanopsin: chicken (*Gallus gallus*) ABX10830.1 (OPN4X); African clawed frog (*Xenouus laevis*) NP_001079143.1 (Opn4x); salmon (*Salmo salar*) AFI61533.1 (Opn4x1a); halibut (*Hippoglossus hippoglossus*) KF941293 (Opn4x2); zebrafish (*Danio rerio*) ENSDARP00000123655 (Opn4x2); cod (*Gadus morhua*) AAO20043.1 (Opn4x1); zebrafish (*Danio rerio*) ENSDARP00000100318 (Opn4x1); cod (*Gadus morhua*) AAM95160.1 (Opn4x2); halibut (*Hippoglossus hippoglossus*) KF941292 (Opn4x1); salmon (*Salmo salar*) AFI61531.1 (Opn4x1b1), AFI61532.1 (Opn4x1b2); elephant shark (*Callorhinchus milii*) AFU50497.1 (Opn4x)

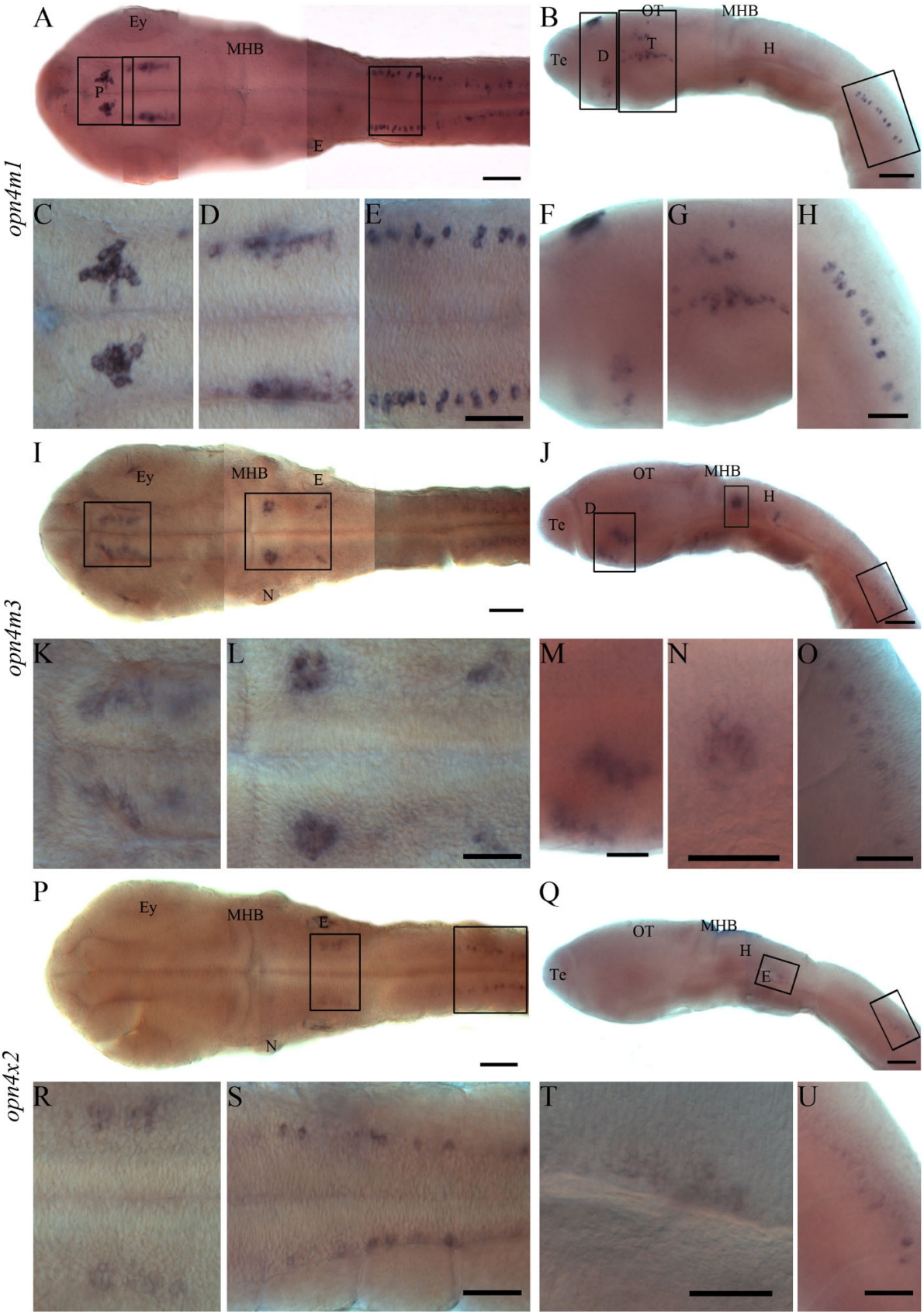
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Figure 3 *In situ* hybridization on whole halibut larvae at the stage of hatching.
Expression in the brain and spinal cord is shown from a dorsal and lateral view with different magnifications. The higher magnifications are indicated in the overview pictures by squares. The overview pictures (A-B, I-J, P-Q) are made by placing several pictures with different focus plane together. A-H: Expression of *opn4m1* close to the pineal (P), ventral in diencephalon (D), in tegmentum (T), optic tectum (OT), ventral to the neuromasts (N) and in the spinal cord. I-O: Expression of *opn4m3* in ventral diencephalon, in the hindbrain (H), medial to the ear (E) and in the spinal cord. P-U: Expression of *opn4x2* medial to the ear and in the spinal cord. Te, telencephalon, MHB, midbrain-hindbrain boundary. Scale bars: 100 μm in A, B, I, J, P, Q, 50 μm C-H, K-Q, R-U

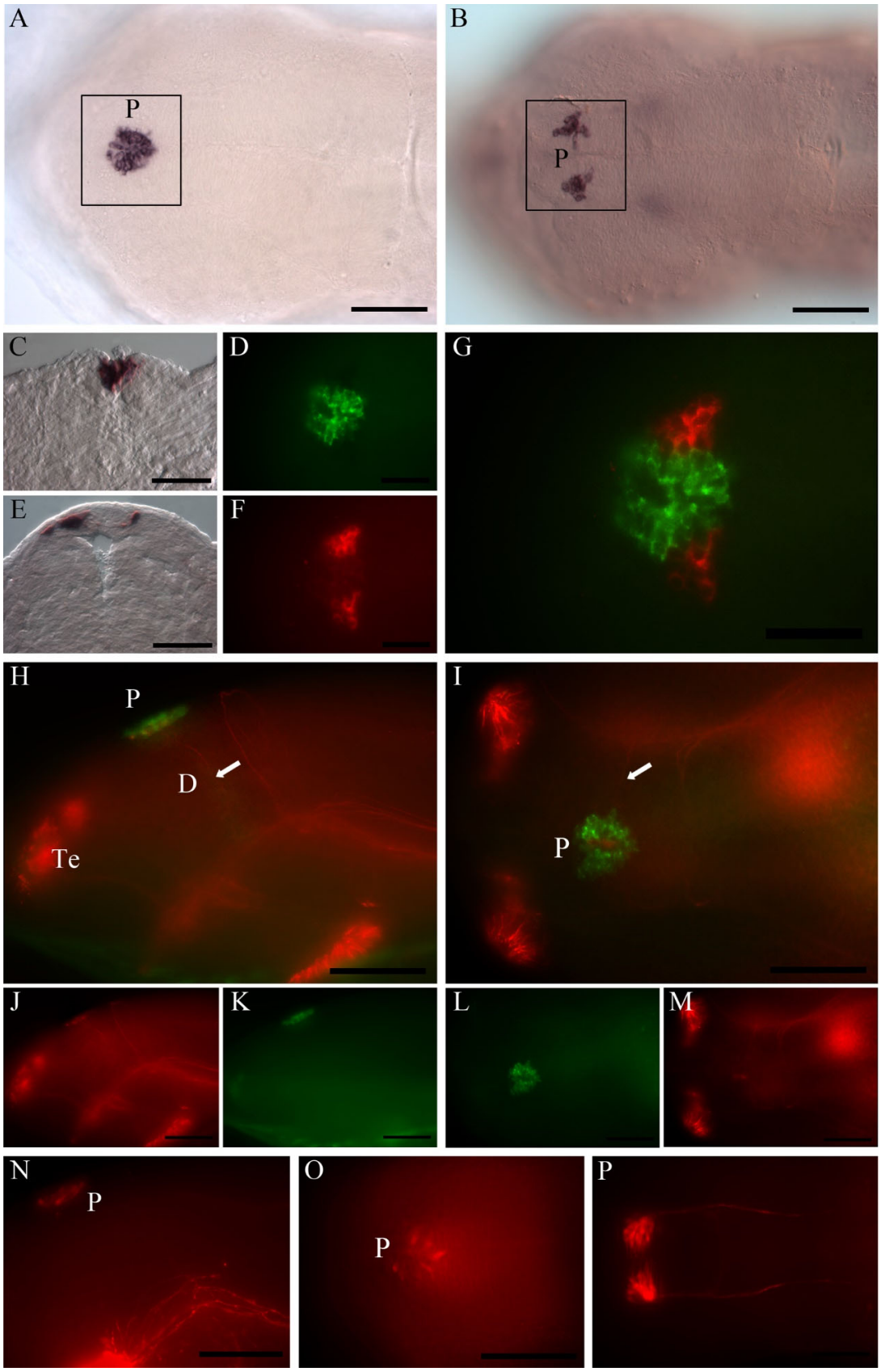
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Figure 4 At the stage of hatching exorhodopsin and melanopsin positive cells are apparent in the pineal region.
A-B: A dorsal view showing the expression of *exorh* (A) and *opn4m1* (B) in the pineal region (P). C and E: Transversal sections through the pineal region demonstrate the localization of *exorh* (C) and *opn4m1* (E) positive cells. D, F, G: Expression of *exorh* (D) (green) and *opn4m1* (F) (red) in the same embryo show that the two genes are expressed in adjacent cells (G). H-M: The neuronal projections (anti-acetylated tubulin) (red) from the exorhodopsin (green) expressing pineal are shown in a lateral (H, J, K) and dorsal view (I, L, M) illustrating that the projections from the pineal descend deep in the brain (white arrows). N-P: Serotonergic positive cells and projections (red) at the stage of hatching shown from a lateral (N) and dorsal view (O, P). Cells are detected in the pineal and the ventral part of diencephalon and midbrain. Te, telencephalon, D, diencephalon. (See Supplementary Figure 1 for magenta/green copy.) Scale bars: 100 μm in A-B and H-P, 50 μm in C-G

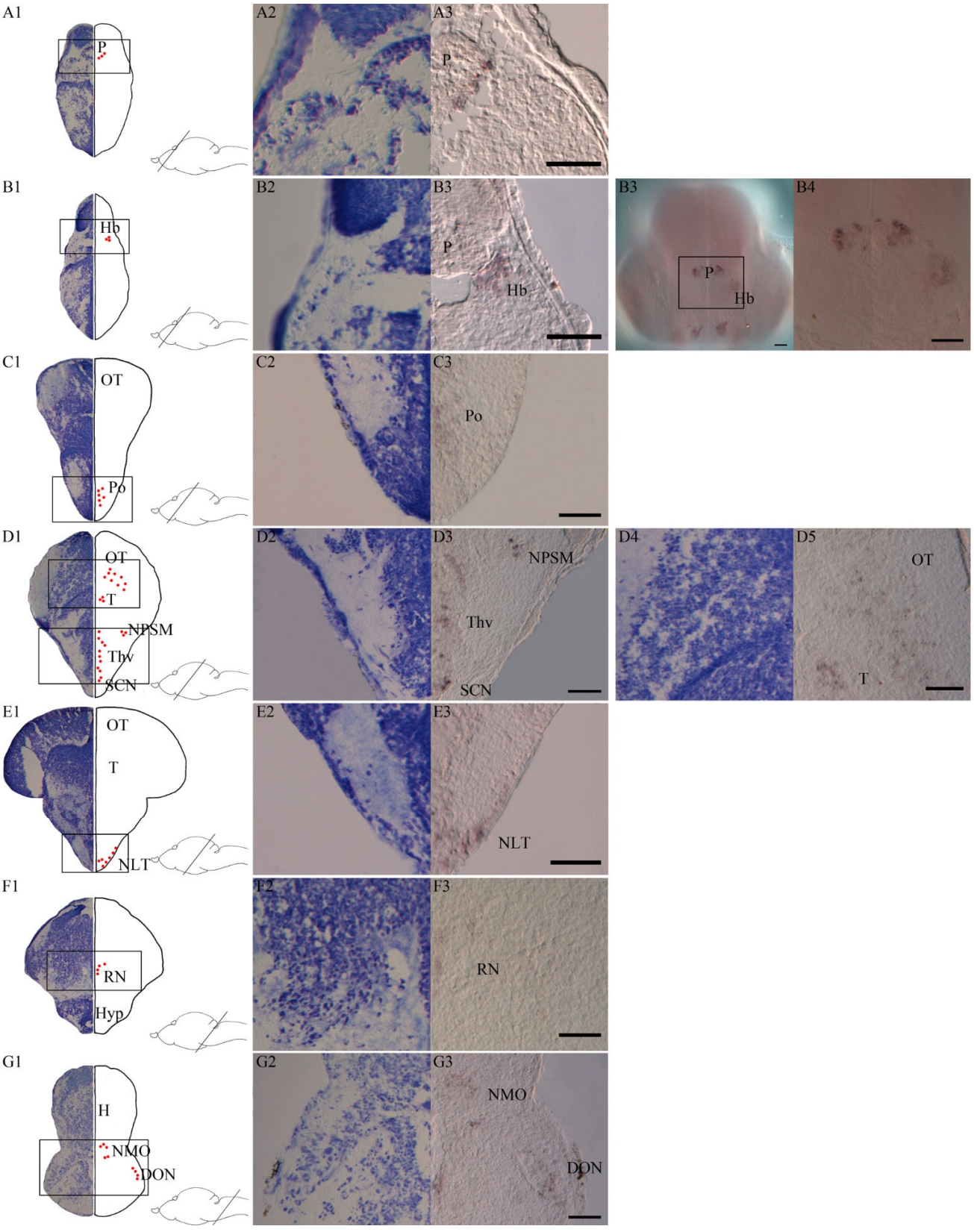
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Figure 5 Melanopsin (*opn4m1*) expression in the brain of Atlantic halibut larvae.
A1-J1: Nissl-stained transversal sections at the equivalent level of melanopsin expressing cells illustrated by red dots in a 47 days post hatching larva. A2-J2 and D4: The Nissl-stained cell populations of interest with a higher magnification. A3-J3 and D5: Melanopsin expression at the same level and with the same high magnification. B4-B5: A whole larva 27 days post fertilization seen from a rostral view, whole head (B4) and the region of pineal and habenula (B5). A1-A3: Expression around the pineal (P). B1-B5: Melanopsin positive cells in the left habenula (Hb). C1-C3: Expression in the preoptic area (Po). D1-D3: *opn4m1* in the suprachiasmatic nucleus (SCN), ventral thalamus (Thv) and presumably in cells of the nucleus pretectalis superficialis magnocellularis (NPSM). D1 and D4-D5: Expression in the dorsal tegmentum (T) and in optic tectum (OT). E1-E3: Expression of *opn4m1* in the nucleus lateralis tuberis (NLT) of the hypothalamus (Hyp). F1-F3: Melanopsin expression most likely in raphe nuclei (RN). G1-G3: Melanopsin positive cells in the hindbrain in cells resembling nucleus medialis octavolateralis (NMO) and descending octaval nucleus (DON). Scale bars 50 μm

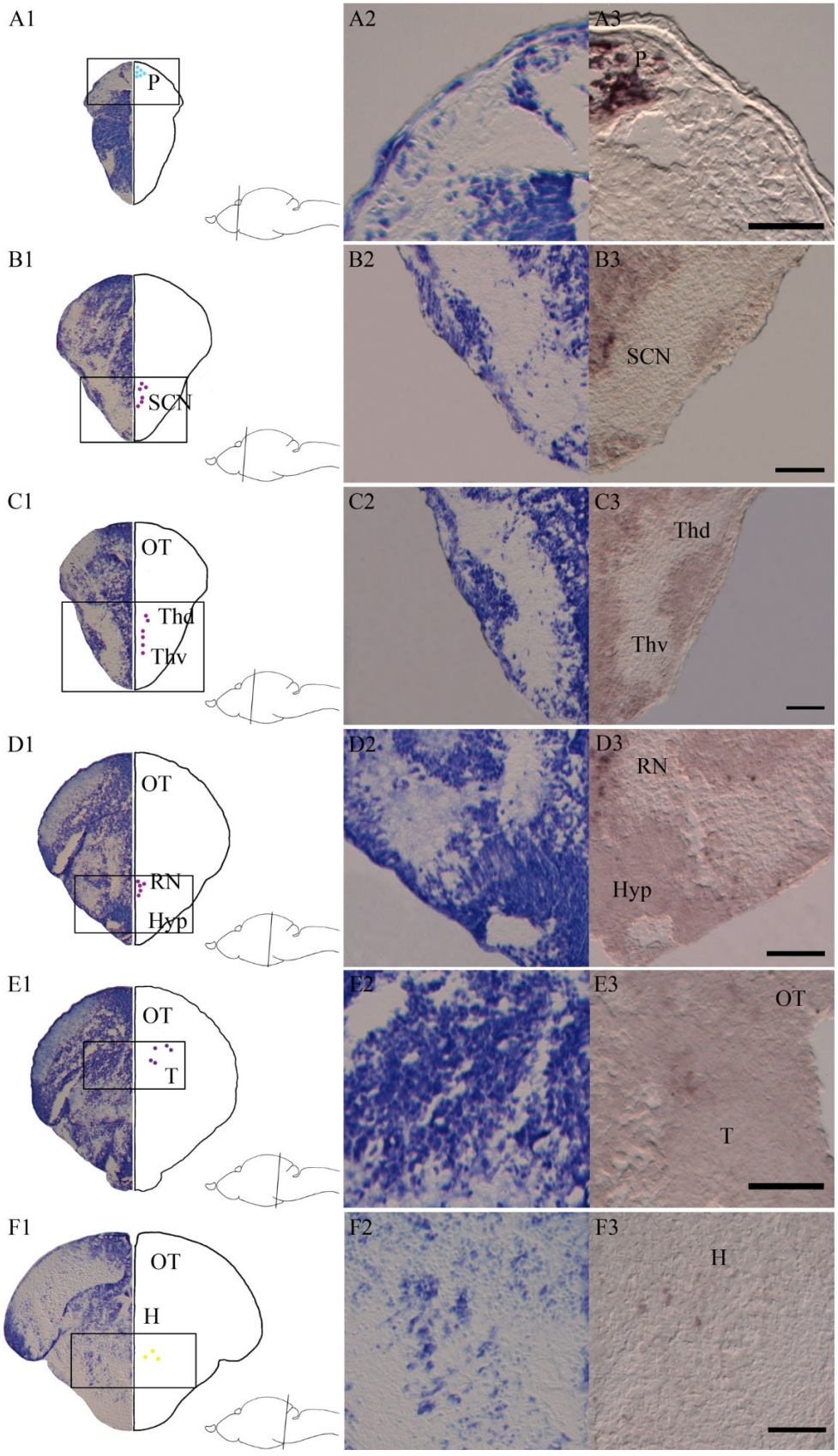
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Figure 6 Melanopsin (*opn4m3* and *opn4x2*) and exorhodopsin expression in the brain of Atlantic halibut larvae at 47 days post hatching.
A1-F1: Nissl-stained transversal sections at the equivalent level of *exorh* expressing cells illustrated by light blue dots (A1), *opn4m3* expressing cells illustrated by purple dots (B1-E1) and *opn4x2* expressing cells illustrated by yellow dots (F1). A2-F2: The Nissl-stained cell populations of interest with a higher magnification. A3-F3: Exorhodopsin and melanopsin expressions at the same level and with the same high magnification. A1-3: Expression of *exorh* in the pineal (P). B1-B3: *opn4m3* in the suprachiasmatic nucleus (SCN). C1-C3: Expression of *opn4m3* in dorsal and ventral thalamus (Thv and Thd). D1-D3: Expression of *opn4m3* presumably in rape nuclei (RN) E1-E3: *opn4m3* positive cells dorsal in tegmentum (T) and in optic tectum (OT). F1-F3: Expression of *opn4x2* medial in rostral hindbrain (H). Hyp, hypothalamus. Scale bars 50 μm

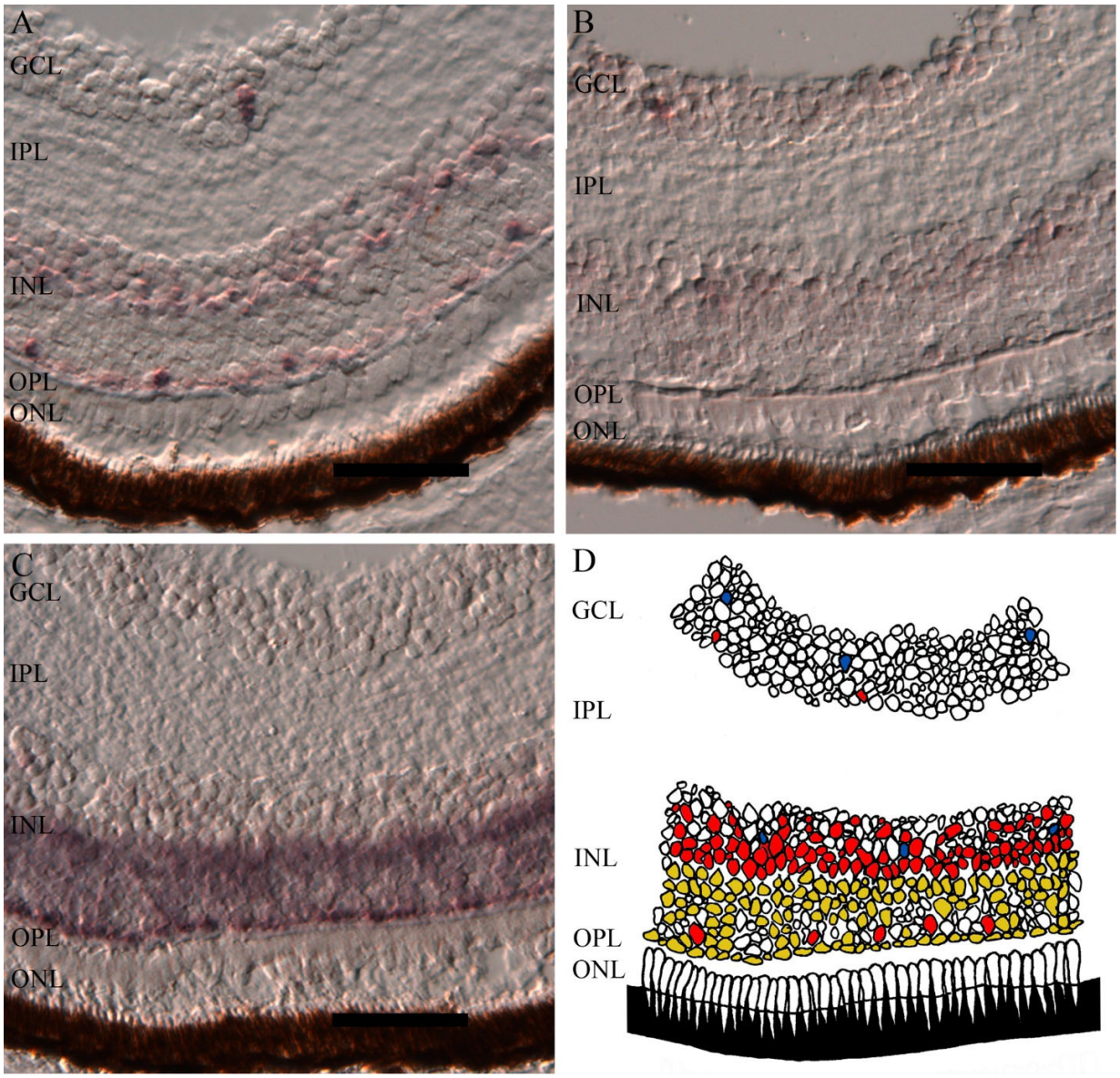


Figure 7 Distribution of melanopsin positive cells in a functional retina at 47 days post hatching. A: The mammalian-like melanopsin *opn4m1* is expressed in the in the ganglion cell layer (GCL) and in the inner nuclear layer (INL) probably in cells resembling amacrine cells and in cells that are most likely horizontal cells. B: Distribution of *opn4x1* in GCL and in INL possibly in cells resembling amacrine cells. C: Expression of *opn4x2* in the INL most likely in horizontal cells close to the outer plexiform layer (OPL) and in a diffuse manner in bipolar cells. D: Schematic drawing of the relative melanopsin expression, *opn4m1* (red), *opn4x1* (blue), *opn4x2* (yellow). IPL, inner plexiform layer, ONL, outer nuclear layer. Scale bars 50 µm

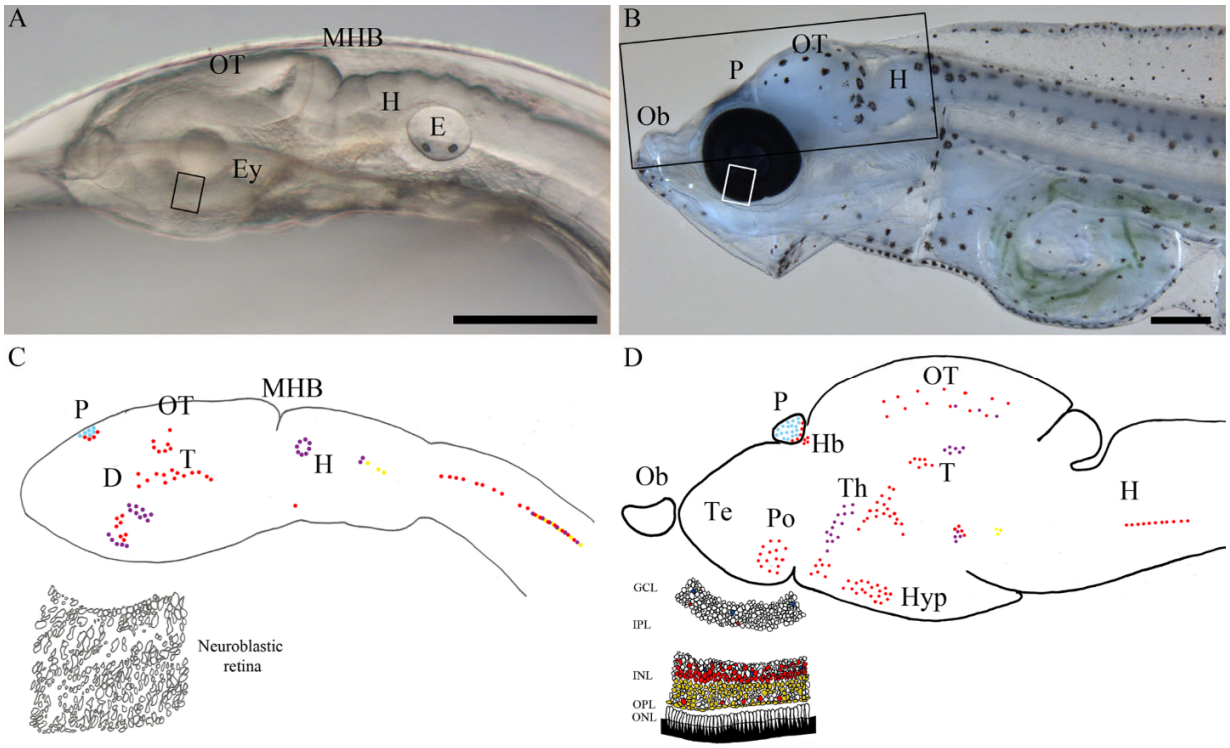
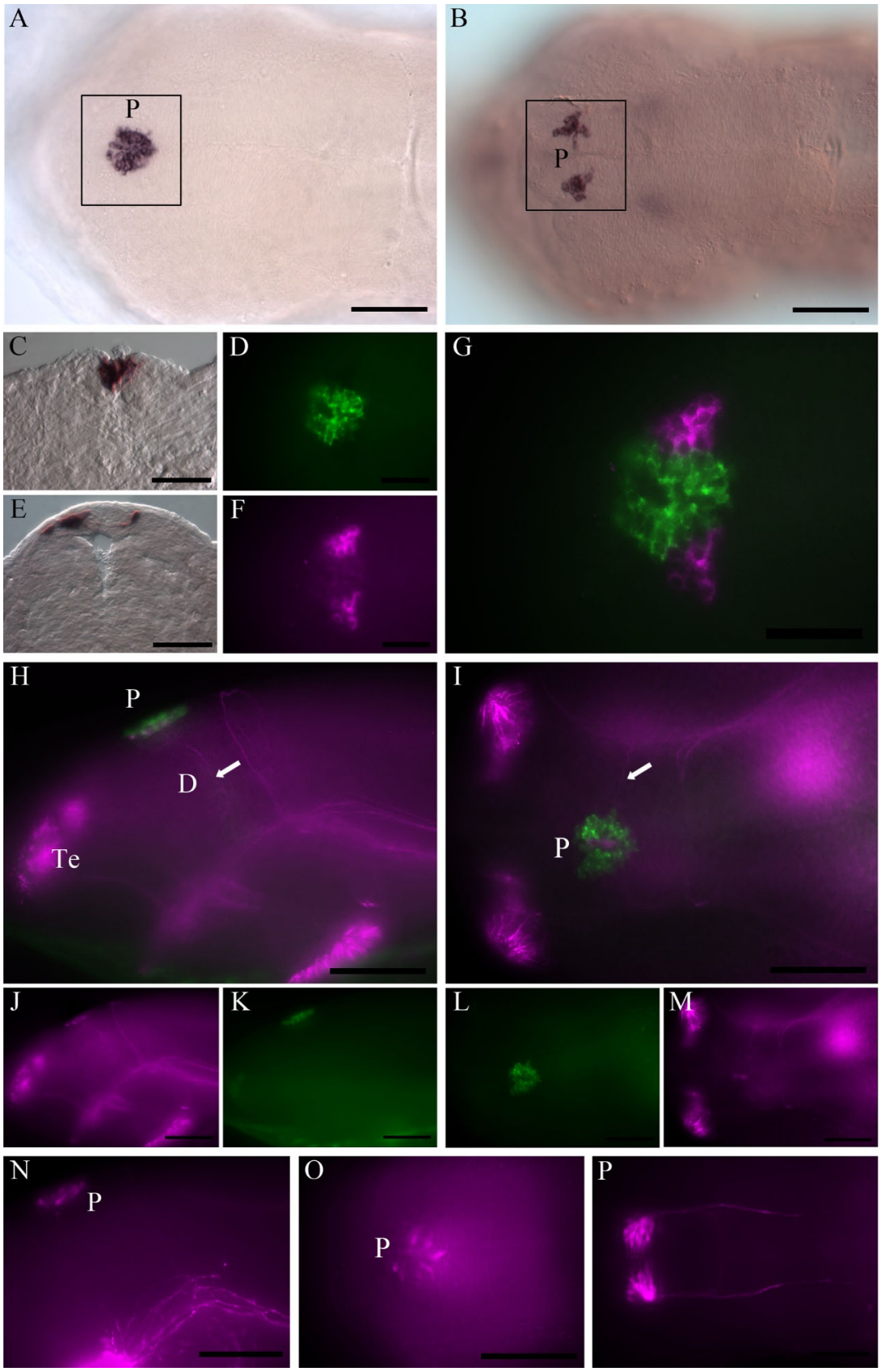


Figure 8 Melanopsin and exorhodopsin expression in the transparent halibut brain at the stage of hatching and at first feeding.

A: Lateral view of an unpigmented halibut embryo inside the eggshell at the stage of hatching. B: Lateral view of a halibut larva with functional eyes at the time of first feeding, showing the pigmented eye and the transparent brain with scattered pigmentation. C: Lateral schematic drawing of the halibut larvae (approximately with the same view as in (A)) at the stage of hatching illustrating the pineal specific exorhodopsin expression and the extensive melanopsin expression in the brain and spinal cord. A drawing of a section through the presumptive retina illustrates the neuroblastic cells. D: Schematic drawing of a halibut brain (lateral) in a larva with functional eyes showing the distribution of exorhodopsin and melanopsin. A drawing of a section through the retina shows the differentiated retinal layers and the relative distribution of melanopsins and exorhodopsin. *exorh* in light blue, *opn4m1* in red, *opn4m3* in purple, *opn4x1* in blue and *opn4x2* in yellow. For abbreviations, see list. Scale bar: 500 µm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Supplementary Figure 1 At the stage of hatching exorhodopsin and melanopsin positive cells are apparent in the pineal region.

A-B: A dorsal view showing the expression of *exorh* (A) and *opn4m1* (B) in the pineal region (P). C and E: Transversal sections through the pineal region demonstrate the localization of *exorh* (C) and *opn4m1* (E) positive cells. D, F, G: Expression of *exorh* (D) (green) and *opn4m1* (F) (magenta) in the same embryo show that the two genes are expressed in adjacent cells (G). H-M: The neuronal projections (anti-acetylated tubulin) (magenta) from the exorhodopsin (green) expressing pineal are shown in a lateral (H, J, K) and dorsal view (I, L, M) illustrating that the projections from the pineal descend deep in the brain (white arrows). N-P: Serotonergic positive cells and projections (magenta) at the stage of hatching shown from a lateral (N) and dorsal view (O, P). Cells are detected in the pineal and the ventral part of diencephalon and midbrain. Te, telencephalon, D, diencephalon. Scale bars: 100 μm in A-B and H-P, 50 μm in C-G