Non-visual photoreceptive systems in early development of Atlantic halibut (*Hippoglossus hippoglossus*)

Mariann Eilertsen

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Scientific environment

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2. Content

3. Abstract

Non-visual photoreception is thought to be involved in a range of behavioural and physiological processes in teleost fishes, such as synchronisation of circadian and circannual rhythms, but also regulation of skin pigmentation, buoyancy in the water, hatching of eggs and phototaxis. Knowledge of the teleost photoreceptive system and the photopigments involved is far from complete. Especially, the function of the photoreceptive system at early stages of development, the neuronal integration of the photoreceptor cells and the role of different photopigments need to be elucidated.

This thesis describes the identification of several non-visual opsins in Atlantic halibut (*Hippoglossus hippoglossus*). Four melanopsins, two isoforms of vertebrate ancient opsin and exorhodopsin have been described. The non-visual opsins identified in this present work show refined and extensive expression from early life stages of Atlantic halibut. Expression studies show non-visual opsin positive cells in a wide range of brain regions and in the spinal cord of halibut. The expression is detected early in development, at the period of the light regulated hatching process, when the embryo is poorly developed without functional eyes. Further on, the extensive expression of non-visual opsins persists in the larval brain at first feeding when the eyes are functional and expression of non-visual opsins are detected in the retina. Several of the brain regions have expression of more than one non-visual opsin, and this work studies the most apparent regions in detail.

In the pineal complex melanopsin positive cells flank the expression of exorhodopsin and the two genes are expressed in cells adjacent to each other. It is suggested that the existence of two non-visual opsins in the pineal complex provides detection of light of different wavelengths and thus may have the possibility to expand the spectral information it can obtain from the environment. Melanopsin may also function as a photoisomerase providing 11-*cis* retinal to exorhodopsin. A transient, bilateral cluster of

cells in the hindbrain is also described in the thesis. This cluster expresses both melanopsin and vertebrate ancient (VA) opsin thought to be of different evolutionary heritage and the work demonstrate for the first time in vertebrates that mRNAs of both photopigments are expressed within the in the same cells.

Further on, the thesis indicates that the transient hindbrain cluster expressing both melanopsin and VA opsin is involved in the light-regulated hatching process of halibut. The results show that the cluster is connected to a neuronal network with projections out in the yolk sac, reaching the narrow belt of hatching gland cells. In addition, studies on light-arrested eggs demonstrate expression of the immediate early gene *c-fos* in the hindbrain cluster and in the hatching glands after dark induced hatching. These results indicate that the photosensitive hindbrain cluster regulates hatching in halibut and that this process is dependent on neural signalling.

The thesis gives an example of a teleost embryo that organises the non-visual system with a combination of dispersed and aggregated photoreceptor cells. The neuronal identity of the photoreceptor cells can either be primary sensory neurons (first-order) or interneurons (high-order). The melanopsin positive cells identified in the spinal cord at the stage of hatching are dispersed, and may represent the first example of dispersed high-order photoreceptor cells. The work has identified several photosensitive clusters in brain regions with retinal and pineal innervations, and they may be interneurons modulating the incoming signal before it is transmitted. In addition, the aggregated cells of the hindbrain cluster may function as primary sensory cells in regulation of hatching or they may be modulating the signal. Photoreceptive organs are well described in the literature, but much less are known about the high-order photoreceptor cells and the early non-visual photoreceptive system in halibut can be a good model of such studies.

4. Nomenclature and abbreviations

aanat, aryl-alkylamine N-acetyltransferase

dpf, days post fertilization

dph, days post hatching

exo-rod, exorhodopsin

Opn3, encephalopsin

Opn4, melanopsin

Opn4m, mammalian-like melanopsin

Opn4x, Xenopus-like melanopsin

Opn5, neuropsin

Otx5, Orthodenticle homeobox 5

Tmt opsin, teleost multiple tissue opsin

VA opsin, vertebrate ancient opsin

5. General introduction

5.1 Detection of light

5.1.1 Light is an important cue for living organisms

Light has an important impact on life. In nature the light changes with the solar cycle and organisms from bacteria to human have adapted to the alterations of light intensity and spectral differences. Biological processes follow the rhythm of day and night (circadian rhythm) and the seasonal changes of the year (circannual rhythm). Circadian rhythms are biological cycles that have a period of about a day and in animals many physiological and behavioral variables exhibit daily oscillations, such as body temperature, hormonal levels, pupil size, heart rate, sleep and cognitive performance (Berson, 2003; Foster, 2002). The biological clock has to be synchronized with the solar day and the irradiance of light at dawn and dusk seems to provide the best indicator for the phase of the day when the intensity and spectral composition of light is changed (Roenneberg and Foster, 1997). In animals such as fish where growth, development and reproduction is in time with seasonal changes, the length of the day is considered to be the most reliable cue for timing seasonally events (Bromage et al., 2001). In nearly all branches of living organisms there exist light sensitive pigments detecting the cues from the solar rhythm. Examples of photopigments are the flavoproteins, including cryptochromes, bilins, including phytochrome, and the retinal-binding opsins, including rod opsin.

5.2.1 Molecular mechanisms of photoreception in retinal-binding opsins

Photopigments that consist of a vitamin A based chromophore (retinal) covalently bound to an opsin protein are known to play a role in bacteria, algae and animals. Vision as well as extra ocular photoreception in animals relies upon this photopigment stored in the enlarged membrane of the photoreceptor cell. The opsin protein belongs to a superfamily

of G-protein coupled receptors (Figure 1). Characteristically, the opsin is a seven alpha-helical transmembrane protein with three extracellular and three intracellular loops. Absorption of a photon by the chromophore results in photoisomerisation from 11-*cis* to all-*trans* configuration. This allows a conformational change in the opsin protein, initiating a G-protein dependent phototransduction cascade leading to detection of light (reviewed in Bockaert and Pin, 1999; Nilsson, 2004; Peirson et al., 2009; Terakita, 2005).

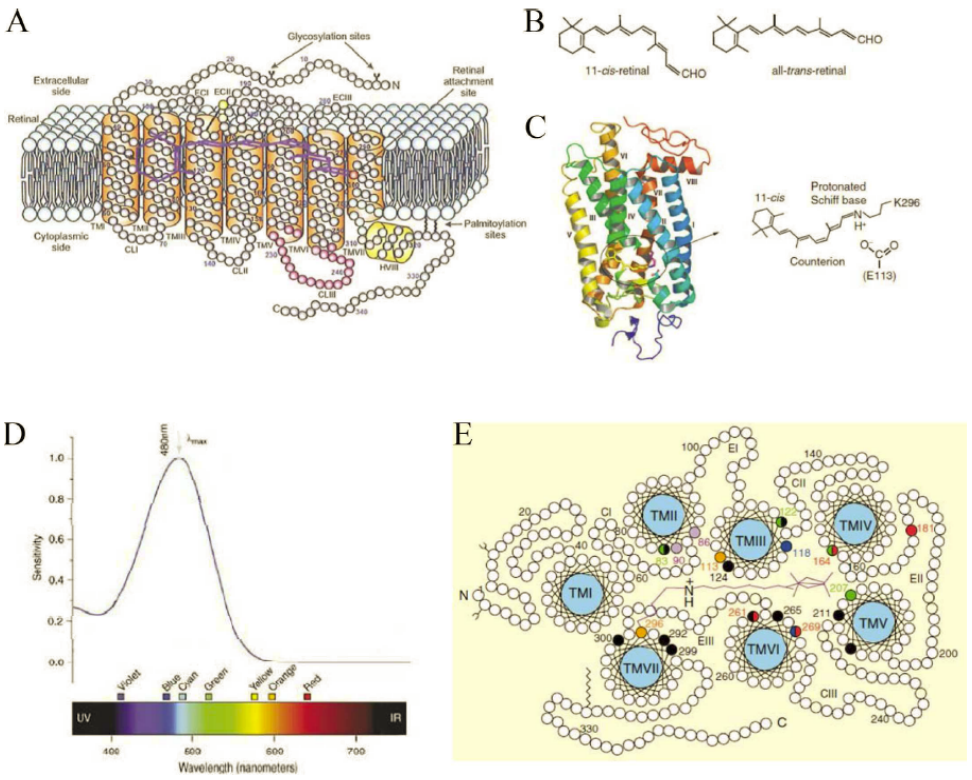

Figure 1 Structure and features of the vertebrate photopigment

A: Predicted secondary structure of the vertebrate photopigment based on bovine rod opsin. The seven transmembrane domains (TMI-VII) of the opsin are highlighted in orange and the 11-*cis* retinal chromophore is shown in purple. A conserved lysine (K-296) (red) in the TMVII is covalently linked to the chromophore through a Schiff base. In many vertebrate photopigments a negative charged residue at

position 113 (usually glutamate) provides the counterion to the protonated Schiff base. (Modified from Davies et al. 2010) B: The chemical structure of 11-*cis* and all-*trans* form of the retinal chromophore (Modified from Terakita et al. 2005). C: The crystal structure of bovine rhodopsin and binding site for the chromophore 11-*cis* retinal outlined in the circle. (Modified from Terakita et al. 2005) D: All opsin-based photopigments binding retinal have a characteristic absorption spectrum. This can be used as a "spectral fingerprint" to decide the photopigment mediating a biological process. Here illustrated by irradiance-dependent responses from a range of species suggesting a consensus opsin template with an absorption maximum around 480 nm. (Modified from Hankins et al. 2008) E: The spectral sensitivity is primarily determined by the interactions between the retinal chromophore and specific amino acids in the ligand binding pocket of the opsin. Amino acids involved in spectral tuning are colour coded with opsin class: LWS red, RH2 green, SWS2 blue, SWS1 violet and RH1 black. Colours that are split indicate amino acids involved in tuning of more than one opsin class. (Figure modified from Bowmaker and Hunt, 2006)

5.2 Evolution of photoreception

5.2.1 Evolution of photoreceptive organs

A model for evolution of the sensory system for detection of light has been proposed by Nilsson 2009. The model is based on a task-punctuated evolution where the sensory system evolved by a sequential achievement of tasks. In this model the old task is faced with different scenarios when a new one is archived. When a new task is gained by modification and elaboration of the old one, the new task can serve with a similar purpose as the old leading to replacement of the old task. This is illustrated by the transition from directional photoreception to spatial vision. In the case of gene duplication an old task can persist even though a new task is achieve, and the continuation of non-directional photoreception monitoring the diel light cycle is an example of this. (Nilsson, 2009). (See Figure 2 for more details.)

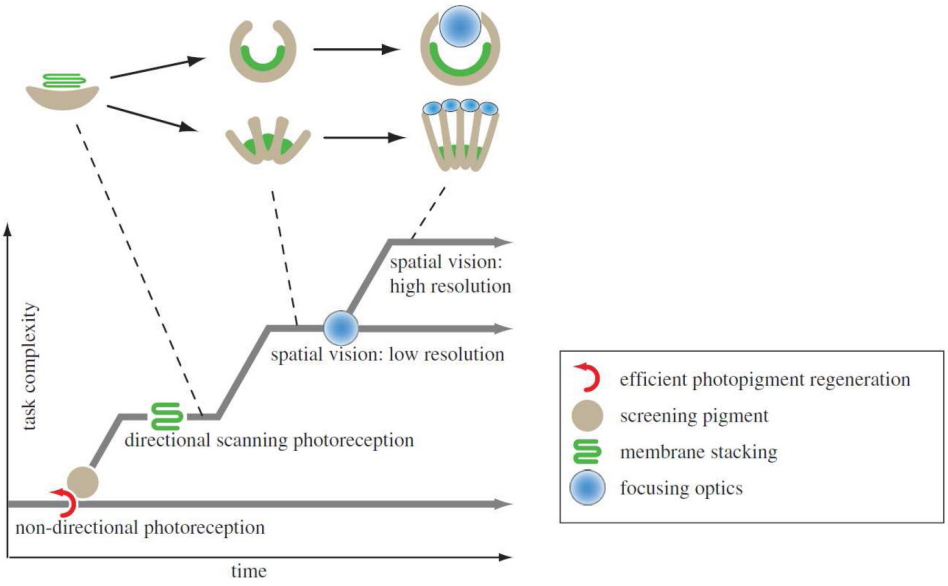

Figure 2 Evolvement of the sensory system for light detection

Starting out with a non-directional photoreception as a single sensory task, the early metazoans had the opportunity to sense the cues from the solar cycle. Gene duplication then led to effective photopigments by generation of a photoisomerase. This event was followed by an introduction of a screening pigment probably established as a derivative of protective body pigmentation. The screening pigment provided directional photoreception. Membrane stacking of the photoreceptor cell gave the opportunity for better contrast discrimination and spatial vision was then accomplished by multiple receptor cells. To accomplish high resolution of the spatial vision a lens was then gradually introduced by tissue filling the cavity of a pigment-eye cup. The light detecting region could then be shifted from the receptor cell to the lens. (From Nilsson 2009)

5.2.2 Evolution of eyes

The evolution of the eye has been debated for centuries and one of the important questions has been how many times the eye has evolved independently in evolution. From morphological comparison of eye anatomy and photoreceptor cells it was proposed that the eye has polyphyletic origin (Mayr, 1961). This view has been dominating until the discovery of the *Pax 6* gene as a universal master control gene in eye development in all metazoans (Gehring and Ikeo, 1999; Halder et al., 1995; Quiring et al., 1994). The discovery a of conserved *Pax 6* and later *Sine-oculus* related genes (Loosli et al., 1999) indicated a monophyletic origin of the eye and a common ancestral prototype of eye has been proposed. Under the control of the *Pax 6* gene a photosensitive cell formed a prototype eye together with a pigment cell. By evolution different eye types have developed, giving rise to the various types of eyes from simple compound eyes to highly evolved vertebrate camera-type eyes (Figure 3) (Gehring, 2002; Gehring and Ikeo, 1999). This prototype eye model has been further elaborated by a "division of labour model" proposed by Arendt et al. (2009). With the base in the theory of a "complexity drain on cells in the evolution of multicellularity" (McShea, 2002) they have proposed that early metazoans had eyes with a multi-functional cell type. The cell combined the functions of a rhabdomeric photoreceptor cell, a shading pigment and a locomotor effector. During evolution of the eye the cell had a division of labour by cell-type functional segregation into cells specialised in photoreception, shading and locomotion (Arendt et al., 2009).

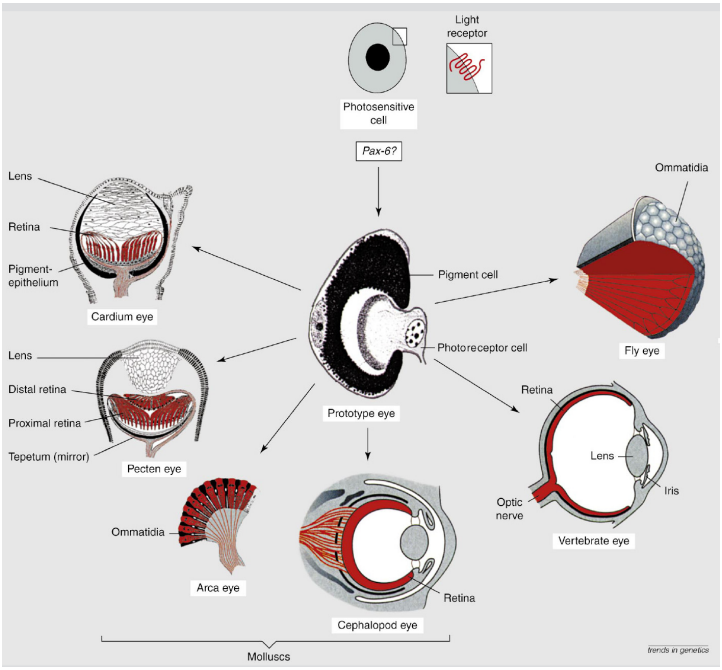

Figure 3 The prototype eye

A model for how various eye types have evolved from a common ancestral prototype. In the beginning a photosensitive cell with an opsin as a light receptor has evolved. A prototype eye has then formed under the control of the *Pax 6* gene when the photosensitive cell has assembled together with a pigment cell. By divergent, parallel and convergent evolution, the different eye-types have arisen. (From Gehring and Ikeo 1999)

5.2.3 Evolution of photoreceptors cells types

Animal photoreceptor cells have two distinct morphologies, the rhabdomeric photoreceptor cells that enlarge their membrane at the apical site and the ciliary photoreceptor cells that fold their cilia membrane (Eakin, 1968; Eakin, 1982). An important issue in the discussion of eye evolution is origin of the different photoreceptor cells. The evolution of photoreceptor cells has been highlight by reconstruction of the

eyes of the Urbilateria (Arendt and Wittbrodt, 2001). By their reconstruction Arendt and Wittbrodt suggested that two distinct photoreceptor cells already existed in the common ancestor of bilaterians, and this theory was further supported when ciliary photoreceptors with a vertebrate-type opsin were found in an invertebrate brain (rag worm *Platynereis dumerilii*) (Arendt et al., 2004). On the basis of their findings in rag worm a scenario for the evolution of the animal photoreceptor cells and eyes was proposed. They suggested that early metazoans had a single type of precursor photoreceptor cell with an ancestral opsin for light detection, and in pre-bilaterian ancestors this opsin has duplicated into two paralogues, c-opsin and r-opsin. This duplication allowed a diversification of the precursor photoreceptor cell into ciliary and rhabdomeric type. Rhabdomeric photoreceptor cells associated with pigment cells to form simple eyes and ciliary photoreceptor cells formed part of the evolving brain, active in non-directional photoreception. The invertebrate rag worm represents this ancestral setting with rhabdomeric photoreceptor cells in the eye and ciliary photoreceptor cells in the brain. During evolution of the vertebrates, both photoreceptor cell types have been incorporated into the retina. The rods and cones, responsible for vision in vertebrates are of ciliary type, while retinal ganglion cells, horizontal cells and amacrine cells have evolved from a common rhabdomeric photoreceptor cell precursor (Arendt, 2003; Arendt et al., 2004).

5.2.4 Phototransduction cascade of rhabdomeric and ciliary photoreceptor cells

Absorption of a photon by the chromophore in the photoreceptor cell results in a conformational change of the chromophore from 11-*cis* retinal to all-*trans* configuration. The subsequently phototransduction cascade differs in the rhabdomeric and ciliary photoreceptor cells (Figure 4) and in order to regenerate the photosensitive 11-*cis* isomer the photoreceptor cells use different approaches. In the ciliary photoreceptor cells the all-*trans* isomer is released form the binding pocket of the opsin protein and regeneration back to 11-*cis* isomer involves the action of retinal/retinol chaperone carrier proteins and

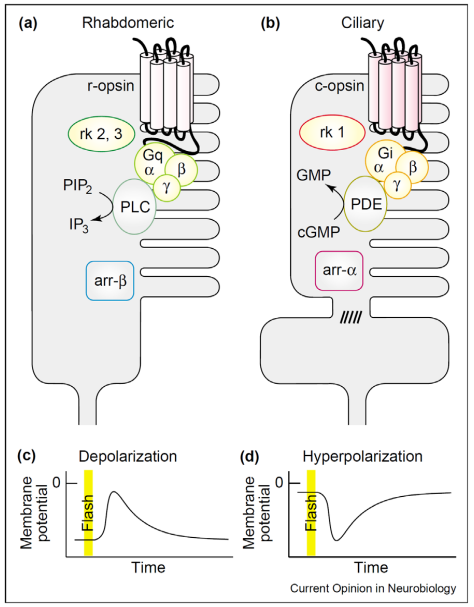

Figure 4 Phototransduction

The two photoreceptor cell types make use of different transduction cascades. A-B: Rhabdomeric photoreceptor cells expressing r-opsin are coupled to Gq protein phototransduction cascade that take use of the a phospholipase C enzyme (PLC) and the cascade leads to depolarization of the membrane. C-D: Ciliary photoreceptor cells expressing c-opsin involve Gi protein and use phosphodiesterase (PDE) in the phototransduction cascade leading to hyperpolarisation of the cell (Arendt, 2003; Arendt and Wittbrodt, 2001). (Figure from Nilsson, 2004).

isomerases, in a dark dependent manner. In rhabdomeric photoreceptor cells the all-*trans* isomer does not leave the binding pocket of the opsin protein but the isomer form a stable association in the all-*trans* state. The rhabdomeric photoreceptor cells are able to undergo photoreversal back to ground state in a light dependent manner either by an accessory photoisomerase or by the intrinsic isomerase activity of the r-opsin photopigment itself (Davies et al., 2010; Koyanagi et al., 2005; Lamb, 2009). A phylogenetic tree comparing

important structural and functional motifs of the opsins shows the phylogenetic course of r-opsin and c-opsin homologues (Figure 6). There are several important residues that differ. For instance the amino acid at position 113 that act as a counterion for the positively charged Schiff base (Sakmar et al., 1989) (covalently binding the opsin protein and the chromophore), the amino acid triad, the ERY motif, critical for G protein interaction (Franke et al., 1992), and the NPxxY$(x)_{5,6}$F motif that works in conjugation with the ERY motif in controlling the structural changes accompanied with photopigment activation (Fritze et al., 2003).

5.2.5 Classification of photoreceptors cells based on distribution and neural identity

Lately an additional classification of photoreceptor cells has been suggested to be able to describe a wider set of light sensitive neurons (Figure 5). The morphological classification with ciliary and rhabdomeric photoreceptor cell types does not fit for all photoreceptor cells. This is exemplified by the dermal photoreceptor cells that do not process either of the morphological modification. A new classification scheme sorting the photoreceptor cells by distribution and neuronal identity is suggested to be able to group all photoreceptor cells. Distribution of cells is described by aggregated or dispersed photoreceptor cells and neuronal identity is divided in first-order and high-order photoreceptor cells. First-order neurons are classified as primary sensory cells that transduce external stimuli into electrical signals and by synapses pass the signal to other neurons. High-order neurons are interneurons that have many synaptic connections and are able to both receive and send electrical signals, in addition they can receive external stimuli directly (Ramirez et al., 2011). In addition, Ramirez et al. (2011) propose three hypotheses for dispersed photoreceptor cell function and evolution that they confirm to some extent by examples from different phyla. First, animals often use dispersed photoreceptor cells for tasks that do not require spatial vision. Second, morphological specialisations such as membrane folding and pigmentation are generally absent in

dispersed photoreceptor cells. Third, dispersed photoreceptor cells have evolved several times in Metazoa and many have co-opted existing phototransduction pathways (Ramirez et al., 2011).

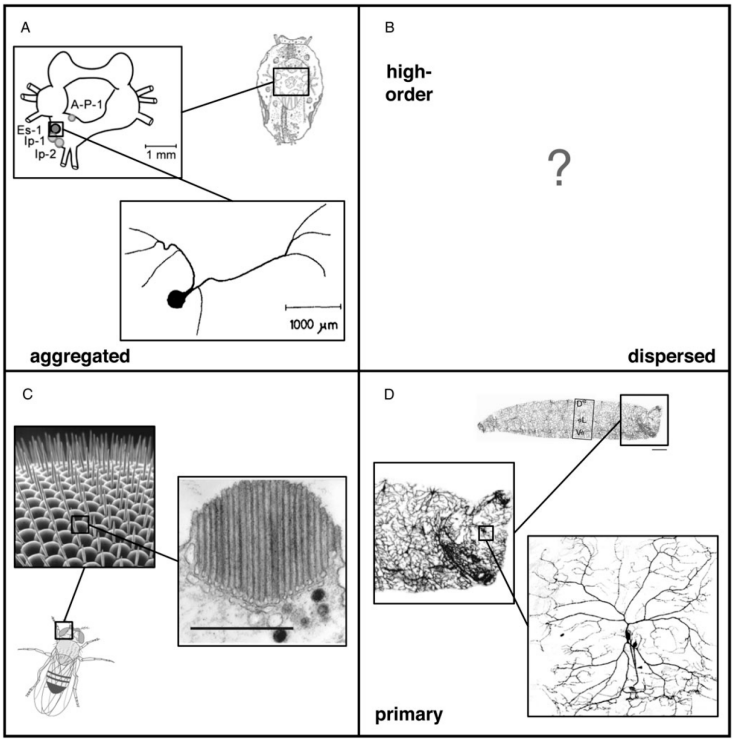

Figure 5 Photoreceptor cells classification by distribution and neuronal identity.
The classification is made up of four quadrants made by an x-axis of cell distribution in animals (aggregated *vs.* dispersed) and a y-axis of position of the cell in a neuronal network (first-order *vs*. high-order). A: Aggregated high-order neuron exemplified by a drawing of *Onchidium verruculatum*, abdominal ganglion with photosensitive neurons. B: Dispersed high-order neurons, no examples of this type described. C: Aggregated primary-order neuron illustrated by a single rhabdomere within an ommatidium of a compound eye of *Drosophila melanogaster* (Scale bar 1 µm). D: Dispersed primary order neurons shown by a single class IV dendritic arborisation neuron in a *Drosophila melanogaster* larva (Scale bar 200 µm). (From Ramirez et al. 2011)

5.3 Diversification of opsin families

5.3.1 Complexity of opsins

A diverse array of photoreceptor cells and photopigments exist in animals to mediate multiple responses to light. Figure 6 shows the different subfamilies of vertebrate opsins. The visual opsins, the cone opsin and rod opsin, located in the cone and rod photoreceptor cells respectively, are marked with a red box and make up just a branch of the c-opsins.

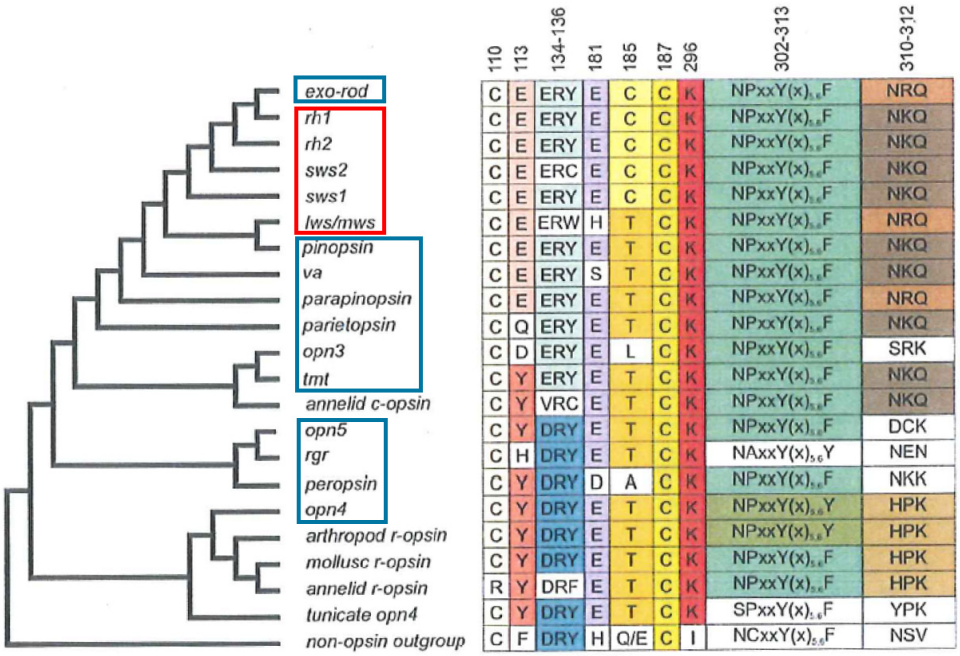

Figure 6 Phylogenetic tree of structural and functional important motifs in opsins
The tree shows the different vertebrate opsin subfamilies and how they branch together with invertebrate opsins of c-opsin or r-opsin origin. Vertebrate visual opsins are highlighted in a red box and vertebrate non-visual opsins in blue boxes. For every opsin clade a consensus sequence is indicated for important motifs, see text for more details. (Modified from Davies et. al 2010)

The other vertebrate opsins are non-visual opsins involved in non-image forming light detection and they are named exorhodopsin (*exo-rod*), pinopsin, vertebrate ancient opsin (*va*), parapinopsin, parietopsin, encephalopsin (*opn3*), teleost multiple tissue opsin (*tmt*), neuropsin (*opn5*), retinal G-protein receptor (*rgr*), peropsin and melanopsin (*opn4*). Of the various non-visual opsins, melanopsin is the only one branching together with the invertebrate r-opsin homologues. This thesis will focus on exorhodopsin, vertebrate ancient opsin and melanopsin and they will be described in detail below.

5.3.2 Exorhodopsin

The pineal is the primary source of the nocturnal hormone melatonin and thereby has an important role in regulation of the circadian clock to ensure that biological rhythms stay in synchronization with the Earth's rotation. In lower vertebrates the pineal contains photoreceptive cells that entrain an endogenous pineal circadian clock (Korf, 1994; Pierce et al., 2008). In the search for the molecular identity of the gene responsible for fish pineal photosensitivity exorhodopsin was identified in zebrafish (Mano et al., 1999). Subsequently the same gene was found in other teleosts, namely the medaka and the European eel (Mano et al., 1999), in Atlantic salmon and puffer fish (Philp et al., 2000a). A later study on zebrafish has elucidated potential roles of exorhodopsin in the pineal of teleosts. Exorhodopsin has an important role in regulating gene transcription in the pineal both for itself and for the melatonin synthesis gene aryl-alkylamine N-acetyl transferase 2 (*aanat2*). The same study showed that exorhodopsin is expressed within a daily rhythm where the expression is higher during night then during the day. Expression of exorhodopsin was found to be activated by the Orthodenticle homeobox 5 (Otx5) transcription factor and the activation of Otx5 was shown to be repressed during the day by the putative circadian clock component Period 3 (Pierce et al., 2008).

Phylogenetic analyses show that exorhodopsin has higher similarities to the vertebrate rod opsin than to non-visual opsin types. It was first assumed that exorhodopsin was a true

paralouge of rod opsin generated by a duplication event within the ray-finned fish lineage (Mano et al., 1999). Subsequent studies of the evolutionary lineage of the teleost exorhodopsin have indicated that exorhodopsin is rather a true orthologue of the vertebrate rod opsin (Bellingham et al., 2003). This is argued by the findings of the teleost rod opsin being intronless (Fitzgibbon et al., 1995) and the teleost exorhodopsin exhibiting the five exon/four intron structure as the rod opsin gene of the vertebrate class (Bellingham et al., 2003). It is further indicated that the intronless teleost rod opsin has been generated through an ancient retrotransposition of a mature mRNA derived from exorhodopsin and that this duplication event occurred early in the evolution of ray-finned fish (Bellingham et al., 2003).

5.3.4 Vertebrate ancient opsin

Vertebrate ancient opsin (VA opsin) was first identified from ocular tissue in Atlantic salmon and phylogenetic analysis suggested it to be a member of a new opsin family that diverged early in the evolution of vertebrate pigments (Soni and Foster, 1997), and thereby the name. More updated phylogenetic analyses of the opsin families known today have shown that even though VA opsin seems to predate for instance the visual opsins other opsin families appear to be more ancestral (e.g. encephalopsin, neuropsin, parapinopsin and teleost multiple tissue opsin) (Davies et al., 2010). VA opsin was subsequently identified in different teleosts (Grone et al., 2007; Jenkins et al., 2003; Kojima et al., 2000; Kojima et al., 2008; Minamoto and Shimizu, 2002; Moutsaki et al., 2000) and searches in the genome databases have identified VA opsin in vertebrate classes such as amphibians, reptiles and birds (Davies et al., 2010; Halford et al., 2009). However database searches for VA opsin in the three main mammalian lineages have not given any results and it has been suggested that the gene was lost early in evolution of modern mammals (Davies et al., 2010). Further on, sequence and phylogenetic analysis have shown that VA opsin is in the same subfamily as the vertebrate visual opsins (Terakita, 2005) and is clearly related to the c-opsin linage of photopigments (Davies et

al., 2010). Many of the VA opsin genes identified have different isoforms, varying in the length of the carboxyl-tail and the isoforms can be describes as *val* (long) and *vas* (short) (Davies et al., 2010). In zebrafish there exist one gene with two isoforms, long (*valopa*) and short (*vas*), and in addition a second VA gene (*valopb*) (Kojima et al., 2000; Kojima et al., 2008).

In situ hybridisation studies have revealed the expression pattern of VA opsin in several species. In Atlantic salmon retina VA opsin is expressed in horizontal and amacrine cells (Soni et al., 1998) and in the brain expression is detected in the pineal, habenula/ subhabenula and dorsal thalamus (Philp et al., 2000b; Sandbakken et al., 2012). Expression studies of different developmental stages in zebrafish showed VA opsin positive cells in the eye, forebrain, midbrain, hindbrain and spinal cord (Kojima et al., 2008). In chicken VA opsin has been detected within a population of hypothalamic neurons with projections to the median eminence. The study has indicated an important role of VA opsin in mediating the photoperiodic response (Halford et al., 2009).

5.3.3 Melanopsin

Melanopsin was first identified from photosensitive dermal melanophores of *Xenopus laevis* in an attempt to identify an opsin that may regulate the melanosome migration (Provencio et al., 1998). Subsequently homologues were identified in mammals and showed to be expressed in retinal ganglion cells projecting to the suprachiasmatic nucleus of the hypothalamus, important for the regulation of the circadian rhythm (Berson et al., 2002; Gooley et al., 2001; Hattar et al., 2002; Provencio et al., 2000). The central role of melanopsin in entrainment of the circadian clock has been further confirmed by several studies. Knockout studies in mice lacking melanopsin photoreceptors have shown that melanopsin seems to be a significant contributor to the photoentrainment in mammals, but are not essential for the circadian clock to receive photic input (Panda et al., 2002; Ruby et al., 2002). However, mice lacking rod, cone and melanopsin photoreceptors fail to

show any significant pupil reflex. They are not able to entrain to the light/dark cycles and they do not show any masking response to light (Hattar et al., 2003; Panda et al., 2003). Sequence analyses have shown that melanopsin share greater homology with invertebrate opsin than with vertebrate opsin and melanopsin is believed to be of r-opsin origin (see Figure 6). This is reflected in several domains thought to have a functional significance. The putative counterion for the chromophore attachment site is tyrosine (Y-103) rather then a glutamate counterion (E-113) common in vertebrate opsins (Provencio et al., 1998) In the cytoplasmatic loops, shown to have important roles in G-protein activation, melanopsin has higher similarities with invertebrate opsin and unlike visual opsins melanopsin has a long cytoplasmatic tail (Nayak et al., 2007).

After its discovery, melanopsin has been described in all vertebrate classes. The identified genes were considered to be orthologues until it was discovered that the vertebrates have evolved two quite separate melanopsins. Based on sequence similarities, chromosome localisation and phylogenetic analysis the melanopsins were grouped as mammalian-like melanopsins (*Opn4m*) and *Xenopus*-like melanopsins (*Opn4x*) (Bellingham et al., 2006). The two melanopsins are believed to have arise after a gene duplication event from a single ancestral melanopsin (Bellingham et al., 2006) presently conserved in a chordate lancelet (Koyanagi et al., 2005). The *Xenopus*-like melanopsin appears to be lost in mammals and further revision suggests that the gene was lost from the mammalian lineage before the placental/marsupial split (Pires et al., 2007). In teleosts the representation of melanopsins is even more complex due to an additional whole-genome duplication event. The duplication is believed to have happened early in the evolution of ray-finned fish, most likely before the derivation of the teleosts (Christoffels et al., 2004; Jaillon et al., 2004; Naruse et al., 2004).

In situ hybridisation studies on different duplicates of melanopsin in teleosts have shown a diverse expression pattern in both retina and brain. In the adult retina of zebrafish melanopsin is expressed in all main cell types and the duplicates show a discrete

expression profile (Davies et al., 2011). Expression of melanopsin in Atlantic cod retina has been detected in amacrine, ganglion and horizontal cells and in the brain melanopsin is expressed in the suprachiasmatic nucleus and in the habenula (Drivenes et al., 2003). A study in Atlantic salmon further elucidates the expression of melanopsin in the suprachiasmatic nucleus and in the habenula of teleosts. In addition the Atlantic salmon brain has melanopsin expressing cells in the dorsal thalamus and in the nucleus lateralis tuberis of the hypothalamus (Sandbakken et al., 2012). (See Figure 11)

5.4 Non-image forming photoreception in vertebrates

5.4.1 Visual and non-visual photoreception

Photoreception is not limited to image formation and vision, but includes also non-image photoreception responsible for non-directional processes such as entrainment of the circadian and circannual rhythms. Mammals are dependent on ocular photoreception to adapt to changes in the ambient light as they seem to have lost their extra ocular photoreceptors during evolution. Non-mammalian vertebrates on the other hand have photoreceptor cells in a wide rage of tissues including dermal melanophores, pineal organ and deep brain cells (Davies et al., 2010). In non-mammalian vertebrates the pineal contains photoreceptive cells that entrain an endogenous pineal circadian clock (Korf, 1994). Figure 7 illustrates how the photoreceptor cells of retina, brain and pineal may interact to entrain the circadian rhythm in teleosts compared to mammals.

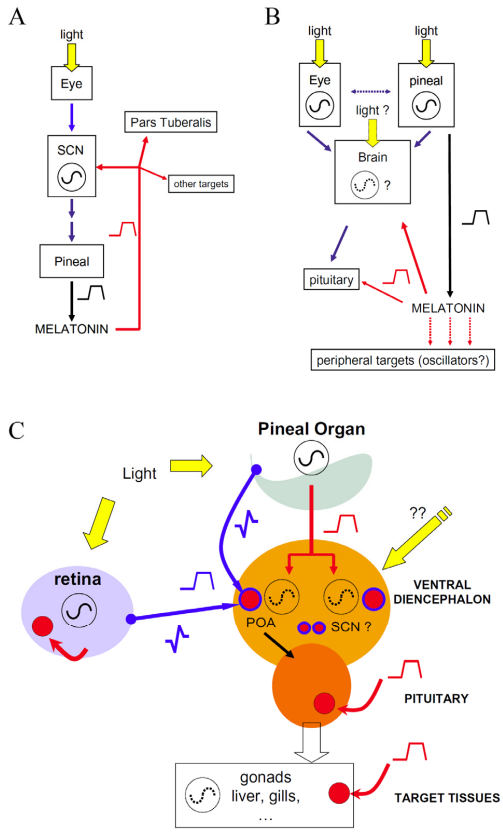

Figure 7 Circadian rhythm

A: In mammals the photic information from non-visual photoreception in the eye reaches the suprachiasmatic nucleus (SCN) through the retinohypotalamic tract (RHT) and the SCN controls the cyclic melatonin secretion from the pineal organ by a multisynaptic pathway. Melatonin feeds back to the SCN and act on the pars tuberalis of the pituitary and other brain targets modulating seasonal neuroendocrine functions. B: In fish photic input comes from multiple pathways, including eye, pineal and most likely the brain. The pineal itself stands for photoreception, production and release of melatonin. C: Photoneurocrine regulation in fish in detail. The photic information reaches the ventral diencephalon from retina and pineal through the RHT and pineal tract, respectively, and in addition perhaps from deep brain photoreceptor cells. The information gives an indication of the length of the day. Production of melatonin is reflected on the length of the day and the season and the hormone information is relayed by melatonin acting on several targets. (From Falcon et al. 2010) POA, preoptic area

5.4.2 The vertebrate retina

The vertebrate retina is an evolutionary conserved photoreceptive organ developed from the embryonic forebrain, consisting of different cell types organized in layers (Pujic and Malicki, 2004) (Figure 8). In the outer nuclear layer of retina the rod and cone photoreceptor cells are situated, mediating image formation and vision. Rod photoreceptor cells have cylinder shaped outer segments, containing rod opsin, which is the visual pigment responsible for dim-light vision. Cone photoreceptor cells have cone-shaped outer segment with cone opsin responsible for bright light vision that enables colour vision. Retinal ganglion cells of the inner retina receive input from the rod and cone photoreceptors and transmit the information to the brain through the optic nerve (Lamb, 2009). A small subset of the retinal ganglion cells are directly photosensitive and express the photopigment melanopsin. In mammals melanopsin is expressed in the cell bodies, in the proximal axons and throughout the dendrites of the intrinsically photosensitive retinal ganglion cells. These cells are known to structure the retinohypothalamic tract innervating the primary circadian peacemaker, the suprachiasmatic nucleus (Berson et al., 2002; Hattar et al., 2002). Bipolar cells of the inner nuclear layer relay information from the rod and cones to the retinal ganglion cells. In addition horizontal and amacrine cells are responsible for the lateral interactions in the retina (Lamb, 2009). In teleost it has been shown that also subsets of horizontal cells are intrinsically photosensitive. The responses to light in these horizontal cells are extremely slow, lasting for minutes. It is suggested that they are involved in a long time scale modulation of the image-forming vision. Both melanopsin and VA opsin are indicated as candidates for the photoreceptive response in the horizontal cells (Cheng et al., 2009; Jenkins et al., 2003) . Lately it has been shown that melanopsin is expressed in all retinal layers in zebrafish and it has been suggested that the teleost retina may be globally light sensitive (Davies et al., 2011).

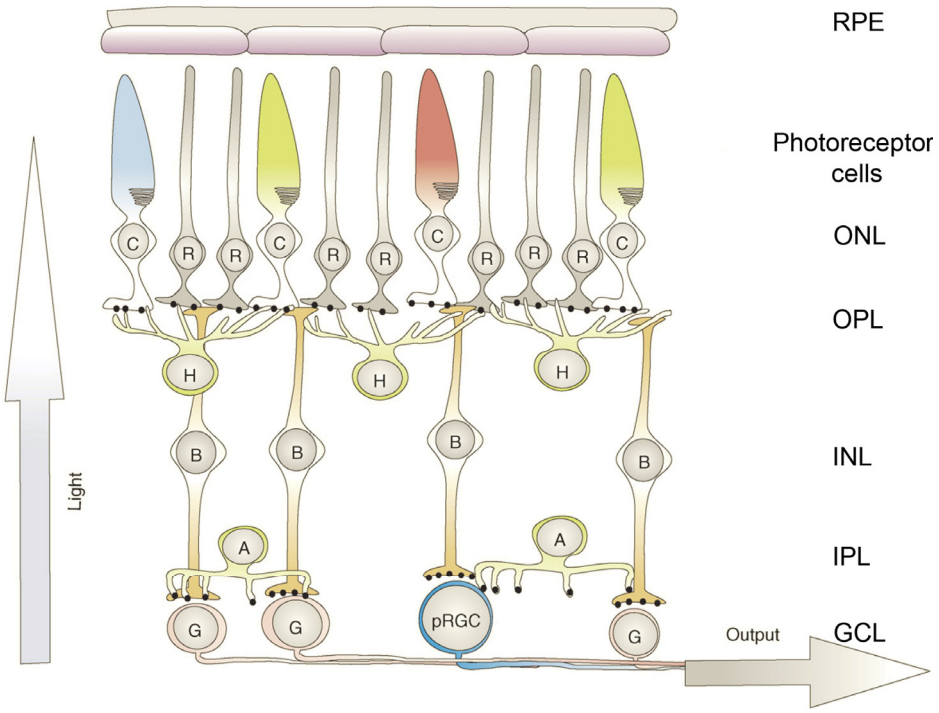

Figure 8 Structure of the vertebrate retina

The light passes through the different retinal layers to reach the rod (R) and cones (C) of the image-forming pathway. The rod and cone photoreceptor cells are located close to the retina pigment epithelium (RPE) and transmit visual information to the ganglion cells (G) via the bipolar cells (B). In addition, the horizontal cells (H) facilitate the connectivity and feedback to the photoreceptor cells and the amacrine cells (A) allowing lateral connections between the bipolar cells and ganglion cells. Axons of the ganglion cells form the optic nerve. A subset of ganglion cells detects light directly using the photopigment melanopsin (see text for detail). (Modified from Hankins et al., 2008) GCL, ganglion cell layer, INL, inner nuclear layer, IPL, inner plexiform layer, ONL,outer nuclear layer, OPL, outer plexiform layer, pRGC, photosensitive retinal ganglion cell.

5.4.3 Photoreception in the pineal organ

In many species the pineal organ is located below a window in the skull where the light can enter and it appears as a vesicle attached to the roof of diencephalon by a stalk. The pineal epithelium of teleosts consists of photoreceptor cells, neurons with projections to the brain and supportive cells (Figure 9) (Ekström and Meissl, 1997; Falcón et al., 2010). Structural and functional the pineal organ of non-mammalian vertebrates resembles a simplified retina. At the level of photoreceptor cells this includes the presence of cone- or rod-like outer segments, molecules of the phototransduction cascade such as opsin, transducin, S-antigen and recoverin, and in addition presence of cyclic GMP-gated channels (Meissl, 1997).

The pineal organ is the site of production and release of the nocturnal hormone melatonin which is a major output of the circadian clocks of vertebrates. Melatonin is produced at night in a dose-dependent manner and is relying on aryl-alkylamine N-acetyltransferase (AANAT) activity. Light inhibits AANAT by proteasomal protelysis, and as AANAT is degradated during daylight the production of melatonin decreases (Falcón et al., 2010). Different opsins have been shown to be expressed in the pineal organ of different teleosts, including VA opsin (Philp et al., 2000b), exorhodopsin (Mano et al., 1999; Philp et al., 2000a), red cone opsin (Mano et al., 1999) and parapinopsin (Koyanagi et al., 2004). Exorhodopsin in zebrafish has been shown to have an important role in regulating gene transcription of *aanat* (Pierce et al., 2008).

Bidirectional connections between the pineal and the brain go trough the pineal stalk and these connections have been elucidated by using anterograde and retrograde tract-tracing techniques in different fish classes. The neural tract-tracing methods have shown bilateral projections to the pretectal and dorsal thalamic cell groups, to ventral thalamic and hypothalamic cell groups, to the habenular nucleus and the preoptic region, and to caudal regions such as mecencephalic tegmentum, torus semicircularis, optic tectum and rostral

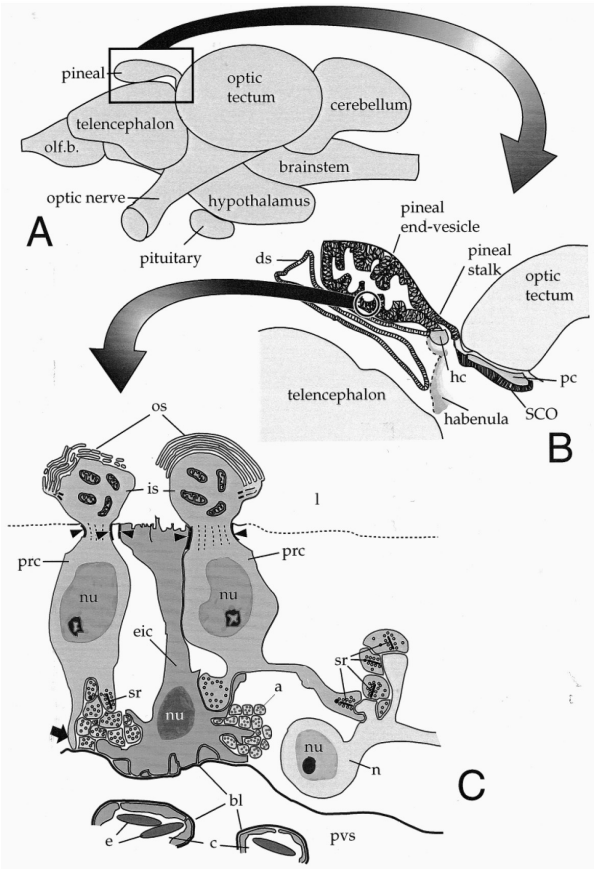

Figure 9 The pineal organ

A: Localization of the pineal organ in the roof of diencephalon. B: The pineal organ is a vesicle attached to the diencephalon through the pineal stalk. Habenula is situated ventral to the pineal. C: Schematic drawing of the cell types in the epithelium of the pineal vesicle. Abbreviations: a, axons of pineal neurons, bl, basal lamina, c, capillaries, ds, dorsal sac, e, erythrocytes, eic, ependymal interstitial cells, hc, habenula commissure, is, photoreceptor inner segment with mitocondria, l, pineal lumen, n, centrally projecting neuron, nu, nucleus, olf. b, olfactory bulb, os, photoreceptor outer segment, pc, posterior commisure, prc, photoreceptor cells, pvs, perivascular space, SCO, subcommissual organ, sr, synaptic ribbons (From Ekstrom and Meissl 1997)

raphe nuclei (Ekström and Meissl, 1997) (Figure 10). Some of these brain regions overlap with areas that also are connected to the retina and makes a possible pathway for exchange of information between the retina and pineal (Ekström and Meissl, 1997; Falcón et al., 2010). Figure 10 illustrates that many of the brain regions with projections from the pineal and retina also have expression of non-visual opsins. The non-visual opsins are exemplified by vertebrate ancient opsin and melanopsin positive cells in the Atlantic salmon brain.

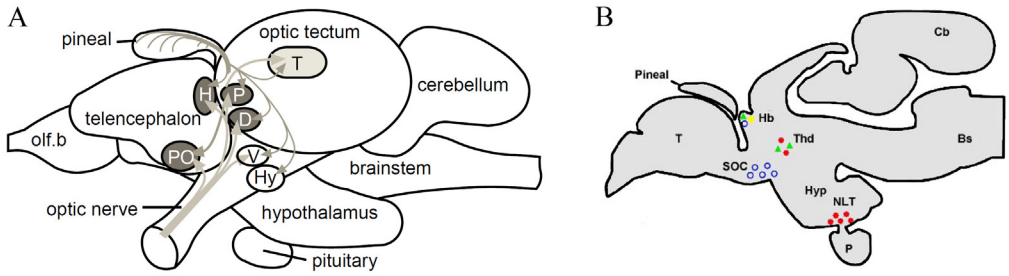

Figure 10 Brain regions with projections from the pineal and retina have expression of non-visual opsins. A: Schematic drawing of the teleost brain showing that there are several brain areas that receive input from both the brain and retina. (The retinotectal pathway is not shown.) In the dark areas, habenula (H), pretectal area (P), dorsal thalamus (D) and the preoptic area (PO), the terminal fields of the pineal and retina axons overlap. (Figure from Ekstrom and Meissel 1997) Hy, perventricular hypothalamus, olf. b, olfactory bulbs, T, mesencephalic tegmentum B: Expression of different melanopsins and vertebrate ancient opsin in the Atlantic salmon brain. The genes are expressed in multiple sites in the brain, Habenula (Hb), dorsal thalamus (Thd), supraoptic/ suprachiasmatic nucleus (SOC) and nucleus lateralis tuberis (NLT). Many of these brain regions also have projections from the pineal and retina. Cells expressing the different opsins are represented by colours: *opn4x1b1*/*opn4x1b2* (blue), *opn4x1a* (yellow), *opn4ma1*/*opn4m1a2* (red), VA opsin (green) (Modified from Sandbakken et al. 2012). Bs, brainstem, Cb, cerebellum, Hyp, hypothalamus, P, pituitary, T, telencephalon

5.4.4 Photoreception in the deep brain cells

The existence of deep brain photoreceptor cells in fish was already indicated in 1911 by Karl von Frisch as a result of studies on blinded and pinealectomized minnows. In the absence of retinal and pineal input, these fishes still changed colour in response to light and the presence of deep diencephalic photoreceptors was suggested (Frisch, 1911). Studies on house sparrows gave the first results that indicated a role of deep brain photoreceptor cells in circadian entrainment, when it was shown that removal of the eyes and pineal did not block photoentrainment (Menaker and Underwood, 1976). More recently studies have identified photoreceptor cells and their photoreceptive pigments in many different regions of the non-mammalian vertebrate brain. In birds vertebrate ancient opsin (VA opsin) has been localized in hypothalamic neurons with projections to the median eminence and a role in mediating photoperiodic response has been indicated (Halford et al., 2009). In fish VA opsin positive cells are found in epithalamic and hypothalamic regions of the Atlantic salmon brain (Philp et al., 2000b). In Atlantic cod melanopsin has been showed to be expressed in the suprachiasmatic nucleus and in the habenula (Drivenes et al., 2003). A recent study in Atlantic salmon demonstrates expression of different melanopsin genes and VA opsin in multiple regions of the brain (see Figure 10). The results show that melanopsin expressing cell populations are located in regions associated with different neuroendocrine systems. It is suggested that melanopsin photoreceptors can be involved in signalling of photoperiodic information trough multiple pathways (Sandbakken et al., 2012). Deep brain photoreceptor cells have also been implicated in behavioural response to light. Previous studies have shown photonegative behaviour in European eel (van Veen et al., 1976) and photo-behavioural responses in catfish (Tabata et al., 1989). Functional studies in zebrafish lacking eyes and pineal have recently identified melanopsin expressing neurons in the preoptic area as the photosensitive region in light-seeking behaviour, triggered by loss of illumination (Fernandes et al., 2012).

5.5 Early life history of Atlantic halibut (*Hippoglossus hippoglossus*)

5.5.1 Advantage of Atlantic halibut in studies of non-visual photoreception

In the study of early non-visual photoreception in teleosts the Atlantic halibut provides a great advantage. Halibut larvae have a period of about a month before the eyes become functional (Kvenseth et al., 1996) providing a good opportunity to study non-visual photoreception at early stages of development without any input from the retina. In addition, the halibut embryos and larvae have been shown to be photosensitive from early stages of development (Flamarique, 2002; Helvik and Walther, 1992; Mangor-Jensen and Waiwood, 1995), offering a good opportunity to relate non-visual opsin expression to behavioural or physiological processes.

5.5.2 Early life stages of Atlantic halibut and influence of light

Atlantic halibut is a valuable species for human consumption and is seen as a very promising species for aquaculture. In 2012 the commercial sale of halibut in Norway was 1741 metric ton (reported by the Norwegian directorate of fisheries). This is a result of a huge research and cultivation effort during the last two decades. In Norwegian coastal waters the flatfish Atlantic halibut spawns at 300-700 meters depth during winter season and the pelagic eggs are gradually moving upwards in the water column after fertilisation (Haug, 1990). At the coast of Northern Norway the highest distribution of eggs were found in 100-250 meters depth at temperatures of 4,5-7,0 °C and salinities of 33,8-35% (Haug et al., 1984). In the dark winter season the eggs are only exposed to low light intensities deep down in the sea. Still it has been shown that light has an important impact of the Atlantic halibut eggs. Light affects buoyancy of the eggs already at four days post fertilisation. In light exposed eggs the density is increased and it is an increase in the perivitelline space compared to eggs reared in darkness. The reason for this phenomena is not clear but it has been proposed that it ensures surface avoidance in order to keep away from turbulence (Mangor-Jensen and Waiwood, 1995). Halibut eggs hatch at an early

developmental stage (approximately 14 days post fertilisation) when the eyes and skin are unpigmented and the hatched embryos have a big yolk sack and a primitive body (Haug, 1990; Lönning et al., 1982). At hatching the unpigmented eyes consist of neuroblastic cells and the larvae develop for 25 days before the eyes become functional (Kvenseth et al., 1996). Studies have shown that halibut eggs hatch in darkness and it has been suggested that light may synchronize hatching in such a way that it occurs at the first night after the larvae reach the developmental competency for hatching. In addition, it has also been shown that if light-arrested eggs are placed in darkness they will hatch rapid and synchronized within 80-140 minutes (Helvik and Walther, 1992). Further on, it has been shown that larvae without functional eyes have a positive phototaxis as a response to light exposure (Flamarique, 2002). Figure 11 gives more details on the ontogeny of Atlantic halibut.

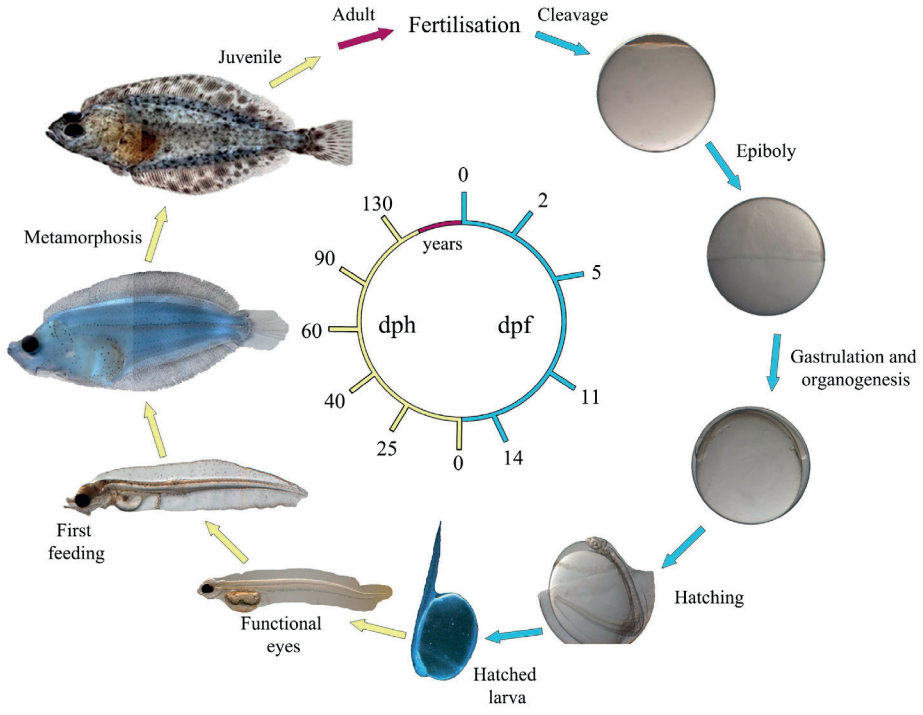

Figure 11 Ontogeny of Atlantic halibut.

After fertilisation the emerging halibut embryo undergoes cleavage, epiboli, gastrulation and organogenesis. Approximately 11 days after fertilisation the rudiment of all main structures in the embryo is visible, including eye, brain, heart and somites. Dependent on the temperature the egg hatches in darkness around 14 days post fertilization (dpf), but the eyes are not fully pigmented and functional before 25 days after hatching (dph). At hatching the halibut embryo has a huge yolk-sac and the yolk-sac phase lasts at least a month after hatching before all the vital organs necessary for becoming a predator are functional. In aquaculture the larva is first feed around 40 dph. The larva undergoes metamorphosis around 60-90 dph becoming a juvenile flatfish around 130 dph (Kvenseth et al., 1996). It takes several years before the halibut is sexually mature, depending on growth rate and size (Haug, 1990).

6. Aims of the study

The overall aim of this work was to give better understanding of non-visual photoreception in teleosts with special focus on early stages of development. The marine flatfish Atlantic halibut has a long pre-retinal period in the open sea when the brain is transparent and unpigmented and the thesis aimed to study the appearance of non-visual systems during this period. Previous studies in teleosts have indicated co-localisation of non-visual opsins of assumed different evolutionary heritage and the thesis also aimed to elucidate this in detail. In halibut the hatching process is shown to be light-regulated and the thesis intended to take advantage of this pre-retinal photosensitive process to study non-visual photoreception.

7. Summary of results

Paper I

In this paper melanopsin and exorhodopsin was identified in Atlantic halibut. Sequence and phylogenetic analyses showed that the four melanopsins identified belong to different subgroups, the mammalian-like melanopsins (*Opn4m*) and the *Xenopus*-like melanopsins (*Opn4x*). The results show that the halibut have two melanopsins in each group and this is in accordance with other teleosts and is probably a result of a whole-genome duplication event early in the evolution of ray-finned fish.

This paper demonstrates a complex expression pattern of melanopsins early in development of Atlantic halibut. Already at the light-regulated hatching process the melanopsin expression is refined and extensive both in the brain and spinal cord and the pineal specific exorhodopsin is also strongly expressed at this stage. Melanopsins were identified in several brain regions at the stage of hatching and the results show melanopsin positive cells in the pineal complex, ventral in diencephalon, in the optic tectum, tegmentum and in rostral and caudal parts of the hindbrain. Further on, the expression of melanopsins and exorhodopsin persist in the brain when the eyes are functional and expression of melanopsins can be detected in different retinal layers. At first feeding when the eyes are functional, melanopsins are detected in habenula, suprachiasmatic nucleus, dorsal thalamus and nucleus lateralis tuberis and these results are consistent with findings in juvenile Atlantic cod (Drivenes et al., 2003) and Atlantic salmon (Sandbakken et al., 2012). In addition melanopsins are detected in the pineal complex, preoptic area, ventral thalamus, optic tectum, tegmentum and the hindbrain of halibut larvae at first feeding.

In the retina, one mammalian-like melanopsin was detected and the expression was localised to the ganglion cell layer and the inner nuclear layer probably in amacrine cells and in horizontal cells. Both *Xenopus*-like melanopsins were expressed in the retina and *opn4x1* was shown to be in the ganglion cell layer and inner nuclear layer while *opn4x2* was detected in the inner nuclear layer. In the inner nuclear layer *opn4x1* expression is most likely in amacrine cells while *opn4x2* is expressed in a diffuse manner in cells resembling bipolar cells and in cells close to the outer plexiform layer that are almost certain horizontal cells.

The results also show that both exorhodopsin and melanopsin are expressed in the pineal complex at hatching and first feeding. Fluorescent double labelling technique demonstrates that the melanopsin positive cells flank the expression of exorhodopsin and that the two genes are expressed in cells adjacent to each other. It is suggested that existence of two different photoreceptor systems in the pineal complex may activate photoreception by different light spectra, or melanopsin may also function as a photoisomerase providing 11-*cis* retinal to exorhodopsin. In addition, an axonal connection between the exorhodopsin expressing pineal and deep brain cells is shown.

Taken together this work demonstrates a complex expression of non-visual opsins already at an early developmental stage and the results show co-localisation of melanopsin and exorhodopsin in the pineal complex of Atlantic halibut. This work strongly pin point the importance of the non-visual system in early stages of teleost when the uncovered and transparent brain is directly exposed to the environmental light.

Paper II

This paper identifies two isoforms of vertebrate ancient opsin (VA opsin) in Atlantic halibut. In consistence with other species the isoforms vary in the length of the carboxyl-tail. The paper does not identify an additional VA opsin gene as seen in zebrafish although extensive search in the halibut genome has been performed.

Results from this paper show that VA opsin is expressed early in development of Atlantic halibut. At the stage of the light-regulated hatching process VA opsin is detected in ventral diencephalon and in a bilateral cluster in the hindbrain. The results show that the hindbrain cluster is ball-shaped with VA opsin negative cells in the middle. At first feeding when the eyes are functional VA opsin is expressed in the dorsal and ventral thalamus, optic tectum, tegmentum and in the hindbrain. In retina VA opsin positive cells are detected in the inner nuclear layer in cells resembling amacrine cells.

This paper shows that both a mammalian-like melanopsin and VA opsin are expressed in the bilateral hindbrain cluster at the stage of hatching. This cluster of aggregated cells is localised just caudal to the midbrain-hindbrain boundary at the same level as the neuromasts. Fluorescent double labelling *in situ* hybridisation shows that the two genes of assumed different evolutionary heritage are expressed in the same cells in the hindbrain cluster, with some cells in the aggregated cluster just expressing one of the opsins. The work demonstrates for the first time in vertebrates that mRNAs of both photopigments are expressed within the in the same cells.

Altogether the work demonstrates that VA opsin is expressed early in development of Atlantic halibut and proves further complexity to the non-visual system in the uncovered and transparent halibut brain. Lately the evolution of photoreceptor cells has been debated and this paper contributes with valuable information, revealing dual photopigment expression in cells of a bilateral hindbrain cluster.

Paper III

This paper describes the bilateral hindbrain cluster expressing both melanopsin and vertebrate ancient (VA) opsin at the light-regulated hatching process of Atlantic halibut. The results demonstrate that the hindbrain cluster is a transient cluster with strong expression before hatching. At hatching the melanopsin expression has started to fade and a few days after hatching the melanopsin expression has disappeared form the cluster. VA opsin expression is strong at hatching but after hatching this expression also scatters and disappears.

Further on, the results show that the hindbrain cluster is connected to a neuronal network with projection out in the yolk sac. The projection is shown to bend off laterally just rostral to the neuromast and then extend out in the yolk sac as a fan. Following the projections it is shown that they reach and extend the narrow belt of hatching glands and they are in close contact with the glands.

Induction of hatching in light-arrested eggs demonstrates that the immediate early gene *c-fos* is activated in the bilateral hindbrain cluster and in the hatching glands. The expression of *c-fos* is detected in eggs sampled 120 minutes after the eggs were put back to darkness. Expression of the immediate early gene *c-fos* is known to be activated quick and transient in response to stimulation of nerve cells and *c-fos* expression in the photosensitive hindbrain cluster and in the hatching glands indicates neural activity as a response to darkness. Further on, the activation of the neural circuit in darkness implies that the net output of melanopsin and VA opsin is inhibitory in the system.

Taken together the work of this paper indicates that hatching in Atlantic halibut is under neuronal regulation and that the photoreceptive transient hindbrain cluster expressing both melanopsin and VA opsin is involved in the light-regulated hatching process of halibut.

8. General discussion

This work aimed to study non-visual photoreception in early life stages of Atlantic halibut and the resulting thesis describes a complex non-visual system from early in development, when the eyes are not functional. The work emphasises the importance of the non-visual system in early life stages of teleosts when the transparent brain is directly exposed to the environmental light. The spatial expression of non-visual opsins in the brain and retina is demonstrated and brain regions with co-localisation of non-visual opsins have been described in detail in this thesis. In addition, a photosensitive hindbrain cluster, expressing two non-visual opsins, is indicated to synchronise the light-regulated hatching process of halibut.

8.1 Non-visual photoreception in teleosts

When this study was initiated the complexity of non-visual opsins in teleosts had just started to emerge. Several subfamilies of non-visual opsins had been described in vertebrates and teleost orthologues had been identified and described to some extent by expression studies (Drivenes et al., 2003; Kojima et al., 2008; Mano et al., 1999; Moutsaki et al., 2003; Philp et al., 2000b). Lately a more detailed characterisation of teleost non-visual opsins with expression domains in the brain (Fischer et al., 2013; Sandbakken et al., 2012) and retina (Davies et al., 2011; Matos-Cruz et al., 2011) has been given. However, little information on early life stages of teleosts when the unpigmented brain is directly exposed to environmental photons can be found in the literature. The information given is mainly on the freshwater species zebrafish (Fernandes et al., 2012; Kojima et al., 2008; Matos-Cruz et al., 2011) but this thesis reveals a complex and extensive expression of non-visual opsins in early life stages of a marine teleost. Further on, functions of the non-visual opsins have mainly been related to mammals and circadian rhythm (Gooley et al., 2001; Hattar et al., 2003; Panda et al.,

2002; Provencio et al., 2000), although the existence of additional photoreceptive capacity in the teleost brain was early indicated. This was shown by studies of blinded and pinealectomized European minnows (Frisch 1911) and later by photonegative behaviour in European eel (van Veen et al., 1976) and photo-behavioural responses in catfish (Tabata et al., 1989). Recently, more detailed knowledge of how non-visual opsins in teleosts contribute to behavioural and physiological responses to light has been reported (Fernandes et al., 2012; Flamarique, 2002; Kokel et al., 2013; Mangor-Jensen and Waiwood, 1995; Shiraki et al., 2010; Villamizar et al., 2013; Villamizar et al., 2012; Zhang et al., 2010) (Table 1). As a continuation, this study indicates that the hindbrain is responsible for the light-regulated hatching process in Atlantic halibut.

Table 1Early behavioural/physiological processes regulated by light

Behavioural/ Physiological process	Region	Opsin	Species (ref)
Light regulated hatching	Hindbrain	VA opsin, melanopsin	Halibut (Paper III)
Regulation of buoyancy of eggs by light			Halibut (Mangor-Jensen and Waiwood 1995)
Positive phototaxis (swimming) as a result of light exposure			Halibut (Flamarique 2002)
Light induced dispersion of skin pigments	Melanophores		Zebrafish (Shiraki et al. 2010)
Background adaptation ("camouflage response")	Pineal or retina		Zebrafish (Zhang et al. 2010)
Light-seeking behaviour as a response to loss of illumination	Preoptic area	melanopsin	Zebrafish (Fernandes et al. 2012)
Photomotor response eclicted by light	Hindbrain		Zebrafish (Kokel et al. 2013)
Diurnal hatching			Zebrafish (Villamizar et al. 2012)
Nocturnal hatching			Senegalese sole (Villamizar et al. 2013)

8.2 Non-visual opsins in teleosts

This thesis has focused on thee subfamilies of the non-visual opsins, the melanopsins with r-opsin origin and the c-opsin homologues vertebrate ancient opsin and exorhodopsin. In non-mammalian vertebrates two distinct groups of melanopsins are described, the mammalian-like melanopsins (*Opn4m*) and the *Xenopus*-like melanopsins (*Opn4x*) (Bellingham et al., 2006). In this thesis two Atlantic halibut melanopsins have been identified in each group (Paper 1). This work has also identified one VA opsin gene with two isoforms differing in the carboxyl-tail (Paper 2) and the pineal specific exorhodopsin in halibut (Paper 1).

8.2.1 Melanopsin, vertebrate ancient opsin and exorhodopsin in the brain of teleosts

Lately a study has demonstrated expression of melanopsins in juvenile Atlantic salmon (Sandbakken et al., 2012) and the results are consistent with studies in juvenile Atlantic cod showing expression in the habenula and suprachiasmatic nucleus (Drivenes et al., 2003). Salmon melanopsin was shown to be co-localised with serotonergic cell populations of left habenula and melanopsin was also detected in dorsal thalamus and in the nucleus lateralis tuberis, known to be the main source of hypothalamic innervations of the pituitary in teleosts (Anglade et al., 1993; Ball, 1981; Sandbakken et al., 2012). In the salmon suprachiasmatic nucleus the melanopsin positive cells were shown to be co-localised with dopaminergic (THir) cell populations known as the main source of dopaminergic innervations of the pituitary (Holmqvist and Ekström, 1995; Sandbakken et al., 2012). These results have led to the suggestion that melanopsin photoreceptors can be involved in signalling of the photoperiodic information of salmon in several pathways (Sandbakken et al., 2012). This thesis has demonstrated that halibut melanopsins are detected in the same brain regions as shown in juvenile salmon already from larval stages (47 dph) (Paper 1) indicating that photoreception by melanopsin positive cells are important from early life stages of teleosts. From a phylogenetic view the Atlantic halibut and Atlantic salmon are distant, suggesting conserved functions of the melanopsins in teleosts.

Further on, vertebrate ancient opsin is detected in the pineal, habenula and dorsal thalamus of juvenile Atlantic salmon (Philp et al., 2000b; Sandbakken et al., 2012). In this thesis VA opsin is shown to be expressed in dorsal and ventral thalamus, dorsal tegmentum and in the hindbrain of halibut larvae at first feeding. The VA opsin expressing cells in the dorsal thalamus are shown to extend caudally and ventrally from the subhabenula region to the posterior commisure in salmon (Philp et al., 2000b), and this is consistent with the expression pattern seen in the extensive dorsal thalamus cluster of halibut (Paper 2). The results indicate that also VA opsin has conserved functions in

the teleosts and that also this non-visual opsin has an important function form early developmental stages. Exorhodopsin is a pineal specific non-visual opsin in teleosts (Mano et al., 1999; Philp et al., 2000a) and expression studies show that exorhodopsin is extensively expressed in the pineal of halibut (Paper 1). The expression pattern of the non-visual opsins in the brain of halibut larvae at first feeding is illustrated in Figure 12 together with the expression pattern at hatching.

8.2.2 Melanopsin and vertebrate ancient opsin in the retina of teleosts

In zebrafish melanopsins are detected in all cell layers and it has been shown that melanopsins are expressed in a robust diurnal rhythm in the retina. As a result, 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; Matos-Cruz et al., 2011). In halibut larvae melanopsin expression is detected both in the ganglion cell layer and in the inner nuclear layer, but no expression is detected in the photoreceptor cell layer as seen in zebrafish. The findings in halibut retina are consistent with results in Atlantic salmon (Sandbakken et al., 2012) and Atlantic cod (Drivenes et al., 2003), as the mammalian-like and *Xenopus*-like melanopsins are expressed in a similar pattern among the species. Vertebrate ancient opsin is detected in the inner nuclear layer in cells resembling amacrine cells. Although the amacrine cells are thought to be of rhabdomeric origin, this result is in consistence with findings in other teleosts (Grone et al., 2007; Minamoto and Shimizu, 2002; Soni et al., 1998).

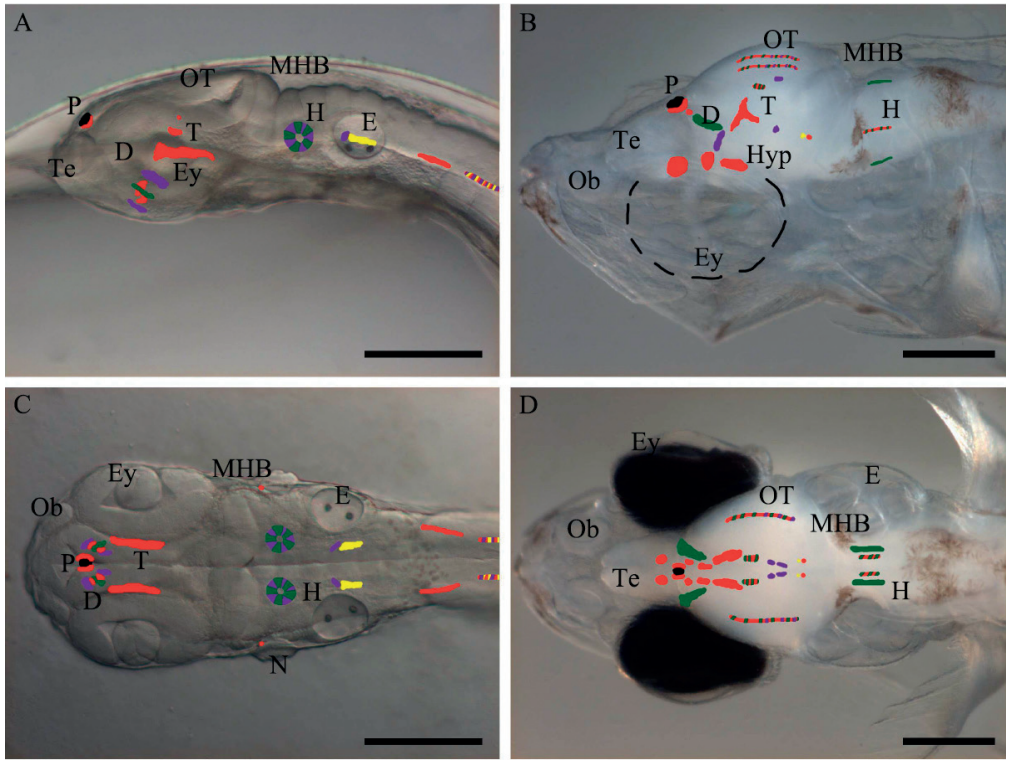

Figure 12 Schematic overview of the non-visual opsins in halibut at the stage of hatching and first feeding. A and C: Lateral and dorsal view of a halibut embryo at the stage of hatching. B and D: Lateral and dorsal view of a halibut larvae at first feeding when the eyes are pigmented and functional. In B) the eyes are removed to reveal the whole brain and indicated by lines. *opn4m1* in red, *opn4m2* in purple, *opn4x2* in yellow, vertebrate ancient opsin in green, exorhodopsin in black. Abbreviation: D, diencephalon E, ear, Ey, eye, H, hindbrain, Hyp, hypothalamus, MHB, midbrain-hindbrain boundary, N, neuromast, Ob, olfactory bulb, OT, optic tectum, P, pineal, T, tegmentum, Te, telencephalon. Scale bars 500 µm.

8.2.3 Complex expression pattern of non-visual opsins at early developmental stages of Atlantic halibut

This thesis aimed to study the appearance of non-visual systems early in development of Atlantic halibut when the brain is transparent and unpigmented. Illustrated by Figure 12 a complex expression pattern of melanopsin, vertebrate ancient opsin and exorhodopsin was detected early in development of halibut. The expression is already refined and extensive at the light-regulated hatching process when the hatching embryos have a primitive body and an undifferentiated retina. The most complex expression was detected for melanopsin showing expression of three paralogues at the stage of hatching. Existence of a non-visual system early in development of halibut supports previous findings demonstrating light-regulated behaviours and physiological processes prior to a functional eye (Flamarique, 2002; Helvik and Walther, 1992; Mangor-Jensen and Waiwood, 1995). Figure 12 also illustrates that the complex expression pattern of non-visual opsins at hatching persists in the halibut larvae at first feeding when the eyes are functional. At this stage the non-visual opsins are also expressed in different retinal layers (Paper 1 and 2). From the figure it is also shown that a bilateral hindbrain cluster is apparent at hatching but not at first feeding. This embryonal specific cluster is shown to be a transient cluster just apparent around hatching (Paper 3).

8.3 Co-localisation of non-visual opsins in different brain regions

Recently the evolution of photoreceptor cells has been debated especially in relation to vertebrate ancient opsin and melanopsin (Davies et al., 2010). Several studies in teleosts indicate that melanopsin of r-opsin origin and vertebrate ancient opsins of c-opsin origin are expressed in the same cell types of retina (Bellingham et al., 2002; Jenkins et al., 2003) and in the same region of the brain (Sandbakken et al., 2012). Further on, VA opsin has been detected in retinal cells thought to be of rhabdomeric origin (Jenkins et al., 2003; Kojima et al., 2000; Soni et al., 1998) and melanopsin is expressed in retinal cells of putative ciliary origin (Davies et al., 2011; Matos-Cruz et al., 2011). This study aimed to

elucidate the previous indications of co-localisation between non-visual opsins of assumed different evolutionary heritage. The results demonstrated that several areas of the halibut brain express more than one non-visual opsin (Figure 12) and by fluorescent double labelling techniques this thesis demonstrates the relative distribution of non-visual opsins in the most apparent areas.

8.3.1 Co-localisation of melanopsin and exorhodopsin in the pineal complex

In the pineal complex the mammalian-like melanopsin (*opn4m1*) and exorhodopsin positive cells are detected and the results show that the two non-visual opsins of assumed different evolutionary heritage are expressed in adjacent cells (Paper 1). The results show that exorhodopsin positive cells are flanked by melanopsin expression, just lateral to the pineal specific exorhodopsin expression. One could speculate why both of them are extensively expressed in pineal complex. One reason could be that they provide detection of light with different wavelengths to the pineal complex, since melanopsins are shown to have maximal sensitivity to blue light (Davies et al., 2011) while exorhodopsin has been suggested to have a maximal sensitivity to green light (Mano et al., 1999). Another possibility is that the melanopsin expressing cells function as a photoisomerase for the exorhodopsin positive cells. Lately a study in zebrafish has shown that not all melanopsins display invertebrate bistability and it has been suggested that the bistable melanopsins in the retina of zebrafish may provide 11-*cis* retinal to the monostable melanopsins in the same retinal layer (Davies et al., 2011). Further on, phylogenetic analysis branches the melanopsin expressed in the pineal complex of halibut together with the zebrafish melanopsins displaying bistability, indicating that also the halibut melanopsin could exhibit bistability.

8.3.2 Co-expression of melanopsin and vertebrate ancient opsin in the hindbrain

In the hindbrain a bilateral ball-shaped cluster of cells is apparent for both vertebrate ancient opsin and the mammalian-like melanopsin (*opn4m2*) at the stage of hatching. In Paper 2 this aggregated hindbrain cluster has been analysed in detail, and fluorescent double labelling *in situ* hybridisation shows that both genes of assumed different evolutionary heritage are expressed in the same cells. In addition, cells expressing just one of the genes are also seen in the cluster.

Based on observations in retina with VA opsin expression in putative rhabdomeric photoreceptor cells and melanopsins in assumed ciliary photoreceptor cells, it has been suggested that the retinal cell types have a common ancestral bimodal cell lineage. This cell lineage is thought to date back to primitive bilaterians that expressed both c-opsin and r-opsin (Davies et al., 2010; Matos-Cruz et al., 2011). One can speculate if this ancestral cell lineage also gives rise to the melanopsin and VA opsin co-expressing cells in the hindbrain, indicating that this photosensitive precursor cell has persisted parallel to the eye evolution of ciliary and rhabdomeric cells. Figure 13 illustrates the potential structure of this ancestral bimodal photoreceptor cell and shows examples from the thesis of potentially different arrangements of cells in the brain. The phototransduction cascade for photoreceptor cells expressing c-opsin or r-opsin is thought to involve different machinery and results in hyperpolarisation or depolarisation respectively (Arendt, 2003). One can further hypothesise which phototransduction machinery the suggested ancient cell lineage employs. Recent studies have indicated that different melanopsins can mediate the phototransduction pathway through Gq or Gi-proteins based on high variability in the coupling specificity of G-proteins (Borges et al., 2012). One can speculate that the melanopsins may not just involve the phototransduction pathway employing Gq-proteins leading to depolarisation. Although uncertain, the cells expressing both melanopsin and VA opsin may also have the possibility to employ both phototransduction cascades based on which opsin that is activated. One can think that

melanopsin has maximum sensitivity to blue light and activates the phototransduction cascade that results in depolarisation while VA opsin can have a maximum sensitivity in the violet light and results in hyperpolarisation. Exposure to light of the whole spectra may activate both opsins and the net membrane potential may not change, leaving the cell at a "resting state".

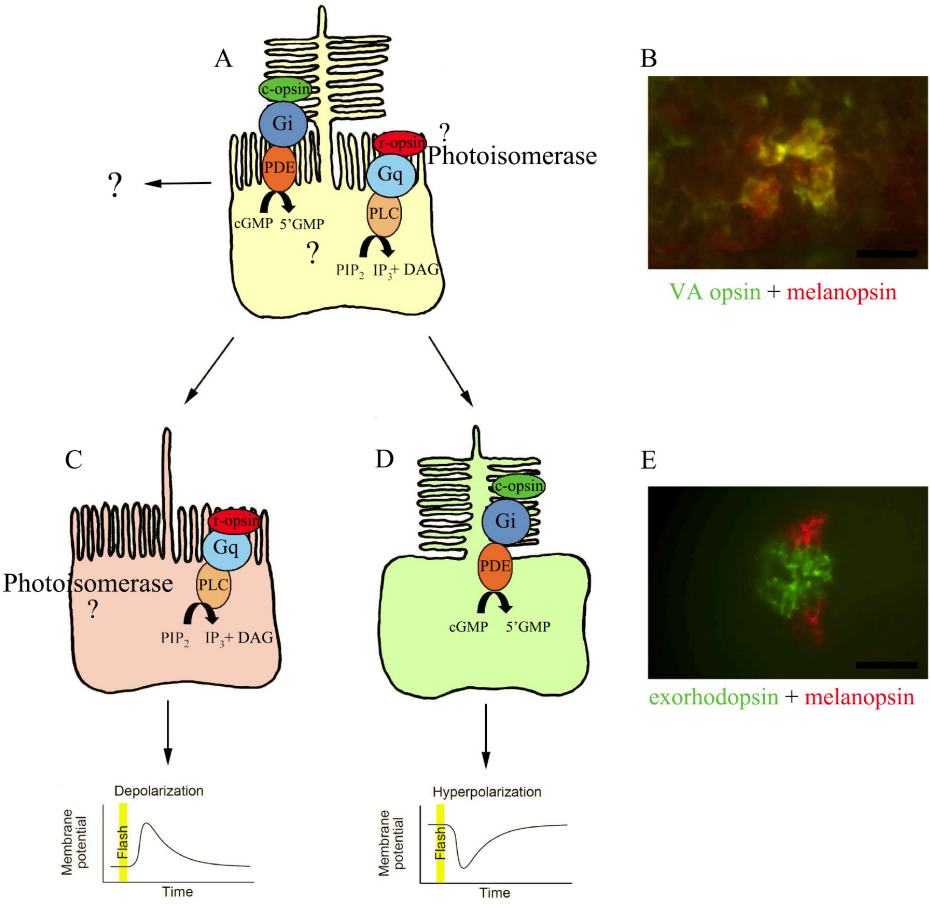

Figure 13 Potential structures and functions of photoreceptor cells.

A) The potential structure of an ancestral bimodal photoreceptor cell expressing both c-opsin and r-opsin. Phototransduction pathways are indicated by some of the molecules known to be involved in the cascades.

Resulting changes in the membrane potential are discussed in the text. The r-opsin may also act as a photoisomerase. B) Expression of two non-visual opsins of assumed different evolutionary heritage in the same cells, shown in this thesis (Paper II). The cells may be an example of the ancestral bimodal photoreceptor cell in the brain. C) Potential structure of a rhabdomeric photoreceptor cell folding the apical membrane of the cell. R-opsin involves the phototransduction machinery leading to depolarisation of the cell and some of the important molecules are shown. The r-opsin may also act as a photoisomerase. D) Potential structure of a ciliary photoreceptor cell folding the membrane of the cilia. C-opsin involves phototransduction cascade leading to hyperpolarisation of the cell and some of the molecules involved are shown. E) An example from the thesis of non-visual opsins of putative different evolutionary heritage expressed in adjacent cells (Paper I). This may represent localisation of rhabdomeric and ciliary photoreceptor cells close to each other in the brain. Abbreviations: cGMP, cyclic guanosine monophosphate, GMP, guanosine monophosphate, PDE, phosphodiesterase, PLC, phospholipase enzyme, PIP_2, phosphatidyl inositol diphosphate, IP_3, inositol triphosphate, DAG, diacylglycerol. Scale bars 50 μm. (Figure based on illustrations in Arendt et al. 2003 and Nilsson 2004)

8.4 Evaluating photoreceptor cells based on distribution and neural identity

Evaluating non-visual opsin expressing photoreceptor cells based on distribution and neural identity can explore more about the functions of the different photoreceptor cells (Ramirez et al., 2011). Clusters of cells expressing non-visual opsins in different brain regions such as the preoptic area, habenula, dorsal thalamus and tegmentum (Paper I and Paper II) are shown to have retinal and pineal innervations (Ekström and Meissl, 1997) (Figure 10) and one can speculate if these cells are aggregated interneurons (high-order) modulating signals from the retina and pineal. They may also be primary photoreceptor cells (first-order) providing photic information as indicated in Figure 7. Recently, it has however been shown that non-visual opsins are expressed in interneurons in the brain of medaka and zebrafish (Fischer et al., 2013). Interestingly, dispersed high-order photoreceptor cells have so far not been identified, but in Paper I we speculate that the dispersed melanopsin positive cells of the spinal cord may be interneurons representing

the first example of dispersed high-order photoreceptor cells. Evaluating the aggregated bilateral hindbrain cluster, the cells may be assessed as first-order photoreceptor cells that function as primary sensory cells in regulation of hatching. The cells could also be high-order photoreceptor cells modulating an incoming signal that then may be transmitted to the hatching glands (Paper 3).

8.5 Hindbrain regulated hatching

In halibut the hatching process is shown to be light-regulated (Helvik and Walther, 1992) and this thesis intended to take advantage of this pre-retinal photosensitive process to study non-visual photoreception. Results show that the bilateral hindbrain cluster with expression of both vertebrate ancient opsin and melanopsin is a transient cluster at hatching (Paper 3). The work also reveals that the hindbrain cluster is connected to a neuronal network with projections out in the yolk sac, reaching the narrow belt of hatching glands. Further on, induction of hatching in light-arrested eggs activates expression of the immediate early gene *c-fos* in the hindbrain cluster and in the hatching glands. Taken together these results demonstrate that it is likely that the hatching process is regulated by the transient cluster of photosensitive cells in the hindbrain and the regulation seems to be driven by neuronal signalling. Further on, the activation of the neural circuit in darkness implies that the net output of melanopsin and VA opsin is inhibitory in the system. Recently the hindbrain was for the first time demonstrated to be responsible for a light-sensing behaviour. In zebrafish a robust and reproducible series of motor behaviours were elicited by visual wavelengths and this “photomotor” response was shown to be driven by the caudal hindbrain (Kokel et al., 2013). This thesis shows that also the rostral hindbrain has photosensitive cells responsible for a light regulated behaviour and unlike the study in zebrafish the thesis identifies the potential responsible photosensitive opsins.

8.4.1 The transient hindbrain cluster may represent an ancient photoreceptor system for regulation of hatching

Animals have an eggshell that surrounds and protects the vulnerable embryo (reviewed in Claw and Swanson, 2012) and parallel with the ancient eggshell, mechanisms for escaping the egg must have evolved. Paper 3 speculates that the transient hindbrain cluster may represent an ancient photoreceptor system that has been kept in evolution to regulate hatching. This is based on the findings in Paper II showing co-expression of opsins of c-opsin and r-opsin heritage in the cells of the hindbrain cluster. Such photoreceptor cells with dual opsins have been postulated to be present in the Urbilateria, prior to the evolution of the ciliated and rhabdomeric photoreceptors cell types (Arendt, 2003; Arendt et al., 2004; Arendt and Wittbrodt, 2001). The paper points out that it is interesting to find a dual photopigment cluster in relation to the hatching mechanism and therefore suggests that the transient hindbrain cluster may represent an ancient photoreceptor system for regulation of hatching.

9. Conclusions and future perspectives

The results presented in this thesis demonstrate the importance of non-visual photoreception in early life stages of teleosts when the uncovered and transparent brain is directly exposed to the environmental light. Several non-visual opsins are shown to be extensively expressed from early developmental stages of Atlantic halibut long before the eyes are functional. Results from the thesis show that this extensive expression persists when the eyes are functional, indicating importance of photoreception directly through the brain even when non-visual opsins are expressed in the retina. Further on, the results detect brain regions with co-localisation of non-visual opsins of assumed different evolutionary heritage. By fluorescent double labelling techniques it is revealed that melanopsin and exorhodopsin are expressed in adjacent cells of the pineal complex, while melanopsin and vertebrate ancient opsin are expressed in the same cells in a bilateral hindbrain cluster. This is the first result demonstrating that putative r-opsin and c-opsin homologues are expressed in the same cells. The thesis also suggests that the hatching process of Atlantic halibut is regulated by the bilateral hindbrain cluster of photosensitive cells and the regulation seems to be driven by neuronal signalling. One may speculate that the hindbrain cluster reflects an old photoreceptor system connected to the light-regulated hatching process, a mechanism that may have appeared early in vertebrate evolution.

In future research on non-visual opsins it is important to keep in mind that studies of gene expression just give an indication of the protein function. Therefore future studies may considerate to include generation of specific antibodies against the non-visual opsins of interest. In addition, it is necessary to demonstrate that the non-visual opsins encode functional photopigments and it is interesting to determine which of the melanopsins that display bistability. It will also be of great interest to determine the peak spectral sensitivity of the non-visual opsins especially in relation to the opsins shown to be co-localised. Future studies may then be able to give more explanations for co-localisation of

non-visual opsins in brain regions such as the pineal complex and the hindbrain cluster. Deeper characterisation of the neuronal network using techniques like retrograde labelling may also be of interest to better understand the connection between the hindbrain cluster and the motor neuronal systems.

So far much of the attention on non-visual photoreception is related to photosensitive organs such as the eye and the pineal. The extensive expression of non-visual opsins in the teleost brain points to that also this should be in focus studying non-visual photoreception. In the future it will be of interest to identify the photosensitive brain cells as first-order (primary sensory cells) or high-order (interneurons) neurons. In this way it may be possible to elucidate their function as primary sensor neurons of light or as modulators of signals from photosensitive organs.

Transgenic approaches using zebrafish may be a way to reveal more about the identity and role of the photosensitive cells in the teleost brain. In addition, a range of photosensitive brain cells are characterised but the resulting behavioural and physiological responses need to be elucidated. In zebrafish nitroreductase ablation techniques give the possibility to remove specific neurons or structures, such as the pineal. It is also possible to perform targeted genetic ablations to remove cells expressing specific genes. Use of morpholino or other knockdown technologies can provide down-regulation of gene expression, potentially showing altered responses to light.

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Paper I

I

Melanopsin and exorhodopsin in the pineal complex of Atlantic halibut (*Hippoglossus hippoglossus*)

Mariann Eilertsen, Øyvind Drivenes, Rolf B. Edvardsen, Clarrisa A. Bradley, Lars O.E. Ebbesson and Jon Vidar Helvik

Manuscript

Melanopsin and exorhodopsin in the pineal complex of Atlantic halibut (*Hippoglossus hippoglossus*)

Mariann Eilertsen, [1] Øyvind Drivenes, [2*] Rolf B. Edvardsen, [3] Clarrisa A. Bradley, [4] Lars O.E. Ebbesson, [1, 5] and Jon Vidar Helvik [1**]

[1] Department of Biology, University of Bergen, N- 5020 Bergen, Norway

[2] Department of Molecular Biology, University of Bergen, N- 5020 Bergen, Norway

[3] Institute of Marine Research, N- 5817 Bergen, Norway

[4] School of Clinical Sciences, University of Bristol, Bristol BS1 3NY, United Kingdom

[5] Uni Research AS, Thormøhlensgt. 49B, N-5006 Bergen, Norway

* Current address: Bergen University College, N- 5020 Bergen, Norway

** Corresponding author and reprint request to:
Jon Vidar Helvik, Department of Biology, University of Bergen, P.O Box 7803, N- 5020 Bergen, Norway, jon.helvik@bio.uib.no

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Abbreviations

BSA, bovine serum albumin

D, diencephalon

DON, descending octaval nucleus

E, ear

Ey, eye

GCL, ganglion cell layer

H, hindbrain

Hb, habenula

Hyp, hypothalamus

INL, inner nuclear layer

IPL, inner plexiform layer

MHB, midbrain hindbrain boundary

N, neuromast

NMO, nucleus medialis octavolateralis

NLT, nucleus lateralis tuberis

NPSM, nucleus pretectalis superficialis magnocellularis

ONL, outer nuclear layer

OPL, outer plexiform layer

OT, optic tectum

P, pineal

Po, preoptic area

RN, raphe nuclei

SCN, suprachiasmatic nucleus

T, tegmentum

Te, telencephalon

Thd, dorsal thalamus

Thv, ventral thalamus

Tw, Tween20

Abstract

The complexity of the non-visual photoreception systems in teleosts has just started to emerge with colocalisation 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. This expression persists in the brain after the eyes become 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 labelling showed that two clusters of melanopsin positive cells are located lateral to the central rosette of exorhodopsin positive cells. The localisation of two different photoreceptor systems in the pineal complex suggests that photoreception may be activated by different light spectra. The melanopsin expressing cells may also function as a photoisomerase providing 11-*cis* retinal to the exorhodopsin positive pineal cells. Appearance of non-visual opsins early in development of halibut provides a new model to study the evolution and functional significance of non-visual opsins. 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.

Introduction

Light has an important impact on life. Almost all animals are dependent on light to modulate their behaviour and physiology and as the intensity of light alters with the solar cycle the animals have to adapt to the changes. The vertebrate eye stands 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 Davies et al., 2010; Peirson et al., 2009). In contrast to mammals that are dependent of photic information from the retinal ganglion cells (Berson et al., 2002), photoreceptors are present in the pineal organ of non-mammalian vertebrates, regulating the melatonin synthesis important for 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 alter 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 behavioural responses to light (Fernandes et al., 2012; Tabata et al., 1989; van Veen et al., 1976) and in birds hypothalamic photoreceptor cells are thought to be responsible for photoperiodic responses (Halford et al., 2009). Numerous photoreceptive cell types and their photosensitive pigments have been identified over the last decades, but how these cells contribute to the behavioural 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 evolution of vertebrate and invertebrate photoreception has been thoroughly debated and during the last decade it has become evident that both "vertebrate and invertebrate photoreceptor cells" exist in the two animal lineages (Arendt et al., 2004). Of special interest in this debate is the invertebrate homologue melanopsin originally identified in dermal melanophores of *Xenopus laevis* (Provencio et al., 1998). Importantly, melanopsin has been shown to have a significant role in entrainment of the circadian rhythm in mammals (Gooley et al., 2001; Hattar et al., 2003; Panda et al., 2002; Provencio et al., 2000) and it is expressed in several extra-ocular tissues in non-mammalian vertebrates such as habenula and suprachiasmatic nucleus of Atlantic cod (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). Until recently, it was believed that invertebrate-like bistability, where the retinal chromophore interchanges between *cis*- and *trans*-isomers in a light-dependent manner, is a general property of the melanopsin family (Koyanagi et al., 2005; Mure et al., 2009; Walker et al., 2008). However, presence of both bistable and monostable forms of melanopsins has been shown in zebrafish retina (Davies et al., 2011). Several studies in teleosts have located melanopsin in the same regions as other opsins of vertebrate origin (Cheng et al., 2009; Jenkins et al., 2003; Sandbakken et al., 2012). By using fluorescent double labelling 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.

The halibut eggs hatch at an early developmental stage and the hatched larvae have a big yolk sac and a primitive larval body (Haug, 1990; Lönning et al., 1982). 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

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 demonstrate a light-seeking behaviour triggered by loss of illumination, and melanopsin expressing cells in the preoptic area were found to regulate this behaviour (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 labelling 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 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 *opnm2*) 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 first 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*, *opn4m2* 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 40x coverage and a contig assembly was made

with the CLC software (CLC bio, Denmark). O*pn4m1m*, *opn4m2* and *opn4x2*, identified by degenerative PCR and RACE PCR, were verified by searching the genome with BLASTN and TBLASTN (NCBI, Bethesda, MD). 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*) and exorhodopsin (*exorh*) were found. Putative opsin genes were predicted based on the BLAST alignments and GENSCAN (Burge and Karlin, 1997), and the annotation was based on BLASTX against GenBank (NCBI, Bethesda, MD) 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 maximum likelihood tree using MEGA version 5 (Tamura et al., 2011) 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).

Riboprobes

Digoxigenin (DIG)-labelled and/or fluorescein-labelled 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 synthesised probes were precipitated by tRNA (Roche Diagnostics, Germany). Sequence alignment

shows that the similarity between the sequence targets of the melanopsin probes does not exceed 75 %.

***In situ* hybridisation 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 hours 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* hybridisation was started by rehydrating the embryos and larvae in methanol (75-25 %) and then rinsing them for 2x5 minutes 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 minutes in1xPBS and then 4x5minutes in 1xPBSTw (0,1 % Tween20 (Sigma, St. Louis, MO), in 1xPBS). The tissue was opened 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 optimalised for the size of the embryos and larvae. After a rinsing step with 1xPBSTw the embryos and larvae were fixated in 4 % paraformaldehyde-buffred PBS (pH 7,4) and then washed 4x5minutes in 1xPBSTw. Pre-hybridisation was carried out at 65 °C for 2 hours in hybridisation solution without probe before incubation with hybridisation solution with probe over night at 65 °C. The hybridisation solution had the composition 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 hybridisation washing series of 2x15 minutes in 50 % formamide (VWR, West Chester, PA) in 2xSSCTw, 1x30 minutes in 2xSSCTw, 2x15 minutes in 2xSSCTw and 2x15 minutes in 0,2xSSCTw were performed at 65 °C. To remove unhybridised probe the embryos and larvae were treated with RNase A (0,02 mg/ml) (Sigma, St. Louis, MO) for 20 minutes at 37 °C before washing with RNase buffer (10 mM Tris-HCl pH 7,5,

0,5 M NaCl, 1 mM EDTA) for 20 minutes 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 hours before over night incubation with Anti-Digoxigenin-Alkaline phosphatase, Fab fragments (1:2000) (Cat. No 11093274910, Roche Diagnostics, Germany) in 2xSSC, 1 % Blocking reagent and 0,3 % TritonX-100. To remove redundant antibody the embryos and larvae were washed 4x20 minutes in 1xPBSTw and 2x10 minutes in visualisation buffer (100 mM Tris-HCl pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$). Visualisation 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 visualisation buffer. All probes were tested in parallel with a sense probe as a control of unspecific binding. Visualisation 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* hybridisation on sectioned embryos and larvae**

Embryos (14 days post fertilisation (dpf)) and larvae (47 days post hatching (dph)) of Atlantic halibut were fixated in 4 % paraformaldehyde-buffred PBS (pH 7,4) for 48 hours 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 over night 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 hour in room temperature and for 10 minutes in 65 °C. One parallel of the 47 dph sectioned larvae was Nissel-stained with 0,5 % Cresyl Fast Violet (Chroma-Gesellschaft, Germany) and the other parallel was stained by *in situ* hybridisation. *In situ* hybridisation was carried out as described in Sandbakken et al. (2012).

***In situ* hybridisation on whole embryos using fluorescent double labelling techniques**

Fluorescent double labelling *in situ* hybridisation was done to identify expression of two genes in the same embryo. Fluorescein-labelled riboprobe for exorhodopsin (*exorh*) and digoxigenin-labelled riboprobe for the mammalian-like melanopsin (*opn4m1*) were used. Preparation and *in situ* hybridisation was done as described above, with the following modifications. At the hybridisation step, both probes were applied. The fluorescein-labelled probe was first visualized by using the antibody Anti-Fluorescein-horse radish peroxidase (POD), Fab fragments (1:400) (Cat. No 11426346910, Roche Diagnostics, Germany) 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) the embryos were blocked for 4 hours in 2 % Blocking reagent (Roche Diagnostics, Germany) and 2xSCC, and the digoxigenin probe was visualised 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 to mark the serotonergic system was performed. Whole embryos at 14 dpf were washed in 2% PBSTX (2 % TritonX-100 (Sigma, St. Louis, MO) in 1xPBS) for 2 days at 4 °C before washing 2x30 minutes with 1,5% PBSTX (1,5 % TritonX-100 in 1xPBS). Incubation with primary antibody for 3 days at 4 °C was done with 1,5 % PBSTX/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) with a concentration of 1:1000. Embryos were washed 2x30 minutes in 1xPBS prior to incubation over night 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). 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 minutes in 1xPBS before mounting in DABCO anti-fading medium (Triethylenediamine, Sigma, St. Louis, MO) and storage in darkness.

Fluorescent *in situ* hybridisation and Immunohistochemistry

Fluorescent *in situ* hybridisation together with immunohistochemistry was done to evaluate the expression pattern in the region of the pineal. *In situ* hybridisation on embryos was performed using the TSA^{TM}Plus Fluorescein System together with fluorescein-labelled exorhodopsin riboprobe as described above. Before immunohistochemistry was done on the fluorescent stained embryos they were incubated for 4 hours 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) was incubated over night in room temperature with 1 % Blocking reagent in 2xSSC. Prior to incubation with secondary antibody the embryos were washed with 1xPBS 3x10 minutes. 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) in 1xPBS/1 % BSA. The incubation took place over night at room temperature before washing 2x30 minutes 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) is a polyclonal antibody against serotonin, and has been showed to have similar staining in classical serotonergic neuronal populations in vertebrates, including (Ebbesson et al., 1992; Ekström and Ebbesson, 1988; Sandbakken et al., 2012). Mouse anti-acetylated tubulin (Cat. No. T7451, Sigma-Aldrich, St. Louis, MO) is a monoclonal antibody that recognizes an epitope located on the α3 isoform of *Chlamydomonas axonemal* α-tubulin. The antibody has been used to

label axon in many organisms including teleosts (Hunter et al., 2011; Ledizet and Piperno, 1991). See Table 3 for details.

Microscopy and pictures

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 an 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 characterisation 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 and exorhodopsin were found by searching the halibut genome using a BLAST algorithm. Two of the melanopsins, *opn4m1* and *opn4m2*, are comparable to the mammalian-like melanopsins (*Opn4m*) and two, *opn4x1* and *opn4x2*, are similar to the *Xenopus*-like type (*Opn4x*). The mammalian-like melanopsin (*opn4m2*) has two splice variants and the difference between them is in the cytoplasmatic tail where one of the isoforms lacks an exon coding for 22 amino acids. GenBank accession number, cDNA length, open reading frame (ORF) and amino acid sequence are listed in Table 2 for the four melanopsins and exorhodopsin.

An alignment (Fig. 1) of the four melanopsins in Atlantic halibut together with published melanopsin sequences of zebrafish (Opn4m) and Atlantic cod (Opn4x) shows that they all span 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 residues tyrosine (Y) (Provencio et al., 1998) and the glutamic acid (E) in the extracellular loop between the TM4 and TM5 are the potential 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 stabilise 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 melanopsin of different species. In addition the visual opsin of halibut and exorhodopsin 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 expected based on the theory of exorhodopsin being the true ortholog of the vertebrate rod opsin (Bellingham et al., 2003).

Expression of melanopsin at the stage of hatching

In situ hybridisation on whole embryos (Fig. 3) showed that the two mammalian-like melanopsins (*opn4m1* and *opn4m2*) 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 negative controls for all the melanopsins and showed no expression (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 optic tectum and a cluster of cells is situated just ventral to optic tectum (Fig. 3B, G). In 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 *opn4m2* 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 *opn4m2* 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 *opn4m2* 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 *opn4m2* was also detected medial to the ear (Fig. 3I, J, L). In the spinal cord distinct cells expressing *opn4m2* were seen just dorsal to the developing notochord but the *opn4m2* 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 *opn4m2* (Fig. 3P, Q, S, U). Staining seen in the ear (Fig. 3P) is artificial and probably due to trapping of probe in the ear.

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

In situ hybridisation 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 labelling 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* hybridisation and immunohistochemistry 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 presence of neural signalling pathways between the pineal and the brain at the time 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 (5'HT) 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.

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 Nissel-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 optic tectum and dorsal in tegmentum (Fig. 5D5). Ventral in diencephalon expression was observed in cells that are most likely the nucleus lateralis tuberis (Fig. 5E3). Ventral in tegmentum *opn4m1* positive cells were detected 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 (*opn4m2*, *opn4x1* and *opn4x2*) and exorhodopsin (*exorh*) was also investigated at 47 dph. Three of them (*opn4m2*, *opn4x2* and *exorh*) had expression in the brain and the spatial expression pattern was compared with adjacent serial Nissel-stained sections (Fig. 6). The pineal specific exorhodopsin is extensively expressed in the pineal at 47 dph (Fig. 6A3). The mammalian-like melanopsin (*opn4m2*) 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 tegmentum and in 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 retina anlage. 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 *opn4m2* 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 a new 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, we detect expression of both melanopsin and exorhodopsin in adjacent cells suggesting that two different photoreceptor systems may be active in the pineal complex and that melanopsin may function as a photoisomerase.

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 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. 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 *opn4m2* 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.

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 may indicate that the complex is able to detect light with different wavelengths and thus expand the spectral information it can obtain from the environment. In other teleosts, the melanopsins are shown to have a maximal sensitivity to blue light (Davies et al., 2011). Exorhodopsin has been suggested to have a maximal sensitivity to green light, since important residues for spectral tuning of green-sensitive rhodopsin are found in zebrafish exorhodopsin (Mano et al., 1999). The flanking melanopsin expressing cells may also function as a photoisomerase for the exorhodopsin expressing pineal cells. Recent evidences 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). 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), indicating that the halibut melanopsin could provide 11-*cis* retinal to the exorhodopsin expressing cell of the pineal. Consistent with previous studies in halibut embryos we show axonal projections from the pineal to the deep brain regions, indicating presence of neural signalling 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*/*opn4m2*). Expression of o*pn4m1* is also detected in tegmentum and optic tectum, while both mammalian-like (*opn4m1*/*opn4m2*) 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 presumably monostable *Xenopus*-like melanopsin is expressed in zebrafish.

This article contributes to 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*/*opn4m2*) 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*/ *opn4m2*) 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 find melanopsin expression presumably in nucleus pretectalis superficialis magnocellularis, in dorsal tegmentum, in the optic tectum and most likely cells of the raphe nuclei. Melanopsin positive cells are also detected in the hindbrain. We indicate that *opn4m1* is expression in the nucleus medialis octavolateralis and in the descending octaval nucleus while *opn4x2* is 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 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. In accordance to juvenile cod and salmon (Drivenes et al., 2003; 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. In zebrafish retina the melanopsins are detected in all cell layers and 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). Comparing the findings in brain and retina it is apparent that the expression of *Xenopus*-like or mammalian-like melanopsins is more conserved among the marine species in the retina than in the brain.

This study shows extensive expression of melanopsins in the larval halibut retina prior to rod development and dim-light vision, which is apparent first after metamorphosis (Kvenseth et al., 1996). Melanopsin positive retinal ganglion cells of mammals have a significant role in entrainment of the circadian rhythm (Gooley et al., 2001; Hattar et al., 2003; Panda et al., 2002; Provencio et al., 2000) and one can speculate about the role of

the extensive melanopsin positive cells in the teleost retina. One possible function of melanopsin could be related to the switch between day-light vision and dim-light vision (mesopic vision) where vision change from cone driven to rod driven vision during dusk, and back to cone driven during dawn. The finding of the melanopsin expressing neurons in a pure cone halibut retina can indicate that this system is not involved in mesopic vision or that it is developed prior to the occurrence of the rods and dim-light vision.

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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.

Table 1 Primer information

Primer name	Sequence (5'–3')	Use
Opn4mFw2	GGGCATCACMGGCATGSTGGGAAACYT	Degenerative primer for *opn4m1* and *opn4m2*
Opn4mFw2-N	ATCTGCTCSATGATCACRCTSAYRTKAT	Nested degenerative primer for *opn4m1* and *opn4m2*
Opn4mRv2	GATGWGTKATGGCRTADATGATGGGGTTGT	Degenerative primer for *opn4m1 and opn4m2*
Opn4mRv2-N	CCARGAGATGACAWAMADCAGTAGSACWAT	Nested degenerative primer for *opn4m1 and opn4m2*
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 *opn4m2*
HhMelF7	TGTGGCCCTCACTGCATTCG	Nested 3'RACE *opn4m2*
HhMelR3	GTAGACCCAGGCAACAGCCAAGA	5'RACE *opn4m2*
HhMelR4	GCTAAGGGCTTTCCTGCGAGACA	Nested 5'RACE *opn4m2*
HMops1F3	GAGGGGCTGATGACGTCTTGT	3'RACE *opn4x2*
HMops1F2	ACGTCTTGTACGTGGGATTACGTC	Nested 3'RACE *opn4x2*
HMops1R3	GATATAAGAGCTCCAGCCGACGA	5'RACE *opn4x2*
HMops1R2	TGATAACCACGTAGCGGTCGAT	Nested 5'RACE *opn4x2*

Table 2 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
opn4m2short	KF941290	1780 bp	1638 bp	545 aa	1 - 1089 bp	1089 bp
opn4m2long	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

Table 3 Antibodies

Antibody	Immunogen	Host species	Manufacturer
Anti-serotonin	Serotonin (5-HT)	Rabbit polyclonal	DiaSorin (Italy), 20080
Anti-acetylated tubulin	Epitope on the α3 isoform of *Chlamydomonas axonemal* α-tubulin within four residues of acetylated Lys-40	Mouse monoclonal, IgG2b	Sigma-Aldrich (St. Louis, MO), T7451

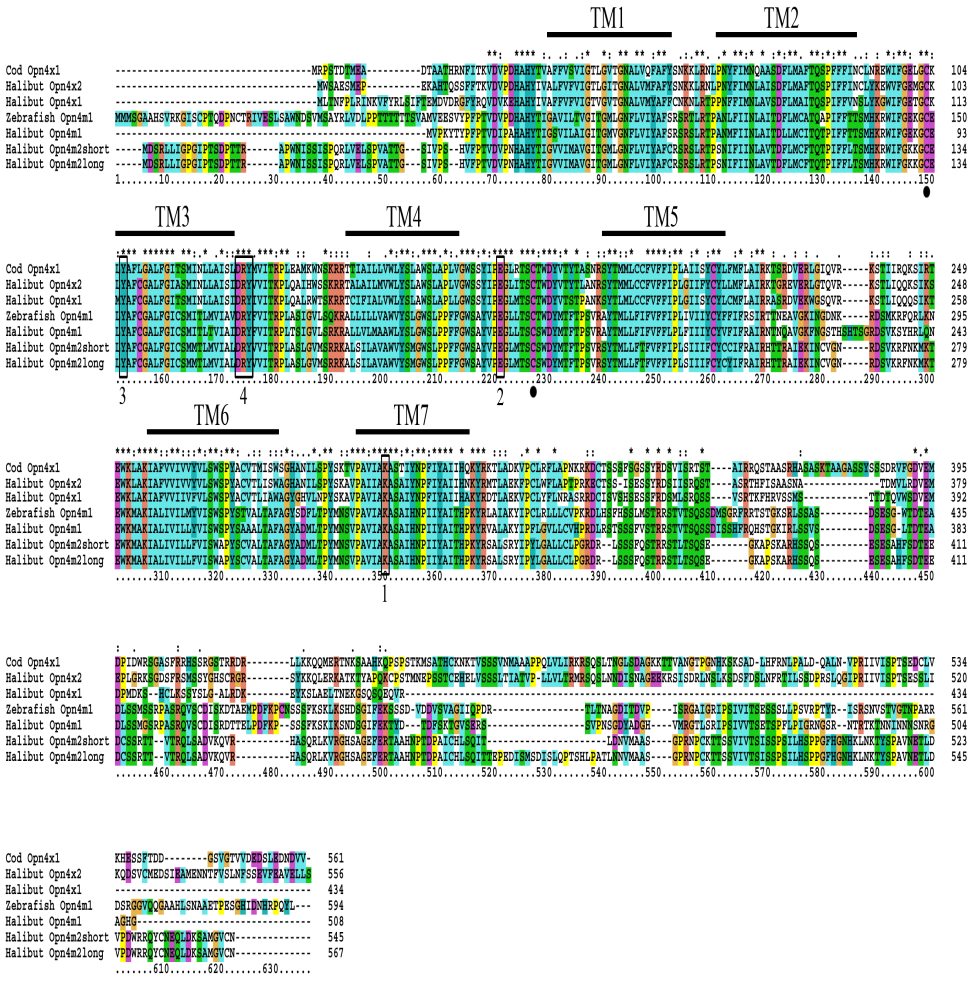

Figure 1 Alignment

Alignment of the deduced amino acid sequence of Atlantic halibut melanopsins against published sequences. The mammalian-like melanopsin is represented by zebrafish Opnm1 and the *Xenopus*-like melanopsin is characterized by cod Opn4x1. The seven transmembrane (TM) domains are indicated by TM1-7 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.

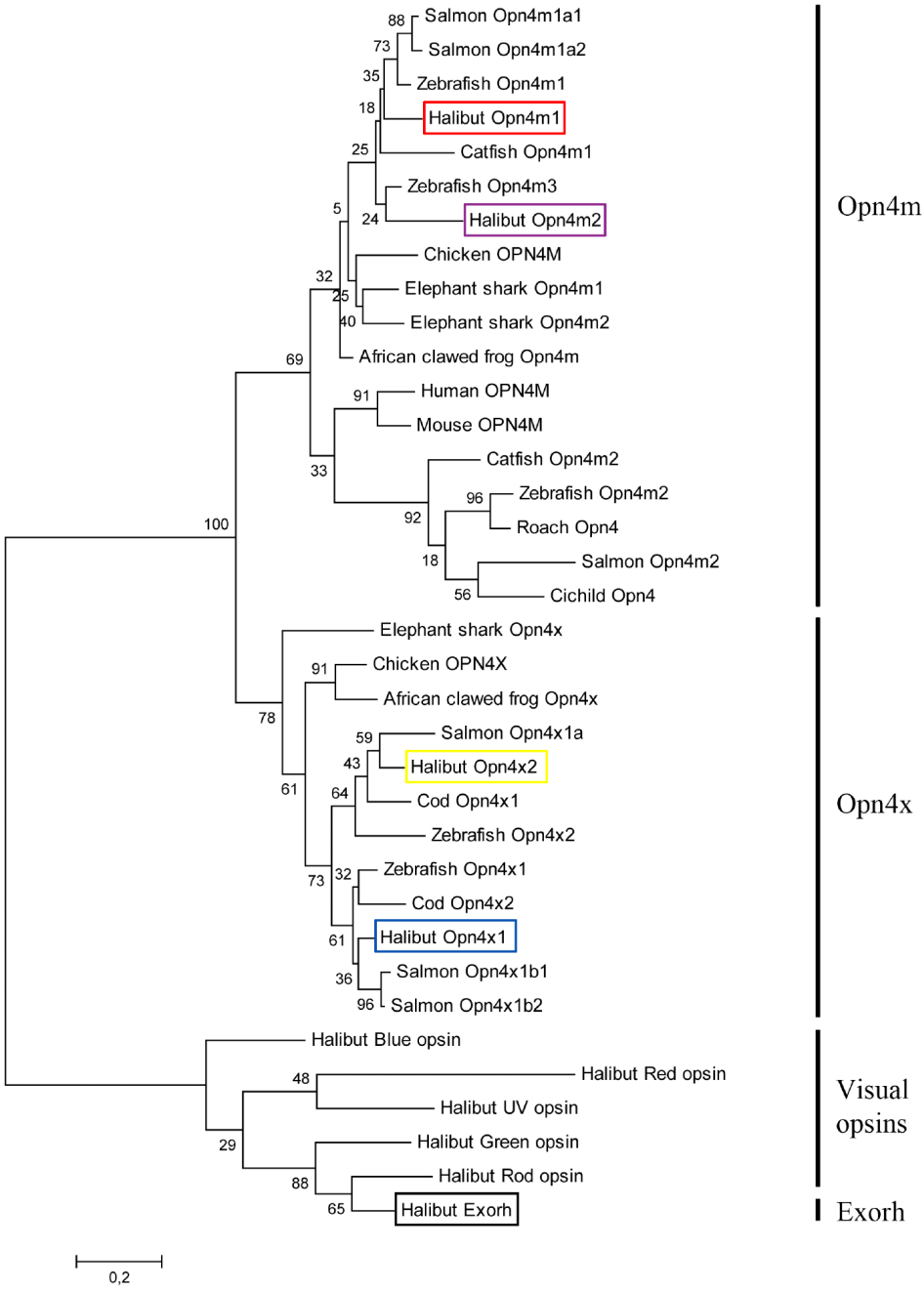
Salmon Opn4m1a1
Salmon Opn4m1a2
Zebrafish Opn4m1
Halibut Opn4m1
Catfish Opn4m1
Zebrafish Opn4m3
Halibut Opn4m2
Chicken OPN4M
Elephant shark Opn4m1
Elephant shark Opn4m2
African clawed frog Opn4m
Human OPN4M
Mouse OPN4M
Catfish Opn4m2
Zebrafish Opn4m2
Roach Opn4
Salmon Opn4m2
Cichild Opn4
Elephant shark Opn4x
Chicken OPN4X
African clawed frog Opn4x
Salmon Opn4x1a
Halibut Opn4x2
Cod Opn4x1
Zebrafish Opn4x2
Zebrafish Opn4x1
Cod Opn4x2
Halibut Opn4x1
Salmon Opn4x1b1
Salmon Opn4x1b2
Halibut Blue opsin
Halibut Red opsin
Halibut UV opsin
Halibut Green opsin
Halibut Rod opsin
Halibut Exorh
0,2
Opn4m
Opn4x
Visual opsins
Exorh

Figure 2 Phylogenetic tree

The maximum likelihood tree shows the phylogenetic relationship between the halibut melanopsins and melanopsins of other species. In addition the halibut visual opsins and exorhodopsin are included. A bootstrap value of 1000 has been applied. The opsin sequences used for generating the tree are: i) Mammalian-like melanopsin: salmon (*Salmo salar*) AFI61534.1 (Opn4m1a1), AFI61536.1 (Opn4m1a2); zebrafish (*Danio rerio*) ENSDARP00000109133 (Opn4m1); halibut (*Hippoglossus hippoglossus*) KF941289 (Opn4m1); zebrafish (*Danio rerio*) ENSDARP00000070530 (Opn4m3); catfish (*Ictalurus punctatus*) ACP43590.1 (Opn4m1); halibut (*Hippoglossus hippoglossus*) KF941291 (Opn4m2); African clawed frog (*Xenouus laevis*) ABD37674.1 (Opn4m); chicken (*Gallus gallus*) ABX10832.1 (OPN4M); elephant shark (*Callorhinchus milii*) AFU50495.1 (Opn4m1), AFU50496.1(Opn4m2); human (*Homo sapiens*) ENSP00000361141 (OPN4M); mouse (Mus musculus) ENSMUSP00000022331 (OPN4M); zebrafish (*Danio rerio*) ENSDARP00000070530 (Opn4m2); Roach (*Rutilus rutilus*) AAO38857.1 (Opn4); catfish (*Ictalurus punctatus*) ACP43591.1(Opn4m2); salmon (*Salmo salar*) JN210550.1 (Opn4m2); Cichild (*Astatotilapia burtoni*) ACB29678.1 (Opn4) ii) *Xenopus*-like melanopsin: elephant shark (*Callorhinchus milii*) AFU50497.1 (Opn4x); 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) iii) Chordate melanopsin (Opn4): lancelet (*Branchiostoma belcheri*) BAE00065.1 (Opn4) iv) Visual opsins: halibut (*Hippoglossus hippoglossus*) AAM17921.1 (Red opsin), AAM17917.1 (UV opsin), AAM17920.1 (Blue opsin), AAM17916.1 (Green opsin), AAM17918.1 (Rod opsin) v) Exorhodopsin: halibut (*Hippoglossus hippoglossus*) KF941294 (Exorh). (Colour boxes highlight the identified opsins in the same colours as used in the schematic drawings in Fig. 5-8).

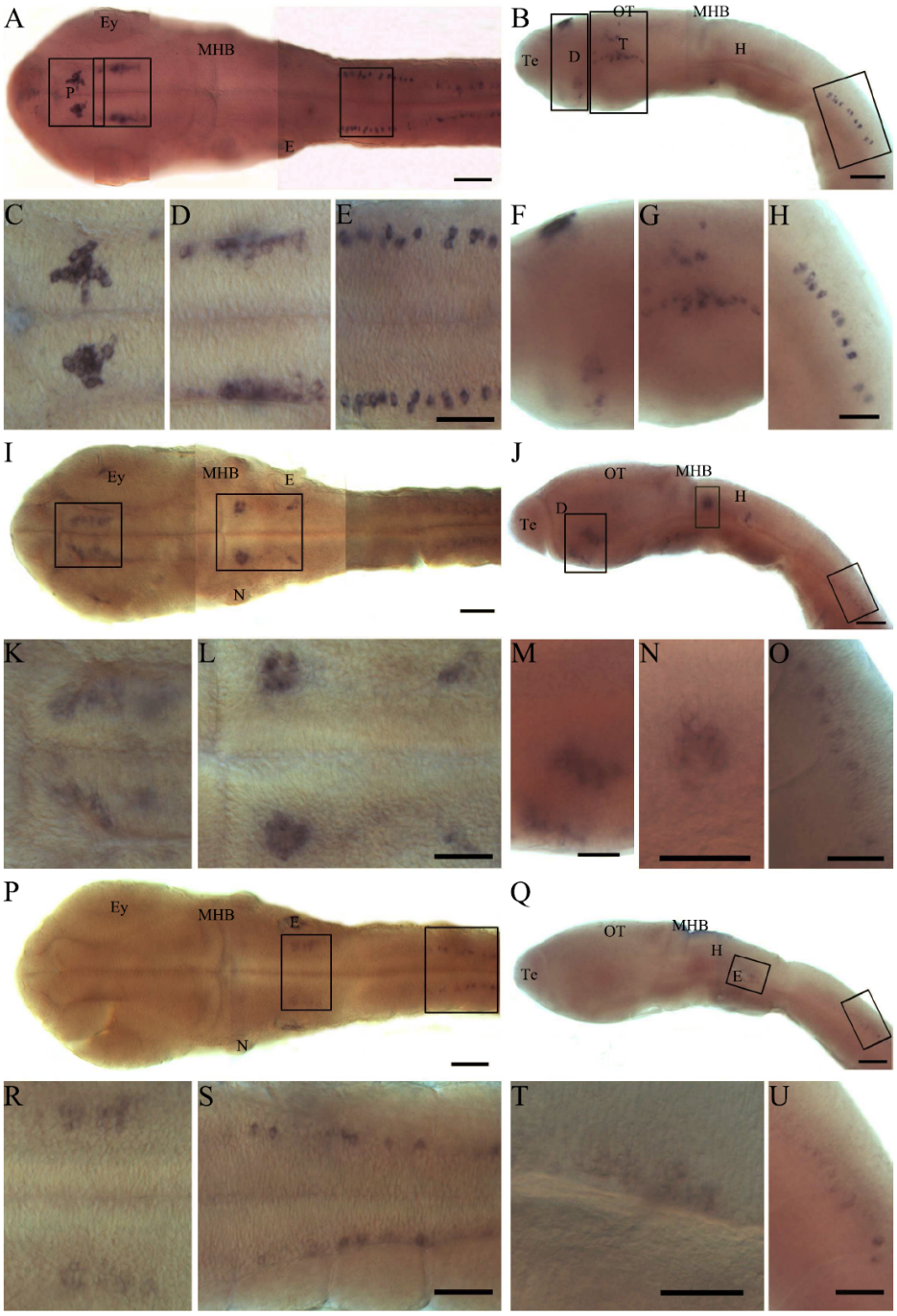
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Figure 3 *In situ* hybridisation on whole halibut larvae at the stage of hatching.
Expression in the brain and spinal cord is shown from a dorsal and lateral view with different magnifications. The overview pictures (A-B, I-J, P-Q) are made by placing 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 *opn4m2* 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

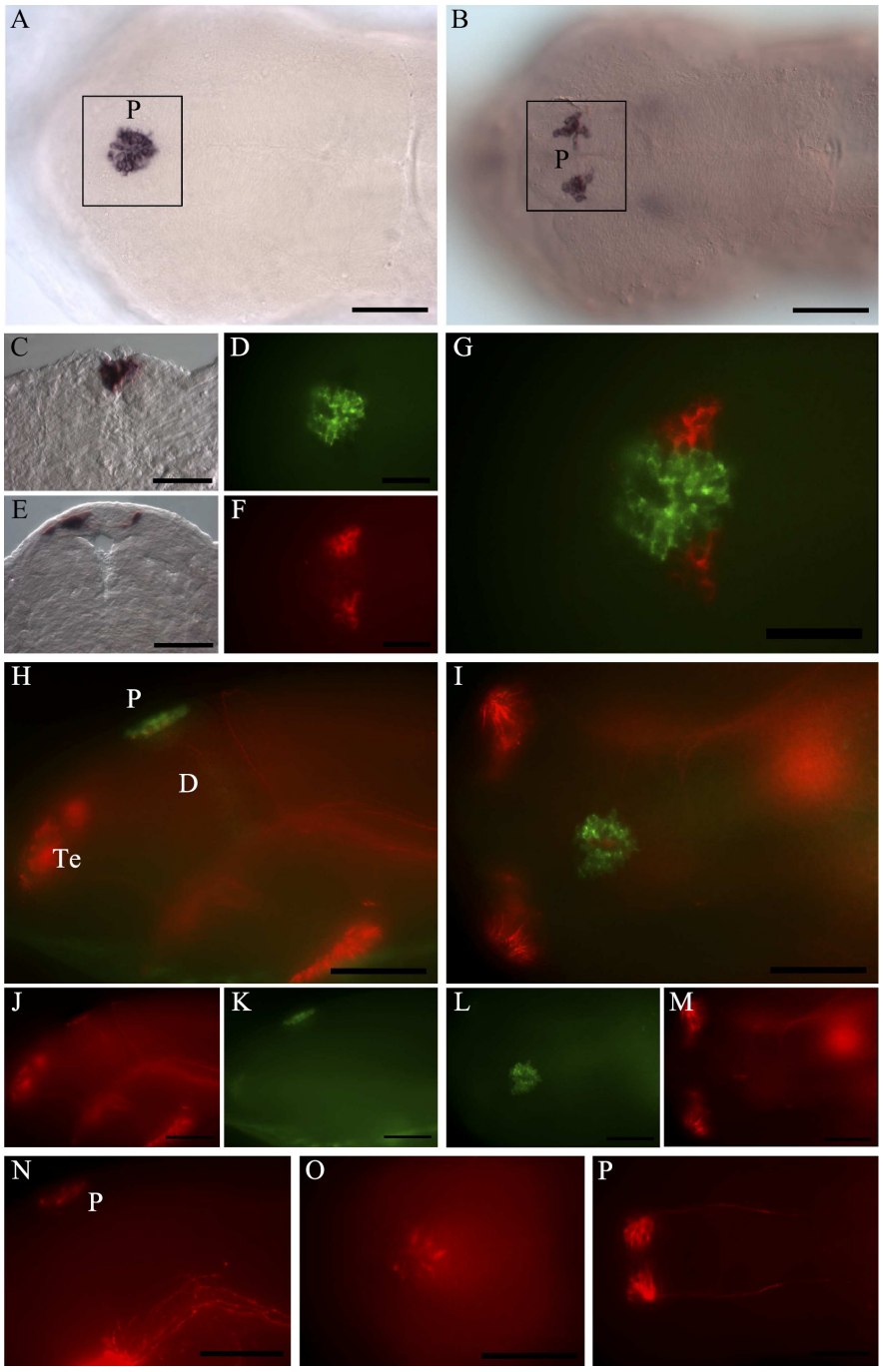
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Figure 4 At the stage of hatching exorhodopsin and melanopsin positive cells are apparent in the pineal region.

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 localisation 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 (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. 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. Scale bars: 100 μm in A-B and H-P, 50 μm in C-G

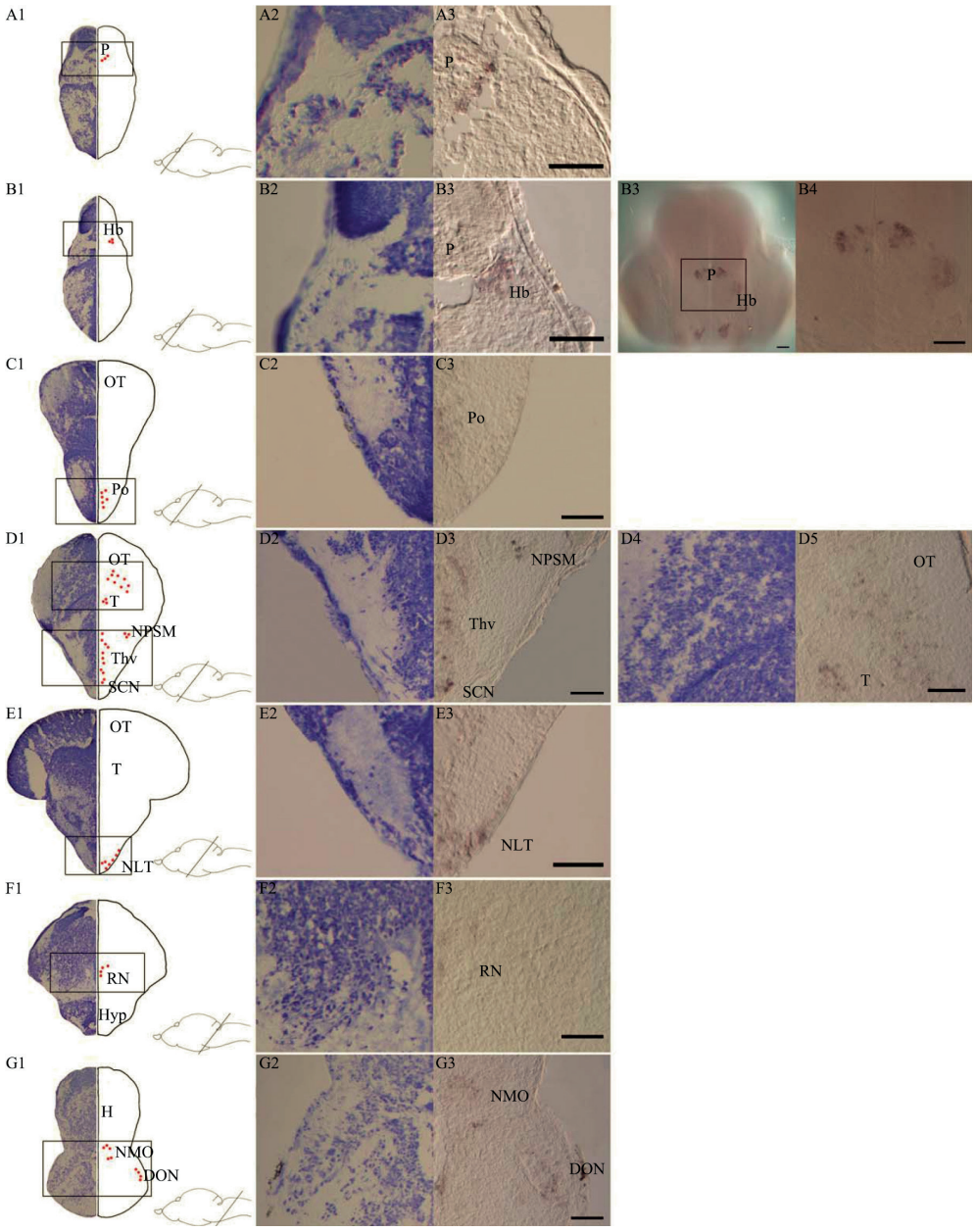
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Figure 5 Melanopsin (*opn4m1*) expression in the brain of Atlantic halibut larvae.
A1-J1: Nissel-stained transversal sections at the equivalent level of melanopsin expressing cells illustrated by red dots in a 47 days post hatching larva. A2-J2 and D4: The Nissel-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

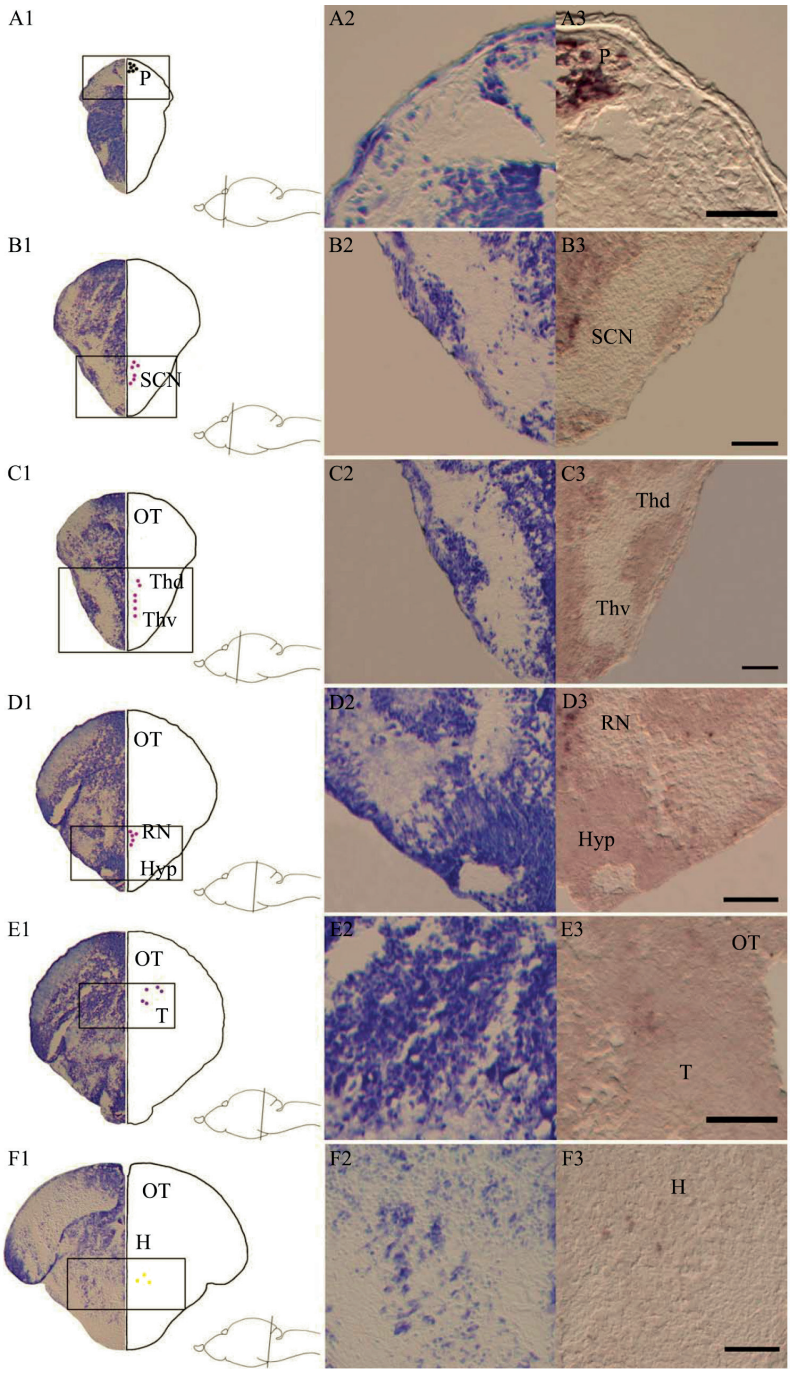
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Figure 6 Melanopsin (*opn4m2* and *opn4x2*) and exorhodopsin expression in the brain of Atlantic halibut larvae at 47 days post hatching.
A1-F1: Nissel-stained transversal sections at the equivalent level of *exorh* expressing cells illustrated by black dots (A1), *opn4m2* expressing cells illustrated by purple dots (B1-E1) and *opn4x2* expressing cells illustrated by yellow dots (F1). A2-F2: The Nissel-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: *opn4m2* in the suprachiasmatic nucleus (SCN). C1-C3: Expression of *opn4m2* in dorsal and ventral thalamus (Thv and Thd). D1-D3: Expression of *opn4m2* presumably in rape nuclei (RN) E1-E3: *opn4m2* 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

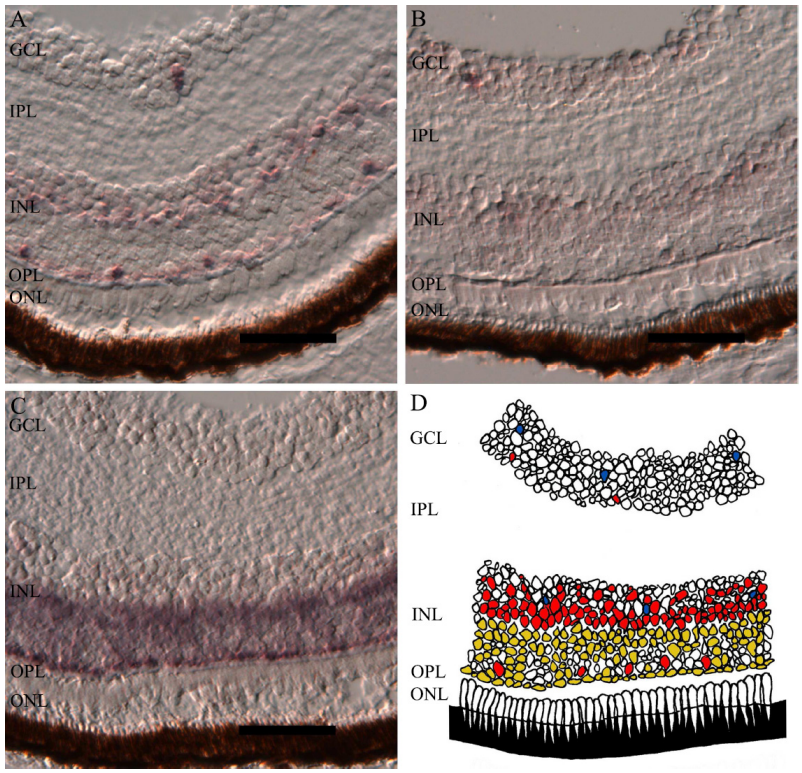

Figure 7 Distribution of melanopsin positive cells in a functional retina at 47 days post hatching. A: The mammalian-like melanopsin *opn4m1* is expressed in the in the ganglion cell layer (GCL) and in the inner nuclear layer (INL) probably in cells resembling amacrine cells and in cells that are most 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

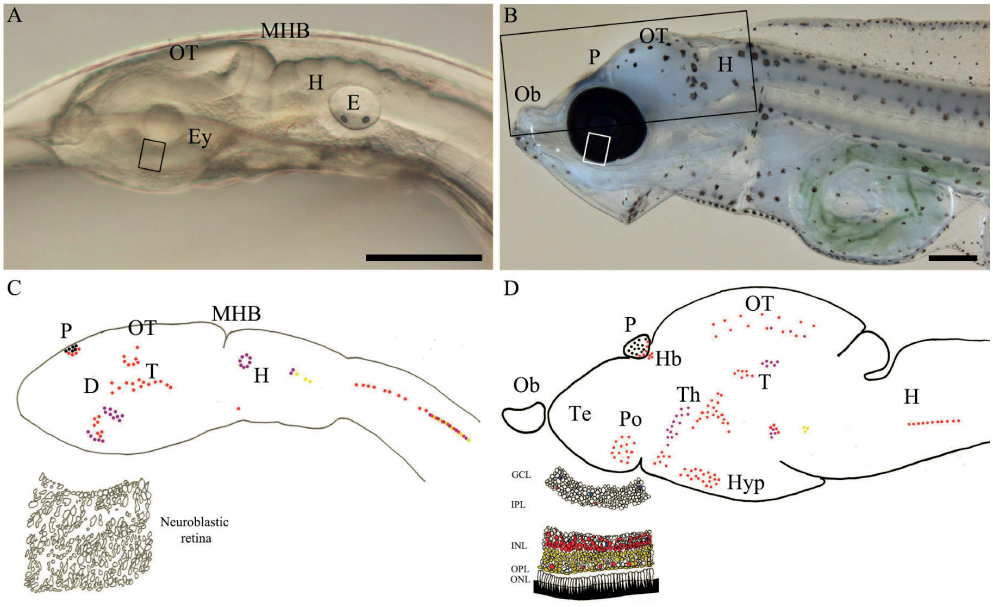

Figure 8 Melanopsin and exorhodopsin expression in the transparent halibut brain at the stage of hatching and at first feeding.

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 black, *opn4m1* in red, *opn4m2* in purple, *opn4x1* in blue and *opn4x2* in yellow. For abbreviations, see list. Scale bar: 500 µm

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Paper II

Cellular co-expression of two photoreceptor pigments of different evolutionary heritage in a hindbrain cluster of Atlantic halibut (*Hippoglossus hippoglossus*)

Mariann Eilertsen, Øyvind Drivenes, Lars O.E. Ebbesson, Jon Vidar Helvik

Manuscript

II

Cellular co-expression of two photoreceptor pigments of different evolutionary heritage in a hindbrain cluster of Atlantic halibut (*Hippoglossus hippoglossus*)

Mariann Eilertsen, [1] Øyvind Drivenes, [2*] Lars O.E. Ebbesson, [1,3] and Jon Vidar Helvik [1**]

[1] Department of Biology, University of Bergen, N- 5020 Bergen, Norway

[2] Department of Molecular Biology, University of Bergen, N- 5020 Bergen, Norway

[3] Uni Research AS, Thormøhlensgt. 49B, N-5006 Bergen, Norway

* Current address: Bergen University College, N- 5020 Bergen, Norway

** Corresponding author and reprint request to:
Jon Vidar Helvik, Department of Biology, University of Bergen, P.O Box 7803, N- 5020 Bergen, Norway, jon.helvik@bio.uib.no

Keywords: melanopsin, vertebrate ancient opsin, non-visual opsin, photoreception

Support: The study was supported by the University of Bergen.

Abbreviations

CeP, cerebella plate
D, diencephalon
E, ear
Ey, eye
FLm, fasciculus longitudialis medialis
GCL, ganglion cell layer
H, hindbrain
Hb, habenula
Hyp, Hypothalamus
INL, inner nuclear layer
IPL, inner plexiform layer
MHB, midbrain hindbrain boundary
N, neuromast
NRetl, nucleus reticularius lateralis
Ob, olfactory bulb
ONL, outer nuclear layer
OPL, outer plexiform layer
OT, optic tectum
P, pineal
Po, preoptic area
T, tegmentum
Te, telencephalon
Th, thalamus
Thd, dorsal thalamus
Thv, ventral thalamus
TS, torus semicircularis

Abstract

The evolution of photoreceptor cells has recently been re-evaluated to better understand the functional significance of newly identified distributions of non-visual opsins, especially in non-mammalian vertebrates. Here we describe the non-visual vertebrate ancient (VA) opsin in the marine teleost Atlantic halibut which has a long pre-retinal photosensitive early life history. The results show that VA opsin is expressed in the halibut brain from early stages of development and persists after the eyes become functional. At the stage of hatching, we find a bilateral hindbrain cluster of cells in halibut expressing both VA opsin and melanopsin. Evolutionary, melanopsin and VA opsin are believed to belong to different classes of photoreceptor pigments that normally are found in rhabdomeric and ciliated photoreceptor cells, respectively. Our data add a new complexity to this understanding, as we here demonstrate for the first time in vertebrates that mRNAs of both photopigments are expressed within the in the same cells in the bilateral hindbrain cluster of Atlantic halibut.

Introduction

Detection of environmental light relies on photoreceptor cells of different properties, the rhabdomeric photoreceptor cells that enlarge their membrane at the apical site and the ciliary photoreceptor cells that fold their cilia membrane (Eakin, 1968; 1982). Recently, the evolution of these photoreceptor cells has been debated (Davies et al., 2010; Ramirez et al., 2011), especially in relation to the non-visual opsins involved in measurements of irradiance and non-directional photoreception (Davies et al., 2010; Matos-Cruz et al., 2011). Taking advantage of the Atlantic halibut, a marine flatfish known to have a dark-dependent hatching process (Helvik and Walther, 1992) and a long pre-retinal stage (Kvenseth et al., 1996), we analyse the composition of non-visual opsins with different evolutionary heritage in the brain.

Evolution of photoreceptor cells has been highlighted by reconstruction of the eyes of the Urbilateria, suggesting that the two distinct photoreceptor cells already existed in the common ancestor of Bilaterians (Arendt and Wittbrodt, 2001). It has been further suggested that in pre-bilateralians a duplication event of an ancestral opsin into c-opsin and r-opsin, allowed a diversification of the precursor photoreceptor cell into ciliary and rhabdomeric type. The ciliary photoreceptor cells are believed to have c-opsin incorporated in their membrane and they involve a Gi-protein dependent phototransduction cascade leading to hyperpolarisation of the cell, while rhabdomeric photoreceptor cells relay on r-opsin, a Gq-protein dependent phototransduction cascade and depolarization of the cell (Arendt, 2003). Evolution of photoreceptor cells has recently been debated, especially in the light of the increasing knowledge of non-visual opsins in non-mammalian vertebrates. In particular, a non-visual opsin protein termed vertebrate ancient opsin (VA opsin) does not fit in to the theory where the rhabdomeric photoreceptor cells express r-opsin and the ciliary photoreceptor cells express c-opsin (Davies et al., 2010). Even though retinal horizontal and amacrine cells are thought to be of rhabdomeric heritage (Arendt, 2003), VA opsin related to c-opsin is shown to be

expressed in both horizontal and amacrine cells of salmon (Philp et al., 2000; Soni et al., 1998) and in horizontal cells of zebrafish (Kojima et al., 2000). In addition, several studies in teleosts indicate that melanopsin of r-opsin origin and VA opsins of c-opsin origin are expressed in the same cell types of retina (Bellingham et al., 2002; Jenkins et al., 2003) and co-localised in some brain regions (Sandbakken et al., 2012).

Recently, a new classification scheme has been suggested for photoreceptor cells (Ramirez et al., 2011). This new approach of grouping photoreceptor cells considers both the anatomical distribution (aggregated *vs.* dispersed) and the position of the photoreceptor cells within a neuronal network (first-order *vs.* high-order). First-ordered photoreceptor cells are considered to function as primary sensory cells transducing external stimuli into electrical signals, passing these signals into other neurons through synapses. High-order photoreceptor cells on the other hand are photosensitive interneurons that are able to both receive and send electrical signals. It is indicated that classification by distribution and neural identity provides the possibility to describe a wider set of light sensitive neurons (Ramirez et al., 2011). Here we use this classification to evaluate photoreceptor cells in halibut.

The present study identifies the debated VA opsin in Atlantic halibut brain at the stage of hatching and first feeding. This work is the first in vertebrates to employ double fluorescent *in situ* hybridisation techniques to reveal the cellular expression of the two opsin genes, melanopsin and VA opsin, of putative different evolutionary heritage. Most interestingly, we find that VA opsin and melanopsin are co-expressed in the same cells of the bilateral hindbrain cluster at the stage of hatching.

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 carried out 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 vertebrate ancient opsin in halibut was done by PCR using degenerative primers as described in (Helvik et al., 2001). To generate full-length of the gene, Rapid Amplification of cDNA Ends (RACE) was done with a nested approach with the annealing temperature of 62,9 °C for 5'RACE and 60 °C for 3'RACE and 35 cycles in the first round. The second round of PCR had a annealing temperature of 66,5 °C for both 5' and 3' RACE and 35 cycles were used. The nested 3'RACE resulted in two PCR products of different length and the assembly of the two RACE products was verified by a PCR with primer binding sites located in the predicted the 5' and 3' UTRs. PCR products were extracted from agarose gel using QIAEX II Gel Extrection 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. Primers are listed in Table 1. The nucleotide sequences were deposited into GeneBank with the accession number KF941295 (*val*) and KF941296 (*vas*).

The halibut genome has recently been sequenced on an Illumina HiSeq200 (Illumina, San Diego, CA) (Pair End, 100bp reads) to 40x coverage and a contig assembly has been made by the CLC software (CLC bio, Denmark). The genome was searched by BLASTN and TBLASTN (NCBI, Bethesda, MD) with the two isoforms of VA opsin to verify the sequenced obtained by PCR. In addition the genome was searched by TBLASTN using available teleost protein opsin sequences as a query in order to obtain a potential duplicate of VA opsin as seen in zebrafish (Kojima et al., 2008).

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. Alignment of the amino acid sequences was made by ClustalX 2.1 (Larkin et al., 2007). Phylogenetic analysis was done by constructing a maximum likelihood tree using MEGA version 5 (Tamura et al., 2011) and 1000 bootstrap replicates were applied to ensure the statistical robustness of each node.

Riboprobes

Preparation of digoxigenin (DIG)-labelled and fluorescein-labelled riboprobes for vertebrate ancient opsin (VA opsin) and digoxigenin (DIG)-labelled riboprobe for melanopsin (*opn4m2*) were done 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 synthesised probes were precipitated by tRNA (Roche Diagnostics, Germany). Sequence alignment shows that the similarity between the sequence targets of the melanopsin and VA opsin probes is approximately 50 % (data not shown).

***In situ* hybridisation**

In situ hybridisation on whole embryos (13 days post fertilisation (dpf) and 47 days post hatching (dph)) was done as described in (Paper I). *In situ* hybridisation on cryosections

(9 dpf, 11 dpf and 13 dpf) was done according to Sandbakken et al. (2012). In addition, parallel cryosections of 47 dph larvae were cut at 10 µm. One parallel was stained by *in situ* hybridisation and the other parallel was Nissel-stained with 0,5 % Cresyl Fast Violet (Chroma-Gesellschaft, Germany).

Fluorescent double labelling *in situ* hybridisation

Fluorescent double labelling *in situ* hybridisation on whole embryos (12 dpf) was done as described in Paper I. Fluorescent double labelling *in situ* hybridisation on cryosections (12 dpf) was performed following the *in situ* hybridisation protocol on cryosections described in Sandbakken et al. (2012), except that both probe were applied at the hybridisation step. In addition, the fluorescein-labelled riboprobe for VA opsin was first visualised by using the antibody Anti-Fluorescein-horse radish peroxidase (POD), Fab fragments (1:400) (Cat. No 11426346910, Roche Diagnostics, Germany) and the TSA^{TM}Plus Fluorescein Systems (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) the sections were blocked for 1 hour in 2 % Blocking reagent (Roche Diagnostics, Germany) in 2xSCC, and the digoxigenin-labelled riboprobe for melanopsin was visualised by use of Fast Red tablets as recommended by the manufacturer (Cat. No 11496549001, Roche Diagnostics, Germany). The stained sections were mounted in DABCO antifading medium (Triethylenediamine, Sigma, St. Louis, MO) and stored in darkness.

Microscopy

Pictures were taken by using a digital camera (Leica DFC 320/ Leica 350 FX) attached to a Leica DM 6000B microscope (Leica Microsystems, Germany) with an ebx75mc-L90 lamp (Leistrungselektronik Jena GmbH, Germany). Confocal images were taken using the Leica TCS SPE confocal system (Leica Microsystems, Germany) together with a Leica DM2500 microscope (Leica Microsystems, Germany). All pictures from the fluorescent

and confocal imaging are single pictures with one focus plane and not a stack of pictures with different focus planes merged together. Adobe Photoshop CS5 (San Jose, CA) was used for adjustments of brightness, contrast, color levels and to sharpen the pictures.

Results

Identification and characterisation of VA opsin in Atlantic halibut

Full-length cDNA of vertebrate ancient opsin (VA opsin) was isolated from an Atlantic halibut cDNA library by PCR and later verified by searching the halibut genome. Two isoforms of VA opsin were identified and an alignment (Fig. 1) shows that the isoforms are identical in the first four exons. The fifth exon is altered in the two isoforms and as in zebrafish (Kojima et al., 2000) the fifth exon provides different length in the cytoplasmatic tail. In zebrafish an additional VA opsin gene has been identified (Kojima et al., 2008) and search for an ortholog in Atlantic halibut has been done by degenerative PCR and later by search in the halibut genome using a BLAST algorithm, with no results. The alignment (Fig. 1) of the putative amino acid sequences of the two isoforms in halibut together with VA opsin in other species shows that they span the seven transmembrane α-helical domains (TM1-7). The isoforms have the lysine (K) in the TM7 that serves as a site for Schiff base linkage of the chromophore 11-*cis* retinal and in the third transmembrane domain the glutamic acid (E) serves as a counterion typical for visual opsins (reviewed in Terakita, 2005). In the interface between TM3 and the second intracellular loop a ERY tripeptide ((E/D)R(Y/W/F)) thought to be critical for G-protein activation is present in the isoforms (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 VA opsin isoforms.

Phylogenetic analysis

The maximum likelihood tree (Fig. 2) based on amino acid sequences shows the putative evolutionary relationship between Atlantic halibut VA opsin and VA opsins of various species. Visual opsins and exorhodopsin of halibut are also included. The melanopsins of Atlantic halibut were used as an outgroup. The two halibut isoforms of VA opsin branch together with zebrafish Valopb and salmon Vas opsin. The phylogenetic tree reveals that VA opsin is more similar to visual opsins than melanopsin, the r-opsin homologue.

Expression of VA opsin in the brain at the stage of hatching

In situ hybridisation on halibut embryos at the stage of hatching showed that VA opsin is expressed in the brain (Fig. 3). To investigate if the two isoforms have different expression pattern riboprobes that discriminate the long and short isoforms were tested. No expression was detected in the short isoform (*vas*) and in addition a riboprobe designed to detect mRNA within the four first exons give the same expression pattern as the riboprobe for *val* (data not shown). Sense probes were included as negative controls and showed no expression (data not shown). VA opsin expression was detected ventral in diencephalon and in the hindbrain at 13 dpf (Fig. 3A-B). In diencephalon, the expression is seen a few cell rows lateral to the midline and in the hindbrain two condense ball shaped clusters of aggregated VA opsin positive cells are seen on each side of the midline at the level of the lateral neuromasts (Fig. 3A). From a lateral view, the diencephalon cluster can be observed as scattered cells descending caudally and ventrally in the diencephalon, while the ball-shaped cluster of cells in the hindbrain is seen caudal to the midbrain-hindbrain boundary in the middle of the hindbrain (Fig. 3B). Sections of the brain at the same developmental stage show the VA opsin positive cells in the diencephalon (Fig. 3C) and in the hindbrain (Fig. 3D). A section through the hindbrain cluster showed that the cells in the middle of the ball are VA opsin negative (Fig. 3D). Sagittal sections of the hindbrain cluster at 9 dpf (Fig. 3E), 11 dpf (Fig. 3F) and 13 dpf (Fig. 3G) show how the expression of VA opsin expands during development.

Co-localisation of VA opsin and melanopsin in the hindbrain

The c-opsin homologue VA opsin and the r-opsin homologue mammalian-like melanopsin (*opn4m2*) are both expressed in the hindbrain at the stage of hatching. The expression is seen bilateral at the level of the lateral neuromasts just caudal to the midbrain-hindbrain boundary (Fig. 4A-B). To verify that the two genes are expressed in the same cells, fluorescent double labelling *in situ* hybridisation was done (Fig. 4C-H). Confocal imaging of a whole embryo showed that the two genes are expressed in the same ball-shaped cluster in the hindbrain (Fig. 4C-E). A sagittal section through the hindbrain cluster showed that VA opsin and melanopsin have overlapping expression within the same cells of the aggregated cluster (Fig. 4 F-H), with some cells in the aggregated cluster just express one of the opsins (data not shown).

VA opsin expression in the brain and retina at first feeding

At the stage of first feeding (47 dph), when the brain regions are more differentiated, VA opsin expression was analysed in whole larvae and in sections (Fig. 5). The spatial distribution of VA positive cells was examined by *in situ* hybridisation on whole larvae (Fig. 5A-B) and on sections the expression pattern was compared with adjacent Nissel-stained sections (Fig. 5D-I). From a dorsal view (Fig. 5A) three bilateral clusters of VA opsin positive cells are apparent. Rostral, a big cluster starts laterally and ends up a few cell rows from the midline. In the midbrain another cluster is seen a few cell rows from the midline while the expression in the hindbrain has a more diffuse pattern. Seen from the side (Fig. 5B) the rostral expression pattern starts dorsal in diencephalon and continues ventrally and caudally. In addition a smaller population of cells is seen ventral to the big rostral group of cells. The cluster of cells in the midbrain is situated dorsal in tegmentum just ventral to the optic tectum and the diffuse pattern in the hindbrain is located dorsally, starting just caudal to the midbrain hindbrain boundary. In the retina, VA opsin is expressed in the inner nuclear layer in cells resembling amacrine cells (Fig. 5C). The transversal sections of the brain show the VA opsin positive cells in more detail (Fig. 5D-I). The big cluster in diencephalon can be seen as single cells in dorsal thalamus (Fig.

5D1-3) and more caudally the same cluster is closer to the midline, and in addition, a small cell population is apparent more ventrally in the ventral thalamus (Fig. 5E1-3). The distribution of cells just ventral to the optic tectum in the tegmentum is shown in Fig. 5F1-3. VA opsin positive cells in the optic tectum and the torus semicircularis are also revealed in transversal sections (Fig. 5G1-3). The diffuse expression seen in the dorsal hindbrain (Fig. 5A-B) is shown to be located just ventral to the cerebella plate in a discrete population of the dorsal hindbrain (Fig. 5H1-3). In addition cells expressing VA opsin are seen medially, just dorsal to the fasciculus longitudinalis medialis and ventrally presumably in the area of nucleus reticularius lateralis (Fig. 5I1-3).

Discussion

Much attention has recently been drawn to the evolution of photoreceptor cells, particularly in relation to the non-visual opsins involved in measurement of irradiance and non-directional photoreception. We add to this discussion by identifying the debated vertebrate ancient opsin in a teleost with a special early life strategy. We take advantage of the Atlantic halibut's early expression of non-visual opsins compared to the later retinal (visual) development. The study demonstrates that the two non-visual opsins with different heritages in photoreceptor evolution, VA opsin and melanopsin, are expressed in the same cells in a unique aggregated hindbrain cluster.

Identification of VA opsin in Atlantic halibut

It is apparent that many of the identified VA opsin genes have different isoforms, varying in the length of the carboxyl-tail (Halford et al., 2009; Jenkins et al., 2003; Kojima et al., 2000; Minamoto and Shimizu, 2002). The isoforms are identical in the first four exons that span the seven transmembrane region while the fifth and last exon differs in the isoforms. The short isoform is generated by a read-through into intron 4 while the long isoform is made by splicing to exon5 (Halford et al., 2009; Kojima et al., 2000). We have identified a long and a short isoform of VA opsin in Atlantic halibut and sequence

alignment (Fig. 1) and searching the halibut genome show that this is also the case for the halibut isoforms. In zebrafish there exists one gene with two isoforms, long (*valopa*) and short (*vas*), and in addition a second VA opsin gene (*valopb*) (Kojima et al., 2000; Kojima et al., 2008). Extensive search in the halibut genome database has not identified any additional VA opsin, indicating that halibut just has one VA opsin gene. This also appears to be the case for other teleosts, where only one VA opsin gene with different isoforms has been reported (Jenkins et al., 2003; Minamoto and Shimizu, 2002). In teleosts, it seems that the VA opsin photoreceptive system rely on either one or two genes, while the melanopsin system uses several genes. This is demonstrated by the existence of four melanopsins in halibut (Paper I), five in zebrafish (Davies et al., 2011; Matos-Cruz et al., 2011) and six in salmon (Sandbakken et al., 2012). Why evolution has expanded the melanopsin family, compare to the VA family is still a puzzle.

Distinct expression of VA opsin at the stage of hatching and first feeding

So far, only a few studies have shown non-visual photoreception at early life stages of teleosts. We have recently identified four melanopsins in Atlantic halibut and demonstrated an extensive expression of the melanopsins at the stage of hatching. Further on we have shown that the expression persists when the eyes are functional around first feeding (Paper I). Here we show that also another non-visual opsin, VA opsin, is expressed from early stages of halibut development. Like melanopsin, VA opsin expression also persists in the brain when the eyes are functional, however the VA opsin expression pattern at hatching is not comparable with that seen at first feeding. Unlike the extensive expression of the melanopsins, the expression of VA opsin is less widespread (Fig. 6). We find the long isoform of VA opsin expressed in the ventral diencephalon and in a bilateral ball-shaped hindbrain clusters at the stage of hatching (Fig. 3), while expression of the short isoform is not apparent. At first feeding the long isoform of VA opsin is extensively expressed in dorsal thalamus, in addition to cells in ventral thalamus, optic tectum, dorsal tegmentum and in dorsal, medial and ventral hindbrain (Fig. 5). The VA opsin expression in the dorsal thalamus at first feeding is consistent with our findings

in Atlantic salmon, while we do not detect expression in the pineal and habenula/subhabenula as shown in salmon (Philp et al., 2000; Sandbakken et al., 2012). Early hindbrain expression of VA opsin is also detected in zebrafish larvae and, as in zebrafish, we detect the expression in the anterior part of the hindbrain (Kojima et al., 2008) (Fig. 3). Figure 6 demonstrates that the VA opsin and melanopsin expression are located in many of the same brain regions, although the extensive VA opsin expression in dorsal thalamus at first feeding does not overlap with any melanopsin expression. The most apparent overlap in expression is the dense cluster of cells in the hindbrain at hatching, but also the ventral diencephalon exhibits melanopsin and VA opsin expression early in development. At first feeding, both melanopsin and VA opsin are expressed in the ventral thalamus, optic tectum, dorsal tegmentum and medial in the hindbrain.

Evolution of photoreceptor cells

In the present study we have demonstrated that both VA opsin and melanopsin, of apparently different evolutionary heritage, are expressed in a bilateral ball-shaped hindbrain cluster at the stage of hatching in halibut (Fig. 4). By fluorescent double labelling techniques we confirm previous indications of co-expression (Bellingham et al., 2002; Jenkins et al., 2003; Sandbakken et al., 2012) by showing for the first time in vertebrates that the mRNAs of both photopigments are expressed in the same cells. It has been suggested that the distinction made between c-opsin and r-opsin expression within vertebrate photoreceptors of rhabdomeric or ciliary heritage needs significant revision (Davies et al., 2010) and our results strongly support this necessity by showing dual expression within photoreceptor cells. In teleost retina it has been proposed that the retinal cell types have a common ancestral bimodal cell lineage dating back to primitive bilaterians that expressed both c-opsin and r-opsin (Davies et al., 2010; Matos-Cruz et al., 2011). This theory is based on results from retina that show melanopsins in photoreceptor cells of assumed ciliary heritage (Davies et al., 2011; Matos-Cruz et al., 2011) and VA opsins in photoreceptors of putative rhabdomeric heritage (Jenkins et al., 2003; Kojima et al., 2000; Soni et al., 1998). We suggest that this ancestral cell lineage may also have

given rise to the dual photopigment expressing cells of the hindbrain in halibut, indicating that this photosensitive precursor cell has persisted parallel to the eye evolution of ciliary and rhabdomeric photoreceptor cells.

Involvement of potential phototransduction cascades

The phototransduction cascade for photoreceptor cells expressing c-opsin or r-opsin is thought to involve different machinery and results in hyperpolarisation or depolarisation respectively (Arendt, 2003). It is therefore unknown how the phototransduction cascade will function in this ancestral cell lineage expressing both opsin types. Recent studies have however indicated that the different paralogs of melanopsin have high variability in the coupling specificity of G-proteins. Although speculative, the different melanopsins may mediate the phototransduction pathway through Gq or Gi-proteins (Borges et al., 2012). One can hypothesise that the melanopsin expressed in the hindbrain cluster not just involve the phototransduction pathway employing Gq-proteins, leading to depolarisation of the cells. Although speculative, the dual photopigment expressing cells of the hindbrain cluster may also have the possibility to employ both phototransduction cascades, based on which opsin that is activated as a response to different spectra of the light.

The cells of the hindbrain cluster may be primary sensory neurons or interneurons

According to the classification of photoreceptor cells based on distribution and neural identity (Ramirez et al., 2011), the melanopsin and VA opsin expressing cells of the hindbrain can be assessed as an aggregated cluster of cells. The aggregated cells may be first-order or high-order photoreceptor cells. One can speculate if the aggregated hindbrain cluster consists of primary sensor neurons directly involved in a photoreceptive response early in development of halibut. Or, the cells may be photosensitive interneurons that can modulate incoming electrical signals before they are transmitted. The neural identity of the cells and the underlying molecular machinery for phototransduction induced by the non-visual photopigments remain to be elucidated. In addition, melanopsin

in the hindbrain cluster may function as a photoisomerase providing 11-*cis* retinal to the monostable VA opsin.

Acknowledgement

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Table 1 Primer information

Primer name	Sequence (5'–3')	Use
OpsinFw	AAGAAGYTCMGTCMACCTCTYAAYT	Degenerative primer
OpsinRv	GTTCATGAAGACRTAGATDAYAGGGTTRTA	Degenerative primer
VAF1	CATCCGACCATCCAACTCGAT	3'RACE
VAR1	TGATGTAGCTGTGAGCCGTCATG	5'RACE
VAF2	CCAAGACAGCCGCTGTCTACAA	Nested 3'RACE
VAR2	GTCAGGCTCACAGGTTGTTCCAATCTT	Nested 5'RACE

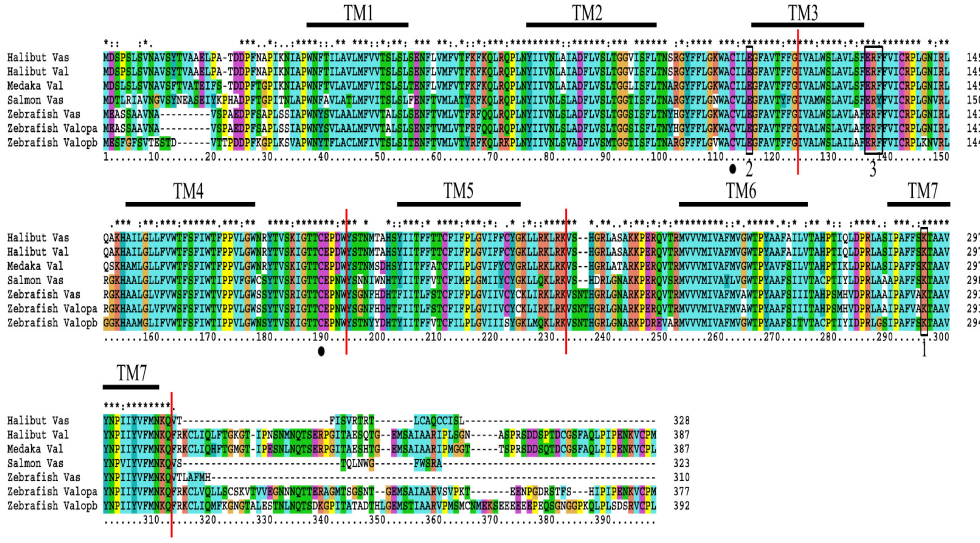


Figure 1 Alignment of the two deduced amino acid sequences of vertebrate ancient opsin (VA opsin) isoforms in Atlantic halibut against published VA opsin sequences.

VA opsin in zebrafish (Valopa and Valopb) and the shorter isoform (Vas) of Valopa are used in the alignment together with VA opsin in medaka (Val) and Atlantic salmon (Vas). The seven transmembrane (TM) domains are indicated by TM1-7 and the alignment shows that the halibut VA opsin isoforms span the domains. The retinal attachment site (K) in TM7 (1), the potential Schiff base counterions E (2) and the ERY tripeptide ((E/D)R(Y/W/F)) (3) are outlined. Cysteins involved in the disulfide bridge formation are indicated with solid circles. Red lines indicate the boundaries between the five exons.

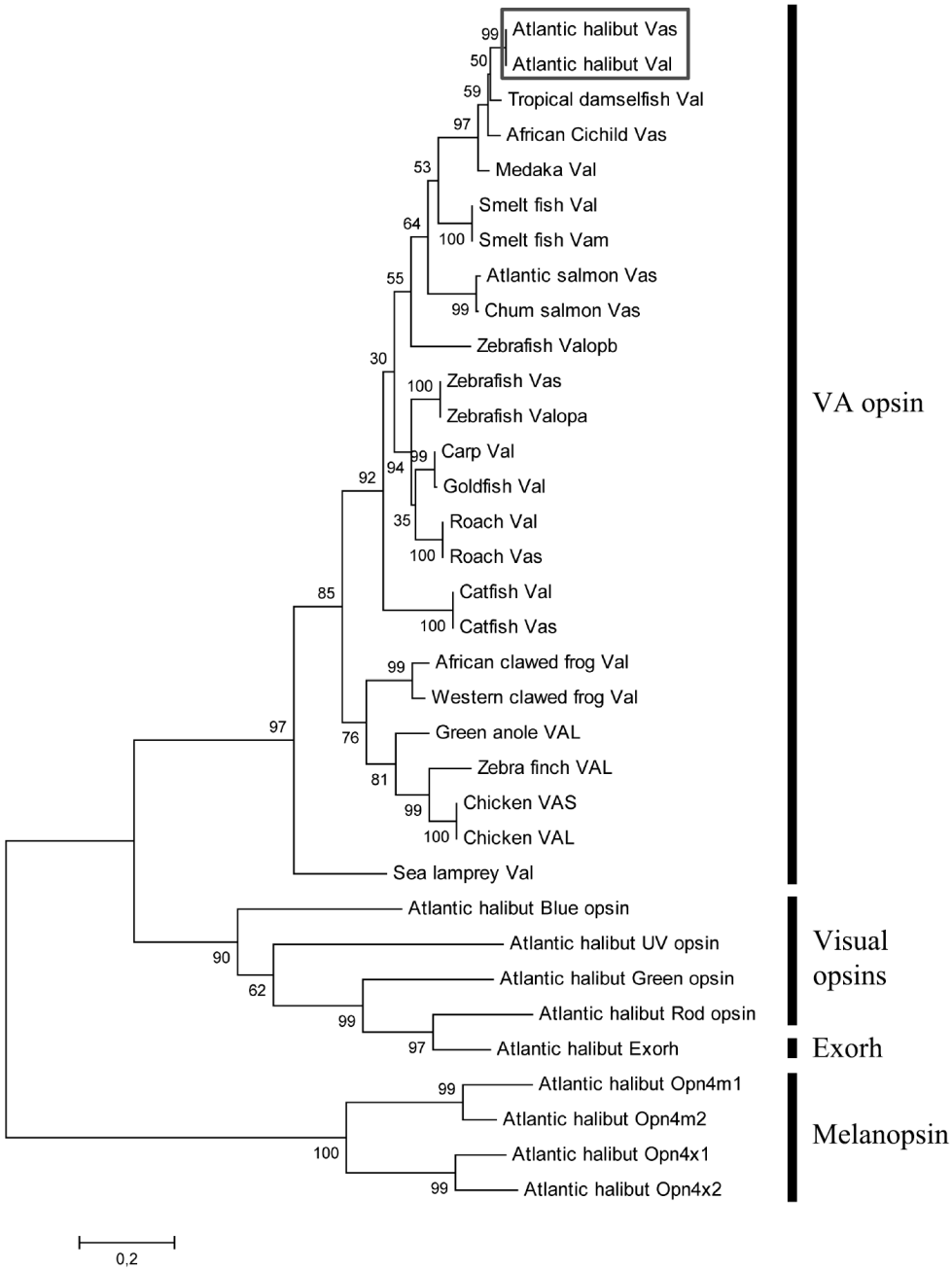

Atlantic halibut Vas
Atlantic halibut Val
Tropical damselfish Val
African Cichild Vas
Medaka Val
Smelt fish Val
Smelt fish Vam
Atlantic salmon Vas
Chum salmon Vas
Zebrafish Valopb
Zebrafish Vas
Zebrafish Valopa
Carp Val
Goldfish Val
Roach Val
Roach Vas
Catfish Val
Catfish Vas
African clawed frog Val
Western clawed frog Val
Green anole VAL
Zebra finch VAL
Chicken VAS
Chicken VAL
Sea lamprey Val
Atlantic halibut Blue opsin
Atlantic halibut UV opsin
Atlantic halibut Green opsin
Atlantic halibut Rod opsin
Atlantic halibut Exorh
Atlantic halibut Opn4m1
Atlantic halibut Opn4m2
Atlantic halibut Opn4x1
Atlantic halibut Opn4x2
VA opsin
Visual opsins
Exorh
Melanopsin
0,2

Figure 2 Phylogenetic tree

The maximum likelihood tree shows the phylogenetic relationship between the Atlantic halibut vertebrate ancient opsin (VA opsin) and VA opsin of other species. The tree also includes the visual opsins and exorhodopsin of halibut and the halibut melanopsins are used as outgroup. A bootstrap value of 1000 has been applied. The opsin sequences used for generating the tree are: i) Vertebrate ancient opsins: Atlantic halibut (*Hippoglossus hippoglossus*) KF941296 (Vas), KF941295 (Val); Tropical damselfish (*Chrysiptera cyanea*) BAK19957.1 (Val); African cichlid (*Haplochromis burtoni*) ACB29677.1 (Vas); Medaka (*Oryzias latipes*) NP001129987.1 (Val); Smelt fish (*Plecoglossus altivelis*) BAB88651.1 (Val), BAB88650.1 (Vam); Atlantic salmon (*Salmon salar*) AAC60124.1 (Vas); Chum salmon (*Oncorhynchus keta*) AAK27833.1 (Vas); Zebrafish (*Danio rerio*) AAY56361.1 (Valopb), BAA94289.1 (Vas), BAA94288.1 (Valopa); Carp (*Cyprinus carpio*) AAF74260.1 (Val); Goldfish (*Carassius auratus*) BAG68692.1 (Val); Roach (*Rutilus rutilus*) AAM77793.1 (Val), AAM77794.1 (Vas); Catfish (*Ictalurus punctatus*) ACP43589.1 (Val), ACP43588.1 (Vas); African clawed frog (*Xenopus laevis*) ACJ61344.1 (Val); Western clawed frog (*Xenopus tropicalis*) ACX32471.1 (Val); Green anole (*Anolis carolinensis*) ACX32472.1 (VAL); Zebra finch (*Taeniopygia guttata*) ACX32473.1 (VAL); Chicken (*Gallus gallus*) ABM66817.2 (VAS), ACX32474.1 (VAL); Sea lamprey (*Petromyzon marinus*) AAC41240.1 (Val) ii) Visual opsins: Atlantic halibut (*Hippoglossus hippoglossus*) AAM17921.1 (Red opsin), AAM17917.1 (UV opsin), AAM17920.1 (Blue opsin), AAM17916.1 (Green opsin), AAM17918.1 (Rod opsin) iii) Exorhodopsin: Atlantic halibut (*Hippoglossus hippoglossus*) KF941294 (Exorh) iv) Melanopsins: Atlantic halibut (*Hippoglossus hippoglossus*) KF941289 (Opn4m1), KF941291 (Opn4m2), KF941292 (Opn4x1), KF941293 (Opn4x2).(A green box highlights the halibut VA opsin isoforms.)

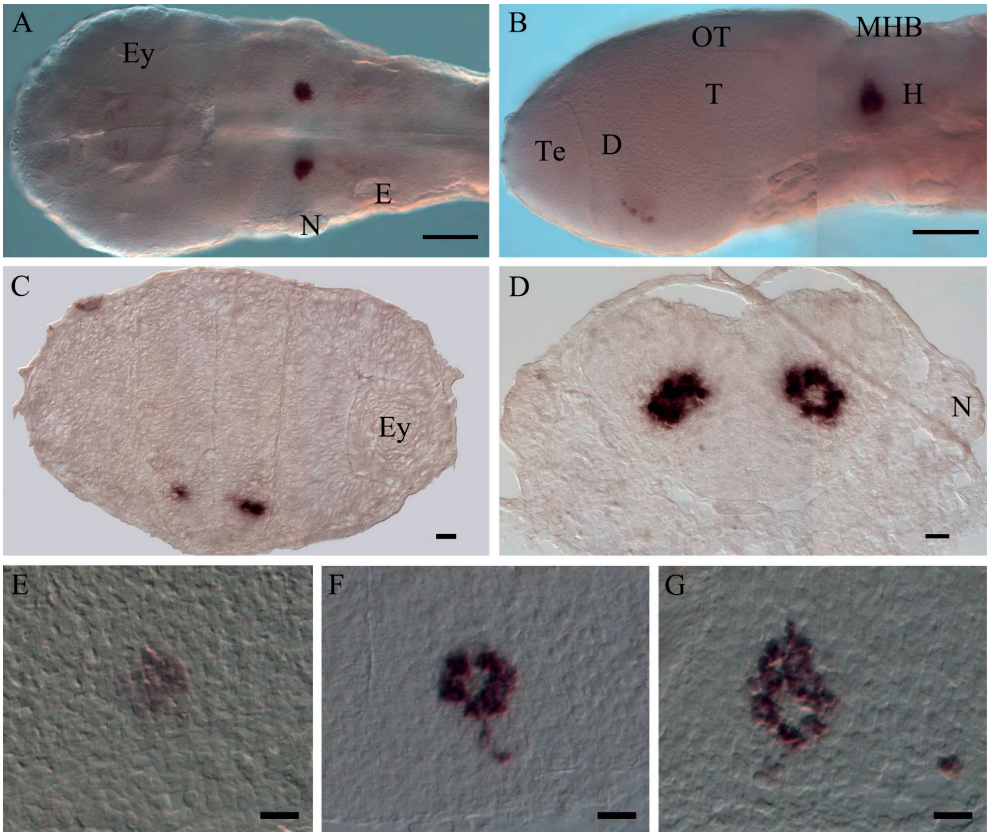


Figure 3 Expression of vertebrate ancient opsin (VA opsin) in the Atlantic halibut brain at early developmental stages.

A-B: A dorsal view (A) and a lateral view (B) of a whole embryo (13 days post fertilisation (dpf)) show VA opsin positive cells in ventral diencephalon and in the hindbrain just caudal to the midbrain-hindbrain boundary. The dorsal and lateral views are made by merging several pictures in different focus planes. C-D: Transversal sections through the brain at the same developmental stage show the expression in diencephalon (C) and the ball-shaped hindbrain clusters with VA opsin negative cells in the middle (D). E-G: Sagittal sections of the hindbrain cluster at 9 dpf, 11 dpf and 13 dpf show how the expression pattern extends. For abbreviations see list. Scale bars: 200 μm in A and B and 20 μm in C-G

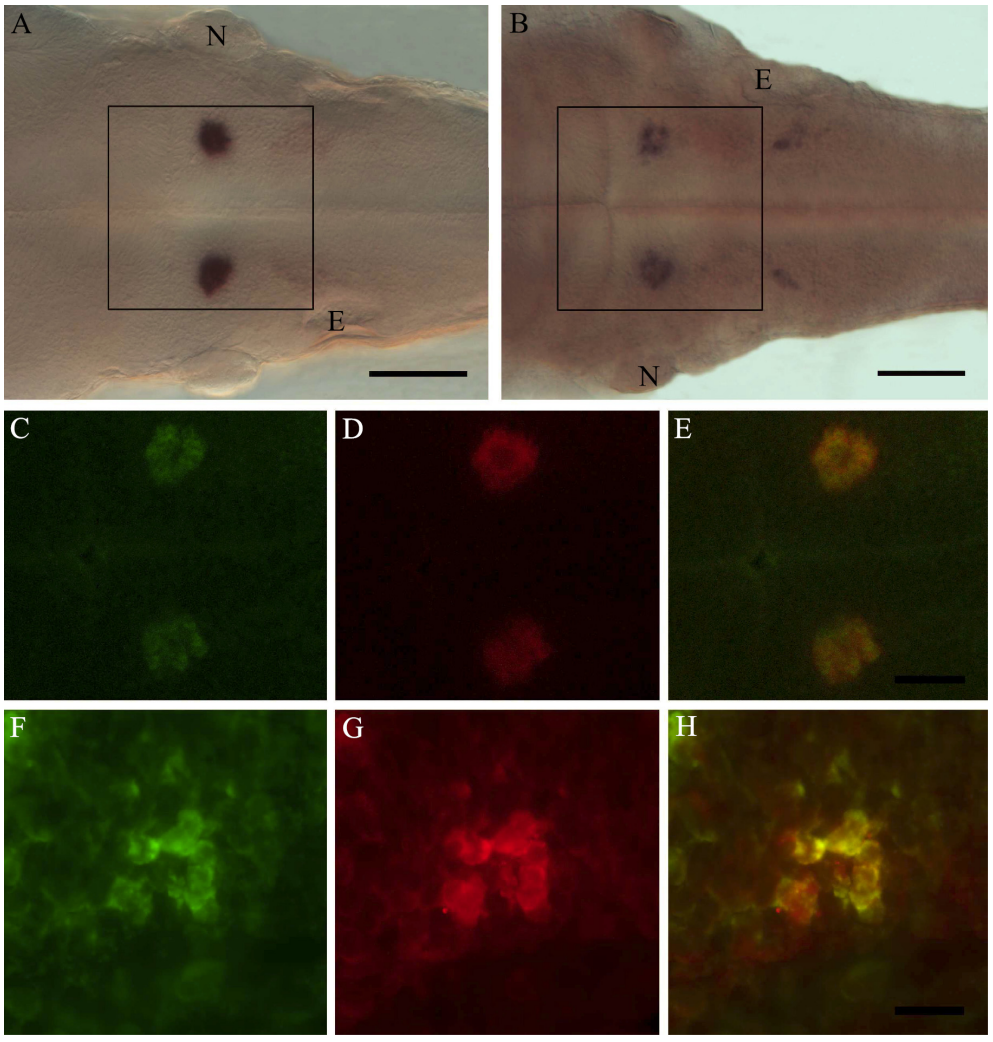
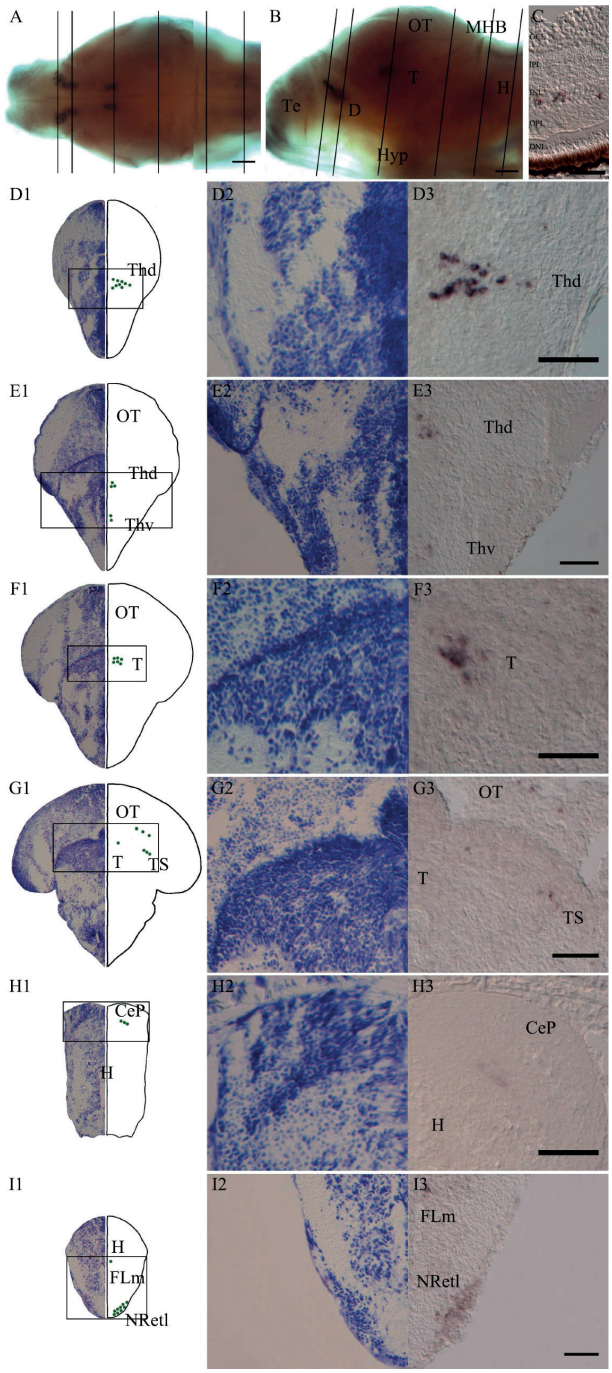


Figure 4 Co- expression of vertebrate ancient opsin and melanopsin in the bilateral hindbrain cluster. A-B: Halibut embryos with vertebrate ancient opsin (VA opsin) (A) and melanopsin (*opn4m2*) (B) expression in ball-shaped clusters in the hindbrain at the stage of hatching. C-E: Confocal imaging of a whole embryo with VA opsin in green (C) and melanopsin in red (D) and the two genes together (E) show that the genes are expressed in the same region. F-H: Fluorescent microscopy of a sagittal section through the hindbrain clusters reveals that VA opsin (F) and melanopsin (G) are in the same cells in the cluster (H). Scale bars: 100 µm in A-B, 50 µm in C-E and 20 µm in F-H.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H1
CeP
H
H2
H3
CeP
H
I1
H
FLm
NRetl
I2
I3
FLm
NRetl

Figure 5 Vertebrate ancient opsin (VA opsin) in brain and retina of halibut larvae with functional eyes. A-B: The spatial distribution of VA opsin in a whole larva (47 days post hatching (dph)) seen from a dorsal (A) and lateral (B) view. The dorsal and view (A) is made by several pictures in different focus planes. Eyes are removed for clarity and lines are drawn to illustrate the transversal sections. C: A transversal section of retina at the same developmental stage. D1-I1: Nissel-stained transversal sections at the equivalent level of VA opsin expressing cells illustrated by green dots. D2-I2: The Nissel-stained cell populations of interest with a higher magnification. D3-I3: VA opsin expression at the same level and with the same high magnification. A: From a dorsal view a big rostral cluster is apparent starting laterally ending up more toward the midline in caudal parts. In the midbrain a cluster of cells is seen a few cell rows from the midline and in hindbrain (H) a diffuse expression pattern is observed. B: From a lateral view the big rostral cluster is located to dorsal diencephalon (D) and in addition a small population of cells is seen ventral to big cluster. The midbrain cluster is in tegmentum (T) just ventral to the optic tectum (OT) and an expression pattern in the hindbrain is located dorsal. C: VA opsin is expressed in the inner nuclear layer of retina in cells similar to amacrine cells. D1-D3: VA opsin expression in dorsal thalamus (THd). E1-E3: Expression in the dorsal and ventral thalamus (THd and THv). F1-F3: Cluster of cells dorsal in tegmentum just ventral to the optic tectum. G1-G3: VA opsin positive cells in optic tectum and torus semicircularis (TS). H1-H3: Diffuse expression dorsal in the hindbrain. I1-I3: Expression medial, just dorsal to the fasciculus longitudinalis medialis (FLm) and ventrally in the area of nucleus reticularius lateralis (NRetl) Scale bars: 100 μm in A-B and 50 μm in C and D3-I3

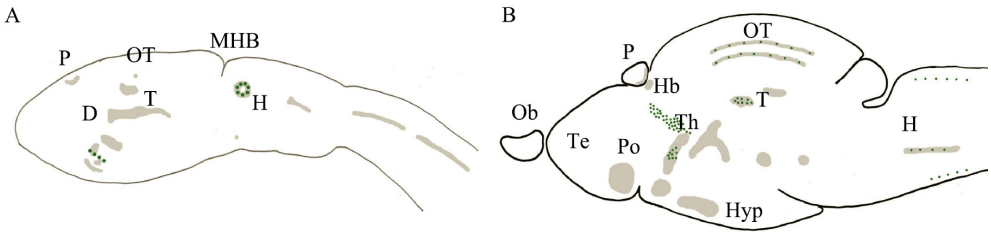

Figure 6 Schematic drawings of vertebrate ancient opsin (VA opsin) and melanopsin positive brain regions.

Regions with expression of the different melanopsins are marked by grey (see paper I) while VA positive areas are marked by green dots. A: At the stage of the light regulated hatching process VA opsin is expressed in the hindbrain and ventral diencephalon together with melanopsin positive cells. B: At first feeding an extensive cluster of VA opsin is located in dorsal thalamus where no melanopsin expression is seen. Brain regions with both VA opsin and melanopsin are ventral thalamus, optic tectum, dorsal tegmentum and medial in the hindbrain. For abbreviations, see list.

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Paper III

Non-visual opsin expressing cells in the hindbrain are involved in the dark-induced hatching process in Atlantic halibut (*Hippoglossus hippoglossus*).

Mariann Eilertsen, Lars O.E. Ebbesson and Jon Vidar Helvik

Manuscript

III

Non-visual opsin expressing cells in the hindbrain are involved in the dark-induced hatching process in Atlantic halibut (*Hippoglossus hippoglossus*).

Mariann Eilertsen, [1] Lars O.E. Ebbesson, [1,2] and Jon Vidar Helvik [1*]

[1] Department of Biology, University of Bergen, N- 5020 Bergen, Norway

[2] Uni Research AS, Thormøhlensgt. 49B, N-5006 Bergen, Norway

* Corresponding author and reprint request to:
Jon Vidar Helvik, Department of Biology, University of Bergen, P.O Box 7803, N- 5020 Bergen, Norway, jon.helvik@bio.uib.no

Keywords: melanopsin, vertebrate ancient opsin, *c-fos*, photoreception, hatching glands, immediate early genes

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Abbreviations

E, ear

Ey, ear

Dpf, days post fertilisation

H, hindbrain

HGC, hatching gland cell

MHB, midbrain-hindbrain boundary

N, neuromast

Ob, olfactory bulb

OT, optic tectum

P, pineal

Te, telencephalon

VA opsin, vertebrate ancient opsin

Y, yolk

Abstract

A wide range of photoreceptor cells and their photosensitive pigments have been identified in teleosts the past decades, but knowledge of how these cells contribute to the behavioural and physiological responses to light is just emerging. Here we describe a transient embryonal cluster of cells in the hindbrain of the marine teleost Atlantic halibut (*Hippoglossus hippoglossus*) expressing both vertebrate ancient (VA) opsin and melanopsin. This bilateral photosensitive cluster of cells is apparent at the stage of the light-regulated hatching of halibut, long before the eyes are functional. We show that the hindbrain cluster is connected to a neuronal network with a projection out in the yolk sac, reaching the narrow belt of hatching glands. In addition, studies with light-arrested eggs demonstrate neural activation in the hindbrain cluster and hatching glands upon dark-induced hatching. Taken together, these results indicate that the photosensitive hindbrain cluster regulates hatching in halibut and that this process is dependent on neural signalling. Moreover the activation of this neural circuit in darkness implies that the net output of melanopsin and VA opsin is inhibitory in the system.

Introduction

Hatching of Atlantic halibut eggs is a dark induced process that provides a unique opportunity to study non-visual photoreception in a teleost. Halibut eggs exposed to light are photo-arrested, but put back to darkness they will hatch rapid and synchronously (Helvik and Walther, 1992). The hatching process takes place at an early developmental stage when the eyes are not functional (Kvenseth et al., 1996). This article takes advantages of the natural pre-retinal state and investigates the non-visual photoreception related to the hatching mechanism.

In contrast to mammals where all detection of light is mediated through photoreceptors in the lateral eyes, non-mammalian vertebrates have photoreceptor cells in several extra-ocular sites including the pineal, parapineal, deep brain cells and dermal melanophores (reviewed Davies et al., 2010; Peirson et al., 2009). While the rod and cones in the eye provide image formation and vision, photoreceptor cells in other layers of retina and in the extra-ocular sites are important for the entrainment of the circadian rhythm, responses to seasonal change and temperature regulation (reviewed in Davies et al., 2010). Many photoreceptive cells and their photosensitive pigments have been identified in the eye and brain, but knowledge of how these cells contribute to the behavioural and physiological responses to light is just emerging. Recently a functional study in zebrafish showed that larvae lacking eyes and pineal do a simple light-seeking behaviour as a response to loss of illumination. The preoptic area was identified as the photosensitive region and melanopsin expressing neurons are thought to mediate the behavioural response (Fernandes et al., 2012). In addition, unhatched pre-retinal zebrafish embryos exposed to intense light have a “photomotor response” and photoreceptors in the hindbrain are indicated to be responsible (Kokel et al., 2013). In the dark dependent pre-retinal hatching process of Atlantic halibut, the pineal organ has been indicated to be important in perceiving and mediating photic information. The pineal contains molecules involved in the phototransduction cascade already before hatching and it has been suggested that the

pineal may influence the time of hatching in halibut (Forsell et al., 1997). Recent studies of non-visual photopigment in Atlantic halibut have however shown a diverse expression pattern in the brain and spinal cord at the stage of hatching (Paper I and II) and other photosensitive regions of the halibut brain may also be important.

In Norwegian coastal waters the Atlantic halibut spawns in the deep sea and the eggs ascend in the water column to 100-250 meters depth, where the hatching process takes place at a very early developmental stage when the hatching embryo has a big yolk sac and a primitive body (Haug et al., 1984; Lönning et al., 1982). At the time of hatching the halibut retina is unpigmented and consists of neuroblastic cells and the eyes are first functional about a month after hatching (Kvenseth et al., 1996; Lönning et al., 1982). Halibut eggs have a specialized hatching process where the hatching gland cells migrate to a narrow belt in the anterior part of the yolk sac (Helvik et al., 1991b). This results in a restricted degradation of the eggshell and cleavage of the eggshell into two separate parts. During the hatching process the posterior parts of the yolk sac contracts and as a result the yolk mass is compressed forward. This ensures the hatching gland cells to be in close contact with the eggshell upon release of the hatching enzyme (Helvik et al., 1991a). In addition to this specialized "rim-hatching" process, hatching of halibut eggs have been shown to be regulated by light. Studies have shown that halibut eggs kept in darkness will hatch within a period of 1,5 day around their natural hatching point approximately 14 days post fertilisation (dpf) (Helvik and Walther, 1992) . But, incubation in light arrests the hatching mechanism and the embryo continues to develop inside the eggshell (Helvik and Walther, 1993). If photo-arrested eggs are placed in darkness after their natural hatching point, they will hatch rapid and synchronized within 80-140 minutes. It has also been showed that darkness is only required for the first 60 minutes. Incubation in light thereafter does not arrest the hatching and the dark signal has induced an irreversible and dark-independent process (Helvik and Walther, 1992). Recently, it was shown that hatching of zebrafish and Senegalese sole are influenced by light. It has been demonstrated that the hatching rhythm of zebrafish is diurnal while Senegalese sole has a

nocturnal hatching rhythm, however the mechanisms of how hatching is regulated by light remains unknown (Villamizar et al., 2013; Villamizar et al., 2012).

Studies on hatching mechanisms in halibut have revealed great detail about the effect of the sensory input from the environment. But even though the pineal has been indicated to be important in perceiving and mediating photic information in the hatching process (Forsell et al., 1997), a connection between the pineal and hatching is still missing. In this study we show that the hatching process is related to a cluster of non-visual opsin expressing neurons in the hindbrain and the ball-shaped cluster of cells is linked to a neuronal network with a projection out in the yolk sac. In addition, studies with photo-arrested eggs show that dark induced hatching activates the immediate early gene *c-fos*. Expression of *c-fos* is seen in the hindbrain cluster and in the hatching glands, indicating that the hatching process is induced through neuronal signalling.

Material and methods

Animals

Eggs and larvae 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.

Identification of c-fos in Atlantic halibut

The halibut genome was lately sequenced on an Illumina HiSeq200 (Illumina, San Diego, CA) (Pair End, 100bp reads) to 40x coverage and a contig assembly was made by the CLC software (CLC bio, Denmark). The genome was searched by TBLASTN (NCBI, Bethesda, MD) using available teleost C-fos protein sequences as query in order to obtain the sequence of the gene in halibut. The putative gene was predicted based on the BLAST alignments and GENSCAN (Burge and Karlin, 1997), and the annotation was based on

BLASTX against GenBank (NCBI, Bethesda, MD) and phylogenetic analysis. Verification of the predicted gene was done by PCR using primers with binding sites in the predicted 5' and 3' UTR and use of cDNA as described in (Paper I). The primers sequences were Hhc-fosF1: 5'GGATCTTCCTACTTGTTGTTGCTG3'and Hhc-fosR1: 5'GTCCATTCCATAGCCCTGTG3'. The nucleotide sequence for *c-fos* was deposited into GeneBank with the accession number KF941297.

Pictures of living Atlantic halibut embryo

Living embryo (16 days post fertilisation (dpf)) in eggshell were mounted in 3% Methylcellulose (Sigma-Aldrich, St. Louis, MO) and oriented in a dorsal and lateral view. Pictures of hatching were taken in seawater using photo-arrested eggs (18 dpf) where hatching was induced by transferring to darkness. Leica M420 macroscope (Leica Microsystems, Germany) and a CoolSNAP-Pro Color Imaging Camera (RS Photomertics, Tucson, AZ) were used for imagine.

Riboprobes

Preparation of digoxigenin (DIG)-labelled and fluorescein-labelled riboprobes for vertebrate ancient opsin (VA opsin) and digoxigenin (DIG)-labelled riboprobe for melanopsin (*opn4m2*) and *c-fos* were done 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 synthesised probes were precipitated by tRNA (Roche Diagnostics, Germany).

***In situ* hybridisation**

In situ hybridisation on whole embryos and larvae of different developmental stages was done as described in (Paper I) using DIG-labelled riboprobe for VA opsin, melanopsin and *c-fos*. Colour staining was performed by 4-Nitro blue tetrazolium chloride (NBT) (Roche Diagnostics, Germany) and 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics, Germany) system.

Immunohistochemistry

To evaluate the neuronal fibre network at the stage of hatching, immunohistochemistry on whole embryos (13 dpf) with monoclonal anti-acetylated tubulin antibody produced in mouse (Clone 6-11 B-1) (1:1000) (Cat. No T7451, Roche Diagnostics, Germany) was done as described in (Paper I), except for some modification. The embryos were incubated in monoclonal anti-acetylated tubulin antibody over night and at room temperature. For visualisation Anti-Mouse IgG (H+L), CF™ 488 antibody produced in goat 2 mg/ml (Cat. No SAB4600042, Sigma-Aldrich, St. Louis, MO) was used.

***In situ* hybridisation and immunohistochemistry in combination**

The combination of *in situ* hybridisation and immunohistochemistry on whole halibut embryo (13 dpf) was performed as explained in (Paper I). Fluorescein-labelled riboprobe was used for VA opsin and the probe was visualised by TSA^{TM}Plus Fluorescein Systems according to the producer's protocol (Cat.No NEL741001KT, Perkin Elmer, Waltham, MA). Monoclonal anti-acetylated tubulin antibody produced in mouse (Clone 6-11 B-1) (1:1000) (T7451, Roche Diagnostics, Germany) and Anti-Mouse IgG (H+L), CF™ 555 antibody produced in goat 2 mg/ml (Cat. No SAB4600066, Sigma-Aldrich, St. Louis, MO) were used for staining of neuronal fibre network.

Antibody characterisation

Mouse anti-acetylated tubulin (Cat. No. T7451, Sigma-Aldrich, St. Louis, MO) is a monoclonal antibody that recognizes an epitope located on the α3 isoform of *Chlamydomonas axonemal* α-tubulin within four residues of acetylated Lys-40. The antibody has been used to label axon in many organisms including teleosts (Hunter et al., 2011; Ledizet and Piperno, 1991).

Functional studies with photo-arrested eggs

To investigate a possible neuronal regulation of hatching, eggs were placed in light before they reached the natural hatching point. The photo-arrested eggs were transferred to

darkness at 18 dpf, a time point after the natural hatching point, to induce the dark-dependent hatching signal. Eggs were sampled by putting them in ice-cold 4% paraformaldehyde after 2, 10, 20, 30, 40, 60 and 120 minutes in the dark. The experiment was conducted using night vision device (Bushnell, Kansas, US), to minimize the light exposure upon sampling. In addition, eggs incubated in light were used as a control. To mark neural activation *in situ* hybridisation with DIG-labelled riboprobe for the immediate early gene *c-fos* was done for all the points sampled including the control kept in light.

Fluorescent double labelling *in situ* hybridisation

Fluorescent double labelling *in situ* hybridisation on whole embryos was done as described in (Paper I) for the 120 minutes in dark group of functional studies with photo-arrested eggs. Fluorescein-labelled riboprobe was used for VA opsin and the probe was visualised by TSA^{TM}Plus Fluorescein Systems according to the producer's protocol (Cat.No NEL741001KT, Perkin Elmer, Waltham, MA). DIG-labelled probe was used for *c-fos* and the probe was visualized by use of Fast Red tablets as recommended by the manufacturer (Cat. No 11496549001, Roche Diagnostics, Germany).

Microscopy

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 an 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). Confocal images were taken using the Leica TCS SPE confocal system (Leica Microsystems, Germany) together with a Leica DM2500 microscope (Leica Microsystems, Germany). Adobe Photoshop CS5 (San Jose, CA) was used for adjustments of brightness, contrast, color levels and to sharpen the pictures.

Results

The hatching halibut embryo and differential expression of vertebrate ancient opsin and melanopsin in the hindbrain

Pictures of a living Atlantic halibut embryo inside the eggshell and at hatching are shown in Figure 1A-D. From a dorsal view the transparent halibut embryo is apparent on the top of a huge yolk sac inside the eggshell. The hatching glands make a narrow belt in the yolk sac starting just caudal to the ear (Fig. 1A). A lateral view of a hatching embryo leaving the eggshell reveals the hatching glands as a ring in the anterior part of the yolk sac (Fig. 1B). Higher magnification of the embryo in Figure 1A from a dorsal (Fig. 1C) and a lateral view (Fig. 1D) show the olfactory bulbs, the unpigmented eyes, the developing brain, the lateral neuromasts and the ears. Both vertebrate ancient opsin (VA opsin) (Fig. 1E-H) and melanopsin (*opn4m2*) (Fig. 1I-K) are expressed in a ball-shaped cluster of cells in the hindbrain at the stage of hatching and Figure 1E-K show the differential development of the hindbrain cluster for the two genes. At 10 dpf VA opsin positive cells are visible in the cluster (Fig. 1E) and at 13 dpf the cluster is even more aggregated (Fig. 1F). After hatching, at 17 dpf the expression is weaker and the VA opsin expressing cells are disintegrated (Fig. 1G) and this is even more apparent at 19 dpf where only a few cells are expressing VA opsin (Fig. 1H). The mammalian-like melanopsin (*opn4m2*) is expressed in an aggregated cluster already at 9 dpf (Fig. 1I) but at 13 dpf the melanopsin expressing cells has started to scatter (Fig. 1J). At 17 dpf the ball-shaped cluster has disappeared (Fig. 1K).

The hindbrain cluster is connected to a neuronal network with projection out in the yolk sac

Newly differentiated neurons and axonal pathways were marked by antibody against acetylated alpha tubulin to study the neuronal networks in the hindbrain in relation to the bilateral VA opsin and melanopsin-expressing cluster (Fig. 2). Figure 2A shows the VA opsin expressing cells in the hindbrain clusters at 13 dpf (Fig. 2A) and Figure 2B shows

the neuronal network in the same embryo. A combination of the pictures demonstrates that projections are in close contact with the hindbrain clusters (Fig. 2C). A closer look on one hindbrain cluster (Fig. 2D) and one of the projections (Fig. 2E) shows that the projection bends laterally at the level of the neuromast and projects out in the yolk sac as a fan just anterior to the neuromast (Fig. 2F). In addition, a connection between the hindbrain cluster and the projection is seen (Fig. 2F). Confocal imaging visualised the connection between the VA opsin cluster (Fig. 2G) and the projection (Fig. 2H) in detail and shows that neurons with connection to the bent projection are located in the hindbrain cluster (Fig. 2I). The fan of projections out in the yolk sac were visualised and related to the narrow belt of hatching glands (Fig. 2J-R). On one side of the embryo a combination of the hatching glands (Fig. 2J) and the projections out in the yolk sac (Fig. 2K) show how the widespread projections are approaching the hatching glands (Fig. 2L). In the fan of projections some of them reach out to the glands and some extends the glands (Fig. 2L). A higher magnification shows how a projection curves and follows the hatching glands (Fig. 2M-O). A close look on some of these hatching glands (Fig. 2P) and the curved projection (Fig. Q) show the close contact between the glands and the projection (Fig. 2R).

Dark-induced hatching of photo-arrested eggs give *c-fos* activation in the hindbrain and in hatching glands

Neural activation in dark-induced hatching after photo-arrest was analysed by expression studies of the immediate early gene *c-fos*. Eggs (18 dpf) sampled after 2, 10, 20, 30, 40, 60 and 120 minutes in dark were analysed and all sampling points except the 120 minutes showed the same expression pattern as the control incubated in light (data not shown). In contrast, at the sampling point 120 minutes expression of *c-fos* was apparent in the hindbrain and in the hatching glands (Fig. 3). Figure 3A shows VA opsin expression in the hindbrain at the same developmental stage as the study with photo-arrested eggs. Appearance of *c-fos* expression in the control is shown in Figure 3B and demonstrates expression in the telencephalon and midbrain. At the sampling point 120 minutes, *c-fos*

expression reveals neural activation in a similar pattern in the telencephalon and midbrain as the control, but in addition *c-fos* is expressed in the hindbrain. The expression in the hindbrain is a bilateral ball-shaped cluster of cells resembling the morphology of the photosensitive hindbrain cluster (Fig. 3C). Fluorescent double labelling in a 120 minutes sampled embryo shows that VA opsin (Fig. 3D) and *c-fos* (Fig. 3E) are expressed in the same bilateral hindbrain cluster (Fig. 3F). Higher magnification of one hindbrain cluster indicates that VA opsin (Fig. 3G) and *c-fos* (Fig. 3H) are express in the same cells (Fig. 3I). Pictures of the yolk sac and hatching glands of an embryo sampled at 120 minutes were set together and illustrate the neural activation in the hatching glands (Fig. 3J). A dorsal view of a living embryo shows the narrow belt of the hatching glands (Fig. 3K). A high magnification of the *c-fos* expression shows the distribution of *c-fos* in the hatching glands (Fig. 3L).

Discussion

The pre-retinal light regulated hatching process of Atlantic halibut has given us a special opportunity to study a physiological response to light in early development of a teleost, independently of the eye. We describe a transient bilateral cluster in the hindbrain expressing non-visual opsins at the stage of hatching in halibut. The cluster is connected to a neuronal network with a projection out in the yolk sac and we demonstrate neural activation in the hindbrain cluster and hatching glands as a response to dark-induced hatching in photo-arrested eggs.

Transient expression of VA opsin and melanopsin around hatching

Recently we have described expression of the non-visual opsins melanopsin, exorhodopsin and VA opsin in the developing halibut embryo at the stage of hatching and at first feeding (Paper I and II). We find extensively expression of the genes in the developing brain and the expression persists when the eyes are functional. An apparent exception is a ball-shaped hindbrain cluster expressing both melanopsin and VA opsin

(Paper I and II). Here we demonstrate that the non-visual opsin expressing hindbrain cluster is transient, with extensive expression of both genes before hatching. The melanopsin expression fades already prior to hatching and a few days after the natural hatching point, the expression has disappeared. The VA opsin positive cells persist a little longer but also this expression scatters and disappears (Fig. 1). Taken together, the hindbrain cluster seems to be a transient embryonal specific cluster just apparent around hatching. Vertebrate ancient opsin and melanopsin in the transient hindbrain cluster are of apparently different evolutionary heritage and thought to involve different phototransduction machinery resulting in hyperpolarisation or depolarisation of the cell, respectively (Arendt, 2003). Activation of the circuit in darkness implies however that the net output of melanopsin and VA opsin is inhibitory in the system. Although speculative, if the two non-visual opsins make use of different phototransduction cascades in the same cell, fading of melanopsin at the time of hatching may allow a VA opsin driven phototransduction cascade.

The hindbrain cluster is connected to a neuronal network with a projection out in the yolk sac

We further demonstrate that the hindbrain cluster is connected to a neuronal network with a projection extending as a fan out in the yolk sac. Some of the projections bend off and are in close contact with the hatching glands, while others extend the narrow belt of hatching glands (Fig. 2). From these findings it can be suggested that the projections provide a signal to the hatching glands, but also contributes to the contraction of the posterior part of the yolk sac, since it has been shown that contractions squeeze the yolk mass forward before hatching (Helvik et al., 1991a). The results demonstrate that there exist a direct connection between the photosensitive hindbrain cluster expressing VA opsin and melanopsin and the region of hatching glands in the yolk sac. In addition, we observe no photosensitive elements of the visual opsins (unpublished results), melanopsins, exorhodopsin and VA opsin in the narrow belt of hatching gland cells,

indicating that there are no direct photoreception of these opsin families in the hatching glands.

Neural activity in the hindbrain cluster and hatching glands

Earlier it has been demonstrated that light serves as a natural cue for hatching in halibut eggs, and it has been suggested that light may synchronize hatching in such a way that it occurs at the first night after the embryos reach the developmental competency for hatching (Helvik and Walther, 1992). Light-regulated hatching has lately also been demonstrated in zebrafish and Senegalese sole (Villamizar et al., 2013; Villamizar et al., 2012). The responsible photoreceptor cells and the mechanisms for regulating hatching have so far not been elucidated. Our results in halibut show however that the immediate early gene *c-fos* is expressed in the hindbrain cluster and hatching glands in a study with dark-induced hatching after photo-arrest. Expression of *c-fos* is known to be activated rapid and transient as a response to a variety of stimuli such as growth factor stimulation and stimulation of nerve cells (Bullitt, 1990; Sheng and Greenberg, 1990) and our results show expression of *c-fos* 120 minutes after the photo-arrested eggs were transfer to darkness. These results suggest that darkness stimulates neural activity that regulates hatching and dark-induced activation of the neural circuit implies that the net output of melanopsin and VA opsin is inhibitory in the system. Further on it is likely that the bilateral photosensitive hindbrain cluster mediates the signal to the hatching glands through the neuronal network with projections out in the yolk sac. In addition, no activation of *c-fos* is detected in the pineal even though the pineal has been suggested to be important in perceiving and mediating photic information in the hatching process (Forsell et al., 1997) and has been shown to express non-visual opsins at the stage of hatching (Paper I).

The photosensitive hindbrain

Recently the hindbrain was for the first time demonstrated to be responsible for a light-sensing behaviour in vertebrates. The caudal hindbrain of zebrafish was shown to drive

series of robust and reproducible motor behaviours as a response to visual wavelengths of light (Kokel et al., 2013). Here we show that also the rostral hindbrain is involved in light-sensing behaviours in vertebrates. Our results indicate that the environmental light is detected directly in the hindbrain providing behavioural and physiological functions driven by neural activity. In zebrafish the response was shown to be dependent of the synthesis of 11-*cis* retinal, but the responsible opsin still needs to be elucidated (Kokel et al., 2013). Our results show that the bilateral cluster of cells in the rostral hindbrain expresses both vertebrate ancient opsin and melanopsin, giving the first identifications of responsible opsins in the hindbrain. Recently, it has been shown that vertebrate ancient opsin and teleost-multiple-tissue opsin are expressed in motor neurons of the hindbrain and in interneurons of the tectum and in rostral tegmental nucleus of teleosts (Fischer et al., 2013). One can only speculate about the cellular identity of the hindbrain cluster in halibut, but according to the classification of photoreceptor cells based on distribution and neural identity (Ramirez et al., 2011), the melanopsin and vertebrate ancient opsin expressing cells of the hindbrain can be assessed as an aggregated cluster of cells either of first or high-order. First-order photoreceptor cells are primary sensory cells and the hindbrain cluster may function as primary sensory in regulation of hatching. The cells of the hindbrain cluster could also be photosensitive interneurons that modulate an incoming electrical signal that may then be transmitted to the hatching glands.

The transient hindbrain cluster may represent an ancient photoreceptor system for regulation of hatching

Animals have an eggshell that surrounds and protect the early vulnerable embryo (reviewed in Claw and Swanson, 2012). Concurrent with the protective eggshell, mechanisms for escaping the egg must have evolved. In fish this mechanism involves digestion of the eggshell by hatching enzyme that is released for the hatching embryo. How different animal groups regulate hatching according to stage of development and environmental cues has got little attention in the literature, but in teleosts such as Atlantic halibut (Helvik and Walther, 1992) and zebrafish (Villamizar et al., 2012) light cues are

central in activation of hatching. In Paper II we show that the cells of the hindbrain cluster co-express opsins of c-opsin and r-opsin heritage and may represent an ancient photoreceptor cell type. Such photoreceptor cells with dual opsins have been postulated to be present in the Urbilateria, prior to the evolution of the ciliated and rhabdomeric photoreceptors cell types (Arendt, 2003; Arendt et al., 2004; Arendt and Wittbrodt, 2001). It is very interesting that these dual hindbrain photoreceptors are transiently activated at hatching, and may therefore be linked to the regulation of hatching. The hindbrain cluster may represent an ancient photoreceptor system that has been kept in evolution to regulate hatching.

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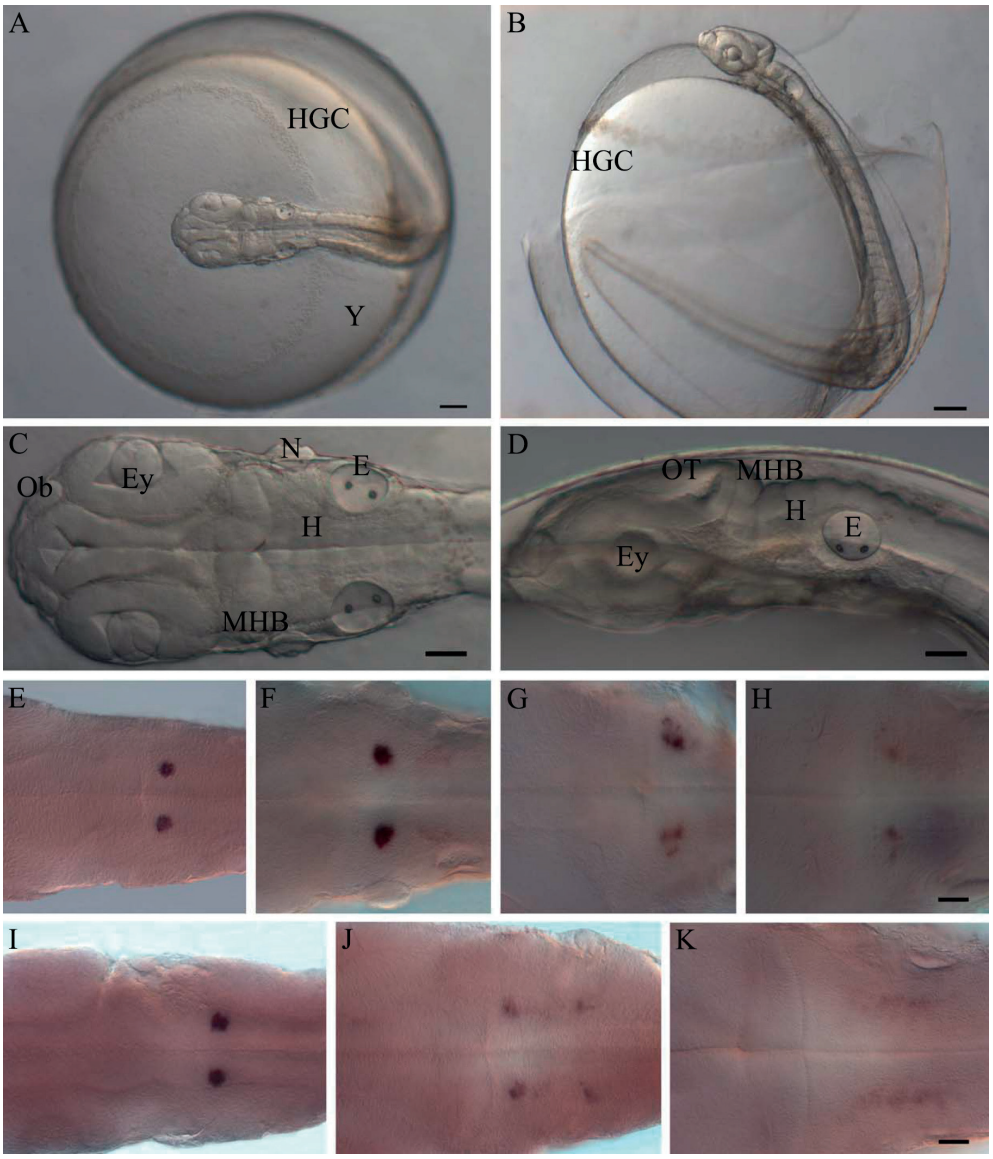

Figure 1 The hatching Atlantic halibut embryo and differential expression of non-visual opsins in a hindbrain cluster.

A: A dorsal view of an embryo in the eggshell on the top of a huge yolk sac (Y) with hatching glands (HGC) in a narrow belt. B: A hatching embryo leaving the eggshell with the hatching glands in an anterior

ring in the yolk sac. C: A closer view of the dorsal embryo in (A) shows the olfactory bulb (Ob), eyes (Ey), midbrain-hindbrain boundary (MHB), hindbrain (H), neuromasts (N) and ears (E). D: A lateral view shows the optic tectum (OT) and the structure of the hindbrain from the side. E: A ball-shaped hindbrain cluster of vertebrate ancient opsin (VA opsin) expressing cells at 10 days post fertilisation (dpf). F: A more condense VA opsin cluster at 13 dpf. G: After hatching at 17 dpf the VA opsin expressing cells are scattered. H: At 19 dpf only a few cells express VA opsin. I: At 9 dpf melanopsin (*opn4m2*) is extensively expressed in the ball-shaped hindbrain cluster. J: At 13 dpf fewer cells in the cluster express melanopsin. K: After hatching (17 dpf) no melanopsin positive cells are observed at the level of the hindbrain cluster. Scale bars: 200 μm in A and B, 100 μm in C and D, 50 μm in E-K.

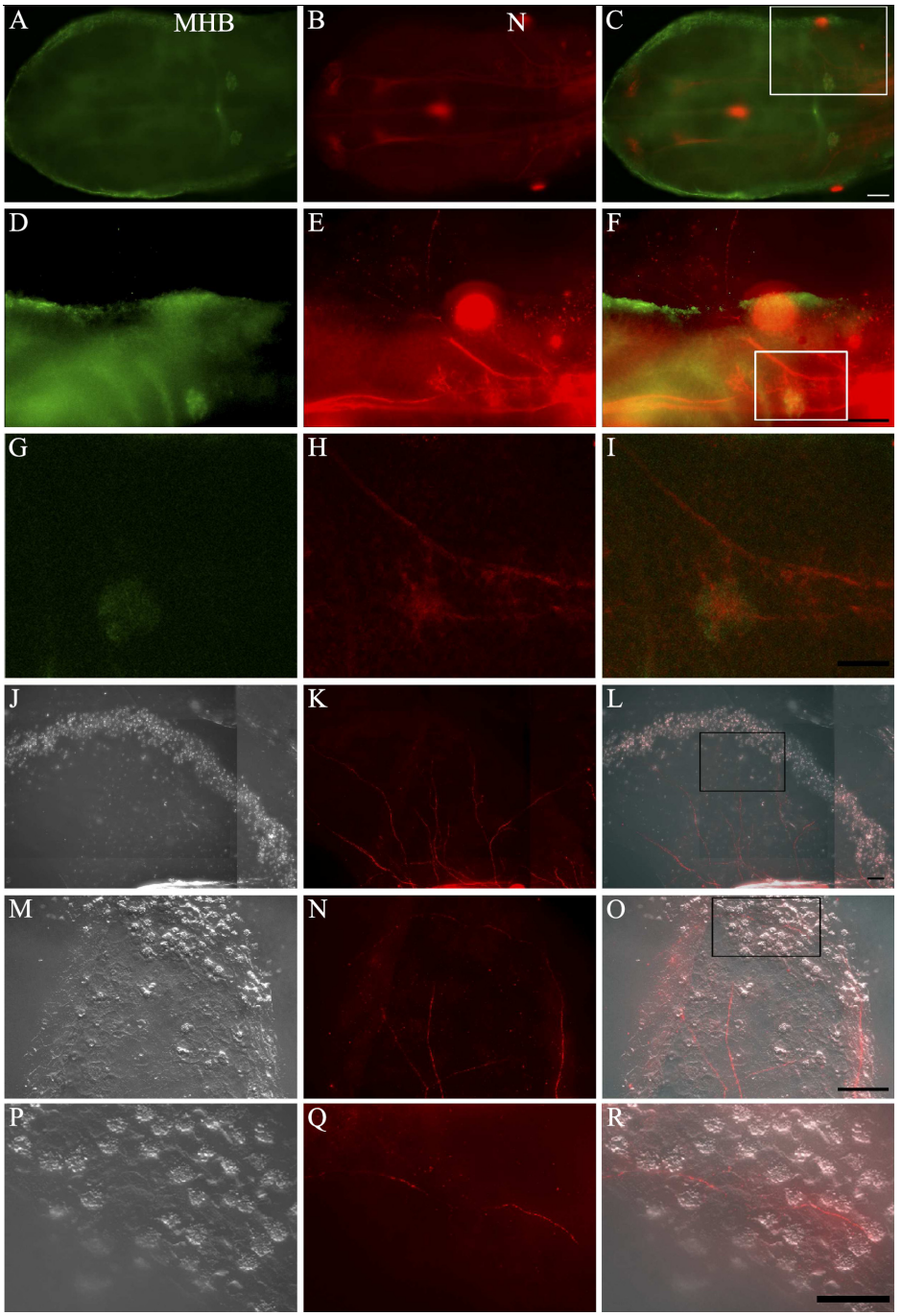
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Figure 2 A neuronal network with a projection out in the yolk sac is connected to the non-visual opsin expressing hindbrain cluster.

A: Vertebrate ancient opsin (VA opsin) positive cells in the hindbrain at 13 days post fertilisation (dpf). B: Newly differentiated neurons and axonal pathways in the same embryo. C: A combination of VA opsin and acetylated alpha tubulin picturing a projection nearby the hindbrain cluster bending at the level of the neuromasts (N). D: Focus on one of the VA opsin expressing hindbrain clusters. E: The bent projection reaches out in the yolk sac as a fan. F: The projection that extends out in the yolk sac is connected to the hindbrain cluster. G: Confocal imaging of the VA opsin positive hindbrain cluster. H: Confocal image of the bent projection. I: A combination of (G) and (H) show how neurons in the hindbrain cluster are connected to the bent projection. J: Hatching glands on one side of a 13 dpf embryo from a dorsal view. The picture is a set of several pictures. K: The fan of projections out in the yolk sac illustrated by the same set of pictures as in (J). L: A combination of (J) and (K) show how the widespread projections reach or extend the hatching glands. M: Higher magnification of the yolk sac and hatching glands. N: Focus on a curved projection. O: A combination of (M) and (N) show that the projection curves at the level of the hatching glands. P: A few hatching glands at high magnification show the structure of the glands in detail. Q: The curved projection in a close up picture. R: Picture (P) and (Q) in combination show the close relation between the hatching glands and the curved projection. Scale bars: 100 μm in A-F and J-O, 50 μm in G-I, 20 μm in P-R. MHB, midbrain-hindbrain boundary

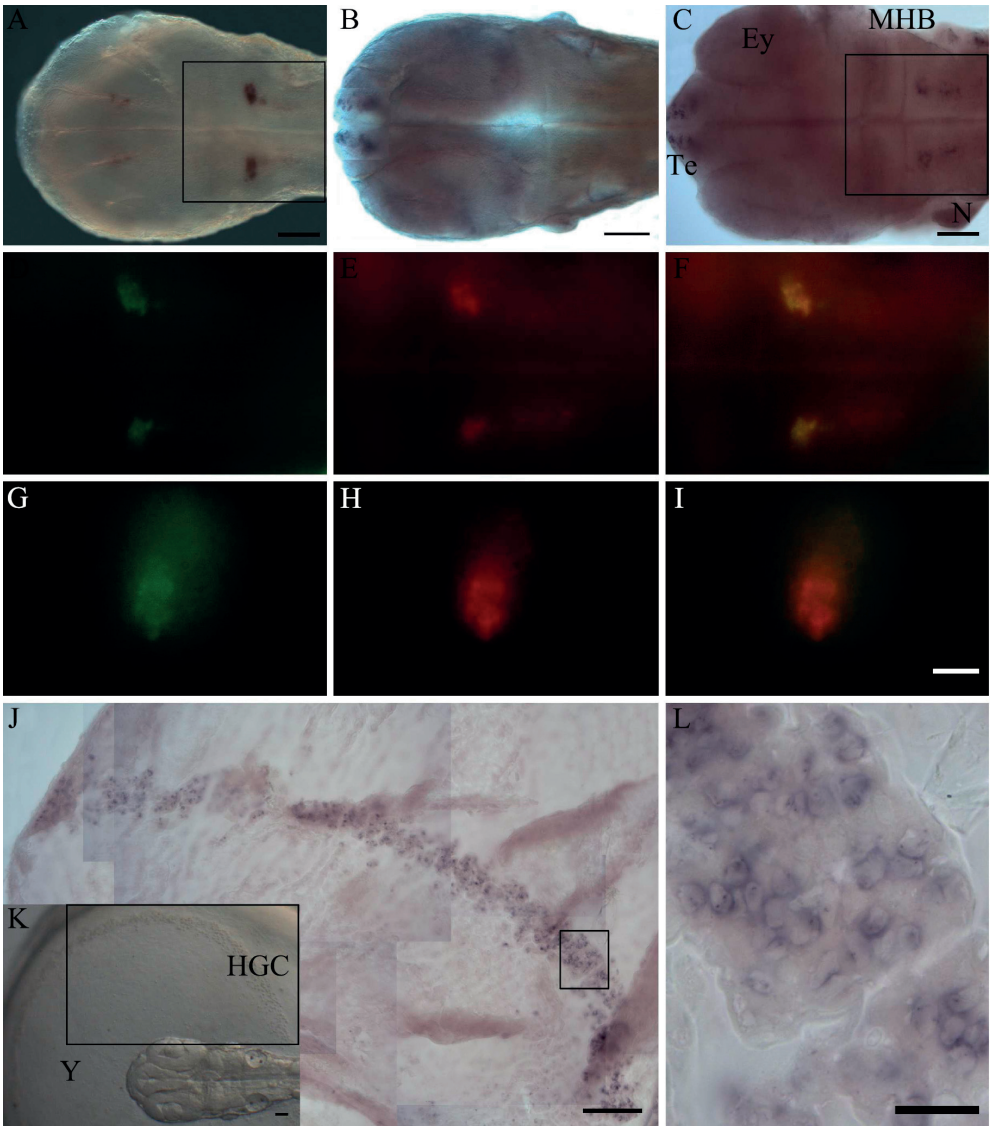

Figure 3 Dark-induced hatching after photo-arrest gives neural activation in the non-visual opsin expressing hindbrain cluster and in the hatching glands.

A: Vertebrate ancient opsin (VA opsin) positive cells in the hindbrain cluster at the same developmental stage as the study with dark-induced hatching after photo-arrest. B: Expression of *c-fos* in a control kept in light demonstrates expression in telencephalon and a diffuse expression in the midbrain. C: Neural

activation is shown by *c-fos* expression in the telencephalon (Te), midbrain and hindbrain at the sampling point 120 minutes. D: VA opsin positive cells in the hindbrain cluster in a 120 minutes sampled embryo. E: Positive cells of *c-fos* in the same embryo. F: VA opsin and *c-fos* in combination show that they are expressed in the same cluster in hindbrain. G: Higher magnification of one VA opsin expressing cluster. H: The same high magnification of *c-fos*. I: A combination of the two genes (G) and (H) indicates that they are expressed in the same cells in the hindbrain cluster. J: Pictures of the yolk sac (Y) and hatching glands (HGC) of 120 minutes sampled embryo put together to illustrate the *c-fos* expression in the hatching glands. K: The narrow belt of hatching glands in a living embryo. L: Detail of the hatching glands expressing *c-fos*. Scale bars: 200 μm in K, 100 μm in A-F and J, 50 μm G-I, 20 μm in L. Eye (Ey), midbrain-hindbrain boundary (MHB), neuromast (N)

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