Transient photoreception in the hindbrain is permissive to the life history transition of hatching in Atlantic halibut

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Abstract

In nonmammalian vertebrates, photoreception takes place in the deep brain already early in development, but knowledge is lacking about the functions of these nonvisual photoreceptive systems. Prior to hatching, Atlantic halibut has a transient bilateral cluster of photoreceptive cells in the hindbrain. The cluster is imbedded in a neuronal network projecting to the narrow belt of hatching glands in the yolk sac. In halibut, hatching is inhibited in light and activated by transfer to darkness and c-*fos* analysis during hatching shows that the hindbrain cluster and hatching glands have neural activation. Unexpectedly, the hindbrain cluster expresses dual photopigments, vertebrate ancient opsin and melanopsin. Evolutionarily, these opsins are believed to belong to different classes of photopigments found in rhabdomeric and ciliary photoreceptors. The concept that an organism develops transient light sensitivity to target critical aspects of life history transitions as hatching provides a fascinating landscape to investigate the timing of other biological events.

Keywords: nonvisual photoreceptor, melanopsin, vertebrate ancient opsin, *c-fos*,

Introduction

Light detection is essential for most animals to coordinate behavioral and physiological processes with photic cues. Photoreception is often related to sensory organs such as the retina and pineal, which provide vision and photic information to the animal throughout life. New complexities to this system have appeared in the last decade as new photoreceptor families have been identified with expression outside the classical photoreceptor areas such as within the deep brain (Peirson et al., 2009; Davies et al., 2010; Fernandes et al., 2013; Davies et al., 2015).

There is a considerable knowledge gap between location and function of deep brain photoreceptor cells. Until now, many photoreceptive cells and their photosensitive pigments have been identified, but how they contribute to light driven behavioral and physiological responses is just emerging. In unhatched zebrafish embryos photoreceptors in the hindbrain are responsible for a "photomotor response" after exposure to intense light (Kokel et al., 2013) and zebrafish larvae lacking eyes and pineal organs demonstrate a light seeking behavior triggered by loss of illumination. Melanopsin-expressing cells in the preoptic area were found to regulate this light seeking behavior (Fernandes et al., 2012). Further, it has been shown that inter- and motorneurons co-express tissue multiple tissue opsin (tmt-opsin) and vertebrate ancient opsin (VA opsin) in zebrafish and medaka brain. Light sensitivity in inter- and motorneurons is suggested to represent a fast possibility to adapt behavior to unpredictable environmental light changes, independent of neurosecretory cells (Fischer et al., 2013). In masu salmon key genes regulating seasonal reproduction and opsins are expressed in the coronet cells of the saccus vasculosus and it has been suggested to be a seasonal sensor in fish sensing changes in day length (Nakane et al., 2013). The marine flat fish Atlantic halibut has a special early life strategy with light-regulated hatching at an early developmental stage long before the eyes are functional (Helvik and Walther, 1992). In Norwegian coastal waters, the 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 (Haug, 1990; Haug et al., 1984). In the dark winter season, the eggs are only exposed to low light intensities deep in the sea.

The aim of this study was to unravel the enigma of how light can regulate hatching in halibut and determine if and how deep brain photoreceptor systems are involved in this early life history transition. Previous studies on the hatching mechanism in halibut have demonstrated in detail the effect of the sensory input from the environment (Helvik et al., 1991; Helvik and

Walther, 1992; 1993). Light serves as a natural cue for hatching in halibut and halibut larvae in nature hatch in darkness (Helvik and Walter, 1992). Further, the hatching glands are located in a narrow belt in the yolk sac and during hatching the yolk sac is reshaped by contraction so that the yolk mass is squeezed anterior before hatching. This ensures close contact between the hatching glands and the eggshell during release of the hatching enzyme, which is necessary for restricted degradation of the eggshell (Helvik et al., 1991). The pineal has been proposed to be important in perceiving and mediating photic information in the hatching process (Forsell et al., 1997), however a connection between the pineal and hatching is still missing. We have revealed diverse photopigment expression patterns in the brain and spinal cord early in development of Atlantic halibut, indicating that other photosensitive regions also may be important in hatching (Eilertsen et al., 2014). From an evolutionary point of view, the nonvisual pigments are of interest since they belong to two different classes of photopigments, melanopsin, as r-opsin, and vertebrate ancient opsin (VA opsin), as c-opsin, normally only found in rhabdomeric and ciliary photoreceptor cells, respectively. The evolutionary origin of photoreceptor cell types is still debated (Davies et al., 2010; Matos-Cruz et al., 2011; Vocking et al., 2017), with the theory of ancient photoreceptors containing duplicated photopigments that subsequently became distinct receptor cells expressing c- and r-opsin (Arendt, 2003). Strikingly, we found a cluster of cells in the hindbrain of Atlantic halibut expressing transiently both melanopsin and VA opsin. This cluster of dual photoreceptors is imbedded in a neuronal network with fibers proceeding out in the yolk sac, reaching the hatching gland cells. Studies with photo-arrested eggs demonstrate that dark induced hatching activates the immediate early gene *c-fos* in the hindbrain cluster and in the hatching glands. Taken together, our data imply how an early life history transition is modulated by transient and direct photoreception in the brain.

Materials and methods

Animals. Eggs 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 of vertebrate ancient opsin. Total RNA was isolated from the retina and brain of juvenile Atlantic halibut 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 VA 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. In the first round with 35 cycles, the annealing temperature was 62.9°C for 5'RACE and 60°C for 3'RACE. The second round of PCR had an 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. The two isoforms are identical in the four first exons, but the fifth exon is altered in the two isoforms as in zebrafish (Kojima et al., 2000) and provides different length in the cytoplasmic tail. As shown in chicken (Halford et al. 2009) the short isoform of VA opsin is generated by a read-through into intron 4 while the long isoforms by splicing to exon 5. 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. Primers are listed in Table 1. The nucleotide sequences were deposited into GeneBank with the accession number KF941295 (*val*) and KF941296 (*vas*).

Verification of VA opsin and identification of *c-fos*. 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, RRID: OMICS_01124). The genome was searched by BLASTN and TBLASTN (NCBI, Bethesda, MD, RRID: nlx_153932 and RRID: OMICS_00999) with the two isoforms of VA opsin to verify the sequence 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). For *c-fos* the genome was searched by TBLASTN 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 (NCBI, Bethesda, MD, RRID: nlx_153933) against GenBank (NCBI, Bethesda, MD, RRID: nif-0000-02873) 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 cloning and sequencing was done as for VA opsin. The nucleotide sequence for *c-fos* was deposited into GeneBank with the accession number KF941297.

Riboprobes. Preparation of digoxigenin (DIG)-labelled and fluorescein-labelled riboprobes for VA opsin (specific for both isoforms) and DIG-labelled riboprobe for melanopsin (*opn4m3*) (specific for both isoforms) 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 synthesized probes were precipitated by LiCl and EtOH together with tRNA (Roche Diagnostics, Germany). Sequence alignment showed that the similarity between the sequence targets of the melanopsin and VA opsin probes is approximately 50% (data not shown), ensuring no cross-hybridization. Sequence information is listed in Table 2.

***In situ* hybridization (ISH).** ISH on whole embryos and larvae of different developmental stages was done as described in Eilertsen et al. (2014) using DIG-labelled riboprobe for VA opsin, melanopsin and *c-fos*. Color staining was performed by 4-Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate system (Roche Diagnostics, Germany). ISH on cryosections (12 and 13 days post fertilization (dpf)) was performed following the preparations and ISH protocol described in Sandbakken et al. (2012). Briefly, the embryos were embedded in Tissue-Tek O.C.T. Compound (Sakura Fintek, Netherlands) and sectioned (10 µm) on a Leica CM 1850 cryostat (Leica Microsystems, Germany). Prior to the hybridization step the tissue was rehydrated in ethanol, permeabilized with proteinase K and treated with triethanolamine (Sigma, St. Louis, MO) and acetic anhydride (Sigma, St. Louis, MO) to reduce background staining. After hybridization the tissue was thoroughly washed and treated with RNase A (Sigma, St. Louis, MO) to remove unhybridized probe. Before applying the antibody (anti-digoxigenin conjugated with alkaline phosphatase, Fab fragments (1:2000) (Cat. No 11093274910, Roche Diagnostics, Germany, RRID: AB_514497)) the tissue was incubated in 2% blocking solution (Roche Diagnostics, Germany) in 2x SSC with 0.05% Triton X-100 (Sigma, St. Louis, MO). The DIG-labelled probe was visualized by 4-Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate system (Roche Diagnostics, Germany). Parallel sections were Nissl-stained with 0,5% cresyl fast violet (Chroma-Gesellschaft, Germany).

Fluorescent double labelling ISH. Fluorescent double labelling ISH on cryosections (12 dpf) was performed following the preparations and ISH protocol described in Sandbakken et al. (2012), except that both probes were applied at the hybridization step. In addition, the fluorescein-labelled riboprobe for VA opsin was first visualized by using the antibody anti-fluorescein conjugated with horse radish peroxidase, Fab fragments (1:400) (Cat. No 11426346910, Roche Diagnostics, Germany, RRID: AB_840257) and the TSA^{TM}Plus Fluorescein Systems (Perkin Elmer, Waltham, MA) according to the producer's protocol. Before applying anti-digoxigenin conjugated with alkaline phosphatase, Fab fragments (1:2000) (Cat. No 11093274910, Roche Diagnostics, Germany, RRID: AB_514497) the sections were blocked for 1 hour in 2% Blocking reagent (Roche Diagnostics, Germany) in 2xSCC, and the DIG-labelled riboprobe for melanopsin was visualized by use of Fast Red tablets as recommended by the manufacturer (Roche Diagnostics, Germany). The stained sections were mounted in DABCO antifading medium (Sigma, St. Louis, MO). Fluorescent double labelling ISH on whole embryos was done as described in Eilertsen et al. (2014). Fluorescein-labelled riboprobe for VA opsin was visualized with the TSA^{TM}Plus Fluorescein System and the DIG-labelled probe for melanopsin or *c-fos* was visualized with Fast Red tablets.

Immunohistochemistry. To evaluate the neuronal fiber 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, RRID: AB_609894) was done as described in Eilertsen et al. (2014), except for the following modifications. The embryos were incubated in monoclonal anti-acetylated tubulin antibody overnight at room temperature and for visualization anti-mouse IgG (H+L), CF™ 488A antibody produced in goat 2 mg/ml (Cat.No SAB4600042, Sigma-Aldrich, St. Louis, MO, RRID: AB_2532075) was used.

ISH and immunohistochemistry in combination. The combination of ISH and immunohistochemistry on whole halibut embryo (13 dpf) was performed as explained in Eilertsen et al. (2014). Fluorescein-labelled riboprobe was used for VA opsin and the probe was visualized by TSA^{TM}Plus Fluorescein Systems (Perkin Elmer, Waltham, MA). Monoclonal anti-acetylated tubulin antibody produced in mouse (Clone 6-11 B-1) (1:1000) (Cat. No T7451, Roche Diagnostics, Germany, RRID: AB_609894) and anti-mouse IgG (H+L), CF™ 555 antibody produced in goat 2 mg/ml (1:100) (Cat. No SAB4600066, Sigma-

Aldrich, St. Louis, MO, RRID: AB_2336060) were used for staining of neuronal fiber network.

Antibody characterization. Mouse anti-acetylated tubulin (Cat. No. T7451, Sigma-Aldrich, St. Louis, MO, RRID: AB_609894) is a monoclonal antibody that recognizes an epitope located on the α3 isoform of *Chlamydomonas axonemal* α-tubulin. The antibody has been shown to recognize a single 55 kDa band (the predicted molecular weight of acetylated tubulin) on western blots of teleost brain extracts (Liu and Lessman, 2007). In addition, the antibody has also been shown to specifically label axons in the developing central nervous system of zebrafish (Chitnis and Kuwada, 1990). The antibody has been used to label axons in many organisms including teleosts (Ledizet and Piperno, 1991; Hunter et al., 2011; Verpy et al., 2011). See Table 3 for details.

Functional studies with photo-arrested eggs. The functional studies with photo-arrested eggs were set up based on the knowledge of a dark-induced hatching process in Atlantic halibut. Incubation in light arrests the hatching mechanism but if photo-arrested eggs are placed in darkness after their natural hatching point, they will hatch in synchrony and rapidly within 80-140 minutes (Helvik and Walther, 1992). To investigate a possible neuronal regulation of hatching, eggs were placed in light (MASTER PL-S 11W/827/2P 1CT, Philips Lighting, Netherlands) 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, Overland Park, KS), to minimize the light exposure upon sampling. In addition, eggs incubated in light were used as a control. To mark neuronal activation ISH with DIG-labelled riboprobe for *c-fos* was done for all the points sampled including the control kept in light.

Pictures of living halibut embryos. Living embryos (16 dpf) in eggshell were mounted in 3% methylcellulose (Sigma-Aldrich, St. Louis, MO) and oriented in a dorsal and lateral view. Photo-arrested eggs in sea-water (18 dpf) were transferred to darkness to induce hatching and pictures were taken using a Leica M420 macroscope (Leica Microsystems, Germany) and a CoolSNAP-Pro Color Imaging Camera (RS Photometrics, Tucson, AZ).

Microscopy and photographic manipulation. Pictures were taken by using a digital camera Leica DFC 320/ Leica 350 FX (Leica Microsystems, Germany) attached to a Leica DM 6000B microscope (Leica Microsystems, Germany) with an ebx75mc-L90 lamp (Leistungselektronik Jena GmbH, Germany). Confocal images were taken using the Leica TCS SPE confocal system together with a Leica DM2500 microscope (Leica Microsystems, Germany). Confocal imaging of acetylated alpha tubulin was done using a Zeiss LSM 510 Meta with 10x Plan-Neofluar 0,3 NA or 20x Plan-Neofluar 0,5 NA (Zeiss. Germany) and the pictures were generated merging a stack of pictures providing a 3D projection. A movie of the pictures from the 3D projection and a movie of different focal levels were also made (See Supplementary Figure 4 and 5). All pictures of the fluorescent double labelling with VA opsin and melanopsin (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

Transient and dual expression of vertebrate ancient opsin and melanopsin in the hindbrain at the time of hatching. The halibut eggs are known to hatch at an early developmental stage when the larvae are transparent and the retina consists of neuroblastic cells (Haug, 1990; Kvenseth et al., 1996). Figure 1A, C-D show a living halibut embryo inside the eggshell around the time of hatching, when the hatching glands make this narrow belt in the yolk sac starting just caudal to the ear (Fig. 1A). At this stage the olfactory bulbs, the unpigmented eyes, the developing brain, the lateral neuromasts and the ears can be seen (Fig. 1C-D). A lateral view of a hatching embryo leaving the eggshell reveals the hatching glands as a ring in the frontal part of the yolk sac showing the restricted digestion of the eggshell (Fig. 1B). In our broad search for photoreceptive elements in the developing halibut embryo we have earlier identified several melanopsins (Eilertsen et al., 2014) and we here report an additional nonvisual opsin, VA opsin. Phylogenetic maximum likelihood analysis verifies that this VA opsin branch together with other VA opsins (data not shown). Our ISH results have detected a ball-shaped cluster of cells in the hindbrain of special interest, expressing both VA opsin (Fig. 1E) and melanopsin (*opn4m3*) (Fig. 1F) around hatching. The expression is seen bilaterally at the level of the lateral neuromasts, most likely in the second rhombomere, caudal to the midbrain-hindbrain boundary. In addition, an oblique band at the

level of the ears, most likely in the fourth rhombomere, is seen for both genes, with strong distinct expression of melanopsin. Nissl staining and ISH with VA opsin probe was done on parallel sections and Fig. 1G-H show transversal sections and Fig. 1I-J show horizontal sections through the hindbrain. The transversal sections show how the hindbrain cluster is located in ventral parts of the hindbrain and the neuromast can be seen in the same section. The horizontal sections reveal the location related to the midbrain-hindbrain boundary and in addition, a positive cell of the oblique band is shown. To verify that the c-opsin homologue VA opsin and the r-opsin homologue melanopsin are expressed in the same cells, fluorescent double labelling ISH was done (Fig. 2A-F). Confocal imaging of a whole embryo showed that the two genes are expressed in the same cluster in the hindbrain (Fig. 2A-C). 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. 2D-F), with some cells in the aggregated cluster just expressing one of the opsins (data not shown). The expression pattern of the two genes has been analyzed around hatching (Fig. 2G-M) and show differences during development of the hindbrain cluster. At 10 dpf VA opsin positive cells are visible in the cluster (Fig. 2G) and at 13 dpf the expression is even more aggregated (Fig. 2H). After hatching, at 17 dpf the expression is weaker and the VA opsin expression is disintegrated (Fig. 2I) and this is even more apparent at 19 dpf where only a few cells are expressing VA opsin (Fig. 2J). The mammalian-like melanopsin (*opn4m3*) is expressed in an aggregated cluster already at 9 dpf (Fig. 2K) but at 13 dpf the melanopsin expression has started to scatter (Fig. 2L). At 17 dpf the ball-shaped expression has disappeared from the neuronal cluster (Fig. 2M).

The hindbrain cluster is imbedded in a neuronal network projecting to the hatching glands. 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 nonvisual opsin expressing cluster (shown by ISH of VA opsin) (Fig. 3). Analyzing the neuronal network of the embryo, it appears that fibers course along the yolk sac and innervate the hatching gland cells (Fig. 3A, C, E). A close look on the hatching glands (Fig. 3B) and a curved fiber (Fig. 3D) show the close connection (Fig. 3F). Analyzing the VA opsin expressing cells in the hindbrain clusters at 13 dpf (Fig. 3G) and the neuronal network (Fig. 3H) demonstrates the localization of the hindbrain clusters in relation to the fibers (Fig. 3I). Further, confocal imaging of acetylated alpha tubulin demonstrates the neuronal network in more detail (Fig. 3J-M), with an overview in Fig. 3J and with focus on the lateral fiber in Fig.

3K-L and the neuronal network of the hindbrain in Fig. 3M. Fig. 3K shows that the fiber (probably the trigeminal sensory axon bundle) bends laterally anterior to the neuromast and splits. Part of the fiber bundle proceeds lateral and exits the brain while others continue dorsorostrally in the brain. Higher magnification and rotated view of the fiber (Fig. 3L) shows the split and fiber extension out in the yolk sac. (See also Supplementary Figure 4 for a movie of the 3D projection in Fig. 3K.) The neuronal network in the hindbrain (Fig. 3M) reveals that the fiber coursing out in the yolk sac is lateral and located more dorsally. (See Supplementary Figure 5 for a movie of the different focal levels in Fig. 3M.) Higher magnification of the pictures in Fig. 3G-I shows the hindbrain cluster (Fig. 3N) and the fibers (Fig. 3P) on one side of the embryo and a combination of the pictures illustrates how the fibers connected to the hindbrain cluster proceed laterally to exit the brain just anterior to the neuromast (Fig. 3R). By confocal imaging the connection between the VA opsin cluster (Fig. 3O) and the fibers (Fig. 3Q) is visualized in detail and the hindbrain cluster appears to be imbedded in a neuronal network reaching the hatching glands (Fig. 3S).

Dark-induced hatching of photo-arrested eggs gives *c-fos* activation in the hindbrain and in hatching glands. Neuronal activation in dark-induced hatching after photo-arrest was analyzed by expression studies of the immediate early gene *c-fos*. Five eggs (18 dpf) per dark stimulation (2, 10, 20, 30, 40, 60 and 120 minutes) were analyzed and all sampling points except the 120 minutes showed the same expression pattern as the control (5 eggs analyzed) 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 of all larvae studied (Fig. 4). A picture of the yolk sac and hatching glands of an embryo sampled at 120 minutes shows the *c-fos* expression in the ring of hatching glands (Fig. 4A) and a high magnification reveals the distribution of *c-fos* expression in single hatching gland cells (Fig. 4B). Figure 4C shows VA opsin expression in the hindbrain at the same developmental stage as the study with photo-arrested eggs. At the sampling point 120 minutes, *c-fos* expression is detected in telencephalon, midbrain and hindbrain (Fig. 4D). The expression in the hindbrain is a bilateral ball-shaped cluster of cells resembling the morphology of the photosensitive hindbrain cluster (Fig. 4D). In the light control, *c-fos* is expressed in a similar pattern in the telencephalon and midbrain as the 120 minutes sampled eggs, but no expression is detected in the hindbrain (Fig. 4E). Fluorescent double labelling ISH in a 120 minutes sampled embryo shows that VA opsin (Fig. 4F, G) and *c-fos* (Fig. 4H, I) are expressed in the same bilateral hindbrain cluster (Fig. 4J, K) in all the embryos studied.

Discussion

We present a unique study that combines the characterization of deep brain photoreceptor cells and functional neuronal activation during hatching of Atlantic halibut eggs. Embryos of halibut hatch at an early developmental stage when the eyes are not functional, yet the development of deep brain nonvisual systems is advanced. This gives a special combination of a nonvisual system, simple neuronal wiring and a light controlled hatching process. Our findings suggest that hatching is under direct control of transient deep brain photoreceptor cells expressing dual photopigments. These results allow the speculation that transient opsin expression may be important to regulate other life history transition events as well.

Transient photoreceptive hindbrain cluster and connections to the hatching glands. In our previous studies on Atlantic halibut (Eilertsen et al., 2014) we have described the early complexity of melanopsins in deep brain photoreceptor cells in the hatching embryo, where we found a bilateral ball-shaped hindbrain cluster expressing *opn4m3*. Remarkably, we found that both types, VA opsin and *opn4m3,* are transiently expressed in this hindbrain cluster (Fig. 1 and 2). Previous studies have indicated a close overlap between VA opsin and melanopsin expression (Bellingham et al., 2002; Jenkins et al., 2003; Sandbakken et al., 2012) or between melanopsin and visual opsins (Davies et al., 2011). Here we show for the first time by fluorescent double labelling ISH that these two opsins, originating from evolutionary distinct heritages, c-opsin and r-opsin, are in fact expressed in the same cells. Earlier, it has been shown that a marine invertebrate in the *Annelida* phylum, *Platynereis dumerilii* co-expresses two opsins of different heritage, the r-opsin and the Go- opsin (Guehmann et al., 2015). Recently, a marine mollusk, *Leptochiton asellus*, is found to co-express a newly discovered xenopsin with r-opsin in a photoreceptor cell having both microvilli and cilia (Vocking et al., 2017). Analyzing the dynamics of the hindbrain cluster, we found that opsins expressions are transient with extensive distributions of both genes before hatching (Fig. 2). After hatching, the expression of VA opsin and melanopsin in the developing cluster scatters and disappears within a few days, indicating that one function of the hindbrain cluster is related to this early life history transition. We further demonstrate that the hindbrain cluster is imbedded in a neuronal network of fibers that extend into the yolk sac (Fig. 3). There exists a connection between the photosensitive hindbrain cluster and the region of hatching glands in the yolk sac, suggesting that the fibers provide a signal to the hatching glands. In addition, we observe no RNA expression of visual opsins, melanopsins, exorhodopsin and VA opsin in the narrow belt

of hatching gland cells (data not shown), indicating that there is no direct photoreception mediated by these opsin families in the hatching glands.

Neural activity in the hindbrain cluster and hatching. Light serves as a natural cue for hatching in halibut eggs, and light synchronize hatching in such a way that it occurs at the first night after the embryos have reached the developmental competency before hatching (Helvik and Walther, 1992). Recently light-regulated hatching has also been demonstrated in zebrafish and Senegalese sole (Villamizar et al., 2012; Villamizar et al., 2013). 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 (Fig. 4). The expression of *c-fos* is activated rapidly and transiently in response to a variety of stimuli such as growth factor stimulation and stimulation of nerve cells (Bullitt, 1990; Sheng and Greenberg, 1990). Our results show expression of *c-fos* 120 minutes after the photo-arrested eggs were transferred to darkness. Although not shown directly, these results indicate that darkness stimulates neural activity that induces hatching, implying that light exerts inhibitory influences on melanopsin- and VA opsin-expressing cells. Our data also suggest that the bilateral photosensitive hindbrain cluster mediates the signal to the hatching glands, through the neuronal network with fibers out in the yolk sac. In addition, even though the pineal organ 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 nonvisual opsins at the stage of hatching (Eilertsen et al., 2014), no *c-fos* activation was detected in the pineal organ here.

Dynamics of the dual photoreceptor cells in the hindbrain cluster and hatching. The melanopsin expression fades already prior to hatching in the hindbrain cluster (Fig. 2). A few days after the natural hatching point, the melanopsin expression has disappeared while the VA opsin expression persists a little longer. Evolutionarily, melanopsin and VA opsin are believed to belong to different classes of photoreceptor pigments normally found in rhabdomeric and ciliary photoreceptor cells and the phototransduction cascades involved are thought to involve different machinery resulting in depolarization or hyperpolarization, respectively (Arendt, 2003; Nilsson, 2004). Chromatic antagonism with a hyperpolarizing light response sensitive to blue light (pinopsin) and a depolarizing light response sensitive to green light (parietopsin) has been described in the parietal-eye photoreceptor cells of lizard,

but in contrast to melanopsin and VA opsin they both belong to the same class of photopigments (Solessio and Engbretson, 1993; Su et al., 2006). However, they involve the same phototransduction cascade leading to an antagonistic response employing different G proteins (Su et al., 2006). In contrast, hyperpolarization response is not detected in the rhabdomeric adult eye of the invertebrate annelid *Platynereis* where Go-opsin is co-expressed with two r-opsins. It has been suggested that Go-opsin does not antagonize the depolarization response of r-opsins in the *Platynereis* eye (Guehmann et al., 2015). It is unknown how the phototransduction cascade will function in the dual photoreceptor cluster of halibut expressing both hyperpolarizing and depolarizing opsin types. One may speculate if light activates both opsin types and balance the membrane potential so that the net charge is unchanged, leaving the cell in a resting state. Fading of melanopsin at the time of hatching may allow a VA opsin-driven cascade. It is conceivable that under the light the activation of the VA opsin hyperpolarizes the photoreceptors and inhibits hatching. Shifting to darkness, the VA hyperpolarized cells will be depolarized due to the dark current, giving a change in the membrane potential allowing an activation of the hatching glands. The developmentally timed down-regulation of melanopsin may therefore contribute to refining the time of hatching.

Hatching, a direct brain photoreceptive control process. Recently the hindbrain was for the first time demonstrated to be responsible for a light-sensing behavior in vertebrates. The caudal hindbrain of zebrafish was shown to drive series of robust and reproducible motor behaviors as a response to visual wavelengths of light (Kokel et al., 2013). Here we suggest that also the rostral hindbrain is involved in light-sensing behaviors in vertebrates. Our results indicate that the environmental light is detected directly in the hindbrain providing behavioral and physiological functions driven by neural activity. In zebrafish, the response was shown to be dependent of the synthesis of 11-*cis* retinal, while 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 VA opsin and melanopsin, giving the first indications of responsible opsins in the hindbrain. Further, we can indicate the neuronal identity of the hindbrain cluster based on the indicated location in the second rhombomere (Fig. 1) and confocal images of the acetylated alpha tubulin (Fig. 3J-M). The location in the second rhombomere is relative lateral (Fig. 1G-J) and corresponds with the location of the rostral trigeminal motor neuron (nVa) located in the second rhombomere of zebrafish (Chandrasekhar et al., 1997; Higashijima et al., 2000). In zebrafish where the neuronal network of the hindbrain is described in more detail, the efferent axon of the nV is demonstrated to join the afferent trigeminal sensory axon

bundle, that turns rostrolaterally and exits the hindbrain in rhombomere 2 (Chandrasekhar et al., 1997). Unusual afferent innervations by the trigeminal ganglion have also been observed in transient glands in other fishes and in amphibians (Roberts and Blight, 1975; Honore and HemmatiBrivanlou, 1996; Pottin et al., 2010). In fish, it has been suggested that the axonal guidance cues probably are provided by the target themselves and both the glands and the fibers have a parallel transient nature related to their function early in development (Pottin et al., 2010). The exact nature of the neuronal regulation of hatching and how photoreceptive neurons, motor neurons and sensory neurons interact to control hatching, are open for further investigations.

Transient photoreceptors may regulate life history transitions. Examples of temporary photoreceptor cells are almost lacking in the literature but studies in invertebrates show that larval eyes degrade during metamorphosis when the adult eyes start to form (Rhode, 1993; Yamaguchi and Seaver, 2013). In sea lamprey reduced dermal photosensitivity has been shown to reflect life history dependent changes in habitat and behavior (Binder et al., 2013), but the responsible cells and photopigments have not been detected. Here we demonstrate a transient photoreceptive cluster of cells in the hindbrain of halibut near hatching and we indicate that this cluster is permissive for this early life history transition. Our results illustrate neurons whose photoreceptive activities are not fixed throughout life and that they can be developmentally regulated toward a biological event. The finding supports a gene-expression signature study of life history transitions in Atlantic salmon where the gene expression of a rod-like opsin (most equivalent to exorhodopsin) is shown to be up- and down-regulated at different life history stages (Aubin-Horth et al., 2009). The concept of transient light sensitivity to target critical aspect of life opens for investigation of other biological events to be regulated by specific transient nonvisual photoreceptor systems.

Acknowledgements

We thank Rolf B. Edvardsen at the Institute of Marine Research for providing the sequence of *c-fos* and for validating our VA opsin sequence data by searching the Atlantic halibut genome. Thanks to Sterling White Halibut for founding the Atlantic halibut genome sequencing and Ragnfrid Mangor-Jensen at the Austevoll Aquaculture Research Station for providing Atlantic halibut eggs. The confocal imaging of acetylated alpha tubulin was performed at the Molecular Imaging Center (MIC) and was thus supported by the Department of Biomedicine and the Faculty of Medicine and Dentistry, at the University of Bergen, and its partners.

References

Arendt D. 2003. Evolution of eyes and photoreceptor cell types. Int J Dev Biol 47(7-8):563-571.

Aubin-Horth N, Letcher BH, Hofmann HA. 2009. Gene-expression signatures of Atlantic salmon's plastic life cycle. Gen Comp Endocrinol 163(3):278-284.

Bellingham J, Whitmore D, Philp AR, Wells DJ, Foster RG. 2002. Zebrafish melanopsin: isolation, tissue localisation and phylogenetic position. Brain Res Mol Brain Res 107(2):128-136.

Binder TR, McDonald DG, Wilkie MP. 2013. Reduced dermal photosensitivity in juvenile sea lampreys (*Petromyzon marinus*) reflects life-history-dependent changes in habitat and behaviour. Can J Zool 91(9):635-639.Bullitt E. 1990. Expression of C-fos-like protein as a marker for neuronal-activity following noxious-stimulation in the rat. Journal of Comparative Neurology 296(4):517-530.

Burge C, Karlin S. 1997. Prediction of complete gene structures in human genomic DNA. J Mol Biol 268(1):78-94.

Chandrasekhar A, Moens CB, Warren JT, Kimmel CB, Kuwada JY. 1997. Development of branchiomotor neurons in zebrafish. Development 124(13):2633-2644.

Chitnis AB, Kuwada JY. 1990. Axonogenesis in the brain of zebrafish embryos. J Neurosci 10(6):1892-1905.

Davies WIL, Tamai TK, Zhen L, Fu JK, Rihel J, Foster RG, Whitmore D, Hankins MW. 2015. An extended family of novel vertebrate photopigments is widely expressed and displays a diversity of function. Genome Res 25(11):1666-1679.

Davies WIL, Zheng L, Hughes S, Tamai TK, Turton M, Halford S, Foster RG, Whitmore D, Hankins MW. 2011. Functional diversity of melanopsins and their global expression in the teleost retina. Cell Mol Life Sci 68(24):4115-4132.

Davies WL, Hankins MW, Foster RG. 2010. Vertebrate ancient opsin and melanopsin: divergent irradiance detectors. Photoch Photobio Sci 9(11):1444-1457.

Eilertsen M, Drivenes Ø, Edvardsen RB, Bradley CA, Ebbesson LOE, Helvik JV. 2014. Exorhodopsin and melanopsin systems in the pineal complex and brain at early developmental stages of Atlantic halibut (*Hippoglossus hippoglossus*). Journal of Comparative Neurology 522(18):4003-4022.

541 Fernandes AM, Fero K, Arrenberg AB, Bergeron SA, Driever W, Burgess HA. 2012. Deep
542 brain photoreceptors control light-seeking behavior in zebrafish larvae. Curr Biol
543 22(21):2042-2047.
544 Fernandes AM, Fero K, Driever W, Burgess HA. 2013. Enlightening the brain: Linking deep
545 brain photoreception with behavior and physiology. Bioessays 35(9):775-779.
546 Fischer RM, Fontinha BM, Kirchmaier S, Steger J, Bloch S, Inoue D, Panda S, Rumpel S,
547 Tessmar-Raible K. 2013. Co-expression of VAL- and TMT-opsins uncovers ancient
548 photosensory interneurons and motorneurons in the vertebrate brain. Plos Biol 11(6).
549 Forsell J, Holmqvist B, Helvik JV, Ekström P. 1997. Role of the pineal organ in the
550 photoregulated hatching of the Atlantic halibut. Int J Dev Biol 41(4):591-595.
551 Guehmann M, Jia H, Randel N, Veraszto C, Bezares-Calderon LA, Michiels NK, Yokoyama
552 S, Jekely G. 2015. Spectral tuning of phototaxis by a Go-opsin in the rhabdomeric
553 eyes of *Platynereis*. Curr Biol 25(17):2265-2271.
554 Halford S, Pires SS, Turton M, Zheng L, Gonzalez-Menendez I, Davies WL, Peirson SN,
555 Garcia-Fernandez JM, Hankins MW, Foster RG. 2009. VA opsin-based
556 photoreceptors in the hypothalamus of birds. Curr Biol 19(16):1396-1402.
557 Haug T. 1990. Biology of the Atlantic halibut, *Hippoglossus hippoglossus* (L, 1758). Adv
558 Mar Biol 26:1-70.
559 Haug T, Kjørsvik E, Solemdal P. 1984. Vertical-distribution of Atlantic halibut
560 (*Hippoglossus-Hippoglossus*) eggs. Can J Fish Aquat Sci 41(5):798-804.
561 Helvik JV, Drivenes Ø, Naess TH, Fjose A, Seo HC. 2001. Molecular cloning and
562 characterization of five opsin genes from the marine flatfish Atlantic halibut
563 (*Hippoglossus hippoglossus*). Visual Neurosci 18(5):767-780.
564 Helvik JV, Oppen-Berntsen DO, Walther BT. 1991. The hatching mechanism in Atlantic
565 halibut (*Hippoglossus hippoglossus*). Int J Dev Biol 35(1):9-16.
566 Helvik JV, Walther BT. 1992. Photo-regulation of the hatching process of halibut
567 (*Hippoglossus hippoglossus*) eggs. J Exp Zool 263(2):204-209.
568 Helvik JV, Walther BT. 1993. Development of hatchability in halibut (*Hippoglossus*
569 *hippoglossus*) embryos. Int J Dev Biol 37(3):487-490.
570 Higashijima S, Hotta Y, Okamoto H. 2000. Visualization of cranial motor neurons in live
571 transgenic zebrafish expressing green fluorescent protein under the control of the *Islet-*
572 *1* promoter/enhancer. J Neurosci 20(1):206-218.
573 Honore E, HemmatiBrivanlou A. 1996. *In vivo* evidence for trigeminal nerve guidance by the
574 cement gland in *Xenopus*. Dev Biol 178(2):363-374.

575 Hunter PR, Nikolaou N, Odermatt B, Williams PR, Drescher U, Meyer MP. 2011.
576 Localization of Cadm2a and Cadm3 proteins during development of the zebrafish
577 nervous system. J Comp Neurol 519(11):2252-2270.
578 Jenkins A, Munoz M, Tarttelin EE, Bellingham J, Foster RG, Hankins MW. 2003. VA opsin,
579 melanopsin, and an inherent light response within retinal interneurons. Curr Biol
580 13(15):1269-1278.
581 Kojima D, Mano H, Fukada Y. 2000. Vertebrate ancient-long opsin: A green-sensitive
582 photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J
583 Neurosci 20(8):2845-2851.
584 Kojima D, Torii M, Fukada Y, Dowling JE. 2008. Differential expression of duplicated VAL-
585 opsin genes in the developing zebrafish. J Neurochem 104(5):1364-1371.
586 Kokel D, Dunn TW, Ahrens MB, Alshut R, Cheung CY, Saint-Amant L, Bruni G, Mateus R,
587 van Ham TJ, Shiraki T, Fukada Y, Kojima D, Yeh JR, Mikut R, von Lintig J, Engert
588 F, Peterson RT. 2013. Identification of nonvisual photomotor response cells in the
589 vertebrate hindbrain. J Neurosci 33(9):3834-3843.
590 Kvenseth AM, Pittman K, Helvik JV. 1996. Eye development in Atlantic halibut
591 (*Hippoglossus hippoglossus*): Differentiation and development of the retina from early
592 yolk sac stages through metamorphosis. Can J Fish Aquat Sci 53(11):2524-2532.
593 Ledizet M, Piperno G. 1991. Detection of acetylated alpha-tubulin by specific antibodies.
594 Method Enzymol 196:264-274.
595 Liu JX, Lessman CA. 2007. Soluble tubulin complexes, gamma-tubulin, and their changing
596 distribution in the zebrafish (*Danio rerio*) ovary, oocyte and embryo. Comp Biochem
597 Physiol B-Biochem Mol Biol 147(1):56-73.Matos-Cruz V, Blasic J, Nickle B,
598 Robinson PR, Hattar S, Halpern ME. 2011. Unexpected diversity and photoperiod
599 dependence of the zebrafish melanopsin system. Plos One 6(9).
600 Nakane Y, Ikegami K, Iigo M, Ono H, Takeda K, Takahashi D, Uesaka M, Kimijima M,
601 Hashimoto R, Arai N, Suga T, Kosuge K, Abe T, Maeda R, Senga T, Amiya N,
602 Azuma T, Amano M, Abe H, Yamamoto N, Yoshimura T. 2013. The saccus
603 vasculosus of fish is a sensor of seasonal changes in day length. Nat Commun 4:7.
604 Nilsson DE. 2004. Eye evolution: a question of genetic promiscuity. Curr Opin Neurobiol
605 14(4):407-414.
606 Peirson SN, Halford S, Foster RG. 2009. The evolution of irradiance detection: melanopsin
607 and the non-visual opsins. Philos T R Soc B 364(1531):2849-2865.

608 Pottin K, Hyacinthe C, Retaux S. 2010. Conservation, development, and function of a cement
609 gland-like structure in the fish *Astyanax mexicanus*. P Natl Acad Sci USA
610 107(40):17256-17261.
611 Rhode B. 1993. Larval and adult eyes in *Capitella spec. I* (Annelida, Polychaeta) J Morphol
612 217(3):327-335.
613 Roberts A, Blight AR. 1975. Anatomy, physiology and behavioral role of sensory nerve-
614 endings in cement gland of embryonic *Xenopus*. Proceedings of the Royal Society
615 Series B-Biological Sciences 192(1106):111-127.
616 Sandbakken M, Ebbesson L, Stefansson S, Helvik JV. 2012. Isolation and characterization of
617 melanopsin photoreceptors of Atlantic salmon (*Salmo salar*). J Comp Neurol
618 520(16):3727-3744.
619 Sheng M, Greenberg ME. 1990. The regulation and function of *c-fos* and other immediate
620 early genes in the nervous-system. Neuron 4(4):477-485.
621 Solessio E, Engbretson GA. 1993. Antagonistic chromatic mechanisms in photoreceptors of
622 the parietal eye of lizards. Nature 364(6436):442-445.
623 Su CY, Luo DG, Terakita A, Shichida Y, Liao HW, Kazmi MA, Sakmar TP, Yau KW. 2006.
624 Parietal-eye phototransduction components and their potential evolutionary
625 implications. Science 311(5767):1617-1621.
626 Thisse C, Thisse B. 2008. High-resolution *in situ* hybridization to whole-mount zebrafish
627 embryos. Nat Protoc 3(1):59-69.
628 Verpy E, Leibovici M, Michalski N, Goodyear RJ, Houdon C, Weil D, Richardson GP, Petit
629 C. 2011. Stereocilin connects outer hair cell stereocilia to one another and to the
630 tectorial membrane. J Comp Neurol 519(2):194-210.
631 Villamizar N, Blanco-Vives B, Oliveira C, Dinis MT, Di Rosa V, Negrini P, Bertolucci C,
632 Sanchez-Vazquez FJ. 2013. Circadian rhythms of embryonic development and
633 hatching in fish: A comparative study of zebrafish (diurnal), Senegalese sole
634 (nocturnal), and Somalian cavefish (blind). Chronobiol Int 30(7):889-900.
635 Villamizar N, Ribas L, Piferrer F, Vera LM, Sanchez-Vazquez FJ. 2012. Impact of daily
636 thermocycles on hatching rhythms, larval performance and sex differentiation of
637 zebrafish. Plos One 7(12).
638 Vocking O, Kourtesis I, Tumu SC, Hausen H. 2017. Co-expression of xenopsin and
639 rhabdomeric opsin in photoreceptors bearing microvilli and cilia. eLife 6.

Yamaguchi E, Seaver EC. 2013. The importance of larval eyes in the polychaete *Capitella teleta*: effects of larval eye deletion on formation of the adult eye. Invertebr Biol 132(4):352-367.

673 **Table 1 Primers vertebrate ancient opsin**

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

674

675 **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
val	KF941295	1743 bp	1164 bp	387 aa	102 - 1182 bp	1081 bp
vas	KF941296	1598 bp	987 bp	328 aa	102 - 1182 bp	1081 bp
opn4m3short	KF941290	1780 bp	1638 bp	545 aa	1 - 1089 bp	1089 bp
opn4m3long	KF941291	1846 bp	1704 bp	567 aa	1 - 1089 bp	1089 bp
c-fos	KF941297	1261 bp	1128 bp	375 aa	1 - 1261 bp	1261 bp

676

677 **Table 3 Antibodies**

Antibody	Immunogen	Manufacturer, host species, mono- vs. polyclonal, catalog number, RRIDs	Dilution
anti-acetylated tubulin	*Chlamydomonas axonemal* α-tubulin within four residudes of acetylated Lys-40	Sigma-Aldrich (St. Louis, MO), Mouse monoclonal, IgG2b, #T7451, RRID: AB_609894	1:1,000
anti-digoxigenin conjugated with alkaline phosphatase, Fab fragments	digoxigenin (DIG)	Roche Diagnostics (Germany), Sheep polyclonal, #11093274910, RRID: AB_514497	1:2,000
anti-fluorescein conjugated with horse radish peroxidase, Fab fragments	fluorescein	Roche Diagnostics (Germany), Sheep polyclonal, #11426346910, RRID: AB_840257	1:400
anti-mouse IgG (H+L), CF™ 488A	mouse IgG (H+L)	Sigma-Aldrich (St. Louis, MO) Goat polyclonal, #SAB4600042, RRID: AB_2532075	1:100
anti-mouse IgG (H+L), CF™ 555	mouse IgG (H+L)	Sigma-Aldrich (St. Louis, MO) Goat polyclonal, #SAB4600066, RRID: AB_2336060	1:100

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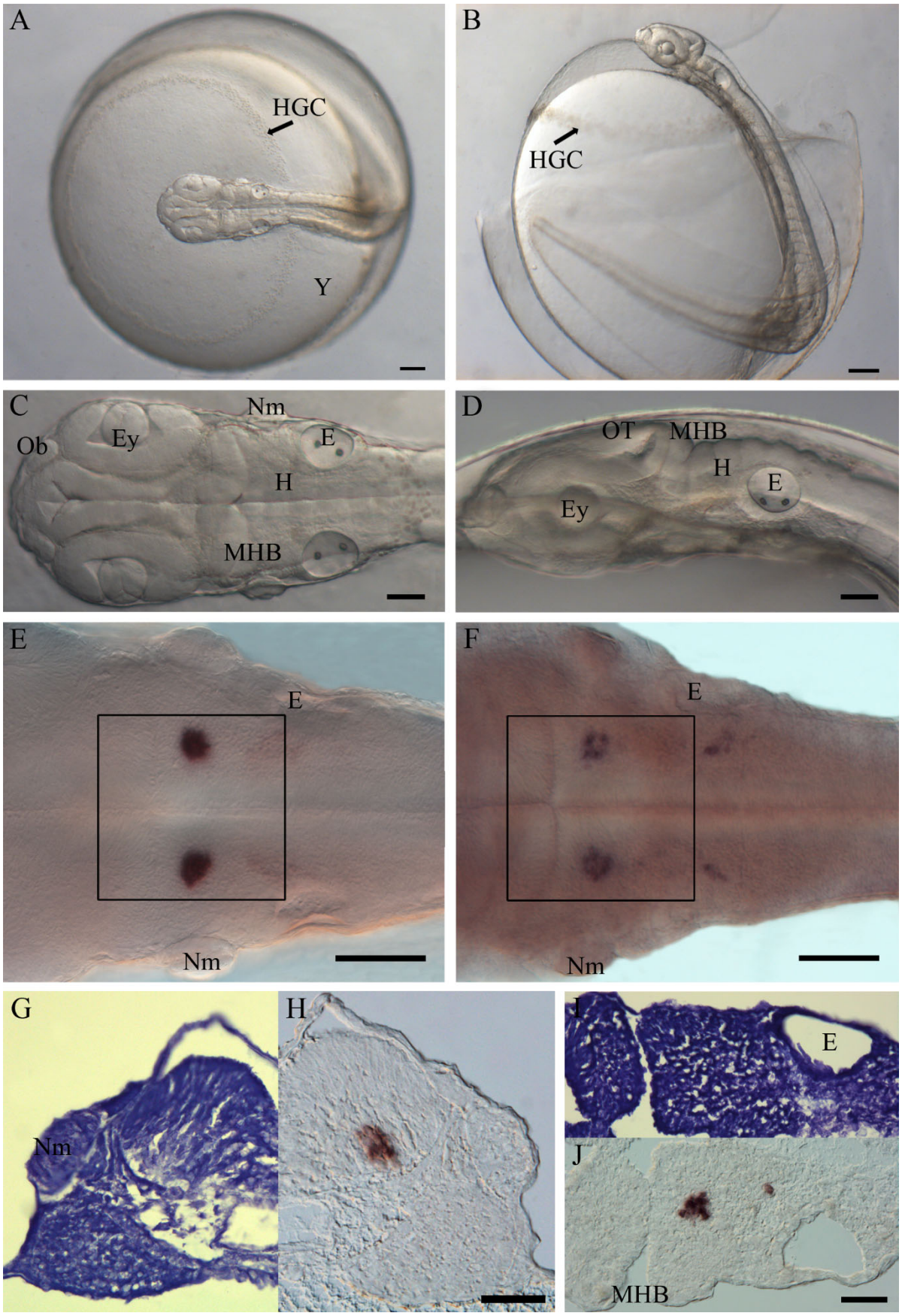


Figure 1 The hatching Atlantic halibut, expression of nonvisual opsins in a bilateral hindbrain cluster. **A:** A dorsal view of an embryo in the eggshell on the top of a huge yolk sac (Y) with

683 hatching glands cells (HGC) in a narrow belt, indicated by a black arrow. **B:** A hatching
684 embryo leaving the eggshell with the hatching glands in a frontal ring (black arrow) showing
685 the restricted digestion of the eggshell. **C:** Higher magnification of dorsal view in (**A**) shows
686 the olfactory bulb (Ob), eyes (Ey), midbrain-hindbrain boundary (MHB), hindbrain (H),
687 neuromasts (Nm) and ears (E). **D:** A lateral view shows the optic tectum (OT) and the
688 structures of the hindbrain including the MHB and the rhombomeres. **E, F:** Halibut embryos
689 with vertebrate ancient opsin (VA opsin) (**E**) and melanopsin (*opn4m3*) (**F**) expression in
690 bilateral ball-shaped clusters and oblique bands in the hindbrain at the stage of hatching. **G-J:**
691 Parallel transversal (**G-H**) and horizontal (**I-J**) sections showing Nissl-staining (**G, I**) and VA
692 opsin expression (**H, J**) of the hindbrain. Scale bars: 200 μm in A-B, 100 μm in C-F, 50 μm in
693 G-J.

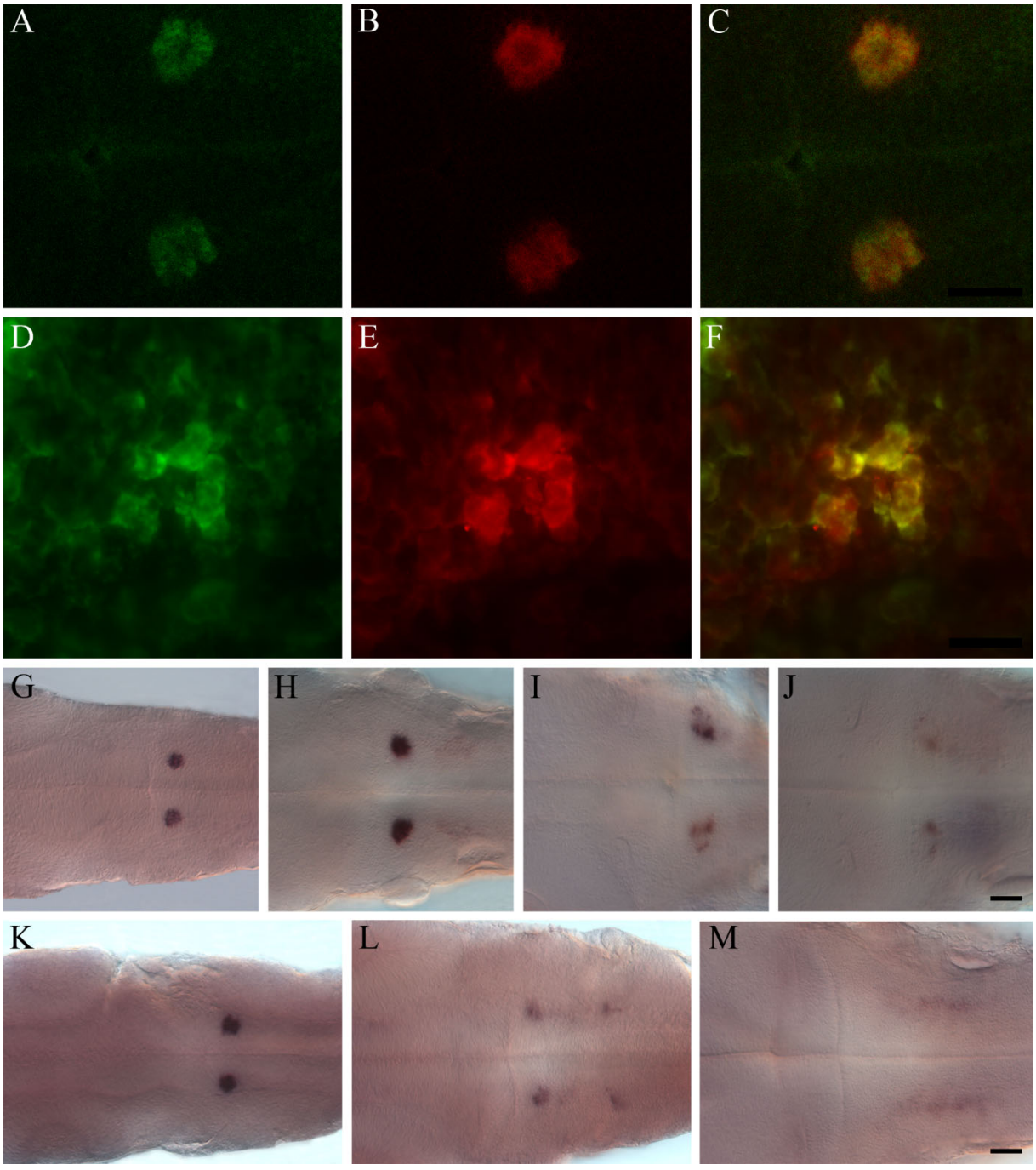


Figure 2 Transient and dual expression of the nonvisual opsins in the hindbrain cluster. **A-C:** Confocal imaging of a whole embryo with VA opsin (**A**) and melanopsin (**B**) and the two genes together (**C**) show that the genes are expressed in the same cluster. **D-F:** Fluorescent microscopy of a sagittal section reveals that VA opsin (**D**) and melanopsin (**E**) are in the same cells in the cluster (**F**). **G-J:** Expression of VA opsin in the developing embryo around hatching. At 10 days post fertilization (dpf) VA opsin is expressed in the hindbrain cluster (**G**) and at 13 dpf the cluster is more condensed (**H**). After hatching at 17 dpf the VA opsin expressing cells scatter (**I**) and at 19 dpf only a few cells express VA opsin (**J**). **K-M:** Expression of melanopsin in the developing embryo around hatching. At 9 dpf melanopsin is

extensively expressed in the hindbrain cluster (**K**) but already at 13 dpf fewer cells in the cluster express melanopsin (**L**). After hatching (17 dpf) no melanopsin positive cells are observed in the hindbrain cluster (**M**). Scale bars: 50 µm in A-C and G-M, 20 µm in D-F. (See Supplementary Figure 1 for magenta/green copy.)

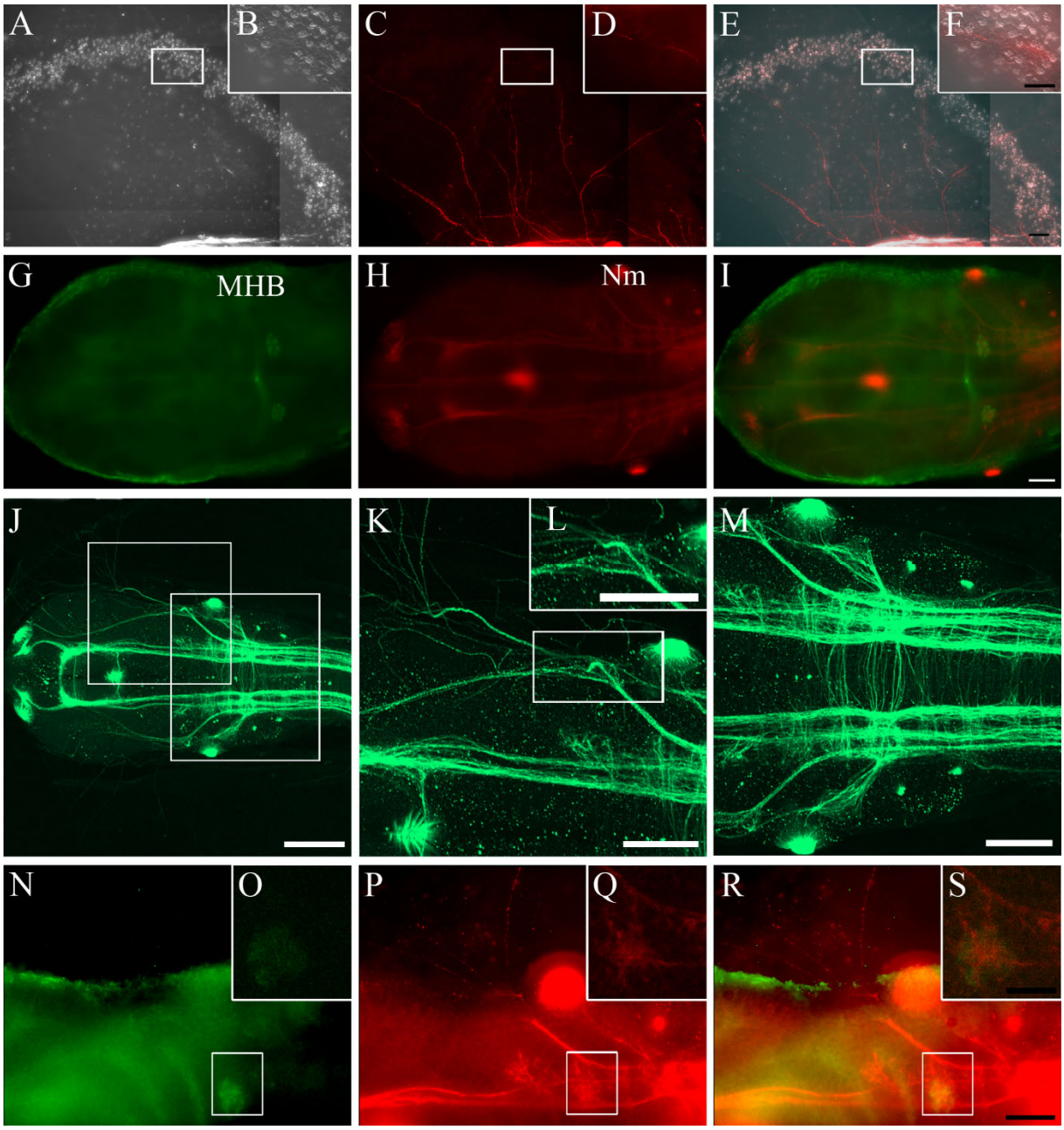


Figure 3 The hindbrain cluster is imbedded in a neuronal network sending fibers out in the yolk sac, innervating the hatching gland cells. **A:** Assembly of dorsal view pictures of hatching glands at 13 days post fertilization (dpf) at one side of the embryo, high magnification of some hatching gland cells (**B**). **C:** Acetylated alpha tubulin illustrates the fibers coursing out in the yolk sac shown by the same collection of pictures as in (**A**), high magnification of a curved fiber (**D**). **E:** A combination of (**A**) and (**C**) shows how the widespread fibers innervate the hatching glands. **F:** (**B**) and (**D**) in combination show the close relation between the hatching glands and the curved fiber. **G:** Vertebrate ancient opsin (VA opsin) positive cells in the hindbrain at 13 dpf located caudal to the midbrain-hindbrain boundary (MHB). **H:** Newly differentiated neurons and axonal pathways in the same embryo

shown by acetylated alpha tubulin. **I:** A combination of VA opsin and acetylated alpha tubulin picturing fibers nearby the hindbrain cluster bending at the level of the neuromasts. **J:** Confocal image of acetylated alpha tubulin showing an overview of the forebrain, midbrain and hindbrain. **K:** Higher magnification of the fiber coursing out of the brain (probably the trigeminal sensory axon bundle) revealed by 3D projection. The picture reveals that the fiber split and one of the fibers proceeds dorsorostrally in the brain while one fiber projects out in the yolk and spreads as a fan. **L:** Focus on the fiber where it splits, with a rotated ventral view of the 3D projection. **M:** Higher magnification of the neuronal network in the hindbrain revealed by a 3D projection. The fiber bending lateral at the level of the neuromast is located laterally. **N:** Focus on one of the VA opsin expressing hindbrain clusters and confocal imaging of the cluster (**O**). **P:** Detail of the fibers proceeding laterally spreading out in the yolk sac as a fan, confocal imaging (**Q**). **R:** The hindbrain cluster is imbedded in the network sending fibers out in the yolk sac. **S:** A combination of the confocal images in (**O**) and (**Q**) shows the connection between the hindbrain cluster and fibers. Scale bars: 200 µm in J, 100 µm in A, C, E, G-I, K-M, N, P, R, 50 µm in O, Q, S, 20 µm in B, D, F. Neuromast (Nm) (See Supplementary Figure 2 for magenta/green copy and Supplementary Figure 4 and 5 for movies of the confocal images of acetylated alpha tubulin.)

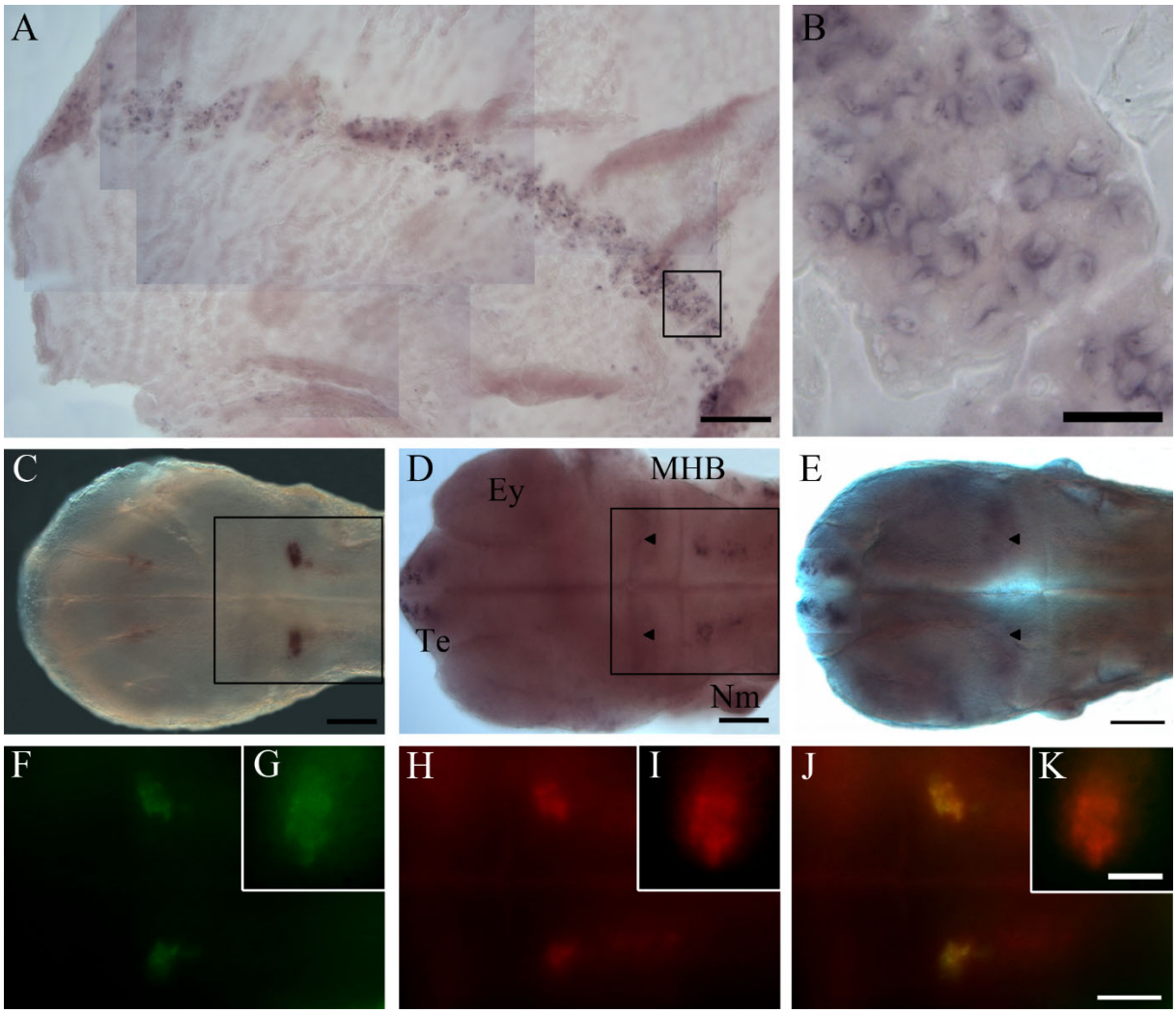
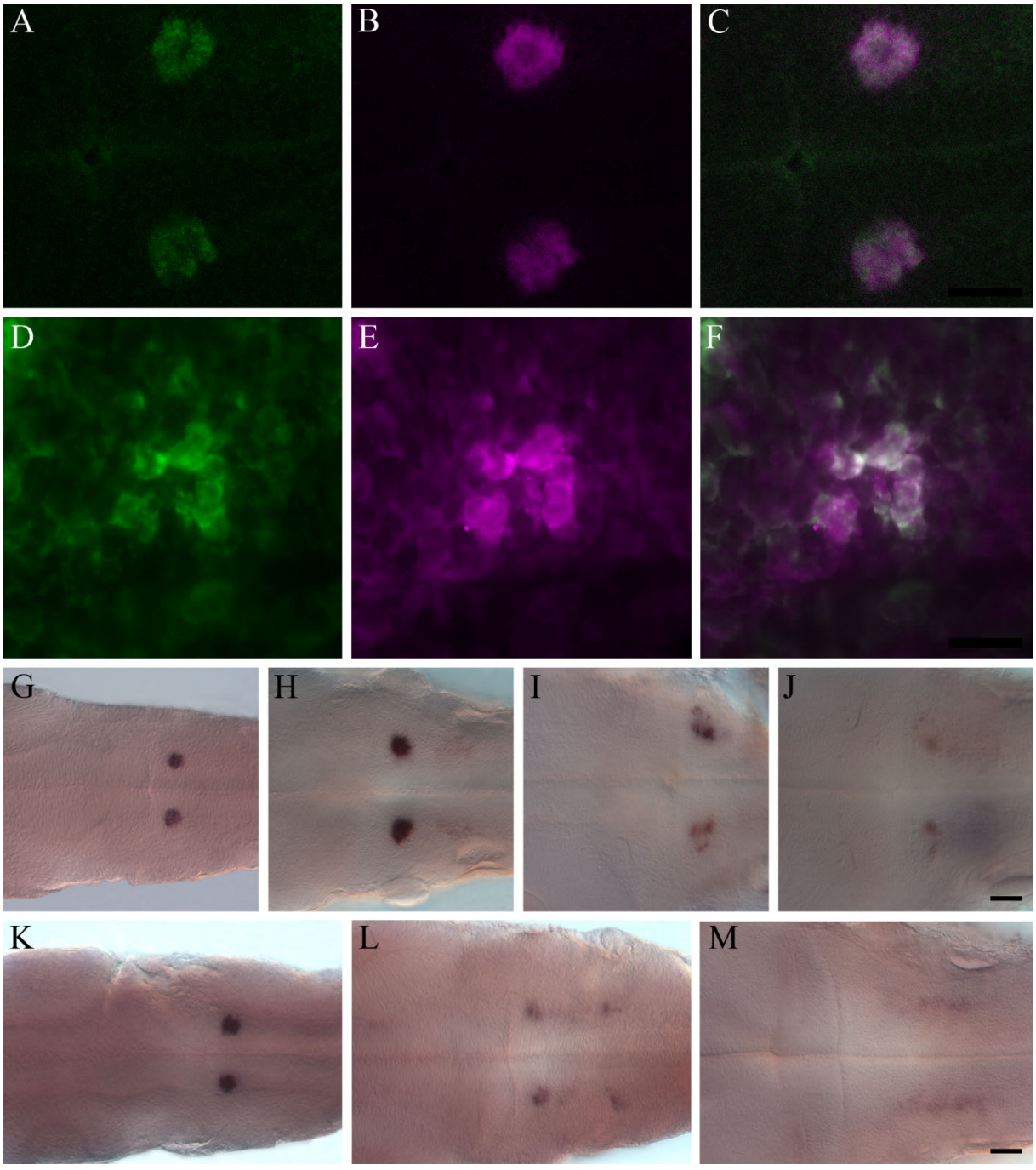
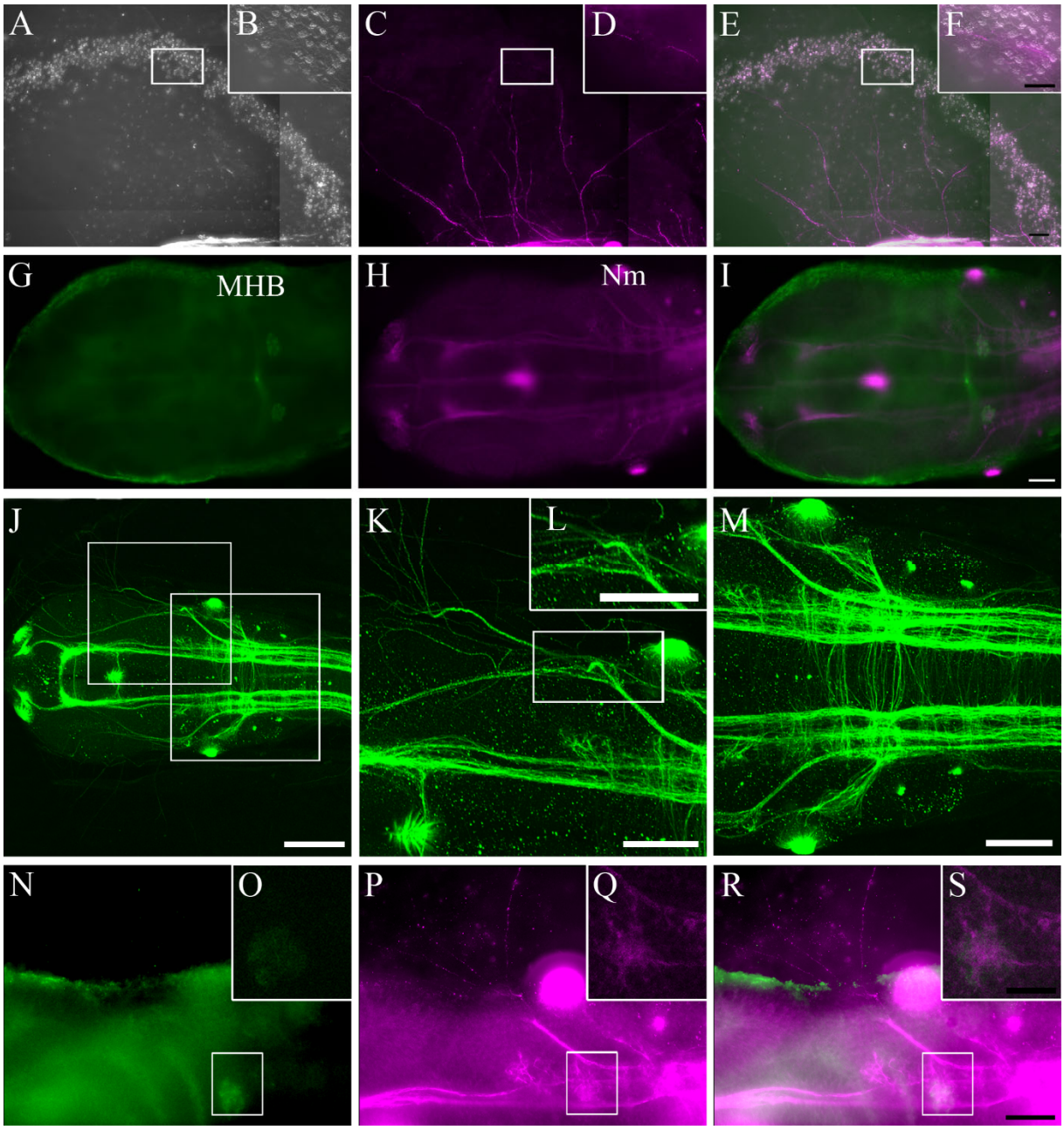


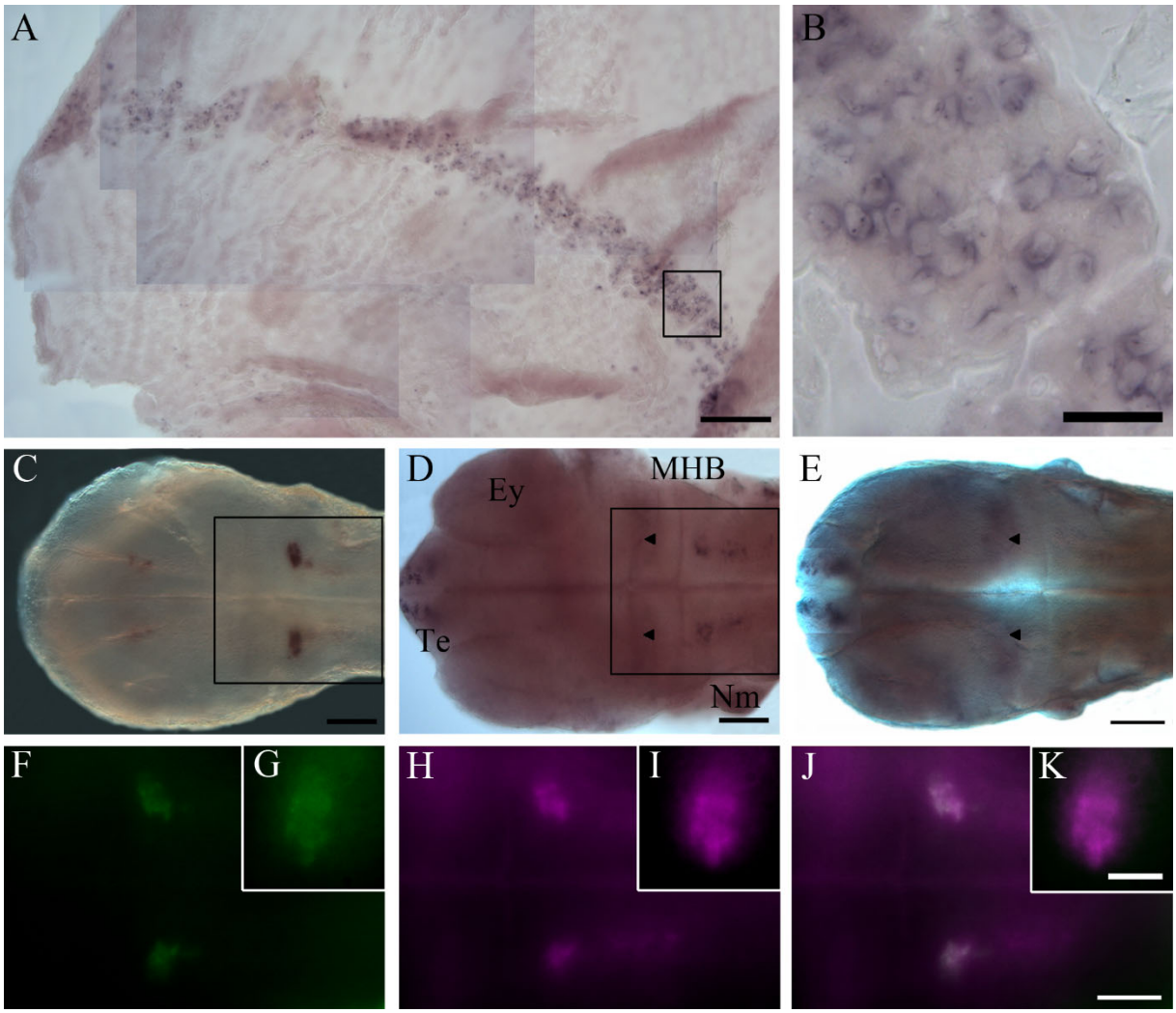
Figure 4 Transfer of photo-arrested eggs to darkness gives neural activation in the hindbrain cluster and hatching glands in the eggs sampled after 120 minutes. **A:** Pictures of the yolk sac and hatching glands of 120 minutes sampled embryo put together to illustrate the *c-fos* expression in the hatching glands. **B:** Detail of the hatching glands expressing *c-fos*. **C:** Vertebrate ancient opsin (VA opsin) in the hindbrain cluster at the same developmental stage as the study with dark-induced hatching after photo-arrest. **D:** At the sampling point 120 minutes neural activation is shown by *c-fos* expression in the telencephalon (Te), midbrain (black arrowhead) and hindbrain. The hindbrain expression is caudal to the midbrain-hindbrain boundary (MHB) and at the level of the neuromast (Nm), most likely in the second rhombomere. **E:** Expression of *c-fos* in a control kept in light shows expression in telencephalon and midbrain (black arrowhead). **F-K** Fluorescent double labelling ISH of the 120 minutes sampled embryo with VA opsin (**F-G**) and *c-fos* (**H-I**) shows that both are expressed in the hindbrain cluster (**J-K**). Scale bars: 100 µm in A, C-E, F, H, J, 50 µm in G, I, K, 20 µm in B. Eye (Ey) (See Supplementary Figure 3 for 1.)



Supplementary figure 1 (See Figure 2 for figure legend)



Supplementary figure 2 (See Figure 3 for figure legend)



Supplementary figure 3 (See Figure 4 for figure legend)

Supplementary Figure 4 Movie of confocal images, view as Figure 3K. The movie is generated by merging a stack of pictures providing 3D projections that are set together as a movie. The movie illustrates the lateral fiber bundle that splits anterior to the neuromast where parts of the fibers course out in the yolk sac and others continue dorsorostrally in the brain.

Supplementary Figure 5 Movie of confocal images, view as Figure 3M. The movie is generated by pictures of different focal levels that are set together as a movie. The movie illustrates the neuronal network in the hindbrain of halibut, from a dorsal to ventral view, revealing that the lateral fiber bundle is located more dorsally.