Gene expression patterns of novel visual and non–visual opsin families in immature and mature Japanese eel males

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This study was carried out to identify and estimate physiological function of a new type of opsin subfamily present in the retina and whole brain tissues of Japanese eel using RNA–Seq transcriptome method. A total of 18 opsin subfamilies were identified through RNA–seq. The visual opsin family included Rh2, SWS2, FWO, DSO, and Exo–Rhod. The non–visual opsin family included four types of melanopsin subfamily (Opn4x1, Opn4x2, Opn4m1, and Opn4m2), peropsin, two types of neuropsin subfamily (Opn5–like, Opn5), Opn3, three types of TMT opsin subfamily (TMT1, 2, 3), VA–opsin, and parapinopsin. In terms of changes in photoreceptor gene expression in the retina of sexually mature and immature male eels, DSO mRNA increased in the maturation group. Analysis of expression of opsin family gene in male eel brain before and after maturation revealed that DSO and SWS2 expression in terms of visual opsin mRNA increased in the sexually mature group. In terms of non–visual opsin mRNA, parapinopsin mRNA increased whereas that of TMT2 decreased in the fore–brain of the sexually mature group. The mRNA for parapinopsin increased in the mid–brain of the sexually mature group, whereas those of TMT1 and TMT3 increased in the hind–brain of the sexually mature group. DSO mRNA also increased in the retina after sexual maturation, and DSO and SWS2 mRNA increased in whole brain part, suggesting that DSO and SWS2 are closely related to sexual maturation.

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Short title: opsin mRNA expression in Japanese eel

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Abstract

This study was carried out to identify and estimate physiological function of a new type of opsin subfamily present in the retina and whole brain tissues of Japanese eel using RNA–Seq transcriptome method. A total of 18 opsin subfamilies were identified through RNA–seq. The visual opsin family included Rh2, SWS2, FWO, DSO, and Exo–Rhod. The non–visual opsin family included four types of melanopsin subfamily (Opn4x1, Opn4x2, Opn4m1, and Opn4m2), peropsin, two types of neuropsin subfamily (Opn5–like, Opn5), Opn3, three types of TMT opsin subfamily (TMT1, 2, 3), VA–opsin, and parapinopsin. In terms of changes in photoreceptor gene expression in the retina of sexually mature and immature male eels, DSO mRNA increased in the maturation group. Analysis of expression of opsin family gene in male eel brain before and after maturation revealed that DSO and SWS2 expression in terms of visual opsin mRNA increased in the sexually mature group. In terms of non–visual opsin mRNA, parapinopsin mRNA increased whereas that of TMT2 decreased in the fore–brain of the sexually mature group. The mRNA for parapinopsin increased in the mid–brain of the sexually mature group, whereas those of TMT1 and TMT3 increased in the hind–brain of the sexually mature group. DSO mRNA also increased

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Keywords: opsin, photoreceptor, Japanese eel, Anguilla japonica, sex maturation

Introduction

Living organisms recognize various environmental information (light, water temperature, salinity, etc.) according to their specific ecology and have ecologically evolved based on the information. Organisms perceive the external environment through light, transmit the information to the brain, synchronize the biological clock operation to activate the metabolism, and control the physiological and ecological functions by inducing the secretion of endocrine hormones such as melatonin (Benoit, 1978; Falcon, 1999, 2007; Campbell et al., 2001). Mammals have two types of photoreceptor proteins (rhodopsin, cone–opsin) that perceive light in the retina of the eye, and different types of photoreceptors recognize the signals of light (wavelength of light, intensity of light, direction of light, and periodicity) (Hestings and Maywood, 2000; Tada et al., 2009). A photoreceptor is a visual sensory cell capable of recognizing light of a specific wavelength. Photoreceptors also refer to an opsin protein receptor that actually absorbs light and converts it into chemical energy. Vertebrate photoreceptors are regulated by opsin, a superfamily of G–protein–coupled receptor (GPCR), opsin, with an inverse agonist 11–*cis* retinal chromophore, covalently bound. Indeed, retinal molecules selectively absorb various spectrum of light depending on the formation of binding with the opsin protein (reviewed in Pugh and Lamb, 2000). Absorption of light at a specific wavelength leads to

conversion into all–*trans* form that binds the opsin and transducing proteins, thereby activating a series of visual sensitive–related cellular signal transduction processes (Terakita, 2005). Opsin superfamily is broadly divided into visual and non–visual opsins. There have been extensive studies on vision function of opsin–based photopigment. However, when the opsin was found in tissues such as avian pineal and amphibian skin, opsins were unofficially divided into visual and non–visual groups (Okano et al., 1994; Kojima and Fukada, 1999; Van Gelder, 2001). As extra–ocular tissues cannot form images, this classification was suggested. Visual opsin initiates the visual transduction cascade, whereas non–visual opsin is involved in circadian entrainment (Doyle et al., 2008) and retinal metabolism (Bellingham et al., 2003).

The habitats of marine organisms, especially fish, vary in depth and region, ranging from freshwater to brackish areas. The light conditions of these habitats are different in terms of turbidity, color, and brightness (Bowmaker, 1994, 2008). For example, in the case of deep–sea snailfish inhabiting relatively deep–water areas, the spectral sensitivities of the rod and cone photoreceptors react to the blue light (Sakata et al., 2015). In contrast, in the case of black bream, shallow–sea fish, cone photoreceptors (Rh2 or MWS), have maximal light absorbance wavelength (λmax) at 545 to 575 nm, which is the dominant light in their habitat (Shand et al., 2002). Thus, it is presumed that animals have obtained a unique visual system that have made them adapt to the light environment of their habitats in the process of evolution.

The Japanese eel, *Anguilla japonica,* has been known to have a dynamic life cycle. It migrates to the seawater areas during spawning season and spawns. Sexually immature eels are yellow whereas sexually mature ones are silver. The leptocephalus undergo metamorphosis into glass eels while moving through ocean currents, migrate to freshwater areas where they spend most the time in their life cycle (Tsukamoto, 1992; Tatsukawa, 2003). Thus, the Japanese eel

93 experiences diverse changes in water environment during its life cycle, which differs greatly
94 from other fish and animals.
95 To date, photoreceptor studies on Anguillid have identified fresh water rhodopsin (FWO)
96 (Zhang et al., 2000), deep–sea rhodopsin (DSO) (Zhang et al., 2000), Rh1d (European eel, *A.*
97 *anguilla* and Japanese eel, *A. japonica* and giant mottled eel, *A. marmorata*) (Wang et al., 2014),
98 Rh2 (European eel and giant mottled eel) (Cottrill et al., 2009), and SWS2 (European eel and
99 giant mottled eel) (Wang et al., 2014). Molecular biological studies on photo sensitivities of
100 these visual pigments and studies on the expression mechanism of photoreceptors according to
101 ecological stages (glass eel, yellow eel, and silver eel) have been actively conducted. However,
102 the presence or function of a subfamily other than the above–mentioned four types of visual
103 opsin or non–visual opsin subfamily in *Anguilla* species has not been reported yet. Physiological
104 studies of photoreceptors in vertebrate animals have reported that pinopsin and VA–opsin
105 (Okano et al., 1994; Soni and Foster, 1997) in the brain of birds and exo–rhodopsin in the pineal
106 gland of zebrafish directly affect body color change and reproductive physiology (Kojima et al.,
107 2000; Collin et al., 2009). Thus, it is considered that other types of photoreceptors, except for the
108 previously reported opsins, may play an important reproductive physiological role in Japanese
109 eel but there has been no further investigation into it.
110 In this study, we investigated the opsin subfamily present in the retina and whole brain tissues
111 of Japanese eel inhabiting Northeast Asia using the RNA–Seq transcriptome. In addition, we
112 examined the opsin subfamily mRNA levels in sexually immature and mature eels using qPCR
113 method. These results identify the physiological role of photoreceptors in the maturation process
114 of Japanese eels and, thus, can be used as a basic material for studies on photoreceptor
115 mechanisms including the effect of environmental factors on maturation and visual adaptation.

116

117 Materials & Methods

118 Experimental fish

119 We purchased Japanese eels, *A. japonica,* inhabiting brackish water at Hadori, Gujwaeup, Jejusi,
120 Jeju, South Korea in September at 2016. The wild fish were kept in Lava seawater center in Jeju
121 Techno-park, Jeju, South Korea (33°N, 126°E). The fish were reared for 1 weeks in acryl tank
122 (800L/capacity) with recirculation system (natural photoperiod= approximately 12L:12D, water
123 temperature 20 ± 1°C). For the study of the maturation induction of the Japanese eel, males were
124 purchased from an eel aquafarm (Hanwool aquafarm, Gwangju, South Korea). The obtained eels
125 were acclimated in the freshwater round acrylic tank (1 ton/capacity) for at least one week. Light
126 conditions were maintained at 12L:12D using fluorescent bulbs (10W, 600 lx, PPFD= 10.0
127 $\mu molm^{-2}s^{-1}$, λp= 545nm) light on at 06:00 and light off at 18:00), and the temperature of the
128 water was maintained at 20 ± 1°C. All experiments were conducted in compliance with the
129 guidelines of Institutional Animal Care and Experimental Committee of the Jeju National
130 University. The protocol was approved by the Animal care and use committee of the Jeju
131 National University (No. 2016–0039).

132

133 For the retina and whole brain RNA–transcriptome analysis (Fig. 1), wild caught Japanese
134 eels (body weight: 233–726 g and body length: 55.3–80.7 cm) were reared for 1 weeks in acryl
135 tank. For the sampling of experimental fish, the retina and the brain were isolated from Japanese
136 eels at 12:00h and 24:00h (n=12, six females and six males) after anesthesia with tricaine
137 methanesulfonate (MS–222, Sigma–Aldrich, ST., USA). The collected tissues were frozen using
138 liquid nitrogen and stored at –80 °C until used for analysis.

For maturation artificially induction of Japanese eels, only males (initial body weight: 186.1 ~ 227.1 g, n=6) were selected and reared in the freshwater tank for at least one week. Later, the water was replaced with sea water for one week, and the fish were reared for eight weeks and intraperitoneally injected with human chorionic gonadotropin (n=6, hCG, 1 IU/g^{-1}) dissolved in saline (150 mM NaCl) at one–week intervals for sexually maturation. During the maturation induction, photoperiod of 12L: 12D (lights on = 07:00, lights off = 19:00) and water temperature of 20 ± 1°C were maintained, and a complete recirculating aquaculture system (800L/capacity). Fluorescent bulbs (20W, approximately 600 lx, 10.0 μmol$m^{-2}s^{-1}$ at 545nm) were situated above on the tank to provide an illuminance at water surface of 600 lx. After eight weeks of intraperitoneal injection, maturation was determined by the presence or absence of spermiation and histological observation of the testis. For analysis of opsin family genes mRNA level changes in the retina and brain part of Japanese eels before and after maturation, the brain was dissected into the fore–brain, mid–brain, and hind–brain (Fig. 2). The extracted tissues were frozen using liquid nitrogen and stored at –80°C until used for analysis.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the retina and three parts of the brain (fore–, mid–, and hind–) using RNA–iso plus (Takara–Bio, Otsu, Japan) according to the manufacturer`s protocol. After isolated the total RNA, quality, and amount–checked on a 2100 bioanalyzer RNA 6000 NANO chip (Bio–Rad, Hercules, CA, USA) and electrophoresis. cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis kit (Roche–diagnostics, Indianapolis, IN, USA) by following the manufacturer`s protocol.

cDNA library construction and massively parallel sequencing

RNA–Seq paired end libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit v2 (catalog #RS–122–2001, Illumina, San Diego, CA). Total RNA was isolated from the retina and brain, respectively. After removal of genomic DNA contamination, RNA quality and quantity were assessed by 2100 bioanalyzer RNA 6000 NANO chip (Bio–Rad). High quality total RNA extracted from retina and brain of X individuals were then pooled, respectively. Starting with total RNA, mRNA purified using poly (A) selection was chemically fragmented and converted into single–stranded cDNA using random hexamer priming. Next, the second strand is generated to create double–stranded cDNA. Library construction begins with generation of blunt–end cDNA fragments from ds–cDNA. Then A–base added to the blunt–end in order to make them ready for ligation of sequencing adapters. After the size selection of ligates, the ligated cDNA fragments which contain adapter sequences are enhanced via PCR using adapter specific primers. The library was quantified with KAPA library quantification kit (Kapa biosystems KK4854) following the manufacturer's instructions. Each library is loaded on Illumina Hiseq2000 platform, and we performed high–throughput sequencing (read length 2×100) to ensure that each sample meets the desired average sequencing depth.

Preprocessing and *de novo* reconstruction of transcriptome,

The bases from 5’ end and 3’end of each read with low quality and adapter sequences were trimmed using Trimmomatic (ver. 0.3.6, Bolger, Lohse & Usadel, 2014), then low averaged quality (Q<25) were removed by PRINSEQ lite (ver. 0.20.4, Schmieder & Edwards, 2011). Cleaned raw reads from retina and brain RNA were pooled, then mapped to the *Anguilla japonica* draft genome sequence (Ref) using tophat2 (ver.2.1.0, C, 2013), then *de novo* transcriptome reconstruction was performed by a genome-guided Trinity (ver. 2.3.2, Grabherr et

al., 2011) with bam mapping result. To remove redundant contigs and create an unigene set, the assembled contigs were clustered and filtered using cd-hit-est with default parameters (CD-HIT package, Li & Godzik, 2006).

Analysis of the opsin DNA sequence

To search opsin family genes from the assembled transcriptome sequences of Japanese eel, the tBlastn program was utilized (E-value < 0.01) on zebrafish opsin protein sequences as queries. The ORF regions of Japanese eel opsin candidates were found through ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), then presumed protein sequences were aligned with teleost opsin family proteins. A phylogenetic tree was constructed by the maximum-likelihood algorithm using RAxML (Stamatakis, 2014). For quantifying the identified opsin family genes expressions, cleaned reads were mapped on reconstructed contigs by Bowtie2 (Langmead & Salzberg, 2012), then the expression levels were estimated using Tigar2 (Nariai et al., 2014).

Quantitative real–time RT–PCR (qPCR)

Real–time qPCR reactions were performed using the Dice real time thermal cycler (TaKaRa–Bio) and SYBR Premix Ex Taq™ II (TaKaRa–Bio). Gene specific primers used for qPCR were designed using Primer3 plus (Primer Biosoft) and are provided in Table 1. Each PCR reaction mix contained 50% of SYBR Premix, 0.2 μM of each forward and reverse primer, and 2 μl of diluted cDNA template by nuclease-free water. The initial 1 min denaturation was followed by 40 cycles of denaturation for 5 s at 95 °C, annealing and extension for 1 min at 60 °C. To ensure the specificity of the PCR amplicons, the temperature of the sample was gradually raised from 60

209 to 95 °C as the last step of the PCR reaction and a melting curve analyzed. The primers were
210 successfully tested in the different cDNA samples of the Japanese eel, evaluating that each
211 primer should amplify a single product, reflected as a single peak in the melting curve analysis.
212 The relative mRNA expression levels of target genes were calculated using the $^{\Delta\Delta}$Ct method, and
213 the reference gene was virtually defined as the average of the threshold cycles (Ct) for EF1α.
214
215 **Histological analysis**
216 The eel testis was fixed in Bouin's fluid. Fixed testis samples were dehydrated through an
217 ethanol series, embedded in paraffin wax, and sectioned to 7–8 μm thickness. Sectioned tissues
218 were stained with Mayer's hematoxylin and eosin. State of the sexual maturation was classified
219 into the following 2 stages: immature stage (spermatogonia and spermatocyte; Fig. 3A) and
220 maturation stage (fully spermatozoa; Fig. 3B).
221
222 **Statistics**
223 All statistical analyses were performed using GraphPad Prism 8.0.2 Software. Comparisons of
224 opsin genes expression levels between sexually immature and mature group were performed by
225 the Unparied t test. In the present study, $P < 0.05$ was accepted as statistically significant.
226
227 ## Results
228 **RNA–Seq transcriptome analysis**
229 Total RNA extracted from the whole brain and retinal tissues of Japanese eels were analyzed
230 using the NGS method. After the adapter trimming and quality filtering, 150,898,925 paired-end
231 reads were survived and used for *de novo* transcriptome reconstruction. As a result of cd-hit-est

clustering, a total of 313,671 contigs (N50 = 965) were obtained. tBlastn and phylogenetic analysis revealed that a total of 18 opsin subfamilies were identified in the retina and the whole brain through RNA–seq (Fig. 4). Among them, the visual opsin families of Japanese eels included rhodopsin2 or middle wave sensitive pigment (Rh2 or MWS), short wavelength–sensitive opsin 2 or blue light sensitive opsin (SWS2), fresh water rhodopsion (FWO), deep–sea water rhodopsin (DSO), and exo–rhodopsion (Exo–Rhod). The non–visual opsin families included four types of melanopsin subfamily (Opn4x1, Opn4x2, Opn4m1, and Opn4m2), peropsin, two types of neuropsin (Opn5–like, Opn5), Opn3 (encephalopsin), three types of teleost multiple tissue opsin (TMT1, TMT2, and TMT3), VA–opsin (vertebrate ancient opsin) and parapinopsin.

Changes of GSI

Sexually immature and mature eels were classified based on the histological observation of testis before and after hCG treatment. In the beginning, spermatogonia was mostly observed in the testis of eel males. After eight weeks of hCG injection, spermiation was found in most of male eels, and spermatozoa was mostly observed in lobules. The gonadosomatic index (GSI) was 0.20 ± 0.01 at the beginning and was 25.7 ± 1.4 after maturation, showing a significant difference ($P > 0.0001$, Fig. 3C)

Changes in opsin family gene expression in the retina between sexually immature and mature eels

Eighteen opsin families identified using the RNA–Seq method were divided into visual opsin (Fig. 5) and non–visual opsin (Fig. 6) families. Then, the mRNA abundance in the retina of

sexually immature and mature eels was analyzed using qPCR. In terms of visual opsin expression, the mRNA abundance of DSO increased in the sexually mature group (Fig. 5B), whereas those of FWO and Rh2 were low in the sexually mature group (Fig. 5C, D). Non–visual opsin mRNA showed no significant difference between sexually immature and mature groups (Fig. 6).

Changes in opsin family gene expression in brain of sexually immature and mature eels

The brains of the sexually immature and mature eels were dissected into the fore–brain, mid–brain, and hind–brain. Then, the opsin families that showed significant differences were investigated as in the retina (P <0.05). In terms of visual opsin expression in the brain, DSO and SWS2 mRNA abundance increased in the fore–brain, mid–brain, and hind–brain of the mature group. Other visual opsin mRNAs did not show significant differences in whole brain part (Fig. 7). In terms of non–visual opsin expression in the brain, mRNA abundance of parapinopsin and Opn4m2 increased in the fore brain of the mature group (Fig. 8J), whereas that of TMT2 was low in the mature group (Fig. 9G). Opn4m2 and parapinopsin mRNA abundance increased in the mid–brain (Fig. 8K, 9Q). TMT1 and TMT3 mRNA abundance increased in the hind–brain of the mature group (Fig. 9F, L).

Discussion

RNA–Seq transcriptome analysis was performed to examine 18 photoreceptor genes in the retina and whole brain of Japanese eels. As a result, two types of cone opsin (SWS2, Rh2) were identified. However, the presence of a long wavelength–sensitive pigment (LWS) in the long wavelength region was not confirmed in this study. In general, organisms must have at least two

cone opsin with different spectra to distinguish colors. Species with one type of cone opsin are considered as “monochromatic vision” or color–blind (Bowmaker et al., 1994). Eels have two or more cone opsin, so they can recognize colors. However, they recognize the wavelength of the narrower region compared with other animals or other fish species. A study suggested that the European eel has two types of cone opsin subfamily, Rh2 (or MWS) and SWS2 cones, so it can distinguish colors (Cottrill et al., 2009). However, the giant mottled eel showed only one type of cone cell that detected a limited range of the optical spectrum (λmax) of 500 nm to 535 nm (Wang et al., 2014). Japanese eels are genetically and ecologically similar European eels; thus, it is presumed that they can recognize colors through two types of cone opsin. In addition, Japanese eels are nocturnal fish and have evolved in a way that they have adapted to dark habitat and/or nocturnal habits. Thus, it is considered that their photoreceptors recognizing the light spectrum of the long wavelength band region may have been functionally atrophied or photoreceptor may have not existed. Similarly, species living in deep–sea or those evolved to adapt to dark environments have been reported to have fewer cone opsins (Mas-Riera, 1991; Pankhurst and Conroy, 1987). SWS1 and LWS gene expression levels were higher in fresh water fish than those in fish inhabiting seawater (Lin et al., 2017). This is because in most freshwater environments, most of the ultraviolet rays penetrating the water surface can be recognized by organisms because of low water depth, while the fish living in deep–sea (50m or more) tend to lose the LWS gene because the long wavelength (red) is not transmitted to deep–sea regions (Lin et al., 2017).

Japanese eel, which was investigated in this study, live in a shallow freshwater region during most of their life span except for spawning. This eel will migrate to deep–sea area of at least 100 m (Cottrill et al., 2009; Tsukamoto, 1992). Regarding this, they may share some genetic

301 characteristics with the deep–sea fish and in this study cone opsin was predicted to be one of the
302 possible genes. The distribution and physiological function of cone opsin appear to be different
303 depending on the level of ecological evolution. In Anguillid sp., only four types opsins (DSO,
304 FWO, Rh2 and SWS2) (Cottrill et al., 2009; Zhang et al., 2000) were studied. Therefore, it is
305 necessary to carry out additional molecular biological and biochemical studies on the range of
306 recognition of color in eels. In this study, opsin families were identified through RNA–seq
307 method, and then highly expressed genes in the retina and brain were analyzed by qPCR. As a
308 result, Parapinopsin mRNA was predominantly expressed in the whole brain, but peropsin and
309 Opn5 were relatively highly expressed in the retina. According to previous studies on opsin
310 expression, the expression of rhodopsin genes in the retina and brain in the ayu (Masuda et al.,
311 2003; Minamoto and Shimize, 2003), Atlantic salmon (Philp et al., 2000), Japanese eel (Zhang et
312 al., 2000), and percomorph fishes (Cortesi et al., 2015), showed different photoreceptor types
313 and expression sites. However, only the limited physiological function of opsin has been
314 reported.

315 Non–visual opsin was named in the 1990s, and it has been known to affect circadian rhythms
316 in mammals, reproduction in birds, light avoidance in amphibian larvae, and neural development
317 during egg development in fish (Beaudry et al., 2017). A study on non–visual opsin showed
318 Opn4 expression in the retinal ganglion in mammals, but Opn4 gene was expressed in the retina,
319 brain and skin in non–mammals (Belingham et al., 2006). In addition, VA–opsin is known to be
320 expressed in the hypothalamus and gonads in birds and fish, and it directly stimulates GnRH in
321 the hypothalamus by recognizing wavelength changes due to photoperiod changes (Davies et al.,
322 2010; Grone et al., 2007). TMT opsin is expressed in most tissues and embryos in the case of
323 zebrafish. In particular, TMT opsin is expressed in cell lines associated with light entrain able

clock (Moutsaki et al., 2003).

In this study, DSO expression increased, and FWO, Opn4m2, VA–opsin, SWS2 and Rh2 expression decreased in the retina during sexual maturation. All of the three brain areas showed the increased DSO and SWS2 expressions. Consistent with the results of this study, a previous study on the photoreceptors of the Japanese eel, reported that DSO expression increased and FWO expression decreased in silver eels (Zhang et al., 2000). In the case of European eel, DSO expression also increased to make eels adapt to the environment before the spawning migration in the early sexual maturation stage. In the late sexual maturation stage, European eels enter deep–sea beyond 100 m depth to spawn, thereby showing a decrease in FWO expression (Zhang et al., 2000). In addition, Japanese conger eel changes its habitat environment from fresh water to open sea while moving from juvenile stage to sexual maturation. To adapt to the changed environment, FWO was mainly expressed in the retina during the juvenile stage, and then DSO expression started to be increased during sexual maturation (Zhang et al., 2002). This may have resulted from Japanese conger eel's adaptation to the environmental change related to light in the process of migrating to the spawning ground. Analysis of opsin family gene mRNA levels in the Japanese eel brain before and after maturation showed that DSO and SWS2 expressions increased after maturation in all three areas of brain. In addition, DSO expression increased in the retina after maturation, suggesting that DSO is closely related to maturation. However, it is unclear whether the increase in DSO and SWS2 expressions in the brain affects maturation.

In recent years, there have been some studies on the reproductive physiological function of VA–opsin belonging to the non–visual opsin (deep brain photoreceptor) family in the brain. VA opsin was cloned from Atlantic salmon (Soni and Foster, 1997), and VA–long (VAL–opsin) was discovered in zebra fish (Kojima et al., 2000). Immunohistochemistry studies on Atlantic salmon

have reported the existence of opsin–like protein in the hypothalamic nucleus magnocellularis preopticus, suggesting its potential gonadal development control function. There have been few studies on non–visual opsin in the brain, especially its relevance to gonadal development. However, photoperiod action did not influence the gonadal development in ayu without both eyes and pineal gland (Suzuki, 1975), and opsin immune–positive fibers passing through basal hypothalamus were observed in the hypothalamus of Atlantic salmon (Philp et al., 2000). These results suggest that the opsin present in the brain directly affects gonadal development. The level of expression of GnRH located at the top of the BPG axis directly affects reproduction. It is unclear as to whether VA–opsin regulates the expression level of GnRH in the hypothalamus of Japanese quail, but VA–opsin, which affected GnRH expression, was identified in GnRH cells (Garcia-Fernadez et al., 2015). Other opsin subfamilies other than VA–opsin may also affect the reproduction system in the hypothalamus, but more research is needed to investigate this hypothesis.

Conclusions

Thousands of opsins have been identified and are divided into eight groups (Terakita, 2005; Yau and Hardie, 2009; Peirson et al., 2009; Terakita et al., 2012). The current data set shows the diversity of opsins in the animal kingdom because the whole genome sequence is determined in many animals. However, there has been a lack of information on the physiological functions other than the molecular structure or biochemical signals in the retina. In particular, the study of ecologically unique species such as Japanese eels is considered as very important in terms of evolution. In this study, 18 types of opsins were identified in the brain and retina of Japanese eels of which 14 types were new opsin genes. Expression of opsins mRNA in the brain and retina was

variable; SWS2 expression was high in all areas of the brain of the sexually mature eels, and TMT3 expression significantly increased in hind–brain. These results suggest that SWS–related shortwave region is directly related to the maturation of Japanese eels. However, follow–up studies are required to demonstrate the relevance. Japanese eels have very unique ecological characteristics, as mentioned above. Unlike other fish species, eco–physiological studies on Japanese eels are necessary to induce artificial maturation through environmental control (light, water temperature etc.), and various studies on the photosensitivity should be continuously carried out.

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Additional Information and Declarations

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

Jun–Hwan Byun, Ji–Yeon Hyeon conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Eun–Su Kim and Yuki Takeuchi conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.

Byeong–Hoon Kim and Miyanishi Hiroshi analyzed the data, contributed reagents/materials/analysis tools.

Kagawa Hirohiko, Se–Jae Kim and Akihiro Takemura conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

Sung–Pyo Hur conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw sequencing reads are publicly available in the Sequence Read Archive (SRA) of the GenBank database under the accession numbers DRR194268 to DRR194271.

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551 ## Figure captions

552 **Fig. 1. Flowchart of the present study.**

553

554 **Fig. 2. Diagram showing the dorsal view (a) and sagittal plane (b) of the eel brain.** Ob,
555 olfactory bulb; Tel, telencephalon; TeO, optic tectum; Cb, cerebellum; Mo, medulla oblongata;
556 P, pineal gland; SD, saccus dorsalis; PON, preoptic nucleus; SV, saccus vasculosus.

557

558 **Fig. 3. Microphotographs of histological sections of different stages of eel testis and changes**
559 **of gonadosomatic index (GSI) after hormonally induced sexual maturation.** (A) immature
560 testis, (B) mature testis, (C) GSI. Scale bar = 200 μm.

561

562 **Fig. 4. Phylogeny of vertebrate visual and non-visual opsins. One thousand bootstrap**
563 **repetitions were performed and values are shown at the inner nodes.** The zebrafish beta 1
564 adrenergic receptor was used as an outgroup to root the tree. Analysis was performed with
565 multiple alignments from the amino acid sequence by using ClustalW program. Bold is indicated
566 the visual and non-visual opsin families of Japanese eel, *A. japonica*.

567

568 **Fig. 5. Visual opsin mRNA level in the retina of sexually immature and mature male**
569 **Japanese eel.** For the artificially induced sexual maturation, hCG was intraperitoneally injected
570 to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was
571 sampled before hCG injection (n=6). Eight weeks after injection, retina was sampled and used
572 for total RNA extraction and cDNA synthesis. The mRNA expression of visual opsin in each
573 sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and

574 third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above
575 each bar indicates significant differences according to the Unparied t test (*$P < 0.05$, **$P <$
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578 **Fig. 6. Non–visual opsin mRNA level in the retina of sexually immature and mature male**
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588 **Fig. 7. Visual opsin mRNA level in the brain of sexually immature and mature male**
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595 indicates significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).
596

Fig. 8. Non–visual opsin mRNA level in the brain of sexually immature and mature male Japanese eel male. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. For the artificially induced sexual maturation, hCG was intraperitoneally injected to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was sampled before hCG injection (n=6). Eight weeks after injection, brain was sampled and used for total RNA extraction and cDNA synthesis. The mRNA expression of non-visual opsin in each sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above each bar indicate significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).

Fig. 9. Non–visual opsin mRNA level in the brain of sexually immature and mature male Japanese eel male. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. For the artificially induced sexual maturation, hCG was intraperitoneally injected to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was sampled before hCG injection (n=6). Eight weeks after injection, brain was sampled and used for total RNA extraction and cDNA synthesis. The mRNA expression of non-visual opsin in each sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above each bar indicate significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).

Figure 1

Flowchart of the present study.

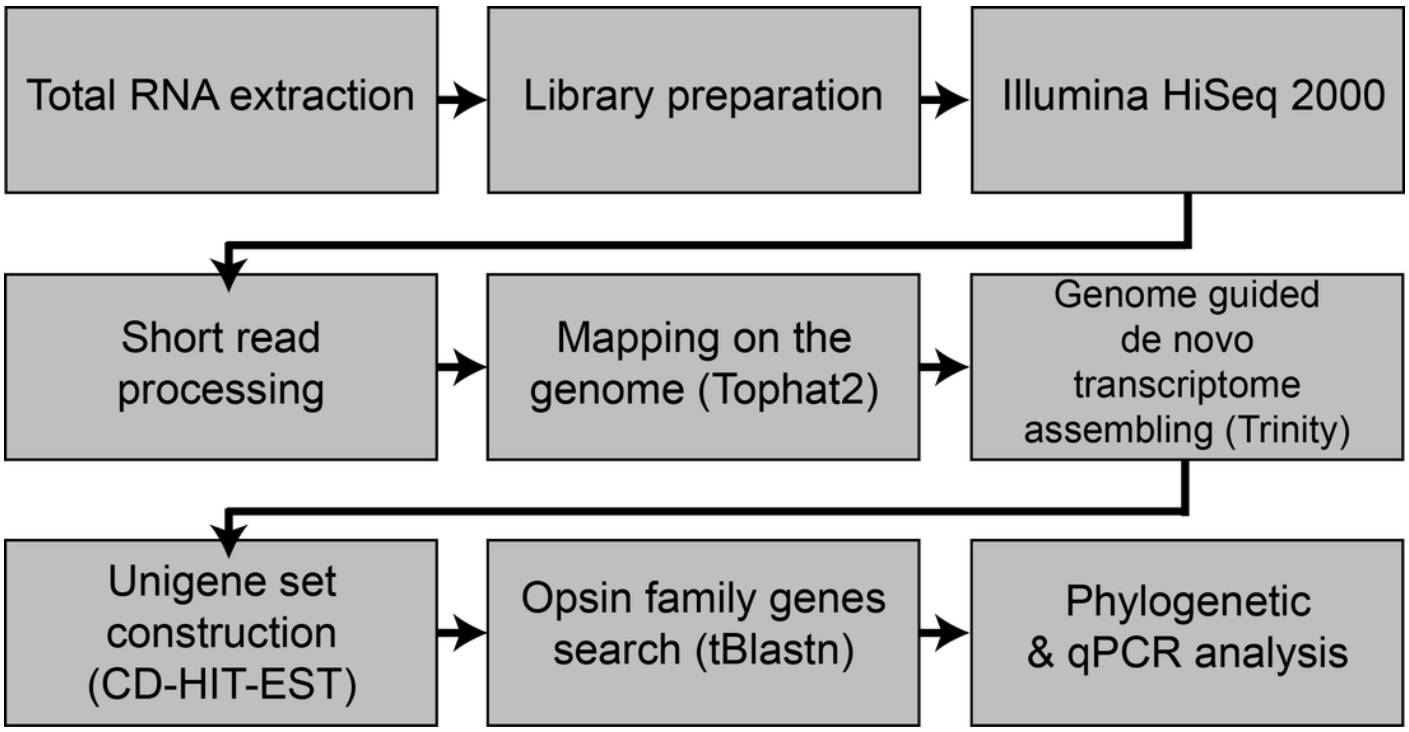

Figure 2

Diagram showing the dorsal view (a) and sagittal plane (b) of the eel brain.

Ob, olfactory bulb; Tel, telencephalon; TeO, optic tectum; Cb, cerebellum; Mo, medulla oblongata; P, pineal gland; SD, saccus dorsalis; PON, preoptic nucleus; SV, saccus vasculosus.

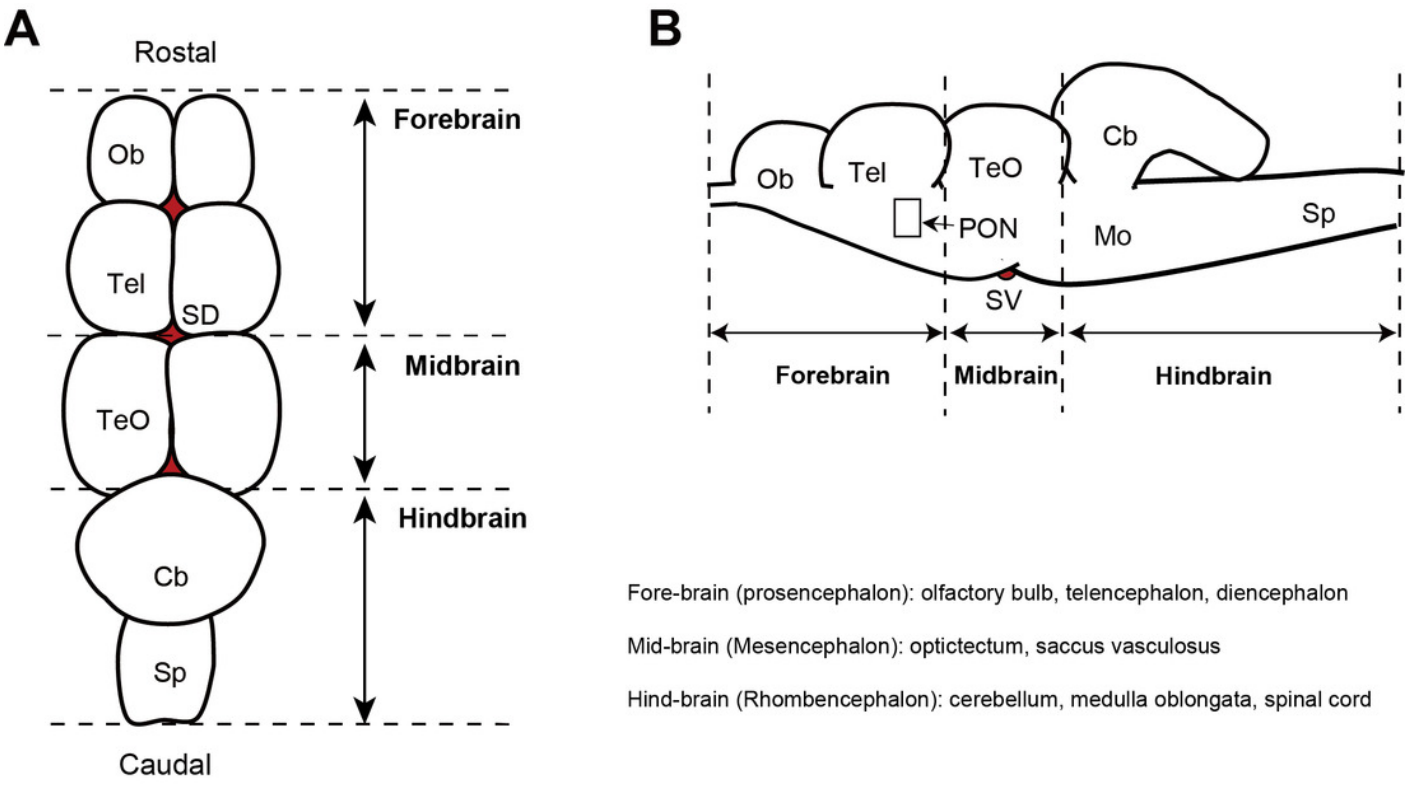

Figure 3

Microphotographs of histological sections of different stages of eel testis and changes of gonadosomatic index (GSI) after hormonally induced sexual maturation.

(A) immature testis, (B) mature testis, (C) GSI. Scale bar = 200 μm.

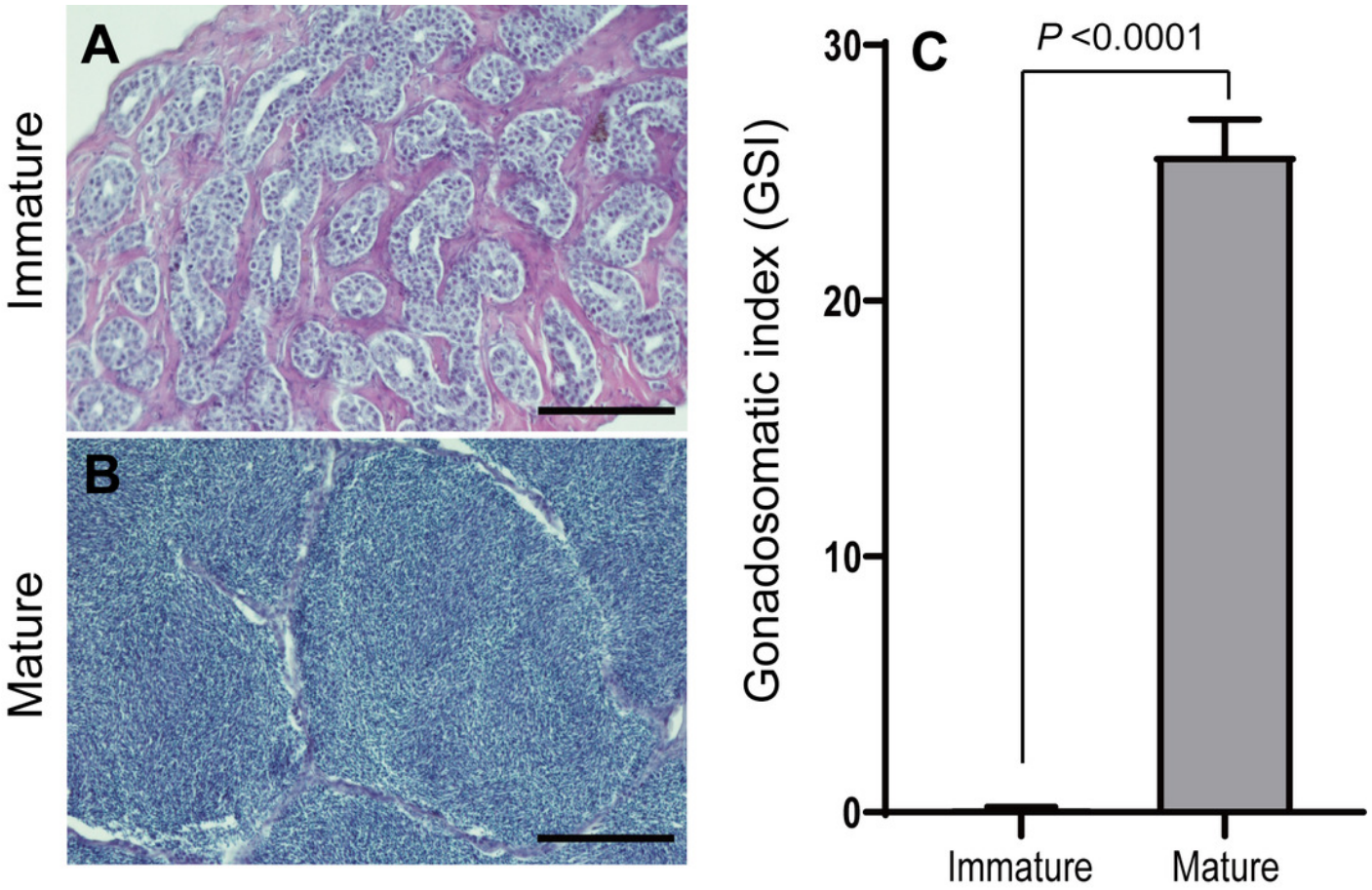

Figure 4

Phylogeny of vertebrate visual and non-visual opsins. One thousand bootstrap repetitions were performed and values are shown at the inner nodes.

The zebrafish beta 1 adrenergic receptor was used as an outgroup to root the tree. Analysis was performed with multiple alignments from the amino acid sequence by using ClustalW program. Bold is indicated the visual and non-visual opsin families of Japanese eel, *A. japonica*.

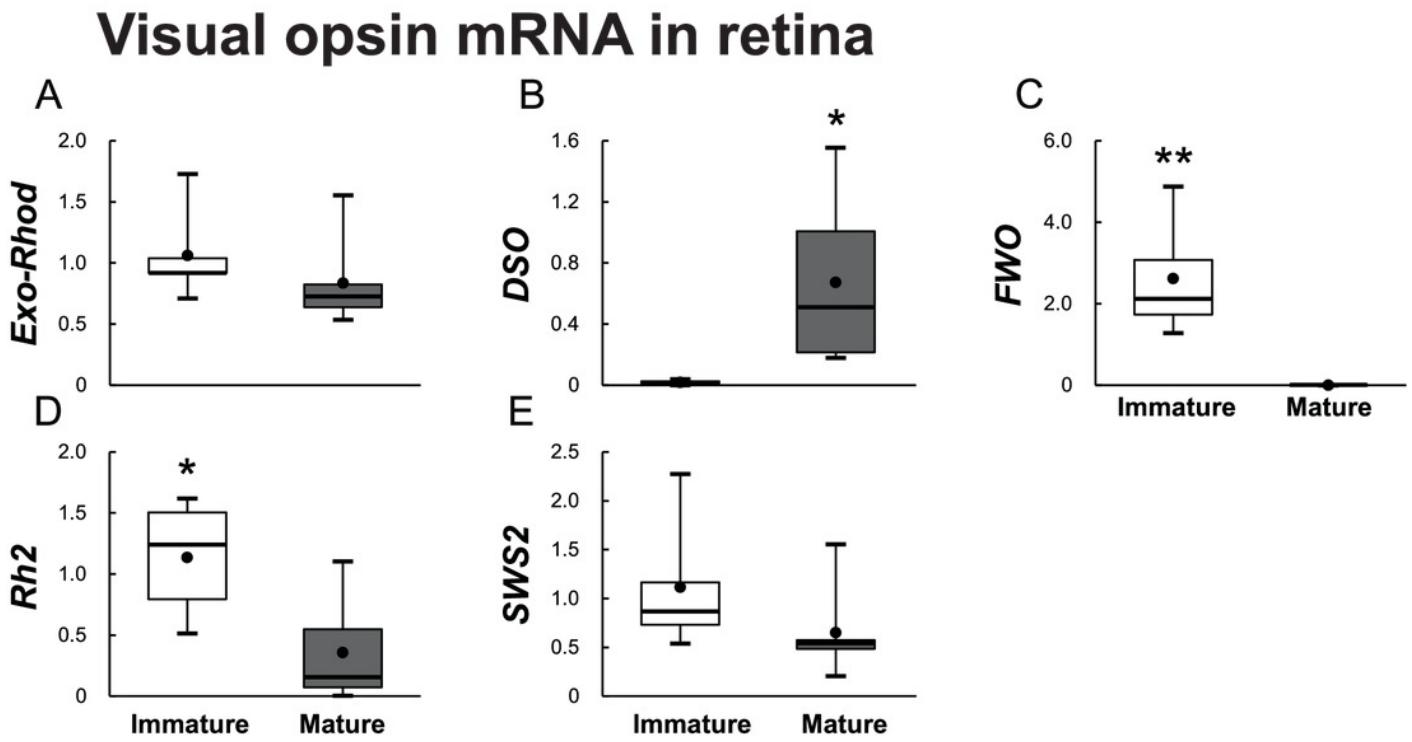

Figure 5

Visual opsin mRNA level in the retina of sexually immature and mature male Japanese eel.

For the artificially induced sexual maturation, hCG was intraperitoneally injected to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was sampled before hCG injection (n=6). Eight weeks after injection, retina was sampled and used for total RNA extraction and cDNA synthesis. The mRNA expression of visual opsin in each sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above each bar indicates significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).

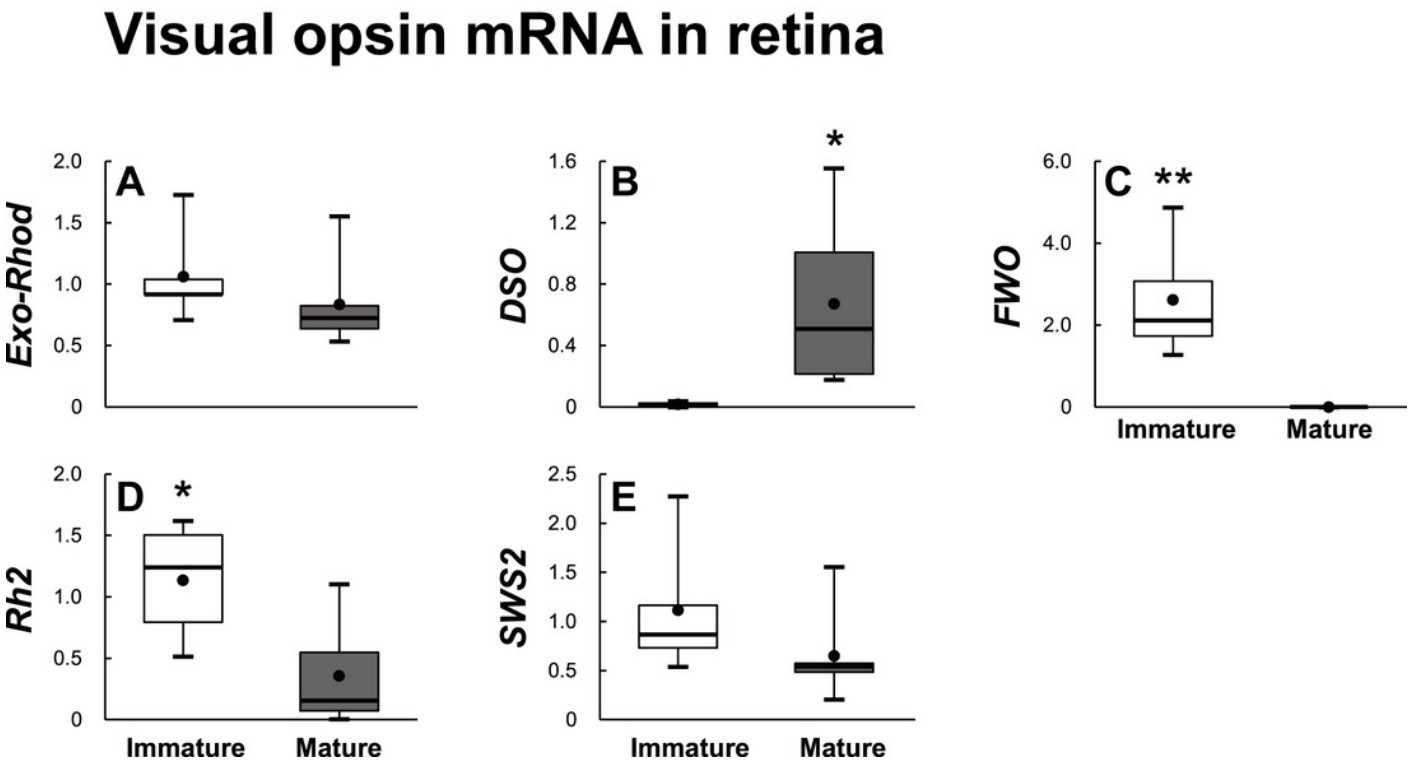

Figure 6

Non–visual opsin mRNA level in the retina of sexually immature and mature male Japanese eel.

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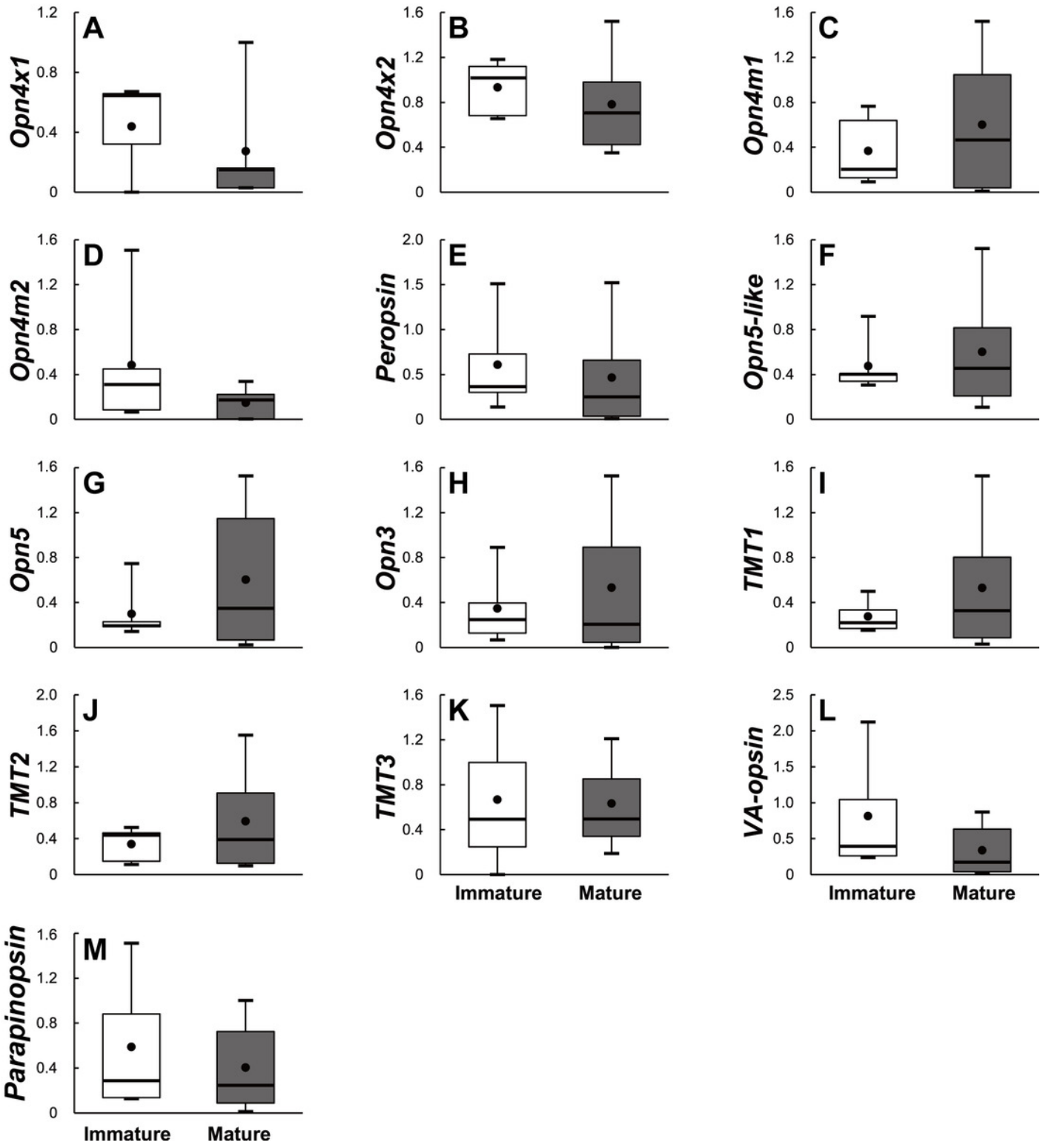

Non-visual opsin mRNA in retina
A
Opn4x1
B
Opn4x2
C
Opn4m1
D
Opn4m2
E
Peropsin
F
Opn5-like
G
Opn5
H
Opn3
I
TMT1
J
TMT2
K
TMT3
L
VA-opsin
M
Parapinopsin
Immature
Mature

Figure 7

Visual opsin mRNA level in the brain of sexually immature and mature male Japanese eel.

For the artificially induced sexual maturation, hCG was intraperitoneally injected to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was sampled before hCG injection (n=6). Eight weeks after injection, brain was sampled and used for total RNA extraction and cDNA synthesis. The mRNA expression of visual opsin in each sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above each bar indicates significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).

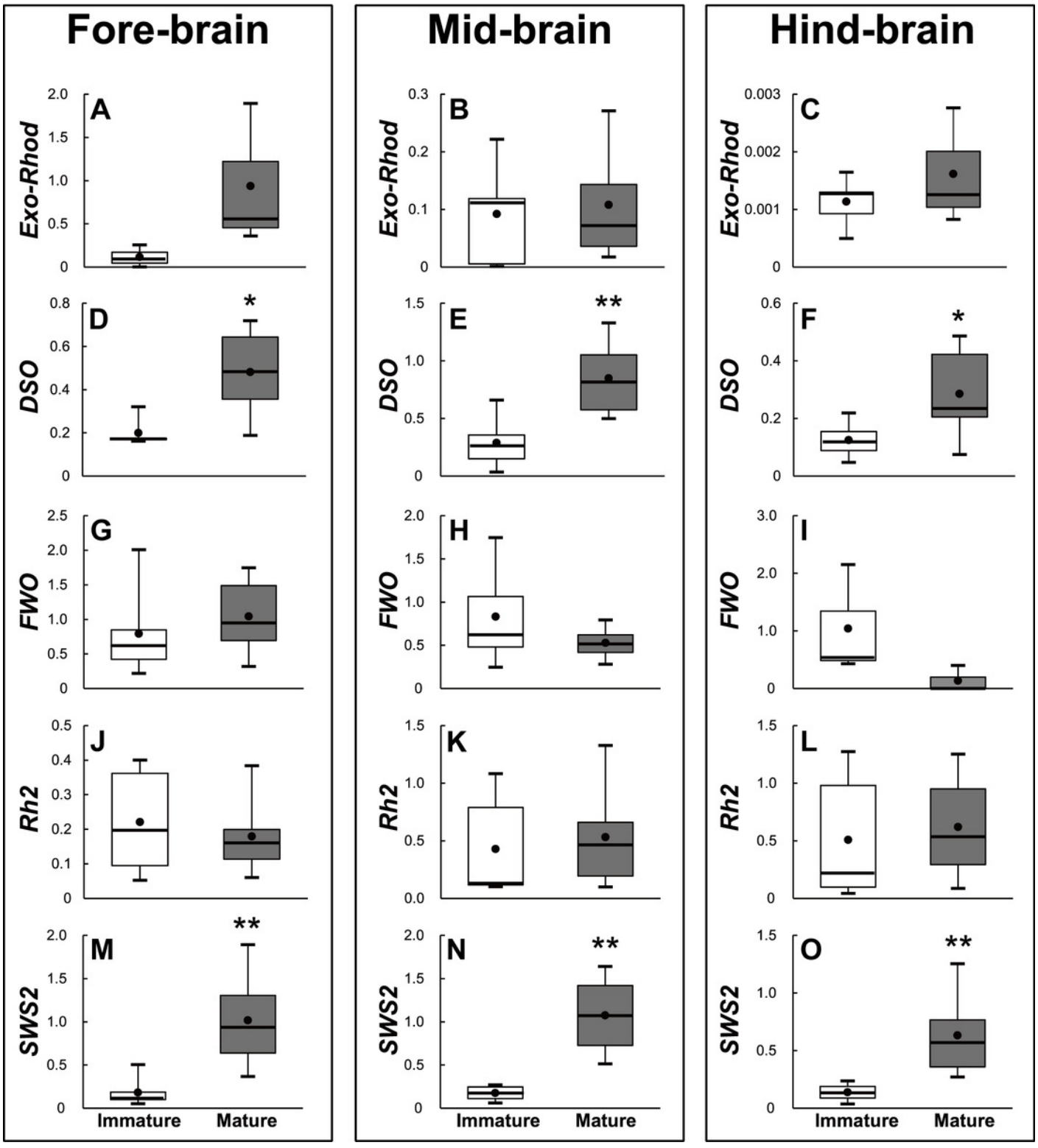
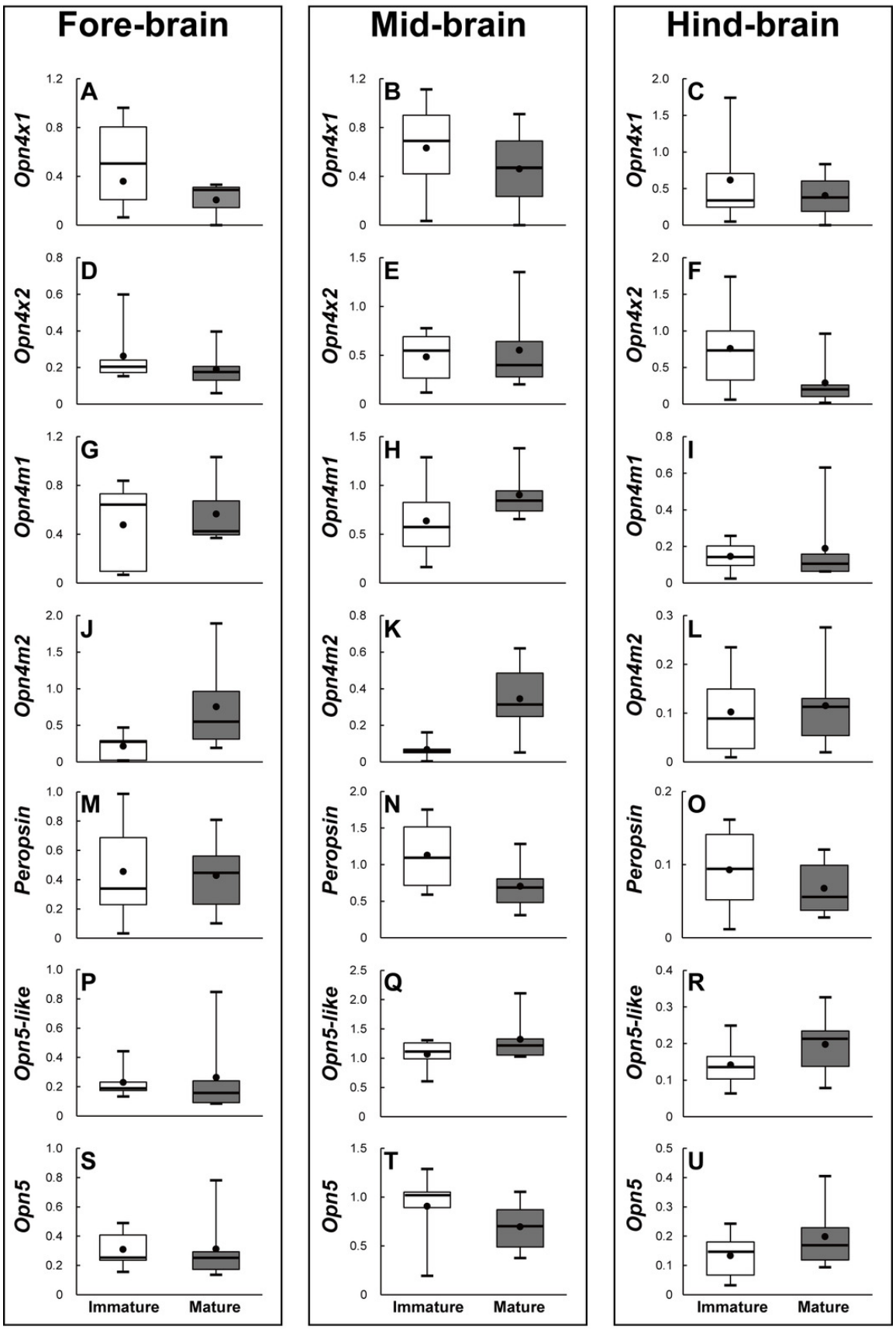

Visual opsin mRNA in brain
Fore-brain
Mid-brain
Hind-brain
Exo-Rhod
DSO
FWO
Rh2
SWS2
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
*
**
Immature
Mature

Figure 8

Non–visual opsin mRNA level in the brain of sexually immature and mature male Japanese eel male.

Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. For the artificially induced sexual maturation, hCG was intraperitoneally injected to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was sampled before hCG injection (n=6). Eight weeks after injection, brain was sampled and used for total RNA extraction and cDNA synthesis. The mRNA expression of non-visual opsin in each sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above each bar indicate significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).


Non-visual opsin mRNA in brain
Fore-brain
Mid-brain
Hind-brain
Opn4x1
Opn4x2
Opn4m1
Opn4m2
Peropsin
Opn5-like
Opn5
A
B
C
D
E
F
G
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L
M
N
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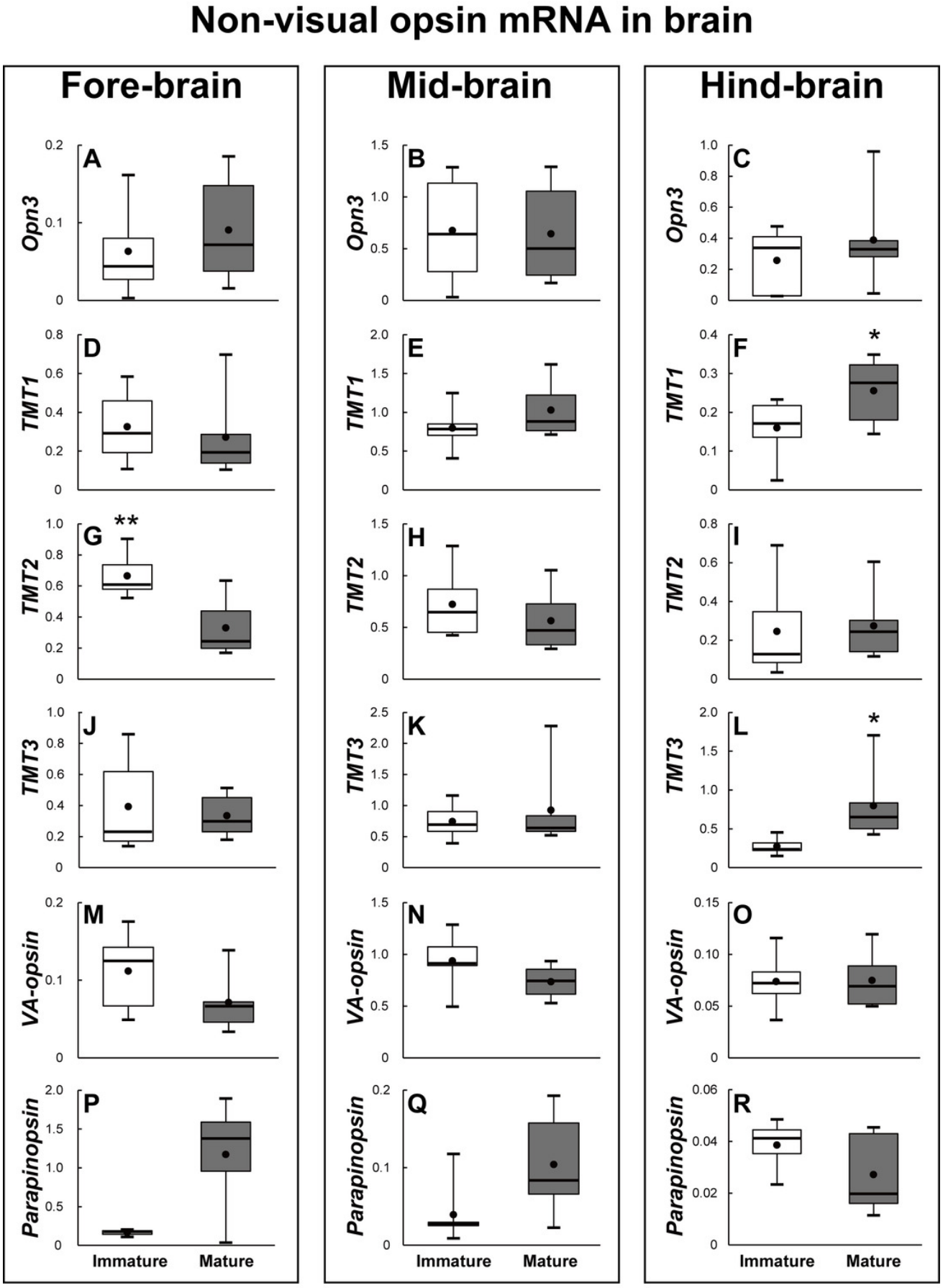
R
S
T
U
Immature
Mature

Figure 9

Non–visual opsin mRNA level in the brain of sexually immature and mature male Japanese eel male.

Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. For the artificially induced sexual maturation, hCG was intraperitoneally injected to the experimental fish group (n=6) at a concentration of 1 IU/g body weight. Immature fish was sampled before hCG injection (n=6). Eight weeks after injection, brain was sampled and used for total RNA extraction and cDNA synthesis. The mRNA expression of non-visual opsin in each sample was measured real-time qPCR. Boxplots show min and max values (whiskers), first and third quartiles (box limits), and median (box inner line) of mRNA levels. The asterisk above each bar indicate significant differences according to the Unparied t test (*$P < 0.05$, **$P < 0.01$).


Non-visual opsin mRNA in brain
Fore-brain
Mid-brain
Hind-brain
A
B
C
D
E
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G
H
I
J
K
L
M
N
O
P
Q
R
Opn3
TMT1
TMT2
TMT3
VA-opsin
Parapinopsin
**
*
*
Immature
Mature

Table 1(on next page)

Primer sets in this study

Table 1. Primer sets in this study

Gene ID	Oligo ID	Sequence	Product size (bp)
EF1–alpha	Forward	5–TCACCCTGGGAGTAAAGCAG–3	222
	Reverse	5–TCCATCCCTTGAACCAGGAC–3	
Opn4x1	Forward	5–GGATCACCTCCATGATCACC–3	189
	Reverse	5–GGCCCTCTGGAATGTATGAA–3	
Opn4x2	Forward	5–GAGTGGGTGTTCGGTGAACT–3	191
	Reverse	5–GAGTACAGCCACACCAGCAG–3	
Opn4m1	Forward	5–AATTCCACCGCATGAAGAAC–3	197
	Reverse	5–GATTATGGGGTTGTGGATGG–3	
Opn4m2	Forward	5–ACTGCAACGGGACATTTAGG–3	226
	Reverse	5–CAGAACGCGTAGATCACCAG–3	
Peropsin	Forward	5–ATGTCTGTGATTGCGGTGAA–3	212
	Reverse	5–AGGCACACAATGGAGTAGGG–3	
Opn5–like	Forward	5–CCAGCCGAGTTCTTCATTGT–3	177
	Reverse	5–TGTGAGGTTGGTCAGACTGC–3	
Opn5	Forward	5–GCCTCCAAATTGTCGAAAGA–3	191
	Reverse	5–GGCTTCCCTGTGACTGTGAT–3	
Opn3	Forward	5–TTGCCTTCACTATCGGAACC–3	194
	Reverse	5–TATCCACCCTCCTTTGATGC–3	
TMT1	Forward	5–TTGGAACTCCGTTCAGCTTT–3	161
	Reverse	5–GGAGGCCATCATGGTACAGT–3	
TMT2	Forward	5–GCTGGGCTGGAGTAGTTACG–3	187
	Reverse	5–GATCTTCCCCACCTGTTTGA–3	
TMT3	Forward	5–TTCGTCTTCTGCCTGTTCCT–3	161
	Reverse	5–AGCAGGTAGCAGGACACCAT–3	
VA–opsin	Forward	5–CAGCTACACCACCAGCAAGA–3	205
	Reverse	5–CGGTTTCCTGGCATTACCTA–3	
Parapinopsin	Forward	5–CTGGAGGGGGTAAAGACCTC–3	227
	Reverse	5–ACAATTACCATGCGGGCTAC–3	
SWS2	Forward	5–AGATGGTGGTGGTGATGGT–3	169
	Reverse	5–GACGTAGATGACGGGGTTG–3	
Rh2	Forward	5–CACCCAGAAAGCAGAGAAGG–3	173
	Reverse	5–ACGCTGAGCTCTTGGAGAAG–3	
Exo–Rhod	Forward	5–GTGGCTGACCTCTTCATGGT–3	189
	Reverse	5–CACAGGCTTGCAGACCACTA–3	
DSO	Forward	5–TCACCATCGAGCACAAGAAG–3	206
	Reverse	5–AACCAGAGACCAGAGCGAAA–3	
FWO	Forward	5–CGATGTACACCTCCATGCAC–3	185
	Reverse	5–CATGATGGCATGGTTCTCAC–3	