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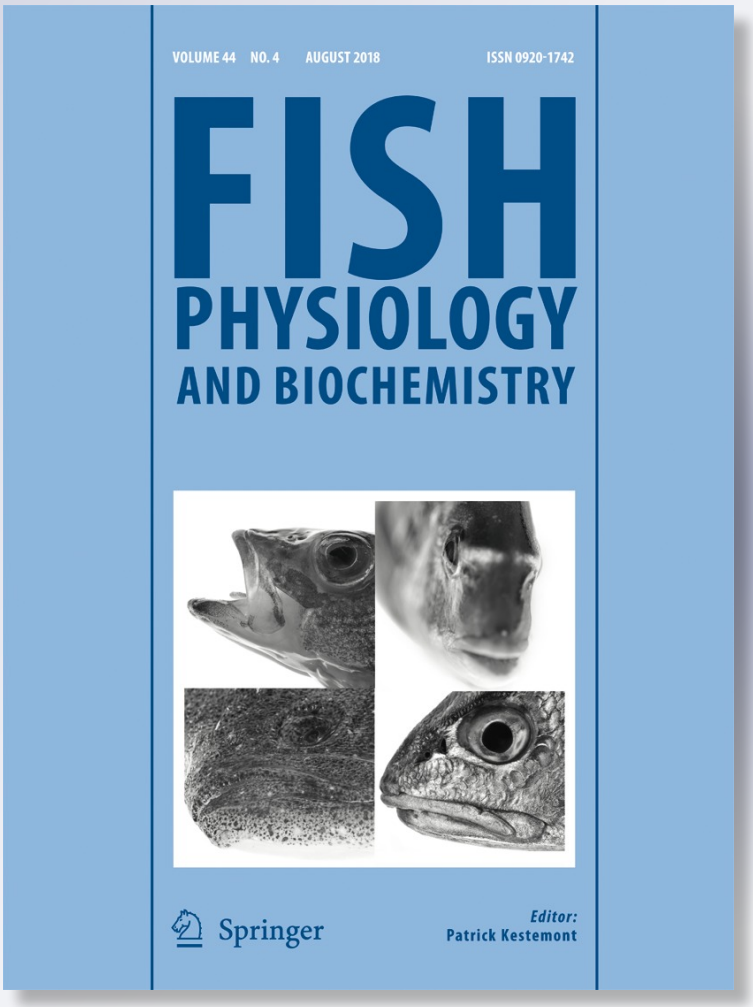

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Effects of recombinant vertebrate ancient long opsin on reproduction in goldfish, *Carassius auratus*: profiling green-wavelength light

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Abstract This study was conducted to identify the possible effect of recombinant vertebrate ancient long (VAL) opsin as a non-visual "photoreceptor" in the deep brain of goldfish, *Carassius auratus*. In addition, we investigated the effects of green-wavelength light on the predictable reproductive function of VAL-opsin as a green-sensitive pigment in the deep brain. To determine this, we quantified changes in gonadotropin hormone (GTH) [GTHα, follicle stimulating hormone (FSH) and luteinizing hormone (LH)] and estrogen receptor (ER; ERα and ERβ) mRNA expression levels associated with goldfish reproduction as well as changes in plasma FSH, LH, and 17β-estradiol (E_2) activities after injection of recombinant VAL-opsin protein in two concentrations (0.1 or 0.5 μg/g body mass) for 4 weeks (injection once weekly) and examined the possible impact of green-wavelength light (500, 520, and 540 nm) on the function of VAL-opsin. As a result, all parameters associated with reproduction significantly increased with time and light-emitting diode (LED) exposure. Based on these results, we suggested that VAL-opsin in the deep brain is involved in goldfish maturation, and it is possible that green-wavelength light improves the ability of VAL-opsin to promote maturation by increasing VAL-opsin expression.

Keywords Goldfish · Green light · Maturation · Reproduction · VAL-opsin

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Abbreviations

E2	17β-estradiol
FSH	Follicle-stimulating hormone
GTH	Gonadotropin hormone
HPG	Hypothalamus-pituitary-gonad
LH	Luteinizing hormone
LED	Light-emitting diode
VAL-opsin	Vertebrate ancient long opsin

Introduction

Light affects physiological phenomena and biorhythms as well as the fertility, growth, and behavior of organisms, such as by inducing or inhibiting hormone secretion by photoreceptors (Migaud et al. 2006; Pierce et al. 2008). Recently, studies on the physiological side effects of light of specific wavelengths on fishes have been performed using light-emitting diodes (LEDs), which are capable of emitting light of a particular wavelength (Villamizar et al. 2009; Choi et al. 2012; Jung et al. 2016). In particular, green-wavelength light has been found to positively affect sexual maturation by stimulating the secretion of sex hormones in fishes (Shin et al. 2011; Kim et al. 2014).

Light signals are absorbed into the body through various pathways. Vertebrates receive light signals of various wavelengths in the retina, the main tissue that absorbs such signals, and transmits them to the target organs to perform various functions (Karakatsouli et al. 2010; Owen et al. 2010). Therefore, fishes have a visual pathway that absorbs light in the visible-wavelength

region (380–780 nm) through the photoreceptor in the retinal pigment epithelial cells (RPEs). This pathway through the eye is a common visual pathway, but in some vertebrates, the presence of a non-visual pathway that senses light signals through the pineal complex, the deep brain, and photoreceptor cells present in the skin has been revealed (Yashikawa and Oishi 1998). Photoreceptors that participate in non-visual pathways from the deep brain include rhodopsin, cone-like opsin, melanopsin, vertebrate ancient (VA) opsin, and VA-long (VAL) opsin (Minamoto and Shimizu 2002; Drivenes et al. 2003). In particular, it has been reported that opsin photoreceptors present in the deep brain are involved in controlling physiological changes, such as skin color change, growth, and reproduction in teleosts (Blanco-Vives et al. 2011; Villamizar et al. 2014). VA-opsin is present in gonadotropin-releasing hormone (GnRH) neurosecretory cells in the brain and is indirectly involved in the sexual maturation of birds via co-expression with GnRH (García-Fernández et al. 2015). VAL-opsin is a variant (ortholog) of VA-opsin, which has the same core sequence, but a longer cytoplasmic tail at the C-terminal region (Soni and Foster 1997; Davies et al. 2012). In other vertebrate animals such as carp, *Cyprinus carpio* and roach, *Rutilus rutilus*, the presence of VAL-opsin as an isoform of VA-opsin has been confirmed (Moutsaki et al. 2000; Jenkins et al. 2003), and a small amount of VAL-opsin has been found in the diencephalic ventricle and retinal horizontal cells of the hypothalamus in zebrafish (Kojima et al. 2008). In particular, VAL-opsin is a green-sensitive pigment that reacts to light in the green-wavelength region through binding to the light-sensitive compound 11-cis-retinal, whereas VA-opsin reacts to light in the blue-wavelength region (Kojima et al. 2000). A study on VAL-opsin has shown that melatonin secretion is regulated in the retina of zebrafish, *Danio rerio*, to control its biological clock, and it is possible that VAL-opsin is involved in the sexual maturation of fishes (Ficher et al. 2013; Song et al. 2016); however, there is no substantial evidence on the relationship between VAL-opsin and sexual maturity or other physiological responses.

In general, sexual maturation of the teleost is regulated through activation of the hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin-releasing hormone secreted from various hypothalamic neurons regulates the synthesis and release of gonadotropin hormones [GTHs; GTHα, follicle-stimulating hormone (FSH), luteinizing hormone (LH)] from the pituitary (Lee et al. 2001; Kobayashi et al. 2006). The stimulation of GTHs induces secretion of 17β-estradiol (E_2), which is a steroid hormone that functions in gonadal development by binding with estrogen receptors (ERs) (Bowman et al. 2002; Davis et al. 2009).

Thus, in the present study, we examined the effect of VAL-opsin protein, a green-sensitive photoreceptor in the deep brain, on the sexual maturity of goldfish, and investigated its function and role, which had not yet been elucidated. To investigate the effects of VAL-opsin on the sexual maturation of immature goldfish and the effect of light of various green wavelengths (500, 520, and 540 nm) on VAL-opsin expression, we determined the concentration of maturation hormones (GTHs, E_2) by injecting two concentrations of recombinant VAL-opsin protein in goldfish.

Materials and methods

Experimental fish and conditions

For each experiment, immature goldfish, *Carassius auratus* ($n = 360$, length, 7.1 ± 0.5 cm; mass, 10.5 ± 0.8 g), were purchased from a commercial aquarium (Busan, Korea) and maintained in five 300-L circulation filter tanks prior to experiments in the laboratory. Fish were reared in duplicate under 12 experimental conditions [four light treatments, one white (control), and three green] × three injection concentrations [0 (control), 0.1, and 0.5 μg/g] with 15 fish per tank (for messenger RNA (mRNA) and protein extraction). Goldfish were reared in automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Buchoen, Korea); the water temperature was maintained at 22 °C, and the fish were allowed to acclimate to the experimental conditions for 24 h.

The light control group was exposed to light from a white fluorescent bulb (27 W, wavelength range 350–650 nm); the light intensity at the water surface was approximately 0.96 W/m^2. The experimental groups were exposed to green-wavelength (500, 520, and 540 nm) LEDs (Daesin LED Co., Kyunggi, Korea); the light intensity at the water surface was approximately 0.5 W/m^2 (Fig. 1). The fish in the control and experimental groups were exposed to a 12-h light:dark photoperiod (lights on at 07:00 h and off at 19:00 h). The fish were reared under these conditions and fed daily with a commercial feed until the day prior to sampling.

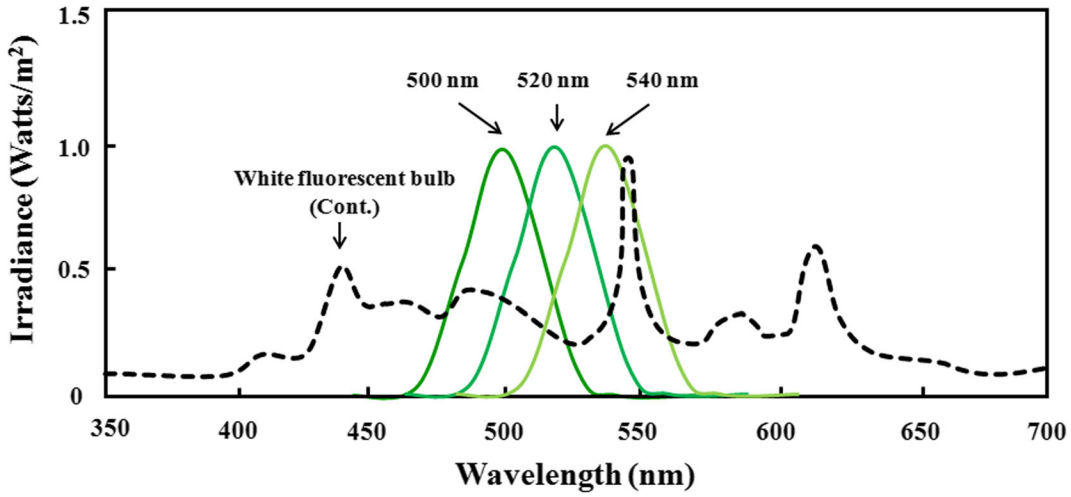

Fig. 1 Spectral profiles of the green (500, 520, and 540 nm) light-emitting diodes (LEDs) used in this study. The dotted line shows the spectral profile of the white fluorescent bulb

The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, CO, USA). Fish were anesthetized with 200 μL/L 2-phenoxyethanol (Daejung Co., Kyunggi, Korea) to minimize stress prior to blood collection. Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 1000×*g*, 10 min) and stored at − 80 °C until analysis. The fish were euthanized by spinal transection (all sampling at 11:00 h) at weekly sampling intervals to collect the brain, pituitary, gonads, and blood under dim light using an attenuated white fluorescent bulb.

VAL-opsin injection

To investigate the effects of VAL-opsin on sexual maturation, fish were anesthetized with 2-phenoxyethanol prior to injection. Injections were administered to the experimental fish using the intraperitoneal (IP) injection method once a week. Recombinant vertebrate ancient opsin (CSB-EP517517SWI; Cusabio, MD, USA) was dissolved in 0.9% physiological saline, and each fish was injected with VAL-opsin [0.1 and 0.5 μg/g, body mass (BM)] at a volume of 10 μL/g BM. The control groups were the same as that of the control of the light experiment. Each tank (each experimental group) included 15 fish. A week after the injection, brain, pituitary, gonad, and blood samples were collected from the fish once a week for 4 weeks (first sampling at 11:00 h).

Total RNA extraction and complementary DNA synthesis

Total RNA was extracted from each sample using TRI reagent® (Molecular Research Center Inc., OH, USA) according to the manufacturer's instructions and DNase-treated total RNA. The integrity of total RNA was confirmed by agarose gel electrophoresis. Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting complementary DNA (cDNA) was diluted and stored at 4 °C for use in quantitative polymerase chain reaction (qPCR).

qPCR

In this study, we considered recommendations regarding the minimum information for publication of quantitative real-time PCR experiments (Bustin et al. 2009). qPCR was conducted to determine the relative expression levels of VAL-opsin, GTHα, FSHβ, LHβ, ERα, ERβ, and β-actin mRNA using cDNA reverse-transcribed from the total RNA extracted from hypothalamus, pituitary, retina, and gonad samples. The primers used for qPCR are shown in Table 1. These primers were designed for each gene using Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure their specificity. PCR amplification was conducted using a

Table 1 Primers used for QPCR amplification

Genes (accession no.)	Primer	DNA sequences
VAL-opsin (AB383149)	Forward	5′-CAC CAC CTG CTT CAT CTT-3′
	Reverse	5′-TCA TCA CAA CCA CCA TAC-3′
GTHa (D86552)	Forward	5′-CTC CTG TCT ATC AGT GTA-3′
	Reverse	5′-ACA AGC ACC CGT TTA ACT-3′
FSH (D88023)	Forward	5′-CCT GGA AAG TGA GGA ATG-3′
	Reverse	5′-GTT CTG GTA AGA CAG CAT CA-3′
LH (D88024)	Forward	5′-TGT CCT ATT CTC TGT AAT TGT CC-3′
	Reverse	5′-GTC TCA TTA ACT GGC TCA CA-3′
ERα (AY055725)	Forward	5′-GGA CTG TGT GGA GGG TAT-3′
	Reverse	5′-AGA GTT GAG CAA GAT GAT GG-3′
ERβ (AF061269)	Forward	5′-GCA GGG TTT CGT GGA TAT-3′
	Reverse	5′-TGT TGG AGT TGA GGA GGA-3′
β-actin (AB039726)	Forward	5′-ATT TGG CAT CAC ACC TTC T-3′
	Reverse	5′-TTC TCC CTG TTG GCT TTG-3′

Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad) with a 20-μL reaction volume containing 10 μL iQ™ SYBR® Green Supermix (Bio-Rad), 5 μL diluted cDNA (50-fold), 300 nM of each 0.5 μL forward and reverse primer, and 4 μL H_2O. The qPCR program was as follows: 95 °C for 5 min, followed by 50 cycles of 95 °C for 20 s and 55 °C for 20 s. Amplification of a single product from PCR was confirmed by a melt curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Amplification efficiencies of the reference and target genes are given in Table 1. As internal controls, the experiments were duplicated with β-actin, and all data are expressed relative to the corresponding β-actin threshold cycle (ΔCt) levels. The calibrated ΔCt values (ΔΔCt) for each sample and the internal controls (β-actin) were calculated using the $2^{-\Delta\Delta Ct}$ method: [ΔΔCt = 2^-($\Delta Ct_{sample} - \Delta Ct_{internal\ control}$)].

Western blot analysis

Fish were euthanized at weekly sampling intervals (first sampling at 11:00 h) to collect gonads for western blot analysis. Total protein isolated from the gonads of goldfish was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. A total of 25 μg of protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used for reference. Samples were electrophoresed at 180 V, and the gels were immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Thereafter, the membranes were blocked with 5% skim milk in 0.04% Tris-buffered saline with Tween (TTBS) for 45 min and subsequently washed in TTBS. Membranes were incubated with ER antibodies (ERα; dilution 1:1000, Sigma; ERβ, dilution 1:4000, Santa Cruz Biotech, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit ERα and mouse ERβ IgG secondary antibodies (dilution 1:5000; Bio-Rad) for 60 min. The internal control used was β-tubulin (dilution, 1:4000; ab6046, Abcam, Cambridge, UK) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:5000; Bio-Rad) for 60 min. Bands were detected using a sensitive electrochemiluminescence system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDoc™ XRS + Systems (Bio-Rad).

Plasma parameter analysis

Plasma samples were separated by centrifugation (4 °C, 1000×*g*, 15 min), and the plasma was analyzed using immunoassays with a follicle-stimulating hormone ELISA kit (catalog no. MBS035576; Mybiosource,

San Diego, CA, USA), fish luteinizing hormone (LH) ELISA kit (MBS283097; Mybiosource, San Diego, CA, USA), and fish estradiol (E_2) ELISA kit (MBS283228; Mybiosource, San Diego, CA, USA). An anti-antibody that was specific to the melatonin antibody was pre-coated onto a microplate. Next, 100 μL tissue supernatant, 50 μL HRP conjugate, and 50 μL antibody were added to each well. These reagents were mixed and then incubated for 2 h at 37 °C. Following the last wash, any remaining wash buffer was aspirated or decanted off, and 50 μL each of substrates A and B were added to each well. These substrate solutions were then incubated for 15 min at 37 °C in the dark, during which time they changed from colorless to dark blue. Following incubation, 50 μL stop solution was added to each well, which changed the color from blue to yellow. The optical density of the solution in each well was then determined within 10 min using a microplate reader set to 450 nm.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., USA). A two-way ANOVA was followed by Tukey's post hoc test to compare differences among groups ($P < 0.05$). Values are expressed as the means ± standard error (SE).

Results

Changes in VAL-opsin mRNA expression in the brain

Expression of VAL-opsin mRNA using cDNA extracted from goldfish brains was significantly higher in the VAL-opsin-injected group than in the control group (non-injection groups) over time (peaking at 4 weeks after injection). In particular, for the green-sensitive pigment, the VAL-opsin expression level in the green-wavelength light groups was higher than that in the white fluorescent groups as the concentration of the injection increased (peaking at 0.5 μg/g BM). However, there were no significant differences between the green-wavelength light groups (Fig. 2).

Changes in GTH mRNA expression levels in the pituitary

Similar to the results of VAL-opsin, expression of GTHα, FSH, and LH mRNA using cDNA extracted from the

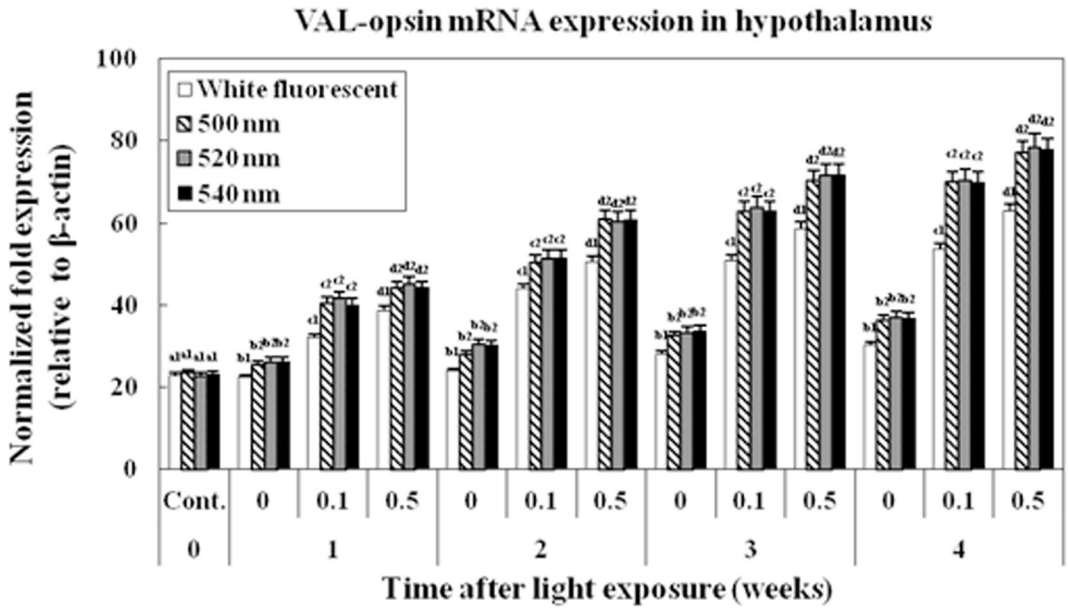

Fig. 2 Changes in the expression levels of vertebrate ancient long opsin (VAL-opsin) mRNA under three green-wavelength lights and a white fluorescent bulb in the hypothalamus following VAL-opsin (0.1 and 0.5 μg/g BM) injection, as measured by quantitative real-time PCR. Results are expressed as the normalized fold change in expression levels with respect to the β-actin levels in the same sample. Different letters indicate significant differences among treatments exposed to different LED spectra at the same time ($P < 0.05$). Different numbers indicate significant differences among time points for the same LED spectrum exposure ($P < 0.05$). All values are represented as means ± SE ($n = 5$)

goldfish pituitary was also higher in the VAL-opsin-injected groups than in the control groups with time (peaking at 4 weeks after injection). In particular, GTHα, FSH, and LH expression in the green-wavelength groups was higher than that in the white fluorescent groups as the concentration of the injections increased (peaking at 0.5 μg/g BM). However, there were no significant differences between the green-wavelength groups (Fig. 3).

Changes in ERα and ERβ mRNA and ER protein expression levels in gonads

ERα and ERβ mRNA expression using cDNA extracted from goldfish gonads was also higher in VAL-opsin-injected than control groups over time (peaking at 4 weeks after injection). In particular, ERα and ERβ expression in the green-wavelength groups was higher than that in the white fluorescent groups as the injection concentration increased (peaking at 0.5 μg/g BM). In

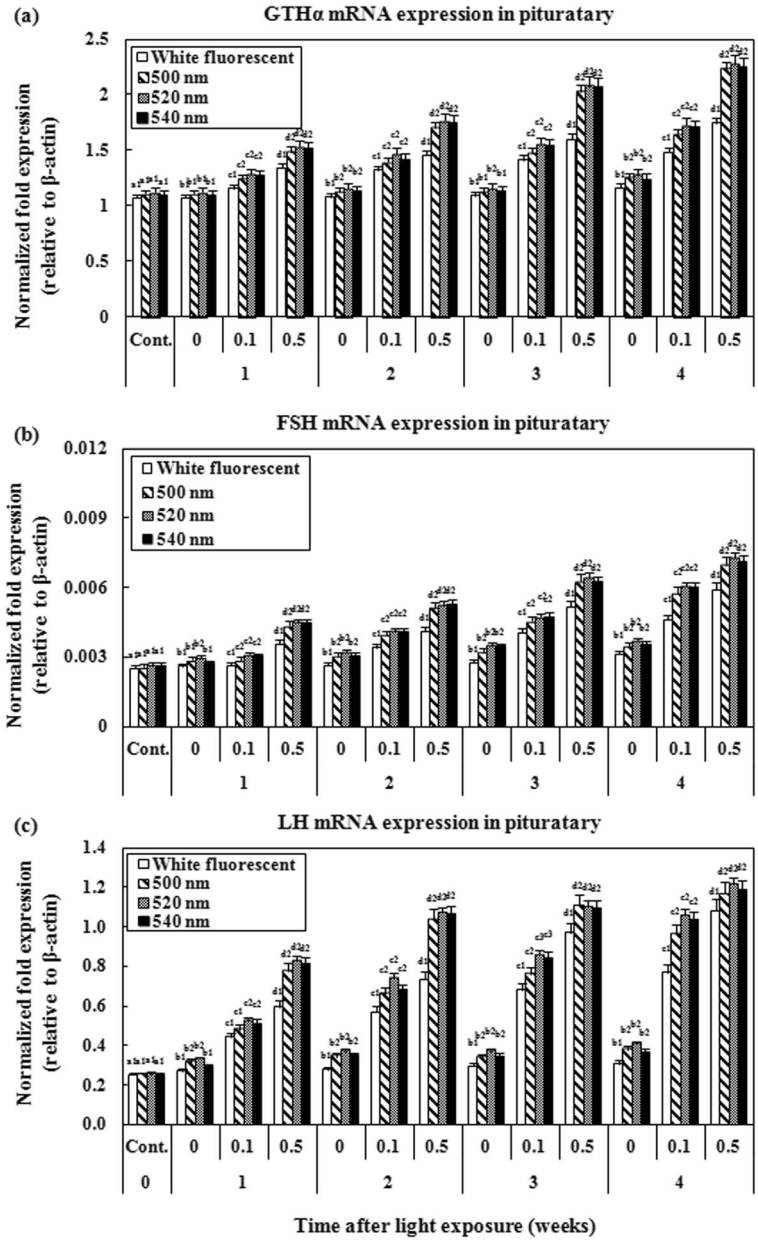

Fig. 3 Changes in the mRNA expression levels of GTHα (**a**), FSH (**b**), and LH (**c**) under three green-wavelength lights and a white fluorescent bulb in the hypothalamus following VAL-opsin (0.1 and 0.5 μg/g BM) injection as measured by quantitative real-time PCR. Results are expressed as the normalized fold change in expression levels with respect to the β-actin levels in the same sample. Different letters indicate significant differences among treatments exposed to different LED spectra at the same time ($P < 0.05$). Different numbers indicate significant differences among time points for the same LED spectrum exposure ($P < 0.05$). All values are represented as means ± SE ($n = 5$)

addition, protein expression of ERα and ERβ was similar to that of the mRNA expression. However, there were no significant differences between the green-wavelength groups (Fig. 4).

Changes in plasma activities of reproductive hormones

Similarly to the results of mRNA expressions of these hormones, the activities of plasma FSH, LH, and E_2 were also higher in VAL-opsin-injected than in control groups over time (peaking at 4 weeks after injection). In particular, the activities in the green-wavelength groups were higher than those in the white fluorescent groups as the injection levels increased (peaking at 0.5 μg/g BM). However, there were no significant differences between the green-wavelength groups (Fig. 5).

Discussion

The expression of VAL-opsin mRNA in the goldfish brain significantly increased with time in all experimental groups for 4 weeks after VAL-opsin injection, and the expression level of VAL-opsin was significantly

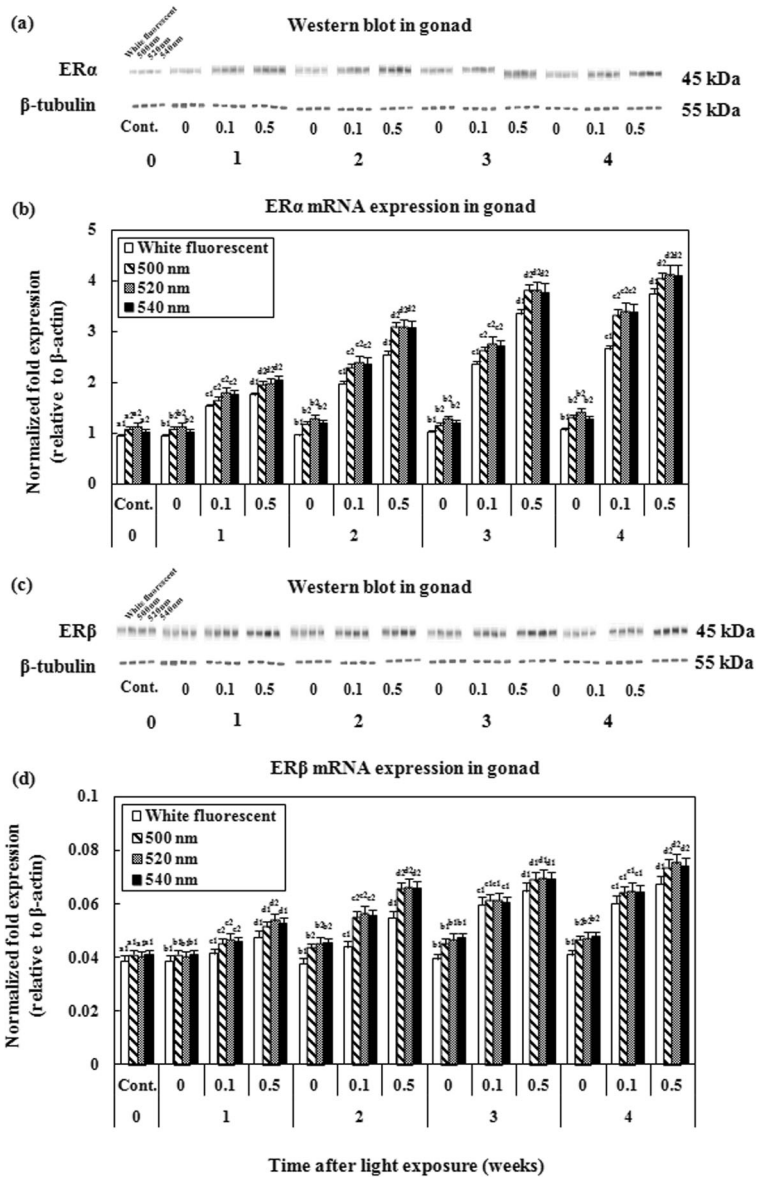

Fig. 4 Changes in the protein expression levels of ERα (**a**), ERβ (**b**), and ERα (**c**), and ERβ (**d**) mRNA expressions in goldfish gonads under three green-wavelength lights and a white fluorescent bulb in the hypothalamus following VAL-opsin (0.1 and 0.5 μg/g BM) injection as measured by quantitative real-time PCR. Results are expressed as the normalized fold change in expression levels with respect to the β-actin levels in the same sample. Different letters indicate significant differences among treatments exposed to different LED spectra at the same time ($P < 0.05$). Different numbers indicate significant differences among time points for the same LED spectrum exposure ($P < 0.05$). All values are represented as means ± SE ($n = 5$)

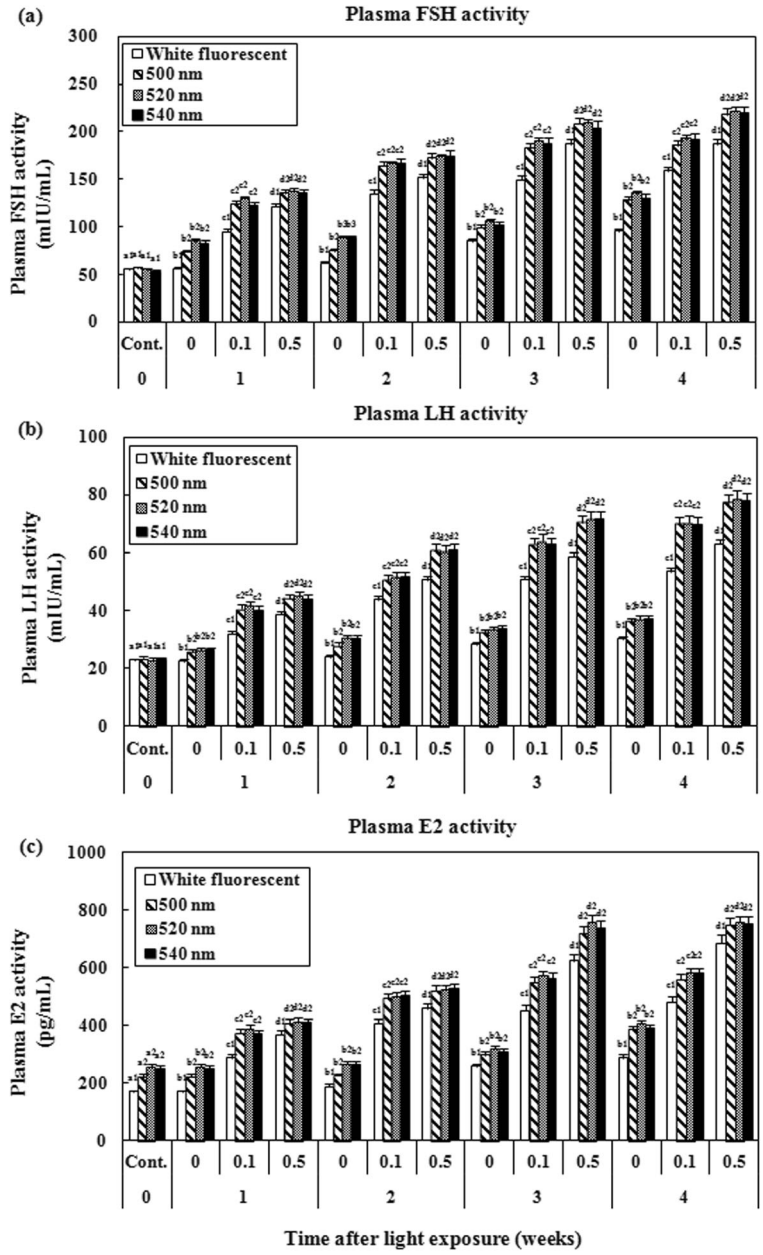

Fig. 5 Changes in the levels of plasma FSH (**a**), LH (**b**), and E_2 (**c**) in goldfish under three green-wavelength lights and a white fluorescent bulb in the hypothalamus following VAL-opsin (0.1 and 0.5 μg/g BM) injection as measured by quantitative real-time PCR. Results are expressed as the normalized fold change in expression levels with respect to the β-actin levels in the same sample. Different letters indicate significant differences among treatments exposed to different LED spectra at the same time ($P < 0.05$). Different numbers indicate significant differences among time points for the same LED spectrum exposure ($P < 0.05$). All values are represented as means ± SE ($n = 5$)

higher with increasing treatment concentrations (0, 0.1, and 0.5 μg/g). In addition, VAL-opsin mRNA expression tended to increase significantly after VAL-opsin injection in all experimental groups examined, as compared to that in the control group injected with VAL-opsin + white fluorescent; however, expression was not significantly different between green-wavelength lights (500, 520, and 540 nm) (Fig. 2).

Kojima et al. (2008) reported that the absorbance of VAL-opsin changes upon binding with 11-cis-retinal, which is a photosensitizing compound, and is a green-sensitive pigment that responds to light in the green wavelengths. Therefore, it is suggested that green-wavelength light promoted the synthesis of VAL-opsin in the deep brain.

In this study, to investigate the relationship between VAL-opsin and various GTHs and E2, which are known as hormones that regulate sexual maturation in the pituitary gland and in the gonads, we compared changes in GTHα, FSH, LH, and ER (ERα and ERβ) mRNA and

protein expression between the VAL-opsin + white fluorescent light group and the experimental groups under green light (the results, shown in Figs. 3 and 4, indicated that after the injection of VAL-opsin, GTHα, FSH, LH, and ER (ERα, ERβ) mRNA and protein expression significantly increased, and the expression levels were significantly higher with increasing treatment concentrations). However, all sex-related hormone mRNA and protein concentrations in the VAL-opsin + green-wavelength experimental groups were significantly higher than those in the VAL-opsin + white fluorescent light-injected experimental group.

To date, few studies on the regulation mechanism of the maturation of VAL-opsin have been conducted. Song et al. (2016) reported that GTH promotes VAL-opsin expression in goldfish. This result suggests that VAL-opsin is related to GTH. In addition, recombinant VAL-opsin significantly increased GTH secretion in this study. Therefore, VAL-opsin and GTH seem to interact with each other through mechanisms that have not yet been revealed.

As noted the above results, we found that VAL-opsin possibly controls the overall HPG axis. As a result, the expression of sex-related hormones was significantly different between the VAL-opsin + white fluorescent light and VAL-opsin + green-wavelengths groups, and the green-wavelength light exhibited a synergistic effect on the regulation of GTHs secretion by VAL-opsin.

The results of this study suggest that (1) VAL-opsin increases the secretion of sex hormones related to the HPG axis in goldfish and that VAL-opsin appears to modulate the activity of the HPG axis based on the finding that the secretion of sex hormones increased at higher concentrations of VAL-opsin. (2) In addition, VAL-opsin was effective in sexual maturation, but it appeared to be synergistic in promoting sexual maturation when irradiated with VAL-opsin + green because VAL-opsin was sensitive to green light. (3) However, VAL-opsin expression at 520-nm wavelength was high in the group irradiated with green light (500, 520, and 540 nm), but no significant difference in expression was observed among groups at different green wavelengths. Therefore, in summary, our results suggest that green-wavelength light effectively enhanced VAL-opsin activity in the deep brain and improved its function and role.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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