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In this study, we examined differences among the gonadotropin-inhibitory hormone (GnIH), kisspeptin 1 (Kiss1), Kiss 1 receptor (G-protein-coupled receptor 54; GPR54), melatonin receptor 1 (MT1), and melatonin levels in brain cells of goldfish *Carassius auratus* exposed to white fluorescent light and three light-emitting diode (LED) wavelength and melatonin treatments in the culture medium. In the green and blue LED treatment groups, GnIH and MT1 mRNA expression levels were significantly lower than in the other groups; conversely, levels significantly increased in the melatonin treatment groups. Additionally, expression levels of Kiss1 and its receptor, GPR54, in the white fluorescent and red LED light groups were significantly lower than the other groups, but levels also significantly decreased in the melatonin treatment groups. These results suggest that white fluorescent and red wavelengths downregulate the production of neurohormones in the brains of *C. auratus* and thus may inhibit sexual maturation in goldfish.

Keywords: G-protein-coupled receptor 54; gonadotropin-inhibitory hormone; kisspeptin; light-emitting diodes; melatonin receptor

1. Introduction

The influence of environmental factors on the growth and reproduction of fish has been extensively studied (Boeuf & Le Bail 1999), and it is well known that light and temperature are among the most important natural environmental factors that regulate reproduction. Lighting characteristics, including wavelength, intensity, and periodicity (daily cycle), are several factors that regulate seasonally dependent changes in the reproductive and growth physiology of fish (Boeuf & Le Bail 1999). The reproductive physiology of fish is strongly influenced by the perception of environmental factors by the sensory systems, which trigger the transduction of signals to the hypothalamus–pituitary–gonadal (HPG) axis (Bromage et al. 2001; Pankhurst & Porter 2003). Furthermore, sexual maturation in teleost fishes is controlled via complex interaction of various nerve control factors, and the HPG axis (Baroiller et al. 1999). One important mechanism involved in this process relates to the production of gonadal GnRH (gonadotropin-releasing hormone) peptides, which directly stimulate the resumption of oocyte meiosis and

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steroidogenesis (Habibi et al. 1988; Pati & Habibi 1998, 2002). A precise balance of HPG hormones is possible because of positive and negative feedback mechanisms. Estrogen is a key gonadal hormone that regulates GnRH production via brain kisspeptins (Kiss), thereby regulating gametogenesis and spawning time (Funes et al. 2003; Seminara et al. 2003).

There are two types of Kiss isoforms, known as Kiss1 and Kiss2, that are present in the brains of teleosts and other vertebrate species (Lee et al. 2009; Um et al. 2010). Both are important in regulating reproduction (Servili et al. 2011), and the neuroendocrine factor Kiss1 is particularly important in regulating sexual maturation (Roa et al. 2011; Servili et al. 2011). In addition, the Kiss-GPR54 signalling system is one of the circuits regulating reproduction by controlling GnRH secretion in the hypothalamus (Colledge 2009). According to histological observations by Irwig et al. (2004), Kiss1 and its receptor, G-protein-coupled receptor 54 (GPR54), are located with GnRH neurons in the hypothalamus; therefore, the interaction between GnRH and Kiss is a function of the GnRH regulator (Irwig et al. 2004; Messager et al. 2005).

In addition, melatonin, the primary hormone secreted by the hypothalamus, affects the gonadotropin-inhibitory hormone (GnIH), Kiss, and GPR54 (Tsutsui et al. 2010; Maitra et al. 2013). Melatonin affects the neurogenic function of GnIH, and interacts with other hypothalamus peptides (GnRH and Kiss) in the reproduction control system via its responses to light levels and photoperiod, and stimulates GnIH synthesis and secretion (Maitra et al. 2013). These actions are mediated via melatonin receptors (MTs), which belong to the G-protein-coupled receptor superfamily (Reppart et al. 1996). A recent report showed that the master circadian clock in teleost fishes may be controlled by the brain and pineal gland and that illumination during the night affected melatonin synthesis in the pineal glands in a chromatic- and intensity-dependent manner (Ekstrzm & Meissl 1997). MTs are distributed in the central nervous system and peripheral tissues of vertebrates (Dubocovich 1995; Reppart et al. 1996), and their expression mediates the various physiological functions of melatonin in these tissues. Vertebrates have three subtypes of MTs: MT1, MT2, and MT3 (Dubocovich 1995; Reppart et al. 1996). MTs can regulate the circadian rhythm in the suprachiasmatic nucleus (SCN) through feedback mechanisms involving the clock genes (Okamura et al. 2002). In addition, high MT1 mRNA expression has been detected in the SCN (Dubocovich et al. 2003).

While there is considerable information on the effect of photoperiod on reproduction (Vlaming 1975; Bromage et al. 2001; Pankhurst & Porter 2003), much less information is available on the importance of light wavelengths and light intensity. Therefore, a goal of this study was to examine the effects of different light spectra on the expression of several neurohormones involved in the regulation of sexual maturation and reproduction.

Light-emitting diodes (LEDs) are a new form of lighting technology that can be designed to output specific wavelengths (Migaud et al. 2007), and the narrow bandwidth light they produce, can be tuned to the environmental sensitivity of a target species (Villamizar et al. 2009). There is evidence that the spectral composition of incidental light is differentially affected in underwater environments, and rapid attenuation occurs with increasing depth (Lythgoe 1979).

In this study, we used molecular and endocrinological methods to investigate the mechanism by which specific light wavelengths affect fish sexual maturation and development. Specifically, we measured changes in expression levels of GnIH, Kiss1,

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GPR54, and MT1 mRNA in goldfish brain cells exposed to fluorescent light, and red, green, and blue LED light, and also measured the levels of melatonin in culture medium under during these experiments.

2. Materials and methods

2.1. Experimental fish and conditions

For each experiment, common goldfish ($n = 20$; length, 6.0 ± 0.5 cm; weight, 12.1 ± 0.4 g) were purchased from a commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for two weeks in eight, 300-L circulation filter tanks in the laboratory. The fish were exposed to light generated by a white fluorescent bulb (27 W), which was also used for the control group, and light intensity near the water surface of the tanks was approximately 0.96 W/m^2. The water temperature and photoperiod were 20 ± 1 °C and 12 h light: 12 h dark (lights on 07:00 h and lights off 19:00 h), respectively. The fish were fed a commercial feed twice daily (at 09:00 and 17:00 h). The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) for minimization of stress prior to brain collection.

2.2. In vitro *culture of brain cells and melatonin treatment*

The culture of goldfish brain neurons was performed using enzymatic and mechanical procedures. Brain tissues were quickly removed and placed in 3 mL of ice-cold dispersion buffer (Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride, and containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone; GIBCOBRL, Rockville, MD). The isolated brain tissues were then transferred to fresh 6 mL dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma). The connective tissues and other impurities were removed, and the brain tissues were chopped into small pieces with a pair of scissors. Brain cells and the minced brain tissue were transferred to a flask and incubated for 10 min at room temperature with slow stirring. The mixture of dispersed brain cells and tissues was filtered, and the culture medium (neurobasal medium, without L-glutamine, containing with 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 1% fetal bovine serum, Gibco-BRL) was added. The cell suspension was centrifuged at 800 × g for 10 min, and the cells were then resuspended in fresh culture medium. Brain cells (1.2 × 106 cells/800 μL/well) were added to a 24-well tissue culture plate. The experiment commenced at 15:00 h and samples (1 mL) were collected at 12 h (03:00), 24 h (15:00), 48 h (15:00), and 96 h (15:00). Fresh culture medium (600 μL) was added to the culture wells at 24 and 48 h.

For the experimental groups, brain cells were exposed to either red (peak at 630 nm), green (530 nm), or blue (450 nm) LED light (Daesin LED Co. Kyunggi, Korea), and to white fluorescent light (Control group, Cont.) (Figure 1). The LEDs were placed 50 cm above the surface of the cell culture plate, and irradiance at the surface of the plate was maintained at approximately 0.9 W/m^2. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA). Melatonin (Sigma, USA), dissolved in 0.9% physiological saline at the appropriate doses (10 and 20 μM), was added to the culture medium at a ratio of 1/1000e (v/v). Cells were treated for 96 h. Each sample was centrifuged (20 °C, 10,000 × g, 15 s), and supernatant was removed and stored at −80 °C until RNA extraction.

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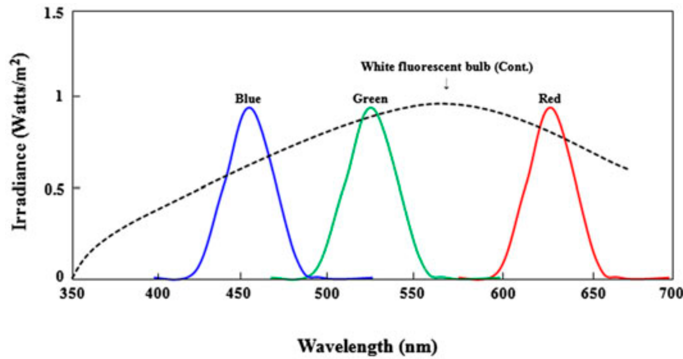

Figure 1. (Colour online) Spectral profiles of red (R, 630 nm), green (G, 530 nm), blue (B, 450 nm) LEDs, and white fluorescent light (Cont.) used in this experiment.
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2.3. *Real-time quantitative PCR*

Total RNA was extracted from the brain cells using a TRIzol kit (Gibco/BRL, USA) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. Real-time quantitative PCR (QPCR) was performed using cDNA. QPCR was conducted to determine the relative expression levels of GnIH (GenBank accession No. **AB078976**), Kiss1 (**FJ236327**), GPR54 (**EU622877**), MT1 (**AB481372**), and β-actin (**AB039726**) mRNA using total RNA extracted from the brain cells. The following QPCR primers were designed with reference to the known sequences of the common goldfish: GnIH forward (5′-CGG AGT CTG GAG ATA GAA GA-3′) and reverse (5′-ACG TGT GTT GGT TTG GTT AT-3′) primers; Kiss1 forward (5′-TGA ACC TAC TTA CCA TAA TTT TGA TG-3′) and reverse (5′-CCT GAG ACC CTG GAG TGA-3′) primers; GPR54 forward (5′-AGT GGT CAT TGT TGT TCT CTT-3′) and reverse (5′-AGG AGT TGG CAT AGG ACA T-3′) primers; MT1 forward (5′-GGT TGG CAG TAG CGA TTT-3′) and reverse (5′-CTC ACG ACG GAA GTT CTG-3′) primers; and β-actin forward (5′-TTC CAG CCA TCC TTC CTA-3′) and reverse (5′-TAC CTC CAG ACA GCA CAG-3′) primers. The PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. The QPCR was performed as follows: 95 °C for 5 min, followed by 35 cycles each of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with β-actin and all data were expressed relative to the corresponding β-actin calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using $2^{-\Delta\Delta Ct}$ Method $[\Delta\Delta Ct = 2^{-(\Delta Ct_{sample} - \Delta Ct_{internalcontrol})}]$.

2.4. *Melatonin determination by ELISA*

To determine the melatonin concentration in plasma, an immunoenzyme assay method was used, with a commercial enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany). Plasma samples were purified with extraction columns in the

centrifuge immediately after defrosting. Next, 50 µL of each sample were added to the wells of an ELISA plate precoated with the capture antibody. The samples were incubated with melatonin-biotin and antiserum solutions for 15 h at 4 °C. The wells

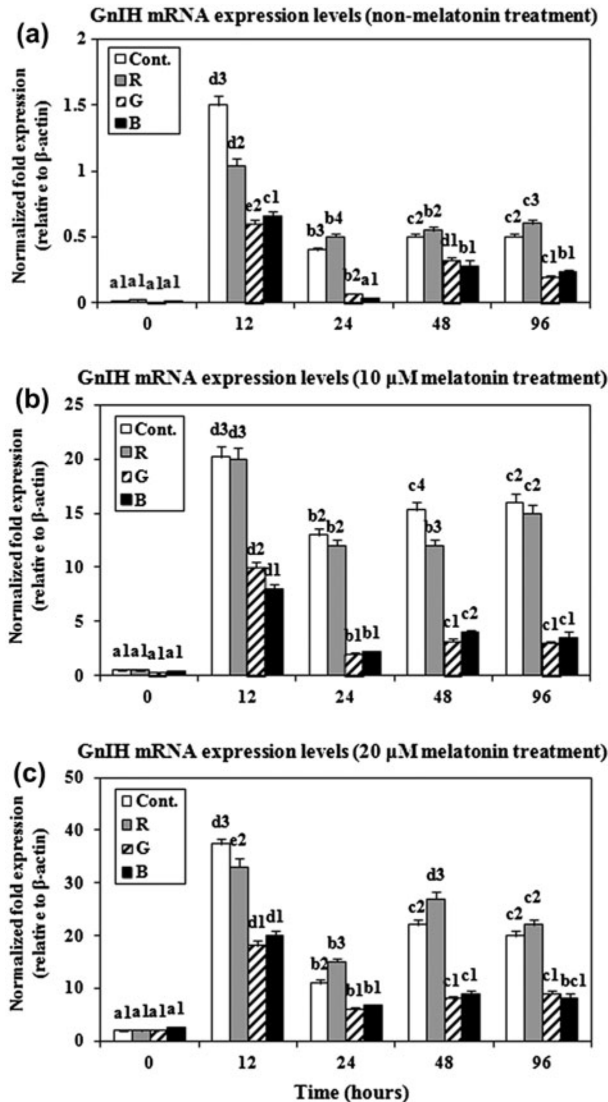

Figure 2. Changes in the expression levels of GnIH mRNA in goldfish brain cells in the control group (a), melatonin treatment group (10 µM) (b), and melatonin treatment group (20 µM) (c), exposed to red (R), green (G), and blue (B) LED light, and white fluorescent light, as measured by QPCR. Total brain cell RNA (2.5 µg) was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values with different characters are significantly different at different sampling time points (hours) in fish exposed to the same light spectrum ($p < 0.05$). The numbers indicate significant differences between different light spectra within the same sampling time point ($p < 0.05$). All values are means ± SD ($n = 5$).

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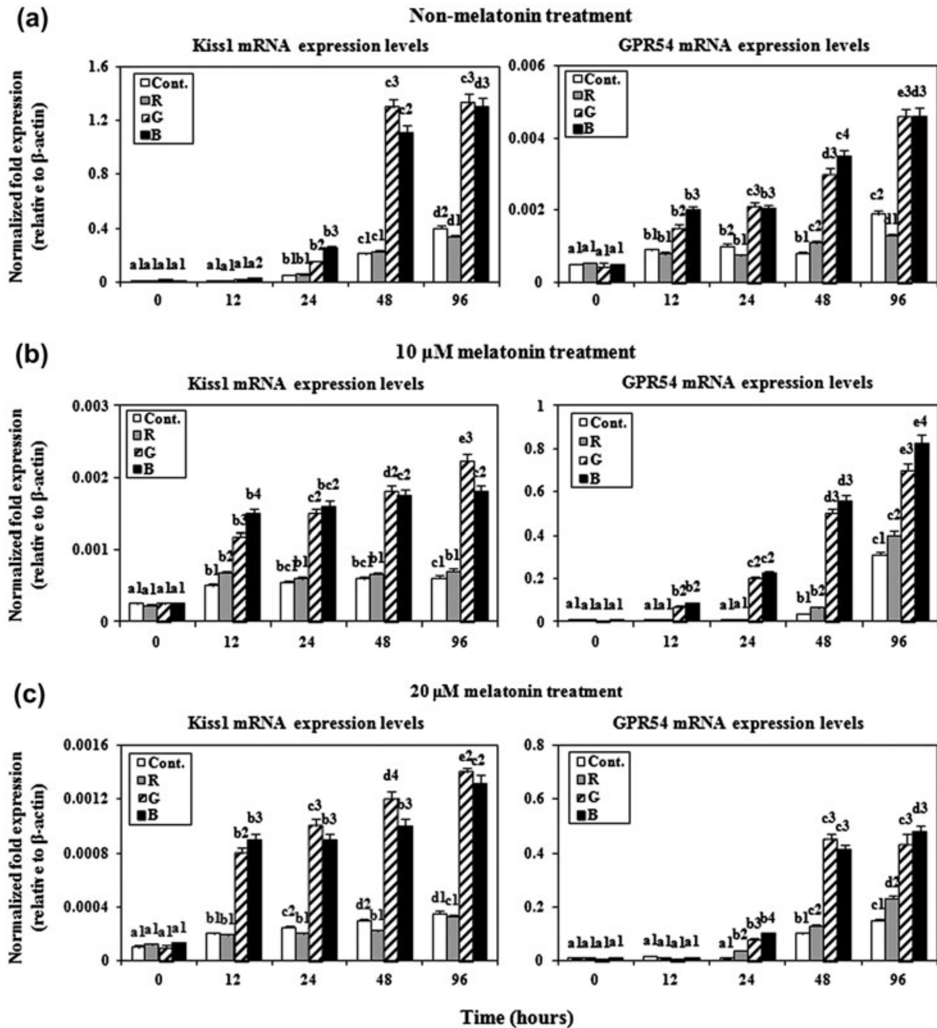

Figure 3. Changes in the expression levels of Kiss1 and GPR54 mRNA in goldfish brain cells in the control group (a), melatonin treatment group (10 μM) (b), and melatonin treatment groups (20 μM) (c), exposed to red (R), green (G), blue (B) LED light, and white fluorescent light, as measured by QPCR. Total RNA (2.5 μg) isolated from the brain cells was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values with different characters are significantly different at different sampling time points (hours) in fish exposed to the same light spectrum ($p < 0.05$). The numbers indicates significant differences between different light spectra within the same sampling time points ($p < 0.05$). All values are means ± SD ($n = 5$).

were then washed with the assay buffer (phosphate buffer with Tween-20 and stabilizer), and the plate was incubated with the enzyme-labeled solution (anti-biotin-alkaline phosphatase in Tris buffer with stabilizers) for 2 h at room temperature and with constant shaking. After washing the plate, it was incubated with a *p*-nitro-phenyl phosphate solution for 30 min, before adding 50 μL of the stop solution (1 N NaOH with 0.25 M EDTA). Absorbance was read at 405 nm.

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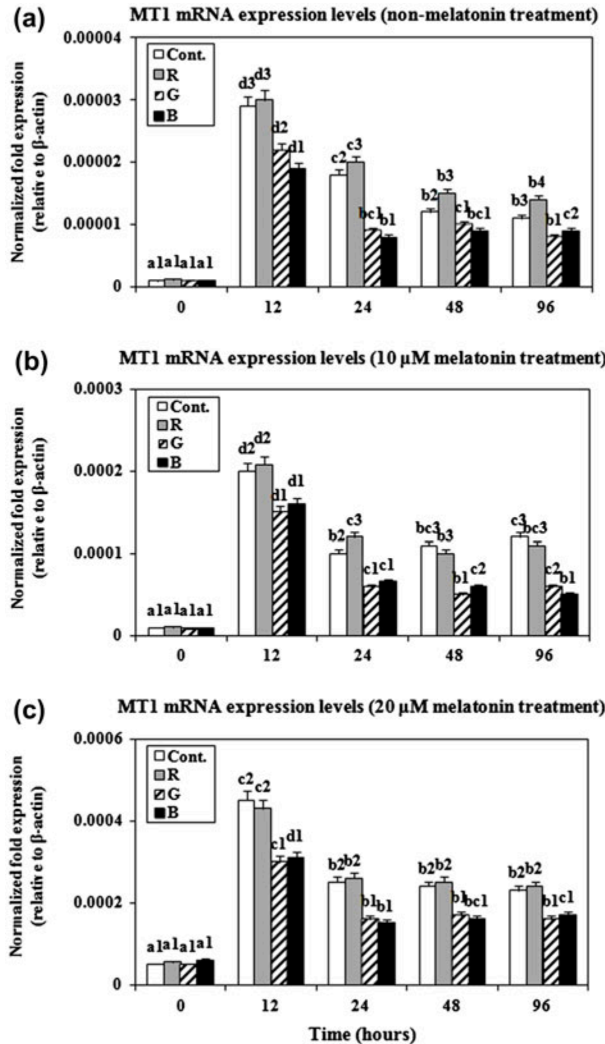

Figure 4. Changes in the expression levels of MT1 mRNA in goldfish brain cells in the control group (a), melatonin treatment group (10 μM) (b), and melatonin treatment group (20 μM) (c), exposed to red (R), green (G), blue (B) LED light, and white fluorescent light, as measured by QPCR. Total RNA (2.5 μg) isolated from the brain cells was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values with different characters are significantly different at different sampling time points (hours) in fish exposed to the same light spectrum ($p < 0.05$). The numbers indicates significant differences between different light spectra within the same sampling time point ($p < 0.05$). All values are means ± SD ($n = 5$).

2.5. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). Two-way ANOVA followed by Tukey's *post hoc* test was used to assess statistically significant differences among different time points in the daily variation and different light spectra. A value of $p < 0.05$ was considered statistically significant.

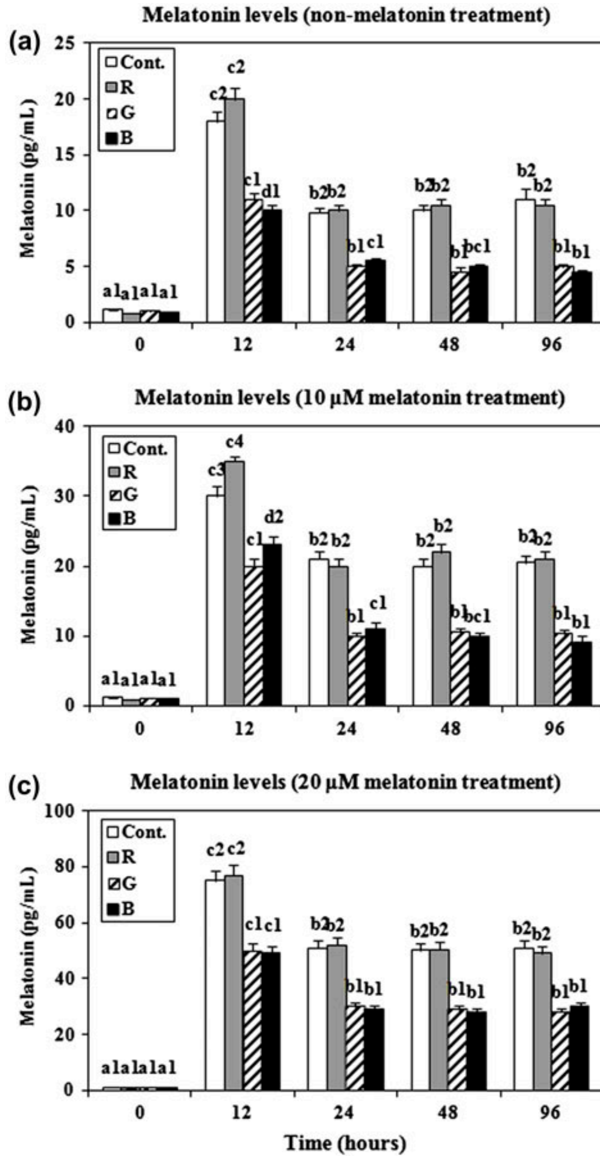

Figure 5. Changes in melatonin levels in the culture medium in the control group (a), melatonin treatment group (10 µM) (b), and melatonin treatment group (20 µM) (c), exposed to red (R), green (G), purple (P) LED light, and white fluorescent light, as measured by a microplate reader. Values with different characters are significantly different at different time points (hours) in fish exposed to the same light spectrum ($p < 0.05$). The numbers indicates significant differences between different light spectra within the same sampling time point ($p < 0.05$). All values are means ± SD ($n = 5$).

3. Results

3.1. GnIH mRNA expression levels

Our experiments detected significant differences in expression of GnIH mRNA in brain cells in response to different light spectra (Figure 2). GnIH mRNA expression levels in

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all experimental groups significantly increased until 96 h, but levels in the groups exposed to white fluorescent and red LED light were significantly higher than in the green and blue LED treatment groups. Furthermore, GnIH mRNA expression levels in the melatonin treatment groups were significantly lower (approximately 10-fold) than the control group (non-melatonin treatment group) (Figure 2(b) and (c)). Additionally, the levels in the 20 μM melatonin treatment group were significantly lower (approximately 2-fold) than in the 10 μM melatonin treatment group (Figure 2(c)).

3.2. Kiss1 and GPR54 mRNA expression levels

Significant differences were also observed in the expression of Kiss1 and GPR54 mRNA in brain cells in response to different light spectra (Figure 3). mRNA expression levels of Kiss1 and its receptor, GPR54, significantly increased in all experimental groups until 96 h. However, expression levels in the groups exposed to white fluorescent and red light were significantly lower than in the green and blue LED light treatment groups. Furthermore, Kiss1 and GPR54 mRNA expression levels in the melatonin treatment groups were significantly lower (approximately 0.5-fold) than in the control group (non-melatonin treatment group) (Figure 3(b) and (c)). Expression levels in the 20 μM melatonin treatment group were also significantly lower (approximately 0.5-fold) than in the 10 μM melatonin treatment group (Figure 3(c)).

3.3. MT1 mRNA expression and melatonin levels

The effects of different light spectra on the expression of MT1 and melatonin levels in brain cells are illustrated in Figures 4 and 5. Levels of MT1 mRNA expression and melatonin in all experimental groups were significantly higher at 12 h (03:00), and then decreased and were maintained at this lower level until 96 h. However, the levels in groups exposed to white fluorescent and red LED light were significantly higher than the green and blue LED treatment groups (Figures 4(a) and 5(a)). Furthermore, expression levels in the melatonin treatment groups were significantly higher than in the control group (non-melatonin treatment group) (Figures 4(b)–(c) and 5(b)–(c)). Finally, expression levels in the 20 μM melatonin treatment group were significantly higher than in the 10 μM melatonin treatment group (Figures 4(c) and 5(c)).

4. Discussion

To investigate the mechanisms by which specific light wavelengths affect sexual maturation and development in fish, we performed experiments using goldfish brain cells cultured under fluorescent light, and red, green, and blue LED light. Specifically, we measured changes in the expression levels of several important endocrinological hormones involved in the regulation of sexual maturation, including GnIH, Kiss1, GPR54, and MT1 mRNA, in response to different light spectra treatments, and also measured the levels of melatonin in culture medium during these experiments.

First, we observed that GnIH mRNA expression levels in cultured goldfish brain cells were higher in groups exposed to fluorescent and red LED light than in the other experimental groups (Figure 2(a)). GnIH plays a role in the interaction between Kiss1 and GPR54 in the hypothalamus (Tsutsui et al. 2010); therefore, we could observe that Kiss1 and GPR54 mRNA expression levels in groups exposed to fluorescent and red LED were significantly lower than that in the other groups (Figure 3). Additionally, Kiss

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and GPR54 play an important role in the physiological regulation of reproductive maturation. Functionally, they participate in the regulation of reproductive function and fertilization metabolism through regulation of the secretion of gonadotropin hormones, feedback actions of sex steroid hormones, and environmental signalling through the acceleration of GnRH neurons during puberty (Parhar et al. 2004; Tena-Sempere et al. 2012). The interaction between GnRH and Kiss1 was previously shown to be a function of the GnRH regulator, which regulates the gonad steroid hormones, and these hormones and Kiss1 interact and are conveyed to the brain through a feedback mechanism (Tsutsui et al. 2010). Kiss stimulates the GnRH neuron through its receptor, GPR54, and this action induces the upregulation of the HPG axis (Colledge 2009; Tsutsui et al. 2010). In a study of Siberian hamsters, Kiss1 mRNA expression levels increased during the process of sex maturation and were related to increased gonadal weight, these findings suggest that an important role for kisspeptin in coordinating and relaying environmentally relevant information to the reproductive axis as well as a role for this peptide in regulating seasonal changes in reproductive function (Greives et al. 2007).

GnIH mRNA expression levels in the melatonin treatment groups were significantly higher than the other groups (Figure 2), and Kiss1 and GPR54 mRNA expression levels were significantly lower in melatonin-treated groups (Figure 3). Previous study reported that melatonin generally controls circadian rhythm and is secreted from the pineal gland, but it also plays a role in the control of hormones secreted from the hypothalamus (Tsutsui et al. 2010). Tsutsui et al. (2010) reported that in vertebrates, melatonin induces neuropeptide synthesis and acts directly on the GnIH neuron to induce GnIH expression via the GnIH receptor. Thus, GnIH could that reproductive status is primarily driven by day length (photoperiod) through melatonin signalling changes. Secreted GnIH also sequentially inhibits the GTH synthesis and secretion, and previous research showed that luteinizing hormone (LH) secretion in mature fish was stimulated in cultured pituitary cells treated by low concentrations of melatonin (Falcón et al. 2010).

We observed that GnIH mRNA expression levels were significantly higher in the group exposed to 20 μM melatonin (Figure 2); conversely, Kiss1 and GPR54 mRNA expression levels were significantly lower (Figure 3). We hypothesized that melatonin induces GnIH expression and then inhibits the expression of Kiss1 and its receptor, GPR54, which play an important role in stimulating reproductive controls. It appears that melatonin affects hormone levels in both the hypothalamus and pituitary gland. Recently, Sébert et al. (2008) reported that follicle-stimulating hormone and LH expression and plasma sex steroid hormones levels decreased in eels exposed to melatonin. Therefore, melatonin and other hypothalamus peptides (GnRH, GnIH, and Kiss) and their interactions affect the functioning of the reproductive control system (Maitra et al. 2013).

Additionally, in this study, Kiss1 and GPR54 mRNA expression levels were significantly lower in cultured brain cells in groups exposed to fluorescence and red LED light; we therefore hypothesized that these light wavelengths inhibit the sexual maturation in fish. Shin et al. (2011) reported that the oxidative, such as expression of SOD, CAT, and GPX mRNA, values in groups exposed to fluorescent and red LED light were significantly higher than that in other light treatment groups, and we therefore hypothesized that fluorescent and red LED light negatively affect fish by inhibiting sexual maturation.

Finally, to investigate whether specific wavelengths affect the regulation of fish maturation, we measured expression of the MT1, which is part of the feedback loop between light wavelength and sex hormones. Our experiments showed that mRNA expression and culture medium melatonin levels were significantly higher at 12 h in all

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experimental groups; furthermore, levels in groups exposed to fluorescent and red LED light were significantly higher than the other groups (Figures 4 and 5). These results indicated that MT1 mRNA expression and plasma melatonin levels measured in samples collected at night-time (03:00) were significantly higher than those collected during day-time. The observation of higher mRNA expression and culture medium melatonin levels in the fluorescent and red LED light treatment groups is also in agreement with previous research. Experiments by Shin et al. (2011) showed that melatonin levels in fluorescent and red LED light treatment groups were significantly higher than green and blue LED treatment groups during exposed LED light. Therefore, we hypothesized that melatonin acts as antioxidant, as highest levels of reactive oxygen species were also observed in the fluorescent and red LED light treatment groups.

In summary, we hypothesize that (1) the interactions of melatonin, GnIH, and Kiss control reproductive hormone levels and induce sexual maturation in fish, and (2) mRNA expression by Kiss1 and GPR54, which stimulate sexual maturation, were induced by exposure to green and blue LED light. This research provides useful and necessary data for further research on endocrinological mechanisms and control of hormones related to reproduction.

Disclosure statement

No potential conflict of interest was reported by the authors.

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