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Melatonin Induces *per1a* Expression in Zebrafish Brain

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Abstract: In vertebrates, the circadian mechanism regulates many physiological and behavioral activities. The major key output of the circadian clock is the secretion of melatonin in the pineal gland. Melatonin secretion remains low during the daytime and increases significantly at night. The rise and fall of melatonin level is a key factor that conveys rhythmic information to the organism to carry out daily and annual physiological rhythms. It also affects the rhythmic oscillation of clock genes in the suprachiasmatic nucleus (SCN) as well as in peripheral tissues. In this study, we have shown that zebrafish brain rhythmically expresses per1a and cry1a in different photoperiodic regimes: light-dark cycle (LD) and continuous light (LL). We have also demonstrated that an acute injection of melatonin in the middle of the subjective day induces *per1a* expression in zebrafish brain. Furthermore, we have also shown that an acute injection of melatonin affects circadian regulation of locomotor activity in zebrafish under LD conditions. These results provide insight into understanding the underlying mechanism of melatonin in regulation of clock genes and locomotor activity in zebrafish.

Keywords: Melatonin; Light; *per1a*; *cry1a*; Zebrafish; Circadian rhythm; SDG 3 Good health and well-being

1. Introduction

As the main pacemaker of the mammalian circadian rhythm, the suprachiasmatic nucleus (SCN) of the hypothalamus is responsible for synchronizing circadian rhythms across the body by coordinating circadian oscillations in peripheral tissues. This internal clock is vital for maintaining the synchronicity of all physiological and behavioral activities

to the 24-hour day-night cycle[1,2]. The SCN carries out this function by receiving photic input from the intrinsically photosensitive retinal ganglion cells (ipRGCs); this signal is transmitted via the retinohypothalamic tract (RHT). The SCN is influenced by hormones such as testosterone, dihydrotestosterone, estradiol, and, most notably, melatonin. Integrating this information, the SCN projects to several brain regions, including the median preoptic area (MnPO), paraventricular nucleus of the hypothalamus (PVN), and ventrolateral preoptic nucleus (VLPO) via the dorsomedial hypothalamus (DMH) to respectively modulate body temperature, influence endocrine release (such as melatonin and glucocorticoids), and regulate sleep[3]. At the molecular level, circadian rhythms in the SCN and peripheral tissues are driven by transcription-translation feedback loops (TTFLs) involving key clock genes, including the brain and muscle Arnt-like protein-1 (*BMAL1*), circadian locomotor output cycles kaput (*CLOCK*), period (*PER1*, *PER2*, and *PER3*), and cryptochrome (*CRY1* and *CRY2*)[4]. When their genes are expressed, the transcription factors BMAL1 and CLOCK heterodimerize, translocate into the nucleus as a complex, and activate the transcription of the *PER* and *CRY* genes. In turn, the PER and CRY proteins form heterodimers, which inhibit their own transcription by repressing the BMAL1:CLOCK complex. This feedback loop generates the standard 24-hour cycle of clock gene expression. Other regulators involved in the circadian mechanism include the REV-ERB receptors and retinoic acid-related orphan receptors (RORs), which, respectively, downregulate and promote the expression of the *BMAL1* gene[5].

As alluded to previously, in addition to acting as an input signal for the SCN, melatonin can also be the outcome of SCN downstream signaling, underscoring its importance in sustaining circadian synchronization across central and peripheral clocks[3,6]. During the day or in the presence of light, signals relayed from the SCN suppress melatonin synthesis, inhibiting its release. Conversely, in the dark, melatonin is secreted by the pineal gland, where it is synthesized from tryptophan via serotonin[7,8]; the rate-limiting enzyme of this synthesis cascade is arylalkylamine *N*-acetyltransferase (AANAT)[9]. Melatonin is then released into the peripheral circulation and exerts its actions in central and peripheral tissues (e.g., adrenal glands, lungs, heart, liver, and kidneys) through interactions with melatonin receptors MT1 and MT2[6,7,10,11]. Centrally, melatonin activates MT1 receptors at the SCN and subsequently lowers the firing rate of SCN neurons to facilitate sleep[12]. On the other hand, activation of MT2 receptors at the SCN leads to phase shifting of the circadian rhythm[13]. Furthermore, melatonin also modulates regions of the default mode network (DMN), especially the precuneus, contributing to fatigue[7]. Peripherally, melatonin is involved in the regulation of fetal development, blood pressure, immune response, gastrointestinal secretions, bone mass, and reproduction[6]. Melatonin has garnered much

interest for its diverse therapeutic potential, not just in disorders related to sleep and circadian rhythm, but also in neurodegenerative disorders as well as cancers due to its reported antioxidant and anti-inflammatory effects[6,11,14]. Indeed, melatonin can impact the gut microbiota[15], the dysbiosis of which has been linked to a variety of disorders, such as autism spectrum disorder[16], obsessive compulsive disorder[17], depression[18], type 2 diabetes mellitus[19], and many others[20].

Given the significance of melatonin in circadian rhythm regulation, the effects of melatonin on clock gene expression have been studied to some extent in mammals. Exogenous melatonin administration advanced the rhythmic expression of *CRY1* but did not significantly alter *PER1* rhythmic expression in the pars tuberalis (PT) of rats[21]. In the PT of Siberian hamsters, *PER1* expression was lowered as a consequence of melatonin administration[22]. Another study demonstrated that the phasic expression of *PER1* in the PT was lost as a result of pinealectomy in Syrian hamsters[23]. These findings suggest that melatonin influences the expression of *CRY1* and *PER1* genes in mammalian brains. However, to the best of our knowledge, no studies have investigated the effects of exogenous melatonin on clock gene expression in healthy adult zebrafish.

The zebrafish model is known for its numerous advantages, including swift development, high reproductive efficiency, ease in breeding, maintenance, handling, and imaging, efficiency in terms of cost, space, and time, capability of high-throughput screening, as well as easily manipulable genetics. Furthermore, zebrafish share high genetic homology and conserved physiological functions with humans[24–27]. In the context of the present study, the zebrafish model has additional utility, such as conserved melatonin function and sleep regulatory system, in addition to its diurnal nature[27]. Despite these similarities, the zebrafish molecular circadian clock differs from that of mammals due to the presence of genome duplications, for instance, zebrafish possess three *clock* genes (*clocka*, *clockb*, and *npas2*), three *bmal* genes (*arnt1a*, *arnt1b*, and *arntl2*), four *per* genes (*per1a*, *per1b*, *per2*, and *per3*), and six *cry* genes (*cry1a*, *cry1b*, *cry2*, *cry3a*, *cry3b*, *cry4*, and *cry5*), however, *cry2*, *cry4*, and *cry5* are not core circadian clock genes. The expression of *clock*, *bmal*, *cry3a*, and *cry3b* genes generally reach their maximum levels during the light-dark transition, whereas *per* genes (besides *per2*) and *cry1b* expression levels culminate in the dark-light transition and early in the light phase respectively. These core circadian genes oscillate regularly in normal light-dark cycle and in continuous darkness[28]. In contrast, the expression of *cry1a* and *per2* genes are dependent on light exposure[27,28]. Another notable difference is that most zebrafish cells can be directly entrained by light, a property that sets them apart from mammalian circadian rhythms[29].

In the present study, we aim to determine the effects of light and melatonin on expression of *cry1a* and *per1a* genes in zebrafish. This study seeks to provide better insight into the underlying mechanisms by which light and melatonin regulate clock gene regulation. Understanding the effects of melatonin in zebrafish, particularly its role in regulating clock gene expression, can shed light on its broader impact on circadian biology. These findings may inform therapeutic approaches for managing sleep disorders, circadian rhythm disruptions, and related health conditions in humans.

2. Materials and Methods

2.1. Animals

In-house bred adult male wild-type zebrafish (*Danio rerio*) of 4 to 5 months were used. They were kept in tanks with dimensions of 36 cm x 26 cm x 22 cm, filled with 10L of dechlorinated water at a temperature of around 27°C. Each tank consisted of 10 to 12 fish. The fish were housed and maintained in the animal facility unit in Monash University. The photoperiodic regime was 14h light (~300 lux) and 10h dark cycle. All fish were fed twice daily to ensure a constant source of nourishment. All experiments followed the guidelines of care and the use of animals with ethical approval by the MONASH Animal Ethical Committee (MARP/2012/092/BC, MARP/2013/112).

2.2. Drug Treatment and Groups

Melatonin (product no. M5250) was dissolved in dimethyl sulfoxide (DMSO), both of which were purchased from Sigma-Aldrich (St. Louis, USA). The mixture was then accordingly diluted with 18.2 MΩ-cm ultrapure water before it was intraperitoneally injected[30] into zebrafish at a dosage of 10mg/kg[31]. For vehicle control, DMSO was diluted to 1% using 18.2 MΩ-cm ultrapure water and injected into zebrafish. In the behavioral study, a total of 32 zebrafish were used. Prior to melatonin administration, zebrafish were randomly divided into two groups (n = 16 each): the first was kept under 14-h light and 10-h dark (LD) conditions (Figure 1A) and the second was kept under 24-h light (LL) conditions (Figure 1B). The beginning of the subjective day (onset of light phase for zebrafish kept in LD conditions) is denoted as CT0, where CT stands for circadian time, and the start of the subjective night (onset of dark phase for fish in LD conditions) is denoted as CT14. Following exposure to their respective photoperiodic regimes, at CT2 on the second subjective day, half of the fish from each photoperiodic regime (n = 8) were given an intraperitoneal (IP) injection of melatonin (10 mg/kg) whilst the other half (vehicle control group) (n = 8) were each given

a dimethyl sulfoxide (DMSO) IP injection. The fish were subsequently allowed 30 minutes for recovery before the commencement of behavioral observation (Figure 1C).

To evaluate the temporal expression of *cry1a* and *per1a* genes in the zebrafish brain in LD conditions, zebrafish, on the second day of LD exposure, were sacrificed and their brains were harvested from CT0 to CT0 the following day at 4-hour intervals (n=4 per timepoint for *cry1a* and *per1a*, total n=28) (Figure 2A). In the next study, in order to determine the effect of melatonin administration on gene expression in zebrafish exposed to LL conditions, zebrafish were divided into two groups, the first group was intraperitoneally injected with melatonin (10mg/kg) while the second group was intraperitoneally injected with vehicle (DMSO) at CT7. Beginning from CT8, zebrafish from both groups were sacrificed with benzocaine and their brains were harvested every 4 hours until CT8 the next day (n=4 per timepoint, total n=56) (Figure 2B). For all brain samples collected, mRNA expression levels for *cry1a* and *per1a* genes were determined. Since melatonin was endogenously produced in zebrafish exposed to LD conditions, the effects of exogenous melatonin administration were not studied in the LD group.

2.3. Observation of Locomotor Activity

The entirety of the behavioral study, from IP injections to observation of locomotor activity, was carried out from CT2 to CT5 during the subjective day. Following the 30-minute recovery period after IP injection of either melatonin (10mg/kg) or vehicle, zebrafish were individually transferred into an empty tank, where their movements were observed for 10 minutes (Figure 1C). The locomotor activity of each zebrafish was analyzed using LoliTrack v2.0 software (Loligo® Systems, Vinborg, Denmark). This protocol used to study the locomotor activity of zebrafish is summarized in Figure 1. After completion of the behavioral study, the zebrafish were humanely sacrificed using benzocaine.

2.4. Brain Sampling

For the gene expression study, zebrafish brains (total n=84) were harvested according to the timelines depicted in Figure 2. Throughout the duration of sampling, the zebrafish were maintained under their respective photoperiodic regime, in LD or LL conditions. Each brain sample was immersed in 200µL of ice cold TRIzol™ reagent (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, USA) and stored at -80°C until the subsequent step.

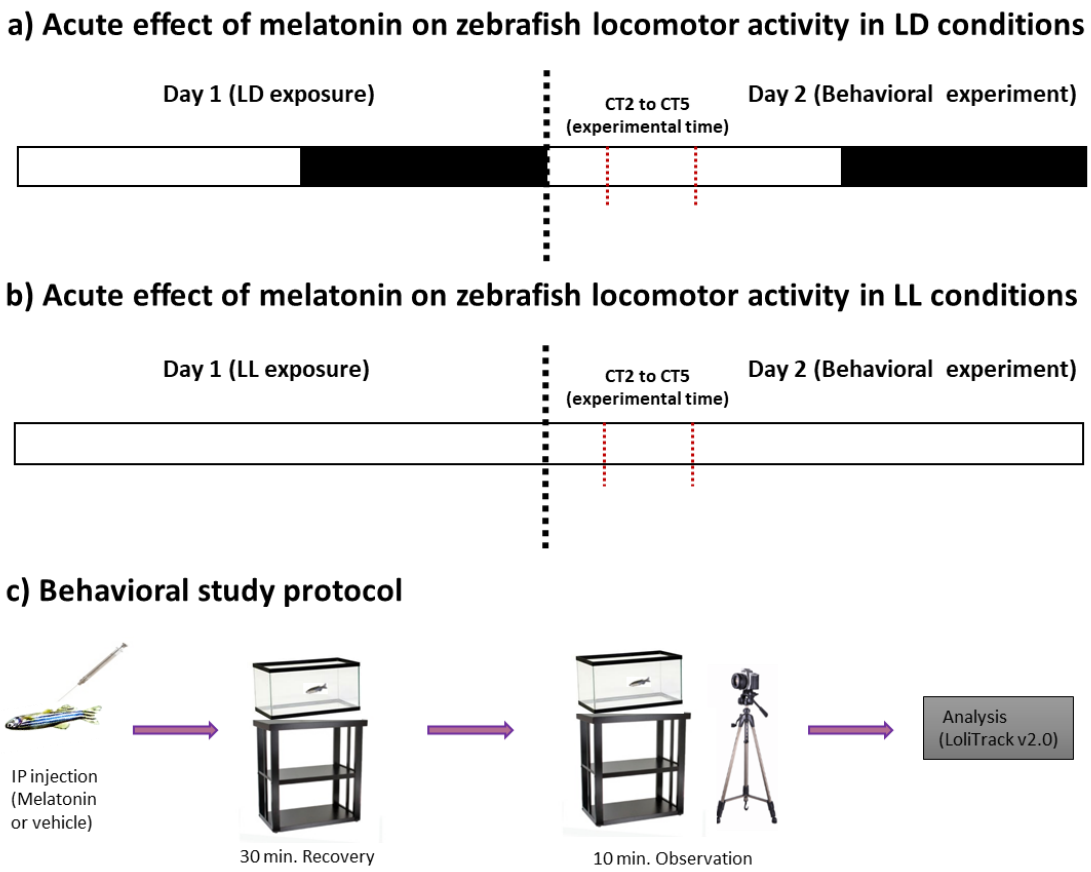

Figure 1. Experiment setup for (a) LD and (b) LL conditions and intraperitoneally injected with melatonin (10 mg/kg) or DMSO (vehicle control group). The procedure of behavioral study shown in (c) was conducted in zebrafish exposed to either photoperiodic regime.

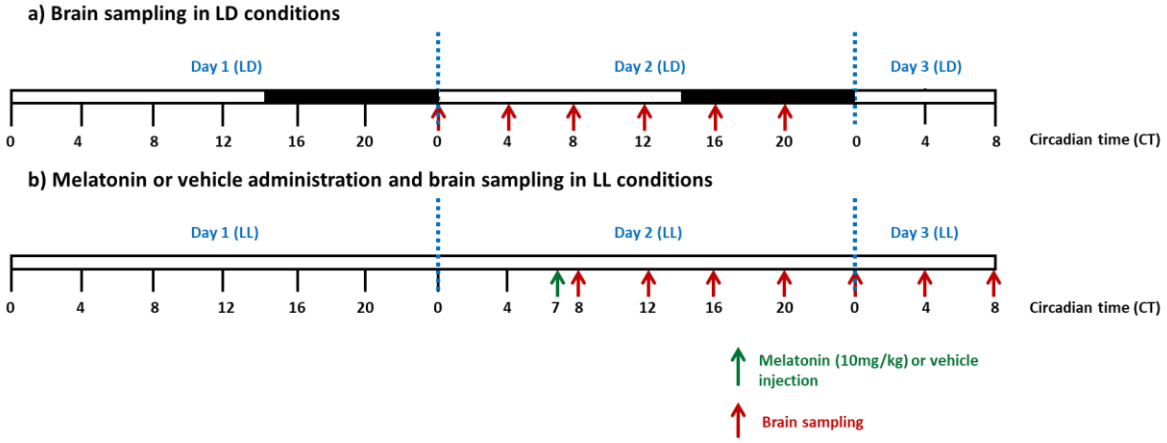

Figure 2. Timeline for gene expression study on zebrafish exposed to (a) LD and (b) LL conditions.

2.5. RNA isolation and synthesis of cDNA

This procedure was adapted from previous studies[30,32,33] and carried out in accordance with the protocol provided by the manufacturer (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, USA). To summarize, all brain samples were first homogenized in TRIzol™ reagent before chloroform was added. The mixture was then centrifuged at 13,500 x *g* for 15 minutes at 4°C. In new Eppendorf tubes, the supernatant was incubated with isopropanol at room temperature, followed by centrifugation at 13,500 x *g* for 10 minutes at 4°C. The resulting pellets were recovered after the supernatant was discarded, and were subsequently washed with 75% ethanol before once again centrifuged at 10,500 x *g* for 5 minutes at 4°C. The pellets were then air dried for up to 10 minutes before they were dissolved in nuclease-free water. A NanoDrop spectrophotometer (Implen NanoPhotometer®) was used to determine the concentration of RNA extracted. Complementary DNA (cDNA) was then synthesized using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) by following to the protocols given by the manufacturer.

2.6. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Quantitative real-time PCR (Q-PCR) was performed to assess the mRNA expression levels of the samples. In this study, the housekeeping gene used was the alpha subunit of the elongation factor-1 complex (ef1-a), along with two target genes of interest: Per1a and Cry1a. Each Q-PCR reaction mixture contained 5.0 µl of 1X Power SYBR Green PCR Master Mix (Applied Biosystems), 3.6 µl of DEPC-treated Milli-Q water, 0.2 µl of each forward and reverse primer (10 µM), and 1.0 µl of the sample. All reactions were performed in duplicate. The samples were loaded into the ABI7500 Real-Time PCR system and subjected to the following thermal cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Afterward, a melt curve analysis was conducted with the following steps: 95°C for 15 seconds, 60°C for 1 minute, 95°C for 30 seconds, and 60°C for 15 seconds. The threshold cycle (CT) values were determined using the system's software, and the mean CT value was calculated for each sample. Relative CT values were obtained by subtracting the CT value of the housekeeping gene (ef1-a) from the CT value of the target genes (Per1a and Cry1a). Relative gene expression was then calculated using the formula: 2^(Housekeeping gene CT – Target gene CT).

Table 1. Sequences of forward and reverse primers for *per1a*, *cry1a* and ef1a.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Accession no.
Per1a	CCTTCCTCAACCGCTTCAGA	GCTGTTCACTCCTTTTGTTCCA	110	NM_001030183.1
Cry1a	GGTGCCGTCCAACCCTAAT	GCGGCATCTGATACCCTGAA	110	NM_001077297.2
ef1a	ACCCTCCTCTTGGTCGCTTT	CCGATTTTCTTCTCAACDCTCTT	115	NM_131263.1

Statistical analysis. Numerical data were analyzed using Student's *t*-test (parametric, unpaired, and assuming unequal variances by applying Welch correction) in GraphPad Prism (v10.1.1) (Dotmatics, Boston, USA) with a significance threshold of $p<0.05$. All results were shown as mean ± standard error of the mean (SEM).

3. Results

3.1. Effects of Light and Acute Melatonin Administration on Zebrafish Locomotor Activity

Under both LD and LL conditions, zebrafish that received DMSO injections swam throughout the entire tank and explored all areas (Figure 3, top and bottom left). In contrast, zebrafish injected with melatonin swam mainly in the upper half of the tank near the surface under LD conditions (Figure 3, top right), whereas zebrafish treated with melatonin under LL conditions (Figure 3, bottom right) exhibited similar locomotion patterns to that of DMSO-injected control zebrafish under LD and LL conditions (Figure 3, top and bottom left).

Melatonin noticeably altered locomotor activity of zebrafish exposed to LD conditions. As opposed to DMSO-injected control zebrafish, which traversed the entire tank, zebrafish treated with melatonin primarily moved around the upper zone of the tank throughout the 10-minute observation period (Figure 3, top left and right). In contrast, melatonin did not appreciably change the swimming patterns of zebrafish under LL conditions (Figure 3, bottom left and right).

DMSO-injected control zebrafish moved a greater distance under LL conditions than under LD conditions (Figure 4). A similar trend was observed in zebrafish administered with melatonin (10 mg/kg) (Figure 4).

Under LD conditions, the administration of melatonin significantly reduced the distance travelled by zebrafish in comparison to DMSO-injected control zebrafish (Figure 4). On the other hand, melatonin treatment did not significantly affect the total distance travelled by zebrafish (Figure 4) under LL conditions.

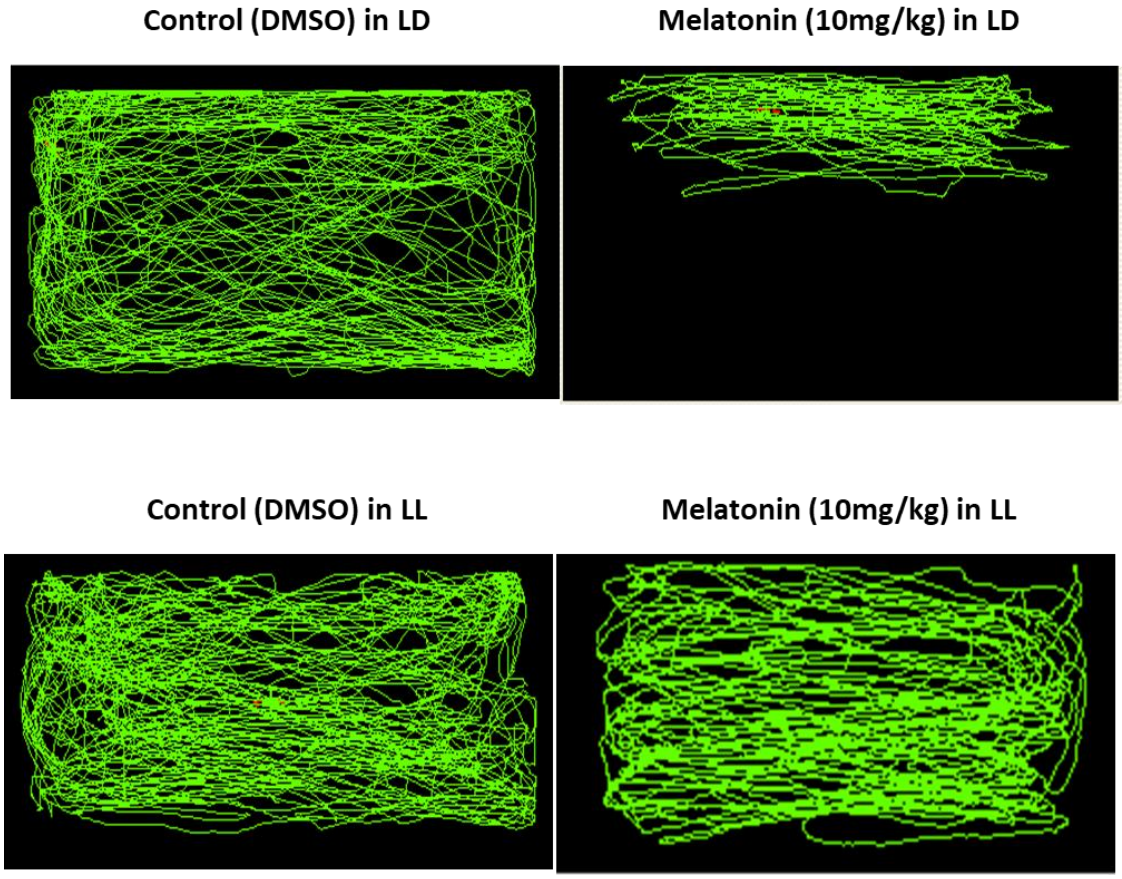

Figure 3. Representative movement traces of individual zebrafish given DMSO (control) and melatonin (10 mg/kg) under LD and LL conditions. LD, 14 h light-10 h dark cycle; LL: constant light condition; DMSO: dimethyl sulfoxide.

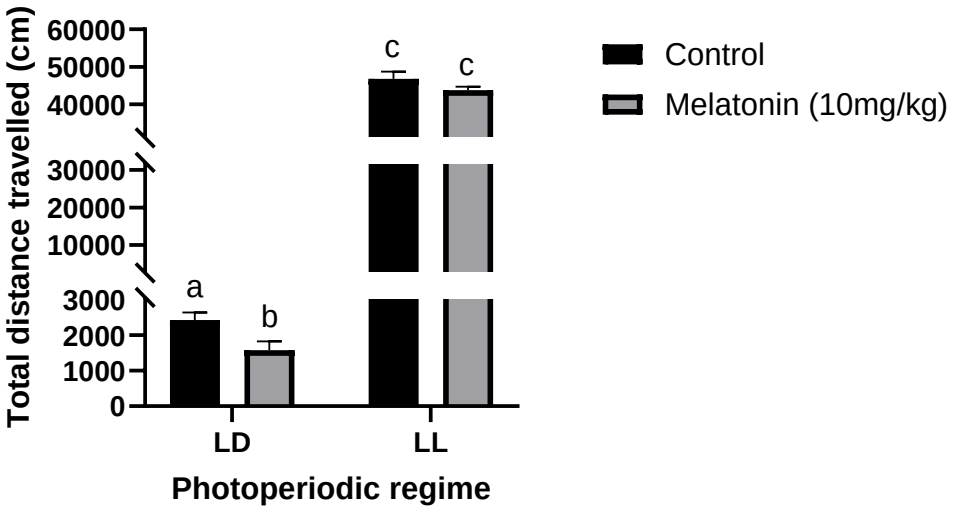

Figure 4. Total distance moved in cm by zebrafish intraperitoneally injected with DMSO (control) and melatonin (10 mg/kg) under LD and LL conditions (n=8 in each group). Each bar represents mean distance travelled (cm) by zebrafish and error bars represent the standard error of the mean (SEM). Data between groups were compared using Student's *t*-test assuming unequal variances and statistical significance ($p<0.05$) was denoted using a, b, and c. Differences in data denoted by different letters are statistically significant ($p<0.05$). LD, 14 h light-10 h dark cycle; LL: constant light condition; DMSO: dimethyl sulfoxide.

3.2. Effects of Light and Acute Melatonin Administration On Expression of cry1a and per1a Genes In Zebrafish

As shown in Figure 5, the expression of *cry1a* in zebrafish exposed to LD conditions increased from CT0 (onset of subjective day) and peaked at CT4. Expression levels then declined, reaching the lowest point at CT20 (six hours after beginning of subjective night) the following day and increasing again at CT0. While in LL conditions, expression of *cry1a* decreases in a similar manner as in LD conditions from CT8 (around middle of subjective day) to CT16 (two hours since commencement of subjective night). Expression levels then showed a general upward trend, with a slight dip at CT0 (beginning of subjective day), and later reaching the highest measured expression level at CT8 on the second day of sampling. Apart from expression levels at CT0 (start of subjective day), *cry1a* expression levels were generally higher (though not significantly) when exposed to LL conditions rather than LD conditions in DMSO-injected control zebrafish (Figure 5).

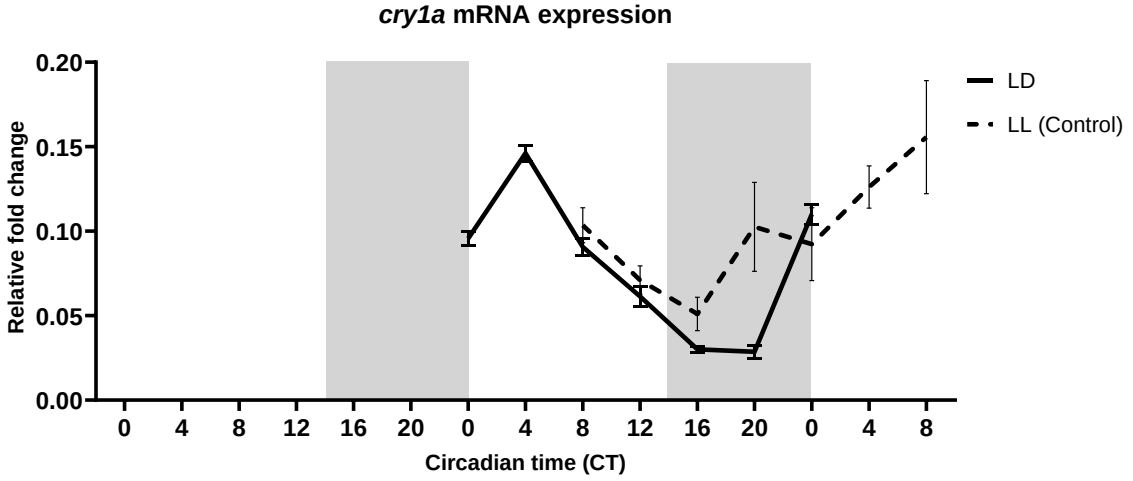

Figure 5. Variation of *cry1a* mRNA expression levels in zebrafish maintained in LD conditions and in DMSO-injected control zebrafish exposed to LL conditions. Relative expression levels of *cry1a* for LL were determined on the second day of exposure to LL conditions. Non-shaded areas represent subjective day, while shaded areas represent subjective night. Each data point represents the mean relative mRNA expression level and error bars represent SEM. LD, 14 h light-10 h dark cycle; LL: constant light condition; DMSO: dimethyl sulfoxide.

As for *per1a*, its expression level in zebrafish under LD conditions fell from the start of the subjective day at CT0 until its lowest level at CT8 and CT12 and then increasing sharply during the subjective night at CT20. Expression of *per1a* in these fish brains fell at the onset of the following subjective day (Figure 6). In DMSO-injected control zebrafish exposed to LL, *per1a* expression decreased from CT8 to its minimum at CT12 before escalating and reaching the pinnacle at CT20 during the subjective night. From there, *per1a* expression generally dropped until the final measurement at CT8 the next subjective day

(Figure 6). Significant differences in *per1a* expression between LD and LL conditions were observed at two time points: CT8 on the first subjective day of sampling and CT0 on the second subjective day of sampling. Peak expression of *per1a* in LD and LL conditions occurred at the same time (CT20) but did not vary significantly from one another (Figure 6).

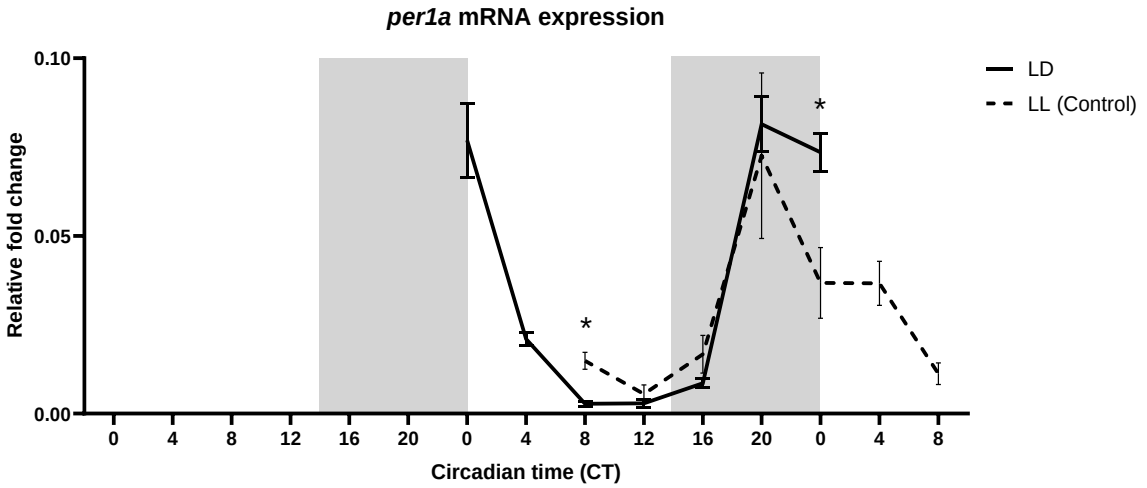

Figure 6. Variation of *per1a* mRNA expression levels in zebrafish maintained in LD conditions and in DMSO-injected control zebrafish exposed to LL conditions. Relative expression levels of *per1a* LL were determined on the second day of exposure to LL conditions. Non-shaded areas represent subjective day, while shaded areas represent subjective night. Each data point represents the mean relative mRNA expression level and error bars represent SEM. The asterisk (*) is used to denote statistical significance between relative mRNA expression levels at the same timepoint (p<0.05). LD, 14 h light-10 h dark cycle; LL: constant light condition; DMSO: dimethyl sulfoxide.

In the LL regime, the expression of *cry1a* in DMSO-injected control zebrafish reduced from CT8 to CT16 before climbing at CT20 and decreasing slightly in the next sampling time at CT0. From CT0 until the final sample at CT8 the next day, *cry1a* expression increased to its highest level measured for DMSO-injected control zebrafish in LL conditions (Figure 7). Meanwhile, in the same lighting protocol, melatonin-treated zebrafish displayed slight increase in *cry1a* expression from CT8 to CT12, which subsequently fell to its minimum at CT16 and inclined to its peak at CT4 and finally, reducing slightly at CT8 (Figure 7). No significant difference in *cry1a* expression levels between DMSO-injected control and melatonin-treated zebrafish were observed at all timepoints (Figure 7).

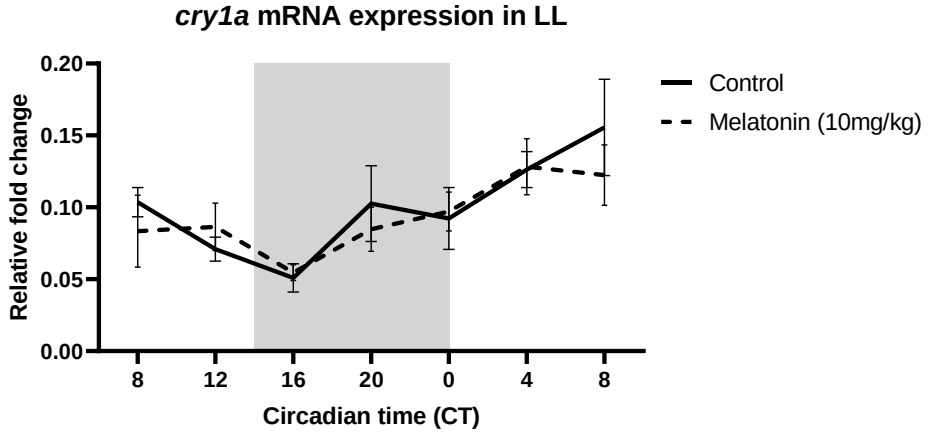

Figure 7. Variation of *cry1a* mRNA expression levels in zebrafish kept in LL conditions and injected with DMSO (control) or with melatonin (10mg/kg). Non-shaded areas represent subjective day, while shaded areas represent subjective night. Each data point represents the mean relative mRNA expression level and error bars represent SEM. LL: constant light condition; DMSO: dimethyl sulfoxide.

Next, *per1a* expression of DMSO-injected control zebrafish in LL fell from CT8 to CT12 and increased rather drastically to its zenith at CT20. The expression then showed a general decline until CT8 (Figure 8). Zebrafish injected with melatonin kept in otherwise the same conditions exhibited an increase in *per1a* expression from CT8 to CT12. A decline in expression level, followed by an incline to the pinnacle at CT0 on the next subjective day was observed. Finally, *per1a* expression in melatonin-treated zebrafish dropped until the end of the sampling duration (Figure 8). Between the DMSO-injected control and melatonin-treated zebrafish, the expression of *per1a* only varied significantly at CT12 on the first subjective day (Figure 8).

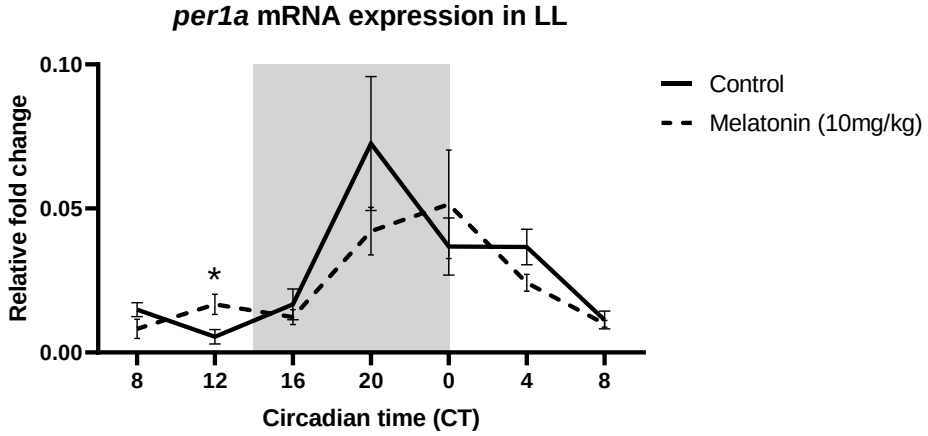

Figure 8. Variation of *per1a* mRNA expression levels in zebrafish kept in LL conditions and injected with DMSO (control) or with melatonin (10mg/kg). Non-shaded areas represent subjective day, while shaded areas represent subjective night. Each data point represents the mean relative mRNA expression level and error bars represent SEM. The asterisk (*) is used to denote statistical significance between relative mRNA expression levels at the same timepoint ($p<0.05$). LL: constant light condition; DMSO: dimethyl sulfoxide.

4. Discussion

As highlighted previously, melatonin is an important signal secreted by the pineal gland in zebrafish during the dark phase to promote sleep[27]. Exposure to LL conditions substantially increased distance travelled by control zebrafish in comparison to control zebrafish kept in the typical LD conditions (Figure 4); this result is consistent with previously published literature that reported diminished sleep in zebrafish subjected to prolonged illumination[34]. This phenomenon of inhibition of zebrafish sleep by light has been known for decades[35]; however, the mechanisms by which light represses sleep in zebrafish remain to be incompletely understood. It is thought that alterations in clock gene expression under extended light exposure[36], along with reduced melatonin secretion and the inherent photosensitivity of most zebrafish cells[35], contribute to this effect[37]. Though unlikely to be the only mechanism by which clock gene expression regulates melatonin secretion, it has been suggested that Per2, the protein product of the light-induced gene *per2*, downregulates *aanat2*, which encodes the key enzyme of melatonin synthesis, thereby suppressing melatonin secretion in zebrafish[38].

Exogenous melatonin evidently changed swimming patterns of zebrafish, predominantly causing them to swim in the top half of the tank (Figure 3). This behavioral change is indicative of the anxiolytic-like effect of melatonin on zebrafish, which is corroborated by past findings[39,40]. Consistent with results of the present study, the locomotor activity of zebrafish was shown to decrease when fish were exposed to melatonin, demonstrating its sedative effects[41]. However, another study observed that melatonin did not significantly affect the distance travelled by zebrafish relative to their control counterparts[40]. This discrepancy could be the result of differences in effective dosage of melatonin administered; in this instance, a dose of 0.232 mg/L was used via bath immersion, a method where the precise concentration absorbed by the organism is difficult to ascertain[42]. Additionally, bath immersion generally results in the lowest absorption levels compared to other drug delivery methods, which could further account for the observed differences[43]. In this study, the melatonin dosage of 10 mg/kg was chosen on the basis of previous reports in rodents[44–47] and zebrafish[31].

On the other hand, in LL, the distance travelled and swimming behavior of melatonin-treated zebrafish were similar to that of control zebrafish (Figures 3 and 4). Under light-dark (LD) conditions, melatonin is rhythmically produced by the pineal gland, with high circulating levels during the night and low levels during the day. However, under constant light (LL) conditions, melatonin production is suppressed[35]. Even with exogenous

melatonin administration, it may take longer to elevate melatonin levels, potentially impacting locomotor activity compared to the typical patterns observed under LD conditions.

Throughout the subjective day, following peak expression soon after light onset, *cry1a* expression in zebrafish under LD conditions declined until reaching its nadir in the subjective night (Figure 5), in agreement with findings from a previous study[48]. Interestingly, prior to the onset of subjective night, *cry1a* expression in LL conditions presented a similar decreasing trend to that of LD conditions, suggesting modulation of *cry1a* expression by the internal circadian clock. Although the relationship between light and *cry1a* expression is better established, some evidence also suggests that *cry1a* expression in zebrafish could also be regulated by the internal circadian clock[49–51].

In LL conditions, however, *cry1a* expression continued to rise after reaching its minimum at CT16, as opposed to LD zebrafish, whose *cry1a* expression remained low until the onset of light phase or subjective day (Figure 5). This change in *cry1a* expression patterns is likely due to differences in lighting conditions. As ample studies have shown that light is a powerful inducer of *cry1a* expression in zebrafish[48,49,52–54], one might expect for *cry1a* expression (especially at CT20) to be significantly higher in LL than in LD, but this significant difference was not observed in the present study as expected (Figure 5). The first potential contributing factor is the variation in light intensities used across different studies, as higher light intensities have been shown to increase the magnitude of *cry1a* induction in zebrafish[48]. Furthermore, previous studies typically studied the effect of light on *cry1a* expression using light pulses rather than continuous light[48,53,54]. One study that employed a sustained light regime also observed insignificant variation in *cry1a* expression in zebrafish exposed to constant light for two to five days, though sampling was only done once a day at the same time[55]. It is therefore likely that *cry1a* expression in zebrafish is regulated by both light and the intrinsic circadian clock, as is the case for *cry1* expression in the pineal gland of rats[56].

In both lighting conditions, the oscillation patterns of *per1a* expression in zebrafish appeared to be in-phase, with *per1a* expression levels peaking at the same time (Figure 6), implying that *per1a* expression is under circadian regulation, in accordance with a past study[57]. This study also showed that *per1a* expression peaks in anticipation of the start of light phase or subjective day[57], as was the case with the present study. However, the oscillatory expression of *per1a* was reported to drastically diminish under continuous light, an effect that was also observed when *cry1a* was experimentally overexpressed[48]. This substantial effect of sustained light on *per1a* expression was not observed in the present study, probably because a higher light intensity was used in the aforementioned study,

leading to increased *cry1a* expression, which more strongly downregulates *per1a* expression via inhibition of CLOCK:BMAL dimer activity[48].

Although oscillatory patterns of *per1a* are generally similar in LD and LL conditions (Figure 6), *per1a* expression significantly differed at two timepoints: CT8 on the first subjective day and CT0 on the second subjective day. Increased *per1* expression in zebrafish cells in response to light exposure has been documented, but this effect was dependent on the duration of light pulse [57]. It was postulated that the light responsiveness of *per1* is decreased when expression levels of *clock*, *bmal1*, and *bmal2* reach their maximum[57] (Zeitgeber time, ZT14 to ZT16, ZT14, and ZT10 respectively in zebrafish brain[58]). This may explain why *per1a* expression at CT8 in LL is significantly higher than that in LD (Figure 6), but not significant in the following timepoints later in the subjective day and beginning of the subjective night. The reciprocal relationship between *cry1a* and *per1a* expressions is evident in LD conditions (Figures 5 and 6): when *cry1a* expression rises, *per1a* expression falls, and vice versa. In LL conditions, however, this relationship does not hold true, especially from CT16 to CT20, where the expressions of both *cry1a* and *per1a* rose (Figures 5 and 6). Hence, we speculate that, since *cry1a* expression affects *per1a* expression[48], there is lag time between *cry1a* expression and its suppressive effects on *per1a* expression. For example, peak expression of *per1a* at CT20 in LL conditions (Figure 6) is due to the trough in *cry1a* expression in the preceding timepoint (Figure 5). Following this line of reasoning, the drop in *per1a* expression at CT0 in LL conditions (Figure 6) is a consequence of the rise in *cry1a* expression at CT20 (Figure 5). The same logic could also be applied to *cry1a* and *per1a* expressions in LD conditions. This could account for the significantly lower *per1a* expression at CT0 in LL conditions than in LD conditions (Figure 6). Further evidence is needed to substantiate this theory, as well as explore the exact timeframe in which changes in *cry1a* expression affect *per1a* expression.

It is a rather complex endeavor to ascribe the factors driving the rhythmic expression of *per1a* in prolonged light, given the complex nature of *per1a* regulation. Not only is *per1a* expression regulated by light itself[48,53] and the expression of *cry1a*[48], *clock*, *bmal1*, and *bmal2*[57], but these circadian genes and their products are also directly or indirectly influenced by each other and other participants in the TTFLs[28]. Furthermore, the expressions of these genes are also, to a certain extent, modulated by light[28]. Nevertheless, unravelling this complex circadian mechanism is important to gain better insight into the mechanism regulating the circadian system.

Melatonin administration did not notably change *cry1a* expression at any timepoint in zebrafish under LL condition (Figure 7), conflicting with results reported in a previous

study done on rats, wherein a single subcutaneous injection of melatonin markedly increased *Cry1* expression levels in the pars tuberalis (PT) of rats[21]. Similarly, increases in *Cry1* expression in response to melatonin have been reported in the PT of other mammals, including soay sheep[59,60], European hamster[61], and Siberian hamster[62]. Melatonin conveys important photoperiodic information to the mammalian PT, which then modulates the hypothalamus and pituitary gland to give rise to the appropriate adaptations (e.g., changes in prolactin and sex and thyroid hormone secretion) to seasonal changes[63,64]. A distinct PT, however, is said to be absent in fish[65]. The saccus vasculosus, which was proposed to detect photoperiodic changes in fish[65], is also absent in zebrafish[66]. Although the mechanisms by which zebrafish detect and respond to photoperiodic stimuli have yet to be fully elucidated, it is plausible that, rather than melatonin signaling, photoperiodic alterations are directly detected by non-visual opsins and extraretinal photoreceptors[67–70]. In addition to findings in the current study, this theory is also supported by another report, which found that melatonin did not significantly affect the acrophase, MESOR (midline estimating statistic of rhythm), and amplitude of *cry1* in zebrafish embryo[71]. Since melatonin has been implicated in reproductive function in zebrafish[72–75], melatonin likely acts as a downstream signaling molecule for photoperiodic regulation in zebrafish, rather than a transducer of photic information from the retina as in mammals[67]. This difference in photoperiodic regulation between species could explain the differences in the response of *cry1a* expression to melatonin administration.

The expression of *per1a* in melatonin-treated zebrafish in LL was significantly increased at one timepoint, CT12, compared to vehicle control (Figure 8). It was previously shown that aged zebrafish had considerably lower night-time melatonin content in the brain than that of their young counterparts[76]. The same study also found that *per1* expression was significantly decreased and was not phase shifted in the eyes of aged zebrafish compared to young zebrafish[76]. Although this change in *per1* expression cannot be solely attributed to the differing levels of melatonin, this correlation may suggest that melatonin potentially promotes *per1* expression in zebrafish.

In stark contrast, subcutaneous melatonin administration to rats led to a phase shift in *per1* expression without a change in amplitude in the rat SCN. Interestingly, one of the major findings of this study was that the effect of melatonin (administered at the end of the subjective day) on clock gene expression was only detected on the subsequent subjective day and no changes were observed on the first subjective night, directly after melatonin administration[77]. In the present study, however, the effects of melatonin became apparent about five hours after melatonin administration. There could be a variety of factors that influenced the effect of melatonin in both studies, for instance, the pharmacokinetic

differences (with respect to absorption, distribution, metabolism, and excretion) of melatonin in rats and zebrafish as well as the dosage of melatonin administered. Furthermore, expression levels in rats were measured specifically in the SCN[77], while in zebrafish, clock gene expression levels were determined for the whole brain. The circadian timing in which melatonin is administered is also known to influence the effect of melatonin on circadian rhythm[4,78–80]. Melatonin in our study was administered to zebrafish at CT7 (seven hours prior to onset of subjective night), whereas Poirel and colleagues injected rats with melatonin half an hour before the start of subjective night[77]. In addition, photoperiod regimes were different in both studies as well, with mRNA expression levels measured in DD by Poirel and colleagues[77] and in LL in the present study.

Another potential reason that may have produced the marked difference in the timing of melatonin effect in rats and zebrafish is the mechanism by which melatonin modulates clock gene expression. Although many possible pathways through which melatonin exerts its effects on the internal circadian clock have been documented[79,81] and proposed[4], the intricacies of the regulation of clock genes by melatonin are not entirely understood[82,83], including the extent to which each pathway contributes to the effects of melatonin and whether there are species differences. One of the leading theories is that melatonin improves the stabilities of key transcription factors CRY, PER, BMAL1, and REV-ERBα in the SCN and PT by inhibiting their degradation via the ubiquitin-proteasome system[4]. This could explain the rather long delay between melatonin administration and melatonin effect, as this pathway utilizes the post-translational mechanism[4,77]. In an in-vitro study with human renal cancer cells, melatonin was revealed to inhibit proteasomal activity[84], but further evidence of proteasomal inhibition by melatonin in the SCN and PT are needed[4]. This model, however, has yet to be substantiated in zebrafish, but a recent study has shown that loss-of-function mutation in the *fbxl3a* gene in zebrafish (encodes for F-box and leucine rich repeat protein 3 (FBXL3), which is an important component of the E3 ubiquitin ligase) interfered with the circadian regulation in zebrafish in terms of locomotor activity, sleep-wake cycles, melatonin secretion rhythm, and circadian oscillations at the molecular level[85].

In zebrafish, microRNAs (miRNAs) were found to regulate circadian gene expression and mediate the effects of light on *aanat2* expression, which controls melatonin synthesis[53,80]. In cancer studies, it is known that melatonin can also modulate miRNAs[86,87], however, regulation of miRNAs by melatonin in zebrafish have yet to be shown. If, for example, this miRNA pathway was more predominant than the proteasome pathway in zebrafish, then this may explain the reason behind the difference in timing of melatonin effect in rats[77] and zebrafish. Though more commonly associated with cancer research[88–91], miRNAs have long been implicated in circadian rhythm regulation, including melatonin

production in mammals[92–94]. Therefore, the role of miRNAs in circadian rhythm regulation in zebrafish could be an avenue worth exploring to further elucidate the complex mechanisms controlling the circadian clock in zebrafish.

In this study, only the acute effects of a single dose of melatonin in zebrafish was investigated. Further inquiry into the acute and delayed effects of repeated administration of melatonin may offer deeper insights into the role of melatonin in circadian rhythm regulation in zebrafish. The lack of uniformity and standardization of experimental procedures across circadian studies in aspects such as photoperiod regime, timing of melatonin administration[4,79,80], melatonin dosage[4], gene subtype[58,95], tissues or regions of interest[95], and sampling time or time intervals between sampling severely complicates comparison and consequent interpretation of results, therefore hindering progress in the study of circadian regulation.

5. Conclusions

In summary, constant illumination alone and in combination with an acute dose of melatonin induced *per1a* expression in zebrafish at specific timepoints, while *cry1a* expression was unaffected. Melatonin administration reduced locomotor activity in LD conditions but did not affect locomotor activity in LL conditions. Further studies are needed to shed more light on the effects of photoperiodic regimes and melatonin on the regulation of the circadian rhythm.

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