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Role of melanocortin system in the locomotor activity rhythms and melatonin secretion as revealed by *agouti-signalling protein* (*asip1*) overexpression in zebrafish

Alejandra Godino-Gimeno[1] | Esther Leal[1] | Mauro Chivite[2] | Elisabeth Tormos[1] | Josep Rotllant[3] | Daniela Vallone[4] | Nicholas S. Foulkes[4] | Jesús M. Míguez[2] | Jose Miguel Cerdá-Reverter[1]

[1]Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal, IATS-CSIC, Fish Neurobehaviour Lab, Castellon, Spain

[2]Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía and Centro de Investigación Mariña, Universidade de Vigo, Vigo, Spain

[3]Department of Biotechnology and Aquaculture, Instituto de Investigaciones Marinas, IIM-CSIC, Vigo, Spain

[4]Institute of Biological and Chemical Systems—Biological Information Processing (IBCS-BIP), Department of Physiological Information Processing, Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany

Correspondence
Jose Miguel Cerdá-Reverter, Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), Ribera de Cabanes, 12595 Castellón, Spain.
Email: jm.cerda.reverter@csic.es

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Abstract

Temporal signals such as light and temperature cycles profoundly modulate animal physiology and behaviour. Via endogenous timing mechanisms which are regulated by these signals, organisms can anticipate cyclic environmental changes and thereby enhance their fitness. The pineal gland in fish, through the secretion of melatonin, appears to play a critical role in the circadian system, most likely acting as an element of the circadian clock system. An important output of this circadian clock is the locomotor activity circadian rhythm which is adapted to the photoperiod and thus determines whether animals are diurnal or nocturnal. By using a genetically modified zebrafish strain known as Tg (*Xla.Eef1a1*:*Cau.asip1*)iim04, which expresses a higher level of the agouti signalling protein 1 (Asip1), an endogenous antagonist of the melanocortin system, we observed a complete disruption of locomotor activity patterns, which correlates with the ablation of the melatonin daily rhythm. Consistent with this, in vitro experiments also demonstrated that Asip1 inhibits melatonin secretion from the zebrafish pineal gland, most likely through the melanocortin receptors expressed in this gland. Asip1 overexpression also disrupted the expression of core clock genes, including *per1a* and *clock1a*, thus blunting circadian oscillation. Collectively, these results

implicate the melanocortin system as playing an important role in modulating pineal physiology and, therefore, circadian organisation in zebrafish.

KEYWORDS
agouti, circadian clock, food intake, melanocyte-stimulating hormone (Msh), pineal, pomc, sleep

1 | INTRODUCTION

Melanocortin peptides are processed from the complex precursor proopiomelanocortin (Pomc) which is mostly synthesised in the pituitary gland. Two small neuronal populations in the arcuate nucleus of the hypothalamus and the nucleus of the solitary tract of the medulla also produce Pomc in mammals, which is processed to yield melanocyte-stimulating hormone (α-Msh) and β-endorphin.[1] Melanocortin peptides bind to five G-protein coupled receptors (Mc1r-Mc5r). Subtype 2 only binds the adrenocorticotropic hormone (Acth) while the remaining four distinctively recognise the different MSH peptides (α-, β- and γ-Msh).[2] Mcrs mediate a wide range of functions including skin and fur pigmentation (Mc1r), energy balance (Mc3r and Mc4r) and stress response (Mc2r).[1] Atypically, Mcrs are also regulated by endogenous antagonists called agouti-signalling protein (Asip) and agouti-related protein (Agrp). Asip is mainly produced in the hair follicle regulating the pigment synthesis in melanocytes by antagonising the effects of α-Msh on Mc1r. Agrp is also synthesised in the arcuate nucleus inhibiting melanocortin signalling at Mc3r and Mc4r to promote food intake.[3]

The teleost-specific genome duplication led to the emergence of paralogous genes for *asip* (agouti signalling protein 1 [*asip1*] and *asip*2) and *agrp* (*agrp*1 and *agrp*2), although the evolutionary history of the agouti family remains unclear (Cortés et al.[2] and Guillot et al.[4]). *asip1* is predominantly expressed in the ventral skin[5] to regulate dorso-ventral pigment patterns[6,7] whereas *agrp1* is expressed in the lateral tuberal nucleus of the hypothalamus, a homologue of the mammalian arcuate nucleus[8] and regulates food intake.[9–13] *agrp*2 is expressed in the retinal pigment epithelium-like (RPE-LC) cells of the zebrafish pineal complex[11,14] and in a small neuronal subpopulation of the preoptic area, the homologous region of the mammalian paraventricular hypothalamic nucleus.[15] Pineal expression is not involved in regulating circadian rhythms of locomotor activity (LA) yet preoptic expression appears to play a role in the stress response.[11] The function of *asip*2 remains unexplored, yet it is overexpressed in the skin of pseudo-albino flatfish.[16] Recent studies have shown that zebrafish overexpressing *asip1* (*asip1-Tg*) exhibit hyperphagia and reduced satiety signalling.[4,17] *asip1-Tg* also shows behavioural disruption, with transgenic animals exhibiting less aggressiveness than their wild-type (WT) counterparts.[18] Intriguingly, *asip1-Tg* show reduced melatonin levels during scotophase.[4] This suggests that transgenic animals may exhibit disrupted LA, since a blockade of the circadian oscillator in the melatonin-producing pineal photoreceptors disrupts LA circadian rhythms,[19] and ablation of melatonin synthesis in the pineal gland disrupts the circadian adjustment of the sleep-wake cycles.[20] Current research shows that nocturnal melatonin secretion in *asip1-Tg* is severely disrupted, which is consistent with nocturnal hyperactive locomotor patterns. Accordingly, daily rhythms of the central serotonin (5-HT) and clock gene expression are also disrupted. Finally, it was demonstrated that Asip1 dose-dependently inhibits in vitro pineal melatonin secretion thus supporting a role for the melanocortin system in pineal gland physiology and the regulation of teleost circadian rhythms.

2 | MATERIAL AND METHODS

2.1 | Animals

WT-Tuebingen (Tu) strain (body weight [BW] = 0.246 ± 0.008) and transgenic [Tg(Xla.Eef1a1:Cau.Asip1)iim04][7] zebrafish (*Danio rerio*) (BW = 0.307 ± 0.015) were kept at the Institute of Aquaculture of Torre de la Sal facilities under standard conditions (27–28°C, 14 h/10 h light [L]/dark [D] cycle [lights on at 7:00 and off at 21:00]). Details are provided in supporting information.

2.2 | Activity experiments

LA studies were carried out in a light and sound-isolated room at 28°C with 12 h:12 h LD cycles (lights on at 9:00 a.m. and off at 21.00). Thirty-day-old zebrafish for each genotype were distributed in six 6-litre tanks ($n = 10$ per tank) equipped with two infrared sensors (E3ZD822, Omron) (Supporting Information S1: Figure S1)

connected to a computer system (AUTOMATA) which recorded sensor activations. Access to the experimental area was prohibited, except for feeding (1.5% BW) once daily at 11.00. After 30 days adjustment, LA was monitored for 28 consecutive days and data integrated at 10 min intervals. Subsequently, the LD cycle was inverted (DL) and then LA was monitored for 12 days. LA was further monitored for 6 days under constant DD following the same experimental protocol. The experiment was performed three independent times on naïve animals. Subsequently, two identical experimental LD replicas were carried out to evaluate clock gene daily expression (replica 1) and melatonin/neurotransmitter determination (replica 2). Sixty fish for each genotype per replica were distributed in twelve 6-litre tanks. Following 30 days, one group of each genotype ($n = 10$) was euthanized by an overdose of anaesthesia (MS222, tricaine methane sulphonate; 300 mg/L) at 4 h intervals. Whole brains were dissected. Nocturnal samplings were carried out under dim red light. Animals fasted for 24 h before sampling.

2.3 | Gene expression studies

qPCR was used to quantify gene expression of one reference gene (β-actin) as normalising factor and five target clock genes, *bmal1*, *clock1a*, *cry1a, n1dr1* and *per1b*. Details are provided in supporting information.

2.4 | Pineal organotypic culture

Zebrafish pineal glands from WT or *asip1-Tg* were dissected under a stereomicroscope and placed in PBS in pools ($n = 3$) for superfusion. See supporting information for more details.

2.5 | Melatonin and monoamine determination

Brain melatonin and neurotransmitters were assayed by HPLC according to Guillot et al.[4] See supporting information for more details.

2.6 | Statistical analyses

Variations in brain gene expression, melatonin and monoamines levels at different 24-h time points and genotypes were analysed by two-way analysis of variance (ANOVA) (GraphPad Prism 8). Variation in melatonin levels of superfusion fractions or average levels were assayed by one-way ANOVA. LA actograms and mean waves were plotted with 'El Temps' software v 1.313 (http://el-temps.com). The rhythmicity in brain gene expression, melatonin and monoamines was examined by cosinor analysis run in the aforementioned software and 'Cosinor Online' software (https://cosinor.online/app/cosinor.php). Variation between average levels of LA during L and D phases in both genotypes were assayed by two-way ANOVA ($p < .05$) whereas variation between average levels of brain melatonin and monoamines in both genotypes were assayed by *t* test. Variation was considered when $p < .05$.

3 | RESULTS

3.1 | Activity patterns

WT fish exposed to 12 h:12 h L (light) D (darkness) photoperiod exhibited robust daily rhythms of LA. When the cycle was inverted to 12 h:12 h DL, WT rapidly adjusted their activity pattern to the new conditions (Figures 1 and 2) (Supporting Information S1: Figures S2–S4). *asip1-Tg* also exhibited circadian LA patterns as revealed by the cosinor model (Figure 2) yet activity levels were much higher than those recorded for WT (Figure 3) (Supporting Information S1: Figures S2–S4) (Supporting Information S2: Table S2). No variation was found in the average levels of LA between light and dark phases in *asip1-Tg* under both LD and DL photoperiods (Figure 3). However, diurnal LA of WT was higher than nocturnal activity yet no variation between the same phases (L or D) was observed when the photoperiod was inverted. *asip-Tg1* animals also exhibit enhanced activity under constant DD (constant darkness) conditions (Supporting Information S1: Figures S5 and S6).

3.2 | Melatonin and monoamine brain levels

Due to the small size of the pineal gland in zebrafish, whole-brain melatonin was assayed together with the central monoamine levels in animals of both genotypes subjected to 12 h:12 h LD photoperiods. WT samples exhibited significant daily variations in melatonin and 5-HT levels yet only melatonin displayed circadian oscillations. However, *asip1-Tg* samples failed to exhibit melatonin or 5-HT rhythmic oscillations (Figure 4). Neither central dopamine nor noradrenaline exhibited significant daily variations. Dopaminergic and serotoninergic activity, as revealed

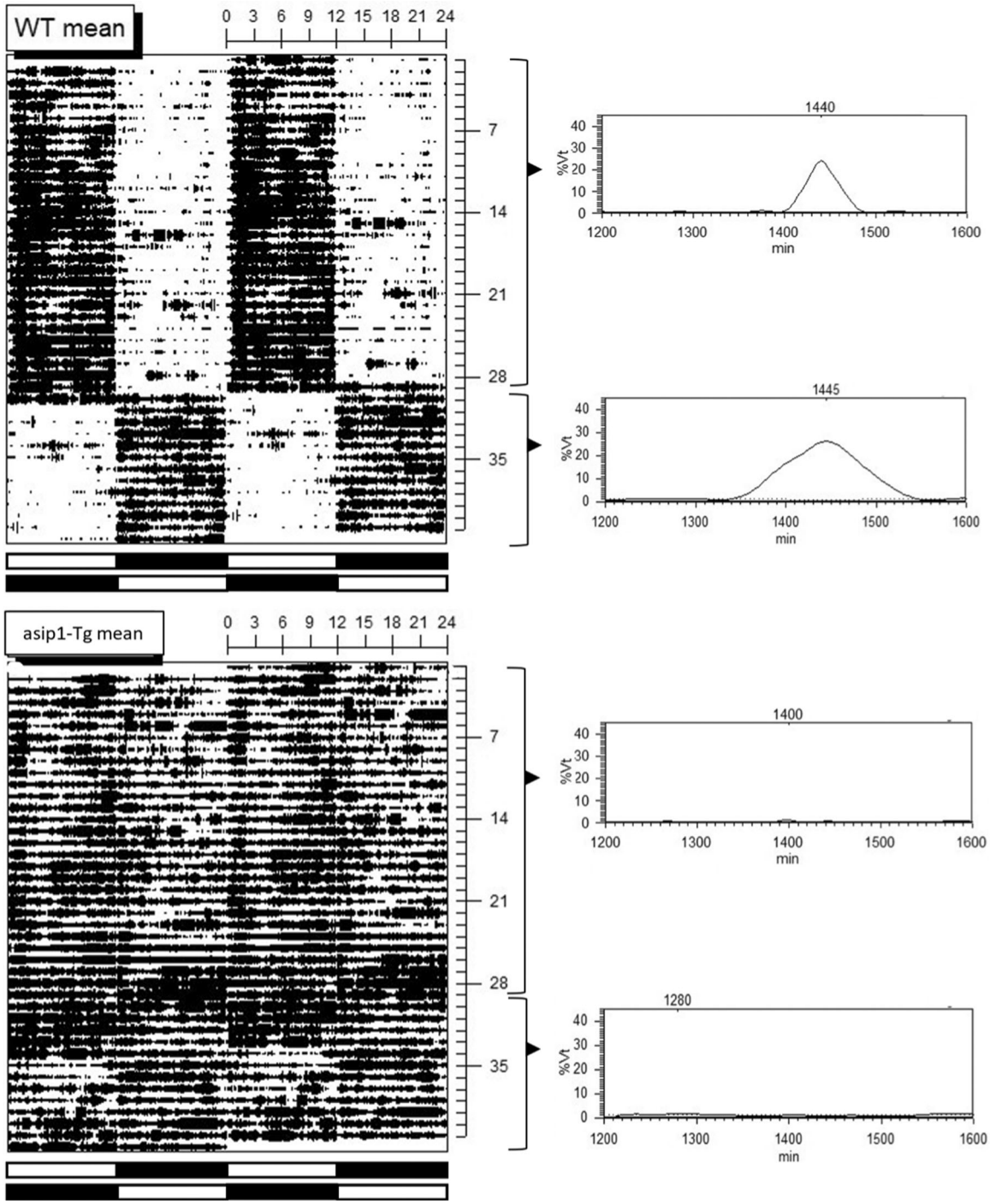

FIGURE 1 Charts (left) show the actograms of locomotor activity of wild-type (WT) (top) and *asip1*-Tg (bottom) animals maintained under a constant LD photoperiod for 28 days. Subsequently, the LD cycle was inverted (DL) and locomotor activity screened for 12 days. Actograms are presented in a dual-plot format (48 h time scale) to facilitate visualisation. Horizontal bars at the bottom represent periods of daylight (in white) and night (in black) for 2 consecutive days. The x-axes indicate time of day (h) and y-axes indicate time (days). Charts (right) show periodogram analysis providing the periodicity of the data with tau values close to 1440 m (24 h) for WT animals under LD and DL. No periodicity was detected in *asip1*-Tg animals.

by DOPAC/dopamine and 5-HIAA/5-HT ratios, displayed significantly inverted fluctuations throughout the LD phases yet only dopaminergic waves fitted to circadian periods in both WT and *asip1-Tg* genotypes (Figure 4). WT exhibited higher melatonin and 5-HT average levels, whereas both average dopaminergic and serotoninergic activity was increased in *asip1-Tg* (Figure 5).

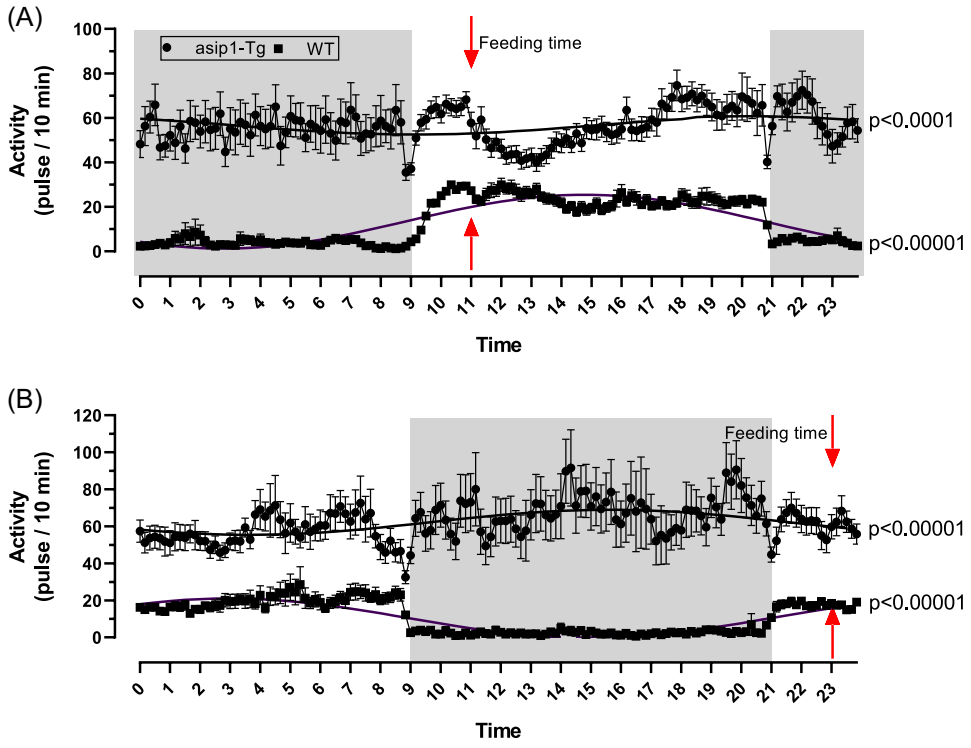

FIGURE 2 The average waveform of locomotor activity (LA) for adult *asip1-Tg* and wild-type zebrafish kept under constant LD (A) or DL (B) photoperiods. LA was recorded by infrared sensors for 28 consecutive days and pulses were integrated every 10 min. Each point represents the mean ± SEM (Standard Error of the Mean) of three 6 L tanks with 10 animals for each genotype. Rhythmicity was checked by cosinor fit run in El Temps v 1.313 (http://el-temps.com) and Cosinor Online (https://cosinor.online/app/cosinor.php) software. The *p* value of the fit is given on the right. The red arrow and grey charts indicate the feeding times and scotophase of the photoperiod, respectively.

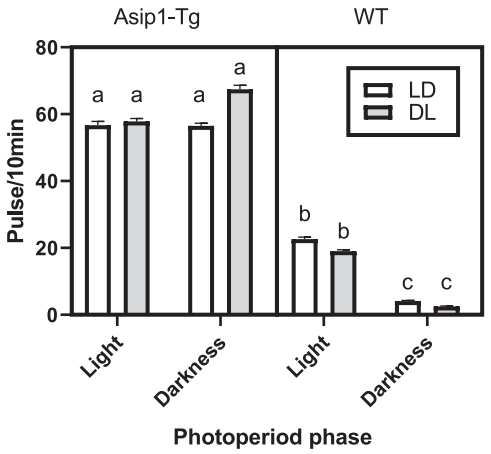

FIGURE 3 Total average locomotor activity (LA) of *asip1* Tg (left chart) and wild-type (right chart) animals kept under LD (28 days) and subsequently inverted DL (12 days) photoperiod during the light and darkness phases. Different letter indicates significant differences after two-way analysis of variance ($p < .05$). See Figure 2 for further details.

3.3 | In vitro effects of melanocortin antagonists on pineal melatonin release

In vivo experiments suggest that *asip1* overexpression can reduce melatonin synthesis and/or secretion. To further test this hypothesis, zebrafish pineal glands were superfused with graded mammalian Asip concentrations. Higher doses of Asip induced a significant time-dependent decrease in melatonin secretion. The effect was detected just after the peptide superperfusion at the lower doses (Figure 6). Accordingly, average melatonin secreted levels were significantly lower in higher Asip-perfused doses (Figure 6).

3.4 | Core clock gene expression

It was hypothesised that the rhythmic expression of central clock genes could be masked in *asip1-Tg*. Transgenic animals lost both *per1b* and *clock1a* gene

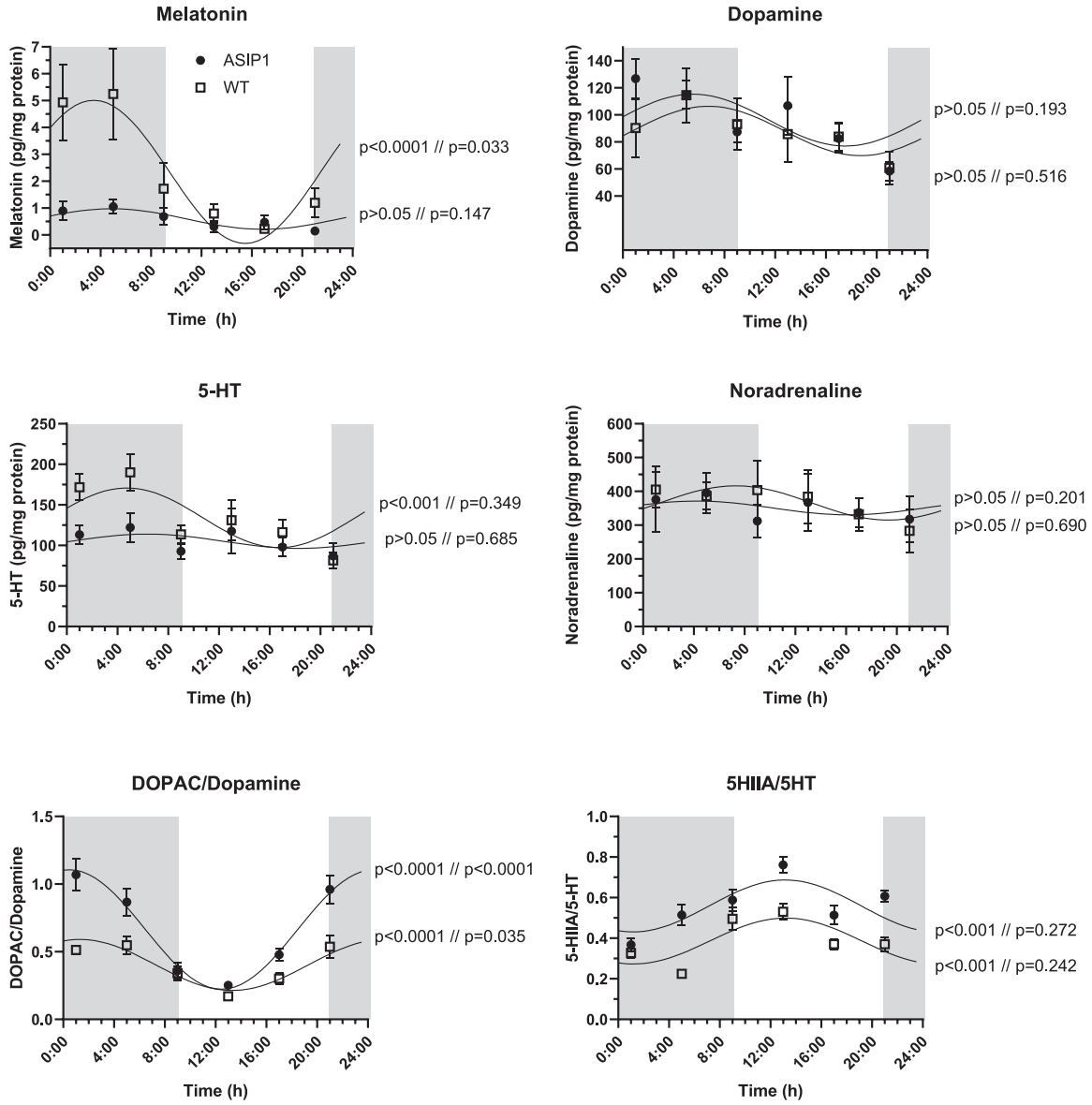

FIGURE 4 Neurotransmitters and melatonin daily rhythms of *asip1* Tg and wild-type animals ($n = 10$) kept under constant LD photoperiod for 30 days. Grey bars indicate the dark phase of the photoperiod. Each point represents the mean ± SEM ($n = 10$). Differences among sampling times of the same genotype were assessed by one-way analysis of variance (ANOVA) and average values fitted to Cosinor curves. Significance values for each analysis are given close to the cosinor curves (ANOVA//Cosinor).

expression rhythmicity which exhibited daily rhythms in WT. *cry1a*, *bmal1a* and *nr1dr1* gene expression all exhibited similar significant daily expression patterns in both genotypes, yet nocturnal expression levels of *nr1d1* were significantly higher in WT (Figure 7).

3.5 | Hypothalamic *pomc* expression

It was further postulated that the increased LA could be associated with anxiety driven by appetite as suggested by increased food intake levels promoted by a disruption in the satiety system in *asip1-Tg*[4,17] and the reduced 5-HT levels in *asip1-Tg*. Expression studies revealed depressed *pomc a* and *b* expression levels during the dark phase of the photoperiod in the transgenic animals. Indeed, daily significant variation in *pomc* expression levels were masked in *asip1-Tg* (Figure 8).

4 | DISCUSSION

Central melanocortin agonists and antagonists are key peptides in controlling food intake and energy expenditure in vertebrates,[3] including fish.[9,11,21] Accordingly, *asip1* overexpression results in increased food intake,

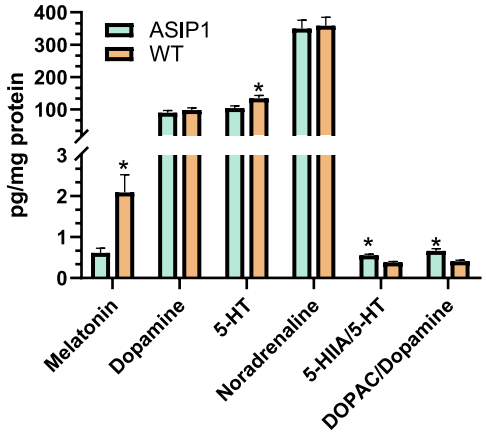

FIGURE 5 Neurotransmitters and melatonin average levels of *asip1* Tg and wild-type animals ($n = 60$) kept under constant LD for 30 days. * indicates significant differences after *t* test ($p < .05$).

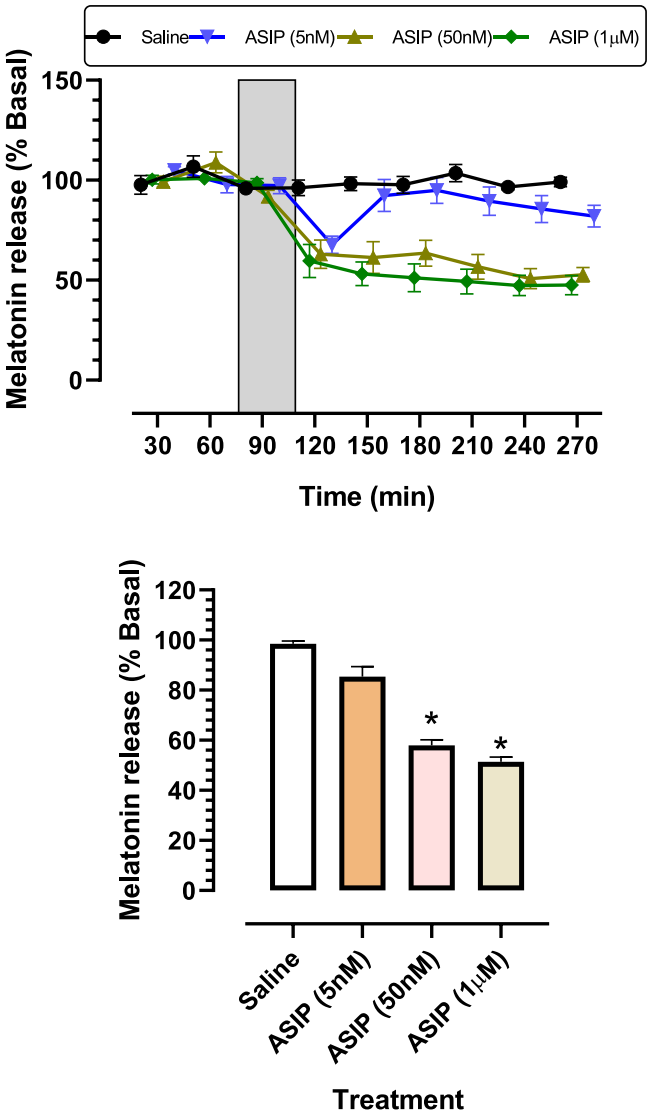

FIGURE 6 Top chart shows melatonin levels secreted from pineal glands superperfused with a constant flow rate of 5 μL/min with saline or melatonin. Fractions were collected at 30 min intervals. Each point represents the mean ± SEM (saline and 1 mM $n = 5$, 5 and 50 nM $n = 6$). Grey bar indicates superfusion period. Experiments were performed in triplicate. Bottom chart displays average levels of melatonin release from pineal glands after superfusion. Each bar represents the mean ± SEM ($n = 10$) from all collected fractions (saline and 1 mM $n = 45$, 5 and 50 nM $n = 54$).

linear growth and food conversion, yet not an obese phenotype.[4,17,22] *agrp1* overexpression results in a similar phenotype.[10–12] Food intake is the final stage of a complex behavioural process which includes food seeking, sensory evaluation, learning and final consumption. Thus, feeding should be associated with daily LA patterns during the active phase of the sleep-wake cycle suggesting a coordinated behavioural regulation. Previous studies showed that *asip1-Tg* have a reduced nocturnal melatonin surge,[4] suggesting a potential role of the melanocortin system in circadian behaviour. LA patterns were examined in *asip1-Tg* as an output of the daily clock under LD conditions, and revealed a severe disruption of LA rhythms due to very high activity during the scotophase. Indeed, *asip1-Tg* consistently showed increased LA during both the photophase and scotophase, yet unlike the WT genotype, no decrease in the activity levels was observed during the nocturnal period. The persistence under constant conditions (DD) suggests an effect on endogenous rhythmicity. These results coincided with a severe disruption of melatonin secretion which completely inhibited the nocturnal pulse, masking its daily oscillation in *asip1-Tg*. Similarly, *asip1* overexpression disrupted 5-HT circadian cycles in the brain by reducing nocturnal levels, suggesting that reduced nocturnal melatonin could be due to reduced 5-HT availability.

The melatonin rhythm is itself an output of the vertebrate circadian clock.[23,24] The molecular architecture of this clock is highly conserved, yet there are significant differences in the organisation of circadian systems. In mammals, the pineal gland is not photosensitive and is indirectly regulated by the master circadian clock in the suprachiasmatic nucleus (SCN), which receives light information from the retina. In birds, the pineal gland is photosensitive but can be regulated in a dual mode, either by SCN afferents or independently by an intrinsic clock. In fish, the pineal gland is directly regulated by light. The SCN has been recognised in most fish brain atlases, including zebrafish,[25] but its functional orthology to the mammalian structure remains controversial. Watanabe and coworkers[26] demonstrated the expression of *per2* in the SCN of flounder, opening up the possibility of a role for the SCN as a circadian pacemaker, but further studies are

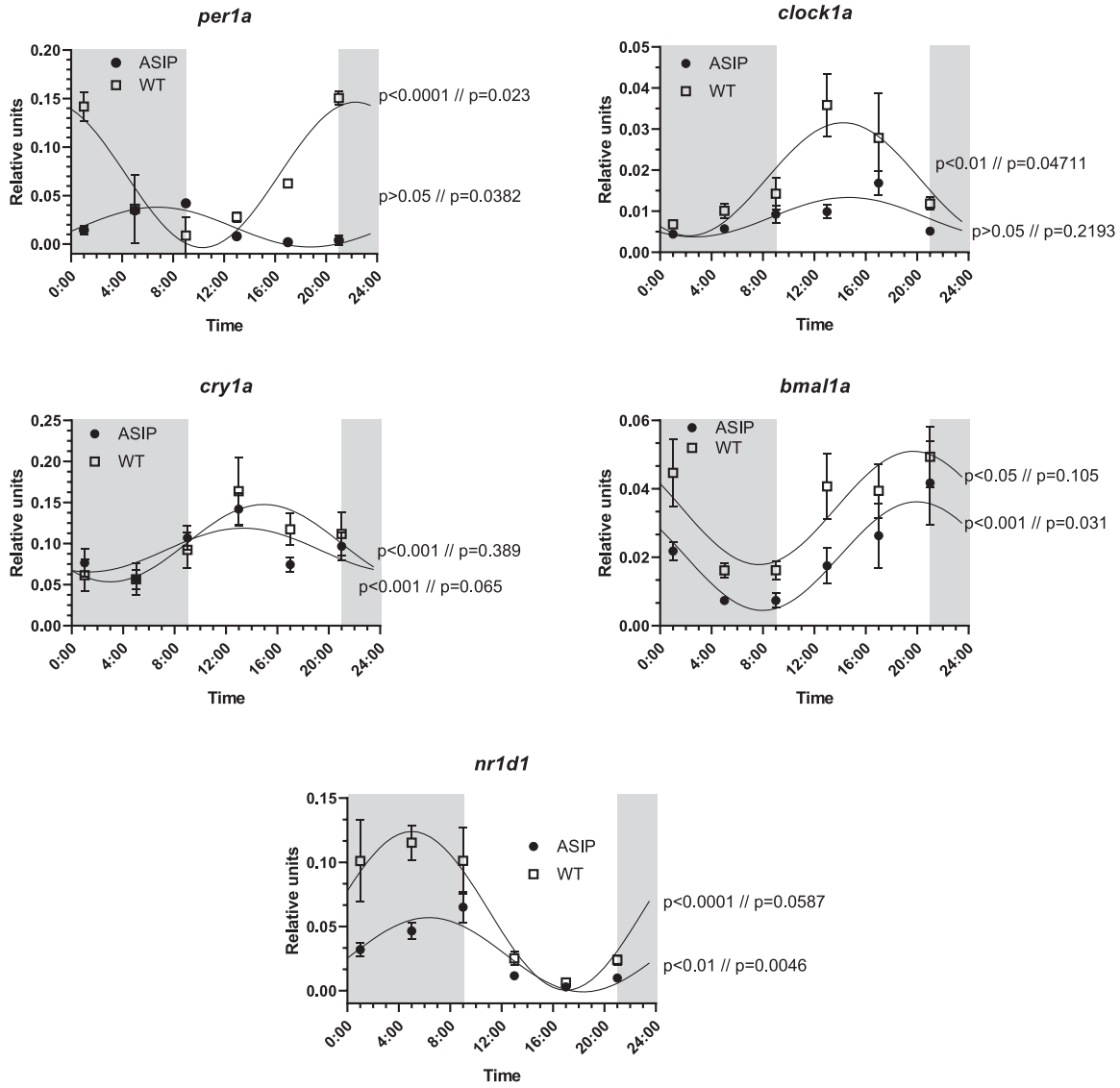

FIGURE 7 Daily rhythms of clock gene expression of *asip1* Tg and wild-type animals ($n = 10$) kept under constant LD photoperiod (12 L/12D, lights on/off at 9 and 21 h, respectively) for 30 days. Grey bars indicate the dark phase of the photoperiod. Each point represents the mean ± SEM ($n = 10$). Difference among sampling times of the same genotype were assessed by one-way analysis of variance (ANOVA) and average values fitted to Cosinor curves. Significance values for each analysis are given close to the Cosinor curves (ANOVA//Cosinor).

needed to demonstrate that some species with specific lifestyles may use the SCN nucleus as a master circadian clock. As no functional equivalent of the SCN has been properly found, the pineal gland is considered to be the master clock organ in fish[27] yet the role of the pineal gland in the oscillator hierarchy may vary between fish species.[28,29] In support of this idea, studies in zebrafish showed that cyclic expression of a reporter gene driven by the *nr1d1* promoter starts in the pineal photoreceptors and then spreads to other brain regions.[30] In addition, *per2* expression has been shown to be higher in the pineal gland than in other regions,[31] as are many accessory clock genes.[32] Pineal-less mutants also show reduced amplitude in behavioural rhythms,[33] a similar effect to that found after blocking its molecular oscillator (Ben-Moshe Livne et al.[19] see below). However, the presence of photoreceptive peripheral oscillators that can be entrained by light,[34] with a similar molecular structure[35,36] in zebrafish, suggests a distributed circadian system that challenges the notion of a master pineal clock at least in this species. To obtain further evidence for the disruption of the circadian system in *asip1-Tg*, the expression of key clock genes in the whole zebrafish brain was examined, including the pineal gland. Once more, not only did *asip1* overexpression disrupt the daily expression of clock genes including *per1a* and *clock1a*, but also reduced the amplitude of *nr1d1* gene expression during the scotophase. The transcription factors Clock

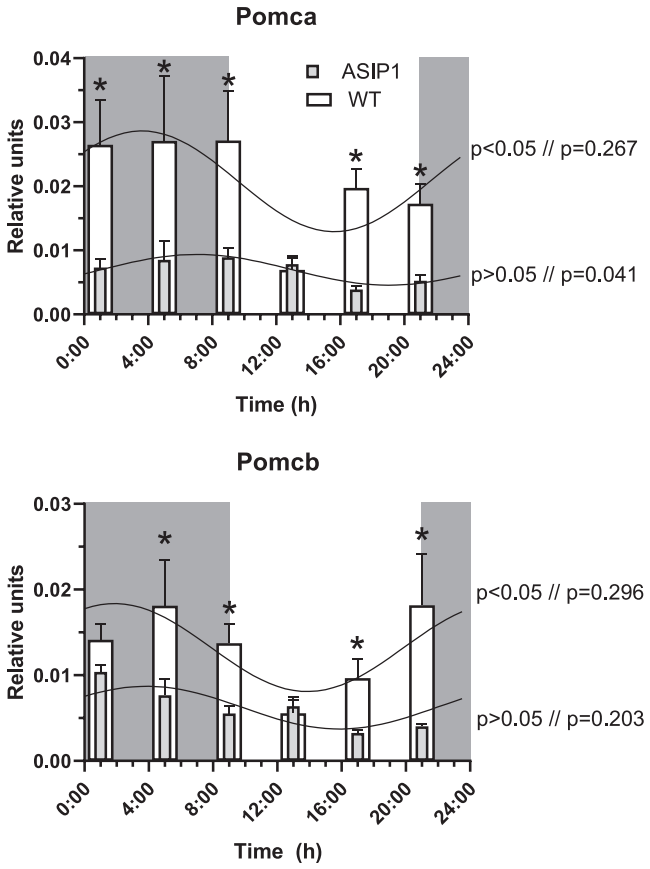

FIGURE 8 Daily rhythms of Pomc a (top) and Pomc b (bottom) encephalic expression of *asip1* Tg and wild-type animals ($n = 10$) kept under constant LD photoperiod for 30 days. Grey bars indicate the dark phase of the photoperiod. Each point represents the mean ± SEM ($n = 10$). Difference among sampling times of the same genotype were assessed by one-way analysis of variance (ANOVA) and average values fitted to Cosinor curves. Significance values for each analysis are given close to the Cosinor curves (ANOVA//Cosinor).

and Bmal in vertebrates bind as heterodimers to E-box enhancers in the promoters of the genes encoding the transcriptional repressors Per and Cry, which in turn repress the transcriptional activity of *clock*/*bmal*, by reducing the expression of their own genes (*per* and *cry*) and thus closing the feedback loop.[36] Studies in zebrafish have shown that the synergistic action of Bmal/Clock regulates the transcriptional activity of *aanat2* via binding to E-box enhancers in the gene promoter, providing a direct molecular mechanism for the effects of Asip1 on melatonin secretion.[37] Results suggest that *asip1* overexpression disrupts the central molecular clock, which subsequently abolishes the melatonin daily rhythm and ultimately leads to a disruption of the LA rhythm. The pineal gland has been proposed as a behaviour-regulating clock centre in zebrafish since a blockade of its molecular oscillator masks circadian rhythms of vertical positioning in the water column under constant dark conditions (DD).[19] However, the LA pattern remained steady under DD in such animals (ΔClock), yet the amplitude was significantly reduced, suggesting that additional central oscillators may play a role in controlling circadian behaviours. Interestingly, ΔClock animals exhibited normal melatonin rhythms under constant LD conditions yet were disrupted under DD conditions. In our model, both the melatonin and LA pattern were entirely disrupted under LD conditions, suggesting that the melanocortin system may rely upon regulation by other central clocks, in addition to the pineal molecular clock, to mask melatonin rhythmicity and behavioural outputs.

It is not immediately obvious how alterations in the dorso-ventral pigment pattern of *asip1* Tg may modify the sensitivity of the pineal gland to light exposure. *asip1* overexpression inhibits dorsal melanogenesis and manages the chromophore fate control, by drastically reducing the number of melanophores.[5–7] Re-evaluation of the dorsal morphology of *asip1-Tg* shows that the reduction in the number of melanocytes solely affects the dorsal trunk, not the dorsal region of the head including the pineal window.[6] Therefore, a morphological explanation of the results is unlikely. This leads to the question concerning how melanocortin encoding information reaches the pineal complex to inhibit the nocturnal melatonin surge and disrupt the circadian LA patterns. The pineal gland is known to express some melanocortin receptors, suggesting a direct role for the melanocortinergic peptides in pineal physiology.[38] To address such a hypothesis, zebrafish pineal glands were dissected and incubated with graded concentrations of mammalian Asip1. Results show that Asip1 can reduce melatonin secretion in a dose-dependent manner which suggests that inhibiting endogenous melanocortinergic activity through specific receptors leads to the inhibition of melatonin secretion. Therefore, these in vitro results mimic those observed in *asip1* Tg, thus further supporting the role of melanocortin system in pineal physiology.

The zebrafish pineal gland is reactive to Agrp antibodies but not to α-MSH,[39] presumably due to cross-reactivity with Agrp2, which is highly expressed in the zebrafish pineal complex.[14] Nevertheless, larval *agrp2* knockouts and ablated RPE-LC, which express *agpr2* in the pineal, exhibit robust circadian LA rhythms under constant dim light conditions. This suggests that Agrp2 and/or RPE-LC cells are irrelevant for the maintenance of locomotor behaviour in zebrafish larvae.[11] Although the experimental conditions and age of the animals differ significantly (larvae vs. adult fish), the agrp2 knockout results suggest that the potential Asip1/Agrp2 structural resemblance cannot explain the

circadian disruption in *asip1-Tg*. However, it should be noted that Agrp2 may play an alternative role in regulating the pineal circadian clock in adult fish.

Asip1 can antagonise all Mcrs with different efficiency[4] yet it is apparently not expressed in the fish pineal gland (data obtained from sea bass *Dicentrarchus labrax*, Agulleiro et al.[40]). *asip1* expression was re-evaluated in our *Drer.asip1-iTol2-eGFP*-BAC transgenic line (Cal et al.[41]) and no expression signal was obtained in the pineal complex, yet animals exhibit robust expression levels in the retina (Supporting Information S1: Figure S7). Furthermore, very limited levels of *asip1* expression were demonstrated in the zebrafish brain.[41] Therefore, it is plausible that the ubiquitous *asip1* expression in our transgenic model may mimic the role of hypothalamic *agrp1*, potentially projecting to the pineal gland. Tracking studies have shown that no pinealopetal cells are labelled in the zebrafish brain after tract-tracing with carbocyanine dyes (Dil)[42] yet the presence of *mc1r*, *mc3r* and *mc4r* mRNAs in the zebrafish pineal has been demonstrated.[38] The absence of pinealopetal neurons has also been reported in several fish species, yet other species including sea bass (*D. labrax*) and sturgeon have an extensive pinealopetal system (see Servili et al.[43] for references). Such a discrepancy could be due to species-specific differences but also to the result of the plasticity of the pinealopetal system in response to different physiological and/or environmental conditions.[43] Once more, the information available does not support the presence of interneurons connecting the tuberal hypothalamus, where both *agrp1* and *pomc* are expressed, and the pineal complex in zebrafish, although the potential plasticity of the pinealopetal system cannot completely rule out this hypothesis. Alternatively, melanocortin agonists and/or antagonists could reach the pineal gland via the systemic circulation. The pineal complex has an extensive choroid plexus, the *saccus dorsalis*, which is highly vascularised and can be accessed by peripheral hormones for regulatory purposes. Indeed, the pineal gland seems to be modulated by systemic hormones in zebrafish since the absence of leptin promotes a decrease in brain melatonin levels.[44]

Constitutive signalling of the MCRs could be another alternative explanation. Previous studies have shown that Mc1r and Mc4r in fish can signal in the absence of agonists with constitutive activity and that both Asip1 and Agrp1 can act as inverse agonists.[4,45,46] It is then plausible that the pineal Mcrs impose a melanocortinergic tone required for the regular functioning of the molecular clock, which is disrupted by ectopic *asip1* overexpression. Indeed, in vitro studies support this hypothesis. The physiological significance of melanocortin-induced inhibition of melatonin secretion needs to be further explored, as does the mechanism by which melanocortins reach the pineal complex. *asip1-Tg* exhibit hyperphagia[4,17] due to a depressed satiety system.[4] Transgenic fish also display subordinate personalities, elevated cortisol levels[18] and high anxiety levels mediated by depressed central 5-HT levels (Guillot et al.[4], Rocha et al.,[18] Godino-Gimeno et al., unpublished results). It has been observed that 5-HT circadian oscillations are masked in *asip1* Tg. 5-HT is an anorexigenic neurotransmitter in vertebrates, including zebrafish (Lei Wee et al.[47] and references therein), which acts partly by regulating hypothalamic Pomc neurons.[48] Moreover, activation of arcuate Agrp neurons inhibits Pomc neurons throughout the GABAergic projections.[49] Our transgenic animals exhibit reduced levels of 5-HT and *pomc a/b* expression, suggesting they may be particularly hungry during the nocturnal period. Unfortunately, we were unable to replicate the zebrafish self-feeding systems reported by del Pozo et al.[50] to estimate nocturnal food intake. It is, therefore, plausible that the high activity level of *asip1* Tg is related to the increased anxiety driven by appetite, which ultimately results in increased foraging behaviour and LA.

Melatonin is also required for controlling sleep, which is regulated by a homoeostatic process that responds to internal and external cues. This homoeostatic regulation is evidenced by a compensatory increase in intensity and duration after deprivation. A circadian process that responds to external cues sets the timing of sleep.[20,23] The timing of sleep in mammals, like other circadian rhythms, is controlled by the SCN, which regulates melatonin secretion in the pineal gland, as well as the rhythmic secretion of neuropeptides and monoamines.[23] Melatonin is a potent sleep-promoting hormone in fish.[51] Since nocturnal melatonin levels in *asip1* Tg are comparable to those of WT during the light period, the obvious question is whether the transgenic animals sleep during the nocturnal period. Adult zebrafish show reduced LA and an increased arousal threshold after short-term forced wakefulness, yet after long-term sleep deprivation many animals become hyperactive even for some time after the deprivation has ended without sleep rebound.[52] It is, therefore, plausible that the hyperactivity observed in *asip1* Tg is driven by the potential sleep deprivation imposed by low nocturnal melatonin levels. As previously suggested, the hyperactivity observed in *asip1* Tg, even during daytime, could reflect the absence of homoeostatic regulation of sleep in adult fish (see Zhdanova[52] and Cirelli and Tonon[53]).

In conclusion, we show that overexpression of endogenous melanocortin antagonists disrupts the central molecular clock in zebrafish, coinciding with the masking of melatonin daily rhythms. Furthermore, in

vitro experiments mimic in vivo Asip1-induced inhibition of melatonin secretion. The absence of nocturnal melatonin correlates positively with the ablation of LA rhythms, suggesting that Asip1-induced disruption of the molecular clock promotes the abolishment of melatonin rhythms and the subsequent ablation of LA daily rhythms. How this downstream sequence from the molecular clock relates to the sleep-wake and feeding cycles is unknown, however, the *asip1* Tg model could help to elucidate the integration between feeding, locomotor behaviour and the circadian timing system.

AUTHOR CONTRIBUTIONS

Alejandra Godino-Gimeno: Methodology; validation; formal and statistical analysis; investigation; data curation; visualisation; reviewing and editing. **Esther Leal**: Methodology; validation; investigation; reviewing and editing. **Mauro Chivite**: Methodology; data curation; visualisation; reviewing and editing. **Elisabeth Tormos**: Methodology; validation; investigation; reviewing and editing. **Josep Rotllant**: Resources; reviewing and editing; material; reviewing and editing. **Daniela Vallone**: Methodology; supervision; reviewing and editing. **Nicholas S. Foulkes**: Supervision; reviewing and editing. **Jesús M. Míguez**: Methodology; supervision; reviewing and editing. **Jose MiguelCerdá-Reverter**: Conceptualisation; methodology; validation; investigation; data curation; formal and statistical analysis; funding acquisition; supervision; writing-original draft; reviewing and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets that support the findings of the study are available from the corresponding author upon reasonable request (jm.cerda.reverter@csic.es).

ORCID

Jose Miguel Cerdá-Reverter http://orcid.org/0000-0003-1405-5750

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