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Novel functions for Period 3 and Exo-rhodopsin in rhythmic transcription and melatonin biosynthesis within the zebrafish pineal organ

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Abstract

Entrainment of circadian clocks to environmental cues such as photoperiod ensures that daily biological rhythms stay in synchronization with the Earth's rotation. The vertebrate pineal organ has a conserved role in circadian regulation as the primary source of the nocturnal hormone melatonin. In lower vertebrates, the pineal has an endogenous circadian clock as well as photoreceptive cells that regulate this clock. The zebrafish opsin protein Exo-rhodopsin (Exorh) is expressed in pineal photoreceptors and is a candidate to mediate the effects of environmental light on pineal rhythms and melatonin synthesis. We demonstrate that Exorh has an important role in regulating gene transcription within the pineal. In developing embryos that lack Exorh, expression of the *exorh* gene itself and of the melatonin synthesis gene *serotonin N-acetyl transferase 2 (aanat2)* are significantly reduced. This suggests that Exorh protein at the cell membrane is part of a signaling pathway that positively regulates transcription of these genes, and ultimately melatonin production, in the pineal. Like many other opsin genes, *exorh* is expressed with a daily rhythm: mRNA levels are higher at night than during the day. We find that the transcription factor Orthodenticle homeobox 5 (Otx5) activates *exorh* transcription, while the putative circadian clock component Period 3 (Per3) represses expression during the day, thereby contributing to the rhythm of transcription. This work identifies novel roles for Exorh and Per3, and gives insight into potential interactions between the sensory and circadian systems within the pineal.

Keywords

Circadian rhythm; Exo-rhodopsin; Orthodenticle homeobox 5; Period 3; Pineal organ; Serotonin N-acetyl transferase; Transcriptional regulation

1. Introduction

Circadian rhythms are physiological and behavioral changes that occur with a period of approximately 24 hours. These oscillations are driven by an intracellular molecular clock and

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are self-sustaining even in the absence of environmental cues. However, the clock is entrained each day by light or other external stimuli. Since the period of circadian clocks is typically slightly longer or shorter than 24 hours, this entrainment has a critical role in ensuring that daily biological rhythms stay in synchronization with the world around.

In vertebrates, the pineal organ has a central role in the regulation of circadian rhythms as the primary source of circulating melatonin [53]. Melatonin is made during the night and acts to regulate circadian and seasonal rhythms, including daily sleep/wake cycles. In mammals, melatonin also feeds back to regulate the primary circadian clock located in the suprachiasmatic nucleus (SCN), and thus serves as a strong entraining factor [54]. Poor regulation of pineal melatonin rhythms has been related to sleep disorders, feelings of fatigue and confusion, and an increase in cancer risk [4,9,43,45].

Entrainment of mammalian pineal rhythms to environmental light is mediated by opsin proteins located in retinal photoreceptor and ganglion cells. These photoreceptive cells entrain the SCN clock, which then controls pineal rhythms through a multisynaptic pathway [30]. In contrast, the pineal of lower vertebrates contains photoreceptive cells that entrain an endogenous pineal circadian clock, likely through an opsin-mediated signaling cascade that is very similar to that found in retinal photoreceptors [30].

In zebrafish, pineal photoreceptors have been implicated in several responses to light, including entrainment of the circadian clock, triggering the onset of pineal rhythms during development, and mediating acute suppression of melatonin by a light pulse during the dark period [11,60, 63–65]. The pineal opsin protein *Exorh* is expressed in pineal photoreceptors from early embryogenesis to adulthood and is thus an excellent candidate to be mediating these light responses [5,38,60]. Consistent with this, the action spectrum of acute melatonin suppression in isolated adult pineal organs suggests that *Exorh* is one of several opsins involved in this process [65].

Many of the vertebrate opsin genes involved in circadian and visual photoreception are rhythmically expressed [6–8,13,17,23–25,29,31,33,35,42,49,50,55,59]. We find that like these other opsin genes, *exorh* is expressed with a significant difference in day and night mRNA levels. Through *in vivo* over-expression and loss-of-function experiments we have identified three proteins that regulate *exorh* transcription. The pineal transcription factor *Otx5* is required to activate *exorh* expression within the pinealocytes. The circadian regulated factor *Period 3* (*Per3*), whose function in mammals has remained elusive, has an important role in influencing the timing of *exorh* expression. Finally, *Exorh* protein is required for expression of its own gene. These findings suggest a model in which tissue-specific factors and clock components act together to control the spatial and temporal pattern of *exorh* expression. In addition, we provide the first direct evidence for an *in vivo* function of *Exorh*. In addition to regulating transcription from its own gene, *Exorh* protein is required for high expression of *aanat2*, which encodes the penultimate enzyme in the melatonin synthesis pathway. Interestingly, the reduced expression of *aanat2* in *Exorh*-depleted embryos closely matched *aanat2* expression in embryos raised in constant darkness. This suggests that *Exorh* could be mediating the light-dependent initiation of *aanat2* transcription and melatonin production in the developing pineal.

2. Results

exorh transcription is rhythmic

The *exorh* gene is expressed in pineal photoreceptors from approximately 18 hours post fertilization (hpf) to adulthood [5,19,38,60]. Vuilleumier and colleagues recently reported that *exorh* is expressed with a daily rhythm, with higher expression during the dark phase of the circadian cycle [60]. However, they found that the day/night differences in expression were

not statistically significant [60]. Consistent with this, we also found differences in the day and night levels of *exorh* transcripts (Figure 1). However, in contrast to this earlier study, we find that the level of *exorh* transcripts were often significantly lower during the day than during the night (Figure 1C and Supplementary Table 1). Since Vuilleumier et al. and this study both used whole mount RNA in situ hybridization (WISH), which is a semi-quantitative technique, this small discrepancy in our results is likely due to subtle differences in experimental conditions.

Otx5 activates *exorh* transcription

The transcription factor Otx5 is expressed in the developing and mature pineal, and has been previously shown to be important for the expression of several circadian-regulated pineal genes [20]. Otx5 is also a strong candidate to regulate the transcription of *exorh*. The *exorh* promoter contains three putative Otx binding sites 5' to the translation start site. Mutation of these sites attenuates the ability of the *exorh* promoter to drive transgene expression in the pineal [5].

To test whether Otx5 is required for *exorh* transcription, embryos were injected with an antisense morpholino (MO) that binds to the translation start site in the *otx5* mRNA (*otx5* MO). This MO has been previously shown to specifically and effectively knock down Otx5 protein levels [20]. Control embryos were injected with a MO containing 4 base pair mismatches (*otx5* MIS) [20]. Injected embryos were raised in a light/dark (LD) cycle, fixed at regular intervals over a 24 hour period, and assayed for *exorh* expression. Control-injected embryos had a rhythmic pattern of *exorh* expression similar to that of uninjected fish (Compare Figure 1B, C with Figure 2A, B). In contrast, Otx5-depleted embryos had severely reduced or undetectable levels of *exorh* mRNA at all time points tested (Figure 2A, B). This finding indicates that Otx5 is required for *exorh* transcription in pineal photoreceptors.

To determine whether Otx5 is capable of driving *exorh* expression in cells outside of the pineal, we took advantage of the *exorh*:Green Fluorescent Protein (*exorh*:GFP) transgenic line [5]. Single blastomeres of 8–16 cell stage zebrafish embryos were co-injected with *otx5* and Red Fluorescent Protein (RFP) mRNA. Control embryos were co-injected with β -galactosidase (β -gal) and RFP mRNA (Figure 2C). As co-injected mRNAs are inherited together, the RFP serves as a tracer to identify the progeny of the injected cell. Embryos were allowed to develop until approximately 24 hpf and then analyzed by fluorescence microscopy.

Embryos injected with RFP mRNA alone developed normally and the RFP-positive cells were scattered throughout the embryo. Further, ectopic GFP expression was present only very rarely in cells outside of the pineal (Figure 2C, Table 1). In embryos over-expressing Otx5, the RFP/Otx5-positive cells tended to be clustered together. Further, many of the red fluorescent cells were also green fluorescent, indicating that the *exorh*:GFP transgene was being expressed (Figure 2C, Table 1). As has been previously reported, *otx5* mRNA injections also caused developmental defects, likely due to the role of Otx proteins in patterning the forebrain (Figure 2C)[20].

Per3 negatively regulates *exorh* transcription during the day

otx5 mRNA is expressed at constitutively high levels, and Otx5 protein is required for the activation of pineal genes expressed at dawn, during daylight, and at night, suggesting that it is active at all times of day [20]. Thus, it is unlikely that Otx5 alone could control the rhythmic expression of *exorh*. Instead, we hypothesized that the timing of *exorh* transcription is regulated by a rhythmically expressed factor, such as a component of the pineal circadian clock.

The putative clock component Per3 is a potential candidate for this role because the *per3* gene is expressed widely in the developing zebrafish brain with a strong daily rhythm that peaks at dawn [18]. To determine whether *per3* expression is present within pinealocytes, the brains of

3 days post fertilization (dpf) fish were divided into left and right halves by a sagittal cut through the midline. At this stage, the pineal has begun to evaginate from the forebrain, and in bisected embryos appears as a distinct domain separated from the rest of the brain by a translucent area that may correspond to the developing saccus dorsalis (Figure 3A) [28,36]. *otx5* expression can be easily distinguished within this domain (Figure 3A). *per3* transcripts were also detected in the pineal domain at dawn, when *per3* expression in the brain is at its highest point (Figure 3A)[18]. In contrast, expression in the pineal was severely decreased at a night time point, when expression in the surrounding neural tissue was also low (Figure 3A)[18].

Per proteins typically regulate the timing of gene expression by repressing gene transcription [44]. Consistent with this, we found that *exorh* transcripts were up-regulated during the day in embryos injected with a *per3* MO (Figure 3B, C). In contrast, *exorh* mRNA levels were unaffected at time points during the dark period or in embryos injected with a control MO containing 5 base pair mismatches (*per3* MIS)(Figure 3B, C). This indicates that Per3 is required to suppress *exorh* mRNA levels during the light period of the circadian cycle.

aanat2 is a circadian-regulated gene that encodes an enzyme required for melatonin biosynthesis in the pineal [22]. *aanat2* is expressed in a similar phase to *exorh*, with high mRNA levels at night and very low levels during the day [20,22]. Further, like *exorh*, *aanat2* transcription in the pineal is activated by Otx5 [20]. However, in contrast to the results for *exorh*, there were no apparent effects on *aanat2* transcription in Per3 depleted embryos (Figure 3B).

These data suggest that Per3 is present in the pineal, and could be acting directly within pinealocytes to influence *exorh* expression. The *per3* promoter is able to drive circadian expression of luciferase in adult zebrafish pineal organs [27]. Thus, it is possible that the role of Per3 in regulating *exorh* rhythms could continue through adulthood.

Loss of Exorh protein reduces transcription from the *exorh* promoter

The early pineal-specific expression of Exorh makes it an excellent candidate to function as a major light-sensing photopigment in the developing pineal [19,64]. Towards testing the function of Exorh, we designed a MO that binds to the exon 1/intron 1 junction of the *exorh* genomic sequence (*exorh* sp MO). Embryos injected with *exorh* MO had severely reduced levels of *exorh* mRNA compared to embryos injected with a control MO (Figure 4A, B). Embryos were analyzed using a probe that binds to the 3' end of the *exorh* open reading frame and recognizes both correctly and incorrectly spliced transcripts [38]. Thus, the reduced expression suggests that *exorh* transcripts were being degraded due to improper splicing, and that the *exorh* MO effectively knocks-down Exorh protein.

The normal onset of rhythmic *exorh* transcription requires exposure of embryos to a light/dark or dark/light transition [60]. Since Exorh protein is likely involved in pineal photoreception, this raises the possibility that Exorh could regulate the expression of its own gene. To test this, we injected *exorh* sp MO into embryos carrying the *exorh*:GFP transgene [5]. As this transgene does not contain the *exorh* sp MO binding site, its expression cannot be directly affected by the injections. Despite this, fluorescence in the pineal was significantly reduced in Exorh depleted embryos compared to embryos injected with a control MO (Figure 4C, D, G).

To verify this result, the experiment was repeated using a second, non-overlapping MO that binds to the translation start site of the *exorh* mRNA (*exorh* atg MO). Since start site MO bind to the target mRNA but do not directly affect the levels of transcripts, the effects of this MO could be assessed by measuring expression of the endogenous gene [40]. Consistent with the *exorh* sp MO results, injection of the *exorh* atg MO caused significantly reduced expression of *exorh* (Figure 4I, J, K, Supplementary Figure 1).

Although loss of opsin proteins can lead to photoreceptor cell death [26,32,34,47], we found no evidence for loss of photoreceptors in the Exorh depleted fish. The embryonic pineal is largely composed of photoreceptors [5,20,21,39]. Therefore, if photoreceptor cells were dying, there would be a significant change in the size of the pineal. However, the length and width of the pineal *otx5* expression domain, which encompasses both photoreceptor cells and projection neurons, was unaltered in fish injected with either exorh MO (Figure 4E, F, H, L). Therefore, it is unlikely that the reduction in *exorh*:GFP transgene expression was due to loss of photoreceptor cells. Instead, this suggests that the decrease was caused by a down-regulation of *exorh* promoter activity.

Exorh does not initiate transcription of red opsin

Being a photoreceptive organ, the pineal in zebrafish expresses a number of opsins besides *exorh*, including the same RGB cone pigments found in the eye [46]. The fact that activation of an Exorh-dependent pathway is important for transcription of *exorh* in the pineal, suggests that initiation of this same pathway could be required for expression of other pineal opsins. To test this, we determined the effect of Exorh depletion on the expression of *opsin 1* (*cone pigments*) *long-wave-sensitive*, 1 (*opn1lw1*), formerly known as *red opsin* [46]. *opn1lw1* expression was not significantly different in exorh atg MO and control injected embryos (Figure 5A, C). In contrast, *exorh* transcripts were severely reduced in exorh atg MO injected embryos from the same experiment (Figure 5A, C).

Exorh is required for high levels of *aanat2* expression

Expression of the melatonin biosynthetic gene *aanat2* is high at night and very low during the day, accounting in part for the vastly higher levels of melatonin made during the dark period of the circadian cycle [22]. Previous work has demonstrated that the onset of *aanat2* transcription in zebrafish requires exposure of embryos to a transition in lighting conditions from dark to light or from light to dark. We found that depletion of Exorh protein caused a significant reduction of the levels of *aanat2* expression in developing embryos (Figure 5A, B). Again, the control experiment done in parallel showed the expected reduction in *exorh* transcripts following Exorh depletion (Figure 5A, B). This suggests that Exorh could be wholly or partially responsible for triggering *aanat2* transcription in response to changes in lighting conditions.

3. Discussion

Exorh protein is important for gene transcription in the zebrafish pineal organ

Exorh protein shares a 70% sequence identity with the photoreceptive retinal opsin protein Rhodopsin, and is predicted to have seven transmembrane domains typical of G-protein coupled receptors [38]. These observations, together with the early pineal-specific expression of *exorh*, are consistent with Exorh functioning as a major light-sensing molecule in the developing zebrafish pineal.

Previous work demonstrates that expression of *exorh* and *aanat2* in the pineal does not initiate normally when embryos are raised in constant darkness [60,64]. For instance, expression of *aanat2* is reduced by 58–68% when embryos are moved to constant darkness shortly after fertilization [64]. Here, we provide some of the first evidence that Exorh is involved in this light response in developing embryos. In particular, MO-mediated depletion of Exorh protein caused a very similar reduction (>50%) as raising embryos in darkness. This suggests a mechanism wherein light received by Exorh protein initiates a signaling cascade that ultimately results in onset of high levels of *aanat2* and *exorh* transcription (Figure 6).

The most likely role for Exorh is as a pineal photopigment. Consistent with the pineal photoreceptors being active at these early stages, other potential components of a pineal phototransduction cascade, such as the components of transducin heterotrimeric G-proteins, are also expressed in the developing pineal [10,37,52]. However, we do not yet know how the phototransduction cascade interfaces with the pineal transcriptional machinery. Previous work on the *aanat2* regulation by Yoav Gothilf and colleagues gives some insight into how this might occur. The light-dependent onset of *aanat2* transcription in the pineal requires Per2 [63,64]. Transcription of the *per2* gene itself is induced by light [12,63,64]. Thus, Per2 has the potential to act as an intermediate between Exorh on the cell membrane and clock components such as Clock/BMAL on the *aanat2* promoter (Figure 6).

The pattern of exorh transcription is controlled by a combination of tissue-specific and rhythmic factors

This study demonstrates that *exorh* expression has a significant daily rhythm in the embryonic and larval pineal photoreceptors of zebrafish. Further, we define the *in vivo* functions of three proteins, Exorh itself, Otx5, and Per3 in the regulation of the *exorh* expression pattern (Figure 6). We find that Exorh protein is required for normal transcription from its own promoter, suggesting that a phototransduction pathway containing Exorh regulates *exorh* transcription. Further, we find that Otx5 and Per3 have complementary roles. Our loss- and gain-of-function experiments strongly suggest that Otx5 functions as an activator to induce *exorh* transcription in the cells of the pineal. In contrast, Per3 influences the phase of expression by suppressing transcription during the day.

Many other opsin genes are also expressed with a strong daily rhythm [6–8,13,17,23–25,29, 31,33,35,42,49,50,55,59]. However, the function of this rhythmic expression is not well understood. One possibility is that transcription is rhythmic in order to drive rhythmic expression of the Exorh protein. For instance, the cyclic pattern of *exorh* closely matches the expression of several cone opsin genes, which peak at dusk and remain highly expressed through the dark period [23,29]. Interestingly, cone cells are not used for vision during this period of high expression. Korenbrot and Fernald proposed that it is metabolically advantageous to accumulate opsin protein during the dark period before rapid turnover begins at dawn [29], and this could be the case for Exorh as well.

Another possibility is that Exorh is required for a critical function during the night. There is some evidence to support this hypothesis, as Exorh has been implicated in the acute suppression of melatonin levels that occurs when adult zebrafish are exposed to a light pulse during the night [65]. However, rhythmic transcription is not always followed by cyclic expression or cyclic activity of the encoded protein. For instance, expression of the *interphotoreceptor retinoid binding protein (irbp)* gene in the zebrafish retina has a strong circadian rhythm, while protein levels remain essentially constant [16]. Similar to the case with cone opsin, it is thought the high expression of *irbp* mRNA during the day compensates for high turnover of IRBP protein.

Otx5 has now been shown to be required for the expression of four rhythmically expressed pineal genes (*exorh*, *aanat2*, *irbp*, and *reverb-alpha*), but not for the expression of three non-rhythmic pineal genes (*otx5*, *cone rod homeobox (crx)*, and *floating head*) ([20] and this study). Together, this suggests that the role of Otx5 is to activate the expression of circadian genes within pinealocytes. Otx5 likely acts by binding to the promoters of its target genes, as the *exorh* and *aanat2* promoters both have three putative Otx binding sites [1,5]. However, we think it unlikely that Otx5 is involved in generating the rhythm of expression. Instead, the evidence suggests that Otx5 is constitutively active. Otx5 depletion causes loss of *exorh* expression at all time points tested. Further, previous work demonstrates that Otx5 is required

for the expression of genes expressed at night (*aanat2*), day (*irbp*) and dawn (*reverb- α*) [20].

In support of a role for Otx5 as a transcriptional activator of pineal genes, ectopic expression of Otx5 induces transcription from the *exorh* and *aanat2* promoters in cells outside of the pineal and eye (this study and [1]). However, there is an interesting exception to the role of Otx5 as a transcriptional activator. Although Otx5 is expressed widely in the developing eye, it appears to have little or no role in regulating retinal genes. For instance, *aanat2* and *exorh* are expressed only in a small subset of the Otx5 positive cells in the eye, indicating that Otx5 is not sufficient to activate expression of these genes in most retinal photoreceptors [20,60]. Similarly, depletion of Otx5 causes severely reduced pineal expression of *irbp* and the *G protein γ 11 subunit* genes, while their retinal transcription remains unaffected [14,20]. Conversely, depletion of the related Otx family member Crx reduces transcription in the eye but not in the pineal [14,20,52]. A probable explanation is that other transcriptional regulators cooperate with Otx5 and Crx to control the tissue specificity of transcription. As evidence for this, Asaoka and colleagues have identified an element (Pineal expression-promoting element; PIPE) within the *exorh* promoter that confers the ability to drive pineal expression on the normally retina-specific *rhodopsin* promoter [5]. The protein(s) that binds to this element has not yet been identified.

Here, we also identify a novel role for Per3 in the regulation of *exorh* transcriptional rhythms, with loss of Per3 causing increased expression specifically during the light period of the circadian cycle. While the functions of vertebrate Per1 and Per2 proteins are well established, the function of Per3 has been difficult to define. In mice, Per1 and Per2 have central roles in the feedback loops of the circadian clock and in resetting the clock in response to environmental light cues [44]. In contrast, *per3* knock out mice have only a slight decrease in the period of their circadian clock [51]. In humans, certain polymorphisms in the *per3* gene and misregulation of *per3* expression have been associated with breast cancer, chronic myeloid leukemia, bipolar disorder, and the structure of the sleep/wake cycle [15,41,58,61,62].

The fact that *per3* mRNA is present within pinealocytes raises the possibility that Per3 protein could be acting cell autonomously to effect these changes. However, we do not yet understand the biochemical basis of Per3 activity in *exorh* regulation. Per proteins typically act to repress the transcriptional activity of Clock/BMAL heterodimers. However, there are no canonical Clock/BMAL binding sites within the functional 1.1 kb *exorh* promoter region identified by Asaoka and colleagues [5]. Further, a search of genomic sequences 5 kb upstream of the *exorh* start codon, all of the *exorh* introns, and the 751 bp of available *exorh* downstream sequence reveals only one canonical Clock/BMAL binding site, located at position -2040 within the *exorh* 5' UTR (data not shown). Although it is possible that this site mediates transcription by Clock/BMAL, it seems unlikely as functional Clock/BMAL binding sites tend to be found in closely spaced clusters. For instance, functional Clock/BMAL sites within the *aanat2* promoter are found within a 257 bp Pineal Restrictive Downstream Module (PRDM) that contains three Otx binding sites and two Clock/BMAL binding sites [1,2]. Similarly, the promoter for the Otx5 target gene *reverb- α* contains a 165 bp region upstream of the translation start site that contains two Clock/BMAL binding sites that are essential for Clock/BMAL mediated transcription in COS-1 cells [56]. One possibility is that there are additional Clock/BMAL sites further upstream or downstream of the coding sequence or non-canonical binding sites that we do not recognize. Alternatively, the lack of a clear Clock/BMAL regulatory unit could indicate that Per3 is acting to suppress *exorh* transcription indirectly through the regulation of another gene or through a mechanism that does not require Clock/BMAL (Figure 6).

In summary, our study makes three important advances in our understanding of pineal rhythms. Importantly, it defines the first *in vivo* role for Exorh protein in the regulation of transcription of its own gene and of *aanat2*. Second, it provides further evidence for the role of Otx5 as a major activator of rhythmic pineal genes. Finally, we establish a new role for Per3 as a key factor controlling the timing of *exorh* expression.

4. Experimental Procedures

Zebrafish

Adult, embryonic, and larval fish were housed at 28.5°C in a 14:10 hr light/dark (LD) cycle. Fish strains included wildtype (WT) fish that were descendants of fish purchased from Scientific Hatcheries (Huntington Beach, CA, USA) or AB (Eugene, OR, USA), and *exorh:GFP* transgenic fish [5]. Embryos were obtained by natural matings and raised in aquatic system water containing 0.003% phenylthiocarbamide to inhibit the development of pigment. All embryos and larva were kept in temperature-controlled circadian incubators with LD cycles that matched parental lighting conditions. Position within the 24 circadian cycle was noted as Zeitgeber Time (ZT), with ZT0 corresponding to the time lights turned on and ZT14 corresponding to the time lights turned off. All procedures were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University, and were designed to minimize pain and discomfort.

MO injections

MO were manufactured by GeneTools, LLC (Philomath, OR, USA), with the following sequences: per3 MO, 5'-AGGAAAGCCGTCTCCCCCTGGCATT-3'; per3 MIS, 5'-AGcAAAcCCGTgTCCCgCTGcCATT-3'; exorh sp MO, 5'-TTGTAGTGTGCTCACCGCCGAGTGT-3'; exorh atg MO 5'-AGTTGGGTCCCTCCGTCCCGTTCAT-3' standard control (ctl) MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'. The sequences for the otx5 MO and otx5 MIS were as previously described [20]. The exorh sp MO was designed using *exorh* genomic sequences obtained from the Sanger Center (Ensemble Gene ID ENSDARG00000046115).

1–2 cell stage WT embryos were injected with either 4 ng otx5 MO/otx5 MIS, 3 ng per3 MO/per3 MIS, 3 ng exorh sp MO/ctl MO or 1.5 ng exorh atg MO in 1× Danieau buffer [40] with a PLI-90 picoinjector (Harvard Apparatus, Holliston, Massachusetts, USA). The injected embryos were then raised and fixed in time courses as described in the figure legends.

WISH

Embryos were fixed in 4% paraformaldehyde in PBS at 4°C for at least 24 hours before being washed with PBS + 20% Tween 20. Samples were stored in 100% methanol at –20°C unless processed by *in situ* hybridization immediately. Transcripts of *exorh* [38], *opn1lw1* [46], *otx5* [20], *per3* [18] and *aanat2* [22] were detected by WISH as previously described [36]. Stained embryos were visualized using a Zeiss Axioplan2 imaging microscope. Digital images were captured with a Zeiss AxioCam HRm camera (Carl Zeiss MicroImaging Inc. Thornwood, NY, USA) in conjunction with OpenLab software (Scientific Software, Inc. Pleasanton, CA, USA) or with a SPOT RTke 7.4 slider Digital Camera along with SPOT Software version 4.5.9.1 (Diagnostic Instruments Sterling Heights, MI, USA).

Quantification and Statistical Analysis

Digital images were converted to 8-bit grayscale using Adobe Photoshop CS2 version 9.0.1 (Apple software Cupertino, CA, USA). The optical density (OD) for a specified area was calculated from digital images of the pineal using ImageJ software version 1.36b (National

Institutes of Health, Bethesda, MD, USA). The quantified area was the same for all samples within a single experiment.

For Figure 1B, the OD of the WISH signal was calculated for three pineal organs per time point. The values in each experiment were normalized by setting the highest OD reading to 100 and all other readings as a percentage thereof. Data were analyzed using one-way ANOVA with Tukey's *post hoc* comparison of means and the Independent Two Sample Student's t-Test in OriginLab 7.5 (OriginLab Corporation, Northampton, MA, USA).

Sectioning

Embryos at 3 dpf were processed for WISH with a probe for *per3* or *otx5*. The brain was then sectioned by a sagittal cut through the midline using a standard disposable scalpel. For image capture, the sample was positioned so that the cut surface of the brain faced the camera.

mRNA injections

The mMessage Machine Kit (Ambion Inc. Austin, Tx, USA) was used to synthesize capped mRNA in vitro from the pCS2+otx5, pCS2+ β -gal, and pCS2+ RFP plasmids [20,48,57]. Single blastomeres of 8–16 cell stage *exorh*:GFP embryos were injected with 0.5–1.5 nl of mRNA solution using a PLI-90 injector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AANAT2, Serotonin N-acetyl transferase 2
 β -gal, β -galactosidase
 Crx, Cone rod homeobox
 dpf, days post fertilization
 Exorh, Extra-ocular rhodopsin
 GFP, Green Fluorescent Protein
 hpf, hours post fertilization
 IRBP, Interphotoreceptor retinoid binding protein
 LD, light/dark
 MIS, missense morpholino
 MO, morpholino
 opn1lw1, opsin 1 (cone pigments) long-wave-sensitive, 1
 Otx5, Orthodenticle homeobox 5
 Per3, Period 3
 RFP, Red Fluorescent Protein
 SCN, suprachiasmatic nucleus
 WISH, whole mount RNA in situ hybridization
 WT, wildtype
 ZT, zeitgeber time

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Figure 1. There are significant changes in *exorh* expression levels between day and night

(A) Dorsal view of the head of a 2 dpf embryo showing the position of the *exorh* positive cells of the pineal (arrow) relative to the eyes (e). (B) Embryos were raised in a 14:10 hour LD cycle, fixed starting at 50 hpf, and processed for WISH with a probe for *exorh*. Zeitgeber (ZT) indicates position within the circadian cycle, with ZT0=lights on and ZT14=lights off. Dorsal views, anterior to top. Experiment was repeated three times with similar results ($n \geq 10$ embryos per time point for the experiment shown). (C) *exorh* mRNA levels were measured and analyzed as described in Experimental Procedures. Normalized data from three independent experiments are included, with each diamond representing the signal from one embryo ($n > 6$ embryos per time point). White diamonds indicate lights were on (ZT2 and ZT8), and black diamonds

indicate lights were off (ZT14 and ZT20). One way ANOVA, which compares all of the sample groups to each other, indicates that the means of samples taken at different points throughout the circadian cycle are not all the same ($p=0.0004$). Letters (a, b, c, d) denote groups of samples that are not significantly different from one another according to Tukey's post-hoc comparison of means ($p<0.05$). The mean and one standard deviation for each group are indicated by the red lines overlaying the data points. To provide further information, the p values for pairwise comparisons between each of the sample groups are listed in Supplementary Table 1. Scale bars= 50 μ m (A) and 20 μ m (B).

Figure 2. Otx5 controls the tissue-specificity of *exorh* transcription

(A) 1–4 cell stage embryos were injected with otx5 MO or otx5 MIS and then raised in a LD cycle. Time points were collected beginning at 50 hpf, and assayed by WISH for *exorh* expression ($n \geq 10$ embryos per time point). Experiment was repeated twice with similar results. Dorsal views, anterior to the top. Scale bar=20 μ m. (B) Quantification of the digital images shown in A (carried out as described in Experimental Procedures). All measurements taken from the otx5 MO samples were pooled into one set and the measurements from the otx5 MIS into a second data set. Analysis using the Independent Two Sample Student's t-Test indicates that the signal in the Otx5 MO injected embryos is significantly lower than in the Otx5 MIS embryos (OD=0.53 $\pm$ 0.09 and 1.01 $\pm$ 0.21 for otx5 MO and otx5 MIS, respectively, $p=0.0004$).

(C) *otx5* or β -gal mRNA was co-injected with RFP mRNA into a single blastomere of 8–16 cell stage *exorh*:GFP embryos. Live or fixed embryos were examined at 24 hpf by fluorescence microscopy. The embryo co-injected with β -gal & RFP mRNA has no ectopic GFP expression and develops normally, with RFP expressing cells scattered throughout the embryo. The embryo overexpressing Otx5 has ectopic GFP expression that co-localizes with the RFP expression. It also has severe developmental abnormalities, as has been previously reported for Otx5 over-expressing embryos [20]. Experiment was repeated three times, and the combined results are shown in Table 1.

Figure 3. Daytime expression of *exorh* is increased in embryos lacking Per3

(A) The brains of embryos fixed 74.5 and 90.5 hpf were processed via WISH and then sectioned along the midline into left and right halves. The border of the pineal could be easily discerned in the sectioned brain (arrows). *otx5* expression is localized within the pineal tissue. *per3* transcripts are expressed strongly throughout the brain, including the pinealocytes, at ZT2.5 but only weakly at ZT18.5. Experiment was repeated two times with similar results, with $n \geq 4$ embryos per time point. Lateral views, dorsal to the top. (B) Embryos were injected at the 1–4 cell stage with either *per3* MO or *per3* MIS. Embryos were fixed at the indicated ZT and processed for WISH ($n \geq 4$ embryos per time point). (C) The OD of the WISH signal was quantified as described in Experimental Procedures. The daytime samples (ZT0, 4 and 8) from

the per3 MO samples were placed into one group and the dusk/nighttime samples (ZT12, 16 and 20) into another, and then each was compared using the Student's t-test to the analogous group from the per3 MIS samples. This demonstrated that *exorh* levels are significantly higher during early daylight hours (ZT 0, 4, 8) in per3 MO injected embryos compared to controls (OD 0.35 ± 0.03 and 0.87 ± 0.16 for per3 MIS and per3 MO, respectively, $p=0.005$). In contrast, there is no significant difference between the per3 MO and per3 MIS embryos in the evening/night time points (ZT12, 16, 20; OD = 0.94 ± 0.13 and 1.15 ± 0.07 for per3 MIS and per3 MO, respectively, $p=0.08$). Scale bars=20 μ m.

Figure 4. Expression from the *exorh* promoter is decreased in embryos lacking Exorh protein
 Embryos were injected with the indicated MO, raised in a 14:10 hr LD cycle, and then (A, B, E, F) fixed and processed for in situ hybridization or (C, D) analyzed by fluorescence microscopy. (A) Control embryos have high levels of *exorh* mRNA, while (B) expression is severely decreased in embryos injected with the *exorh* MO. Quantification of the in situ signal confirms that there is a significant decrease in the *exorh* sp MO embryos versus controls (OD is 9.19 ± 2.96 for control MO and 6.69 ± 2.39 for *exorh* sp MO, $n \geq 10$ embryos between 33–72 hpf, $p < 0.05$). (C) In *exorh:GFP* transgenic fish, GFP is expressed in a large region that likely encompasses all pineal photoreceptors cells. (D) Transgene expression in Exorh-depleted fish is restricted to a smaller domain. (E) Control injected fish and (F) Exorh-deficient fish had indistinguishable patterns of *otx5* expression in the pineal (p) and left-sided parapineal (pp). (G) The dimensions (length and width) of the fluorescent domain within the pineal were measured from digital images such as those shown in panels C and D. There is a significant difference in both dimensions between the two groups of samples ($n \geq 20$, $p < 0.0001$ and 0.003 for length and width, respectively). (H) The dimensions (length and width) of the *otx5* expression domain in control and *exorh* MO-injected fish were measured from digital images such as those shown in panels E and F. There was no significant change in the dimensions of the *otx5* expression domain upon depletion of Exorh ($n \geq 10$ embryos, $p = 0.30$ and 0.93 for length and width, respectively). (I) Expression of the endogenous *exorh* gene is high in fish injected with a control MO, and (J) much lower in fish injected with the *exorh* atg MO. (K) Quantification of the in situ signal indicates that expression of the endogenous *exorh* gene was severely reduced in *exorh* atg MO injected embryos compared to controls ($n = 10$, $p < 0.001$). (L) The length and width of the *otx5* expression domain is not different between control and *exorh* atg MO-injected fish ($n \geq 9$, $p = 0.70$ and 0.58 for length and width, respectively).

Experiments were repeated three times and representative images are shown. All images are dorsal views, anterior to the top. Embryos are 33 hpf (A, B), 72 hpf (C–F), and 64 hpf (I–J). Scale bars=20 μ m.

Figure 5. Exorh is required for high levels of *aanat2* transcription

(A) Embryos were injected at the 1–4 cell stage with either *exorh* MO or a standard control MO. Control and experimental embryos were processed in tandem for WISH ($n \geq 5$ embryos per condition). While a separate *exorh* control was used for each of the *aanat2* or *opn1lw1* experiments, only one example is shown. For *aanat2*: Embryos were fixed at 2dpf ZT 18. Similar results were observed in 3 independent replicants using the *exorh* atg MO (shown) and 2 independent repeats using the *exorh* sp blocking MO (data not shown). Pineal size was confirmed through *otx5* staining and no change was detected (similar to Fig 4 E,F data not shown). For *opn1lw1*: Embryos were fixed at 2dpf ZT 0. Results were confirmed in two independent experiments using the *exorh* atg MO. Dorsal views, anterior to the top. Scale

bars=25 μ m. **(B)** *exorh* transcription is significantly lower in embryos depleted of Exorh ($p=0.002$, similar to Fig 4 I,J,K). Similarly, *aanat2* expression is significantly reduced when Exorh is depleted ($p=0.006$). **(C)** Robust *exorh* expression is lost when Exorh protein levels are minimized ($p<0.001$). In contrast, high *opn1lw1* levels are unchanged in the presence of the MO ($p=0.08$). The OD of the WISH signal was quantified as described in Experimental Procedures. The Two Sample Independent t-Test was used to determine significance. Each bar is the normalized value of 6 embryos total from 2 different experiments.

Figure 6. Model for the regulation of *exorh* expression

***exorh* regulation:** During the day, Otx5 is present to drive *exorh* transcription in the pineal. However, the level of *exorh* expression is low due to the presence of Per3, which suppresses *exorh* transcription through an unknown mechanism. During the night, Per3 levels fall. Thus, Otx5 protein is free to induce high levels of *exorh* expression. Our results indicate that Exorh also positively influences the strength of transcription from the Exorh promoter, potentially through a multi-step signaling cascade that leads from the cell membrane to the nucleus.

***aanat2* regulation:** Previous work demonstrates that *aanat2* expression depends upon binding of Clock/BMAL and Otx5 to an enhancer region downstream of the coding region [1,3]. We have shown that Exorh protein also positively regulates *aanat2* transcription. One possibility is that Exorh acts through Per2, which is also required for the light-dependent onset of *aanat2* transcription in the pineal [63,64].

Table 1

Overexpression of Otx5 induces ectopic expression of the exorh:GFP transgene

mRNA injected	Pattern of ectopic GFP expression		
	Colocalized with RFP	Not colocalized with RFP	No GFP
240 pg β -gal & 240 pg RFP	0	0	24
360 pg β -gal & 360 pg RFP	0	1	38
120 pg otx5 & 120 pg RFP	4	0	9
240 pg otx5 & 240 pg RFP	18	1	13
360 pg otx5 & 360 pg RFP	3	0	2

The indicated mixtures of mRNAs were injected into single blastomeres of exorh:GFP embryos at the 8-16 cell stage. Live or lightly fixed embryos were examined by fluorescence microscopy at approximately 24 hpf.