Melanopsin elevates locomotor activity during the wake state of the diurnal zebrafish

Marcus P S Dekens1,*†, Bruno M Fontinha1‡, Miguel Gallach1,2,†, Sandra Pflügler3‡ & Kristin Tessmar-Raible1,3‡

Abstract

Mammalian and fish pineals play a key role in adapting behaviour to the ambient light conditions through the release of melatonin. In mice, light inhibits nocturnal locomotor activity via the non-visual photoreceptor Melanopsin. In contrast to the extensively studied function of Melanopsin in the indirect regulation of the rodent pineal, its role in the intrinsically photosensitive zebrafish pineal has not been elucidated. Therefore, it is not evident if the light signalling mechanism is conserved between distant vertebrates, and how Melanopsin could affect diurnal behaviour. A double knockout of melanopsins (opn4.1-opn4xb) was generated in the diurnal zebrafish, which manifests attenuated locomotor activity during the wake state. Transcriptome sequencing gave insight into pathways downstream of Melanopsin, implying that sustained repression of the melatonin pathway is required to elevate locomotor activity during the diurnal wake state. Moreover, we show that light induces locomotor activity during the diurnal wake state in an intensity-dependent manner. These observations suggest a common Melanopsin-driven mechanism between zebrafish and mammals, while the diurnal and nocturnal chronotypes are inversely regulated downstream of melatonin.

Keywords | behavioural genetics; melatonin; neurogenetics; photobiology; pineal
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Introduction

To adapt to the ambient light conditions, activity is regulated directly by light and indirectly through the circadian clock (Dunlap et al., 2004), which anticipates daily recurring events. In nocturnal mice, the non-visual photoreceptor Melanopsin (OPN4) functions in circadian clock entrainment (Panda et al., 2002; Ruby et al., 2002) and in the direct induction of sleep by light (Mrosovsky & Hattar, 2003; Lupi et al., 2008; Tsai et al., 2009). In contrast to nocturnal mice, where Opn4 is solely expressed in the intrinsically photoreceptive retinal ganglion cells (ipRGCs) (Hatori & Panda, 2010), several diurnal species also express Opn4 in the brain. Opn4 is transcribed in many domains of the human brain (Hawrylycz et al., 2012; Nissilä et al., 2017), the zebrafish larval brain (Davies et al., 2011; Matos-Cruz et al., 2011), the chicken pineal (Holthues et al., 2004; Bailey & Cassone, 2005; Chaurasia et al., 2005) and in the pineal of the Atlantic halibut (Eilertsen et al., 2014). In vertebrates, the pineal gland or epiphysis cerebri plays a key role in synchronising behaviour and physiology to the environmental light–dark (LD) cycles through the rhythmic production of the indolamine melatonin (Sapédé & Cau, 2013). The melatonin level reaches its peak during the night in both diurnal and nocturnal animals (Challet, 2007), thus melatonin is a dark phase indicator. In mice, ipRGCs provide the central clock located in the suprachiasmatic nucleus (SCN) with photic information, which in succession regulates the pineal via the paraventricular nucleus (PVN) (Simonneaux & Ribeleyga, 2003). In contrast to the mammalian pineal, which receives indirect photic input through sympathetic norepinephrine (NE) innervation (Simonneaux & Ribeleyga, 2003), the pineals of many fish, amphibians, reptiles and birds display all characteristics of a photoreceptive organ (Korf et al., 1998; Ziv et al., 2007; Sapédé & Cau, 2013; Ben-Moshe Livne et al., 2016; Bertolesi & McFarlane, 2018) and generate endogenous endocrine rhythms, combining input and output functions in the photoreceptor cell (Falcon et al., 2007). Moreover, NE does not play a role in regulating melatonin synthesis in zebrafish (Cahill, 1997).

Melatonin is synthesised by four enzymes: Tryptophan hydroxylase (TPH) catalyses the oxidation of the amino acid tryptophan to 5-hydroxy-L-tryptophan (5-HTP) followed by removal of a carboxyl group by Dopa decarboxylase (DDC) to produce serotonin. Arylsulphynylamine N-acetyltransferase (AANAT) adds an acetyl group to produce N-acetylserotonin, which is then methylated by Acetylserotonin O-methyltransferase (ASMT) into melatonin. The nightly release of NE in the rodent pineal activates the cAMP pathway resulting in the phosphorylation of cAMP response element-binding protein (CREB), which induces Aanat transcription (Rohde et al., 2014a). Homeodomain transcription factors control pinealocyte
specific gene expression by binding to highly conserved pineal regulatory elements (PIRE) (Li et al., 1998), thereby permitting pCREB to regulate these genes through CRE elements. The transcription factor Cone-rod homeobox factor (CRX) induces Aanat (Li et al., 1998; Rohde et al., 2014b), Tph1 and Asmt (Rohde et al., 2019) transcription in the mature rat pineal. Furthermore, Orthodenticle homebox 2 (OTX2) transactivates Crx (Nishida et al., 2003; Rohde et al., 2019) and induces Tph1, Aanat and Asmt (Rohde et al., 2019), and LIM homebox 4 (LHX4) induces Aanat (Hertz et al., 2020). In zebrafish, the homeodomain transcription factor Otx5 has been reported to regulate aanat2 (Gamse et al., 2002). As the aanat2 promoter contains CRE elements (Falcon et al., 2007), Creb could also play a role in regulating the zebrafish melatonin pathway. The rate of melatonin synthesis mainly depends on AANAT (Klein & Weller, 1970; Klein et al., 1997). However, the melatonin level is effectively raised by inducing multiple components of the melatonin pathway (Liu & Borjigin, 2005; Rohde et al., 2019).

Melatonin has been demonstrated to promote sleep in diurnal vertebrates ranging from zebrafish (Zhdanova et al., 2001) to humans (Dollins et al., 1994; Mintz et al., 1998; Zhdanova et al., 2002; Brzezinski et al., 2005; Lek et al., 2019), and a light pulse in the night suppresses melatonin production with the strongest effect at the wavelength where Opn4/OPN4 has its absorption optimum in both zebrafish (Ziv et al., 2007) and humans (Lewy et al., 1980; Czeisler et al., 1995; Cajochen et al., 2000; Lockley et al., 2003). The reduction in the melatonin level is proportional to the intensity of the light pulse (Max & Menaker, 1992; Zachmann et al., 1992b; Bolliet et al., 1995) implying regulation by a light intensity detector. Therefore, OPN4 is a primary candidate in this process (Wong et al., 2005; Mure et al., 2016). Several human studies have shown that bright light during daytime increases activity and vigilance (Cajochen et al., 2000; Phipps-Nelson et al., 2003; Lockley et al., 2006; Smolders et al., 2012; Smolders & de Kort, 2014; Knaier et al., 2016). These data suggest that in diurnal vertebrates, suppression of
melatonin production by light may also play a role in regulating activity levels during the wake state. To investigate how zebrafish adapt to ambient light conditions and the role of Opn4 in this process, a double knockout (dko) was generated of opn4.1 and opn4xb which are coexpressed in the pineal. This study implies that locomotor activity during the wake state is regulated by an Melanopsin-driven mechanism that is common between mammals and zebrafish despite the differences in light input and chronotypes. The Opn4-dependent transcriptome also suggests that Opn4 influences the immune system and the cell cycle in addition to the control it exerts over genes that encode melatonin synthesis enzymes. These findings alter the perspective of a photoreceptor that has until now solely been associated with behaviour to one that adapts diverse functions to the environment.

Results

**opn4 expression in the brain and knockout strategy**

The expression patterns of all five opn4 homologs were characterised in the adult zebrafish brain by in situ hybridisation (ish). The opn4 genes show broad expression in the adult brain (Fig EV1A–E, Table EV1), which we documented in accordance with the neuroanatomical terminology established by Wullimann (Wullimann et al, 1996; Baeuml et al, 2019). opn4.1 and opn4xb stand out as the opn4 genes that are strongly expressed in the pineal (Fig 1A and B). To knockout opn4.1 and opn4xb the transcription activator-like effector nuclease (TALEN) genome editing technique (Cermak et al, 2011; Bedell et al, 2012) was applied. A premature stop codon was introduced close to the start codon in each gene (Fig 1C and D, Table 1). Opn4 is a seven transmembrane G-protein coupled receptor with a light absorbing moiety, the chromophore retinal, bound to helix seven (Fig 1E). The mutated Opn4.1 has lost all transmembrane helices, and only the first and second transmembrane helices remain in the mutated Opn4xb. Thus, from the structure–function relationship, it can be deduced that neither of the mutated opn4 genes encodes a functional photoreceptor. A homozygous double knockout (dko) was generated, as both Melanopsins are likely to have redundant functions given their conspicuous coexpression.

**Expression profiling reveals pathways downstream of Opn4**

Transcriptome sequencing was applied on cDNA from eyes and brain parts of wild-type and opn4.1 and opn4xb with a light absorbing moiety, the chromophore retinal, bound to helix seven (Fig 1E). The mutated Opn4.1 has lost all transmembrane helices, and only the first and second transmembrane helices remain in the mutated Opn4xb. Thus, from the structure–function relationship, it can be deduced that neither of the mutated opn4 genes encodes a functional photoreceptor. A homozygous double knockout (dko) was generated, as both Melanopsins are likely to have redundant functions given their conspicuous coexpression.

Table 1. Transcription activator-like effector DNA-binding domains.

| Ensembl ID | Gene | Target Site | TAL1 DNA Binding Domain | TAL2 DNA Binding Domain |
|------------|------|-------------|-------------------------|------------------------|
| ENSDARG00000007553 | opn4.1 | proximal to START codon | tcactgtgacccctgaggacat | tcccaagaattctcaaaag |
| ENSDARG000000103259 | opn4xb | exon 2 | ttcctcaactctttctca | ttcctcaacatccact |

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Figure 2. Expression profiling reveals pathways downstream of Opn4.

A  Representation of the zebrafish adult brain shows the lateral and dorsal view with abbreviations for the following domains: olfactory bulb [OB], telencephalon [Tel], epiphysis cerebellum [Ce], medulla oblongata [MO], hypothalamus [H] and hypophysis or pituitary [Pit]. Transcriptome sequencing was performed on cDNA from wild-type and opn4 dko eyes and brains, sampled in the light phase (ZT4-6). The brains were separated in an anterior part, which includes part of the forebrain and the pineal, and a posterior part, which includes part of the forebrain and the whole mid- and hindbrain. The cut lines through the depicted brain indicate where the brain was partitioned and which parts were subjected to transcriptome sequencing. The blue (anterior brain) and grey (posterior brain) arrows connect the brain parts in (A) with the differentially expressed genes in (B).

B  Venn diagram shows the number of differentially expressed genes in the opn4 dko anterior brain (dark blue circle), posterior brain (grey circle) and eye (light blue circle). The cutoff for differential expression was set at the significance level of $\alpha = 0.05$. Heat maps of all the differentially expressed genes are presented in Appendix Fig S1A–C. The number of differentially expressed genes that are shared between the data sets is indicated where the datasets overlap.

C  Fisher’s exact test shows that the shared differentially expressed genes between data sets are unlikely to be the result of coincidence. $P$-values for the common differentially expressed genes are indicated where the datasets overlap. The large number of common differentially expressed genes between the data sets derived from the anterior and posterior brain parts is likely due to forebrain regions that are shared between these parts.

D  Pie chart shows the number of differentially expressed genes in the opn4 dko anterior brain that were assigned with KEGG software to phototransduction (dark blue), metabolic (orange) and other pathways. Of the metabolic pathway, 5 genes were assigned to the subcategory tryptophan metabolism (red), which encode all the enzymes that convert tryptophan into melatonin. Note that 198 genes were not assigned to a pathway.

E  Ancestor charts show the gene ontology attributes to which the differentially expressed genes in the opn4 dko anterior brain were assigned with FuncAssociate software (cutoff: $\alpha = 0.05$). The number of genes assigned to an attribute is indicated in the upper right corner. All retrieved biological processes and molecular functions are associated with light, consistent with knockout of a photoreceptor.

Source data are available online for this figure.

[Thisse et al, ZFIN direct data submission], grk7a (Rinner et al, 2005), grk7b (Rinner et al, 2005), rcvrm1 (Zang et al, 2015), rcvrm2 (Zang et al, 2015), rcvrm3 (Zang et al, 2015), gnat1 (Lagman et al, 2015), rbp3 (P = 6.87 x 10^{-15}) (Nickerson et al, 2006), trbpl (P = 0.012) (Nickerson et al, 2006), unc119.2 (P = 0.0003) (Toyama et al, 2009) and pp2 (P = 1.23 x 10^{-4}) (Koyanagi et al, 2015). Also ot5x (P = 0.001) and lbx4 (P = 0.003) (Appendix Fig S1A,G,I) have been demonstrated to be expressed in the pineal (Gamse et al, 2002; Weger et al, 2016). Note that all the genes that are associated with the pineal are overexpressed in the opn4 dko anterior brain (Appendix Fig S1A). As the pineal produces melatonin and detects light (Klein, 2004; Sapède & Cau, 2013), the differentially expressed genes assigned to the melatonin and phototransduction pathways in the opn4 dko anterior brain (Fig 2D and E, Appendix Fig S1A) point to a defect in this gland. In addition to these pathways, five differentially expressed genes in the opn4 dko anterior brain were assigned to the mitogen-activated protein kinase (MAPK) pathway: ngfra (P = 0.042), disp7 (P = 0.002), ppm1na (P = 1.92 x 10^{-6}), dazx (P = 0.006), junf (P = 0.048) and several
two differentially expressed genes in the genes were assigned to a range of pathways with diverse biological functions (Source Data). Two differentially expressed genes in the eye: mhc1uba (P = 7.06 × 10^-9) and mhc1uka (P = 3.05 × 10^-7) (Appendix Fig S1C, P and Q) were both associated with the GO attributes of antigen processing and presentation of peptide antigen (GO:0048002) and antigen binding (GO:003823). Importantly, a substantial number of differentially expressed genes in the opn4 dko function in the immune response: mhc1uba (P_a = 3.46 × 10^-15, P_p = 7.89 × 10^-216), mhc1uka (P_a = 0.04), caspb (P_a = 0.0005, P_p = 4.89 × 10^-9), caspbl (P_a = 0.046), ly6pg (P_a = 3.10 × 10^-9), nrp1b (P_a = 0.027), mpeg1.1 (P_a = 0.049), mrc1 (P_a = 0.012), pigf (P_a = 0.042), dicp3.3 (P_a = 0.0008), tap2a (P_a = 0.037), trim4 (P_a = 0.002), cd74b (P_a = 0.015), b2m (P_a = 0.047) and cell division: mcm7 (P_a = 2.15 × 10^-13, P_p = 2.30 × 10^-31) cdk9 (P_a = 3.45 × 10^-11, P_p = 2.47 × 10^-5), crrna2 (P_a = 0.040), cdk20 (P_a = 0.038) (Appendix Fig S1A–C, K–Q), implying that Opn4 controls diverse processes and confirming previous reports of cell cycle regulation by light (Dekens et al., 2003; Kowalska et al., 2013), regulation of the immune/inflammation response by the rat pineal (Bailey et al., 2009) and the role of melatonin in buffering the immune system (Carrillo-Vico et al., 2013).

**asmt is overexpressed in the opn4 dko pineal**

To validate the transcriptome sequencing data, we repeated the same entainment and sampling procedure and determined the levels of ddc and asmt mRNA by qPCR, which confirmed significant overexpression of ddc (Fig 3B U-test: P = 4.42 × 10^-5) and asmt (Fig 3D U-test: P = 0.0004) in the mature opn4 dko anterior brain. Both ddc and asmt transcripts are expressed in the mature wild-type and opn4 dko pineals, as demonstrated by qPCR (Fig EV2A–D). The elevated asmt mRNA levels detected in the opn4 dko brain (Fig 3C and D) can be attributed to the pineal, as asmt is solely expressed in this gland and not ectopically expressed in the opn4 dko brain. Note that asmt is not overexpressed in the eye (Fig 3C and D).

**opn4 dko larvae show attenuated locomotor activity in the wake state**

As larvae are routinely subjected to behaviour and neuropharmacology studies (Basnet et al., 2019) and the commercially available behaviour systems have been designed for larvae, we investigated locomotor activity at the larval stage. The characteristics of mature...
swimming emerge on the 4th day post fertilisation (dpf) when the swim bladder becomes functional. Thereafter, larvae synchronise locomotor activity to the light-dark phases (Hurd & Cahill, 2002). Locomotor activity or velocity is defined as the rate of change of a larva’s position in a unit time. Wild-type and opn4 dko larvae were entrained to a 12:12 h LD regime in the DanioVision automated observation chamber from the 1st dpf (~30 hpf) onwards, and recorded and tracked on the 5th, 6th and 7th dpf. opn4 dko larvae display significantly lower locomotor activity than wild-type during the light phase (wake state) at 6 dpf (Fig 4G, P = 0.009, d = −0.932, 7 dpf: P = 0.020, d = −0.828). The significance and effect size independently indicate a very high probability that the observed differences in locomotor activity between wild-type and opn4 dko are the result of the phenotype. We also compared the visual motor response (VMR), which is defined as the brief increase in activity after loss of illumination. The VMR consists of two components: large-angle turns (O-bend spike) followed by routine turns (R-turns) (Burgess & Granato, 2007; Fernandes et al., 2012). The opn4 dko showed significant more movement in the large-angle turns compared to wild-type (Fig 4F, 5 dpf: P = 0.008, d = 0.955, 6 dpf: P = 0.001, d = 1.216, 7 dpf: P = 8.93 × 10⁻⁵, d = 1.509), while the routine turns were not affected on the 6th and 7th dpf (Fig EV3A–C and G).

We further investigated opn4 dko larvae on the molecular level. Larvae were entrained to a 12:12 h LD regime and asmt transcript levels were measured by qPCR in whole wild-type and opn4 dko larvae sampled on the 6th dpf. The asmt mRNA levels did not show a significant difference (Figs 4G and EV4C). However, the expression of asmt in the eye and pineal returns the sum of the dissimilar relative expression levels. If a much higher asmt transcript level is present in the eye than in the pineal, an unaffected asmt transcript level in opn4 dko eyes could potentially mask an increase in the asmt transcript level in the opn4 dko pineal when comparing RNA extracts from whole wild type with opn4 dko larvae. Therefore, wild-type and opn4 dko larvae were entrained to the LD regime, sampled and ophthalmectomised at 6 dpf between ZT3 and ZT4, and the asmt transcript levels were determined by qPCR in ophthalmectomised larvae and their eyes separately. This showed significant overexpression of asmt in ophthalmectomised opn4 dko larvae compared to wild-type larvae (Fig 4H, U-test: P = 0.017) and similar asmt expression levels in the eyes (Fig 4I). Whole mount ish for asmt in opn4 dko 6 dpf larvae revealed that asmt is not ectopically expressed (Fig 4J), demonstrating that the increase in asmt transcript level in the ophthalmectomised opn4 dko larvae can be solely attributed to the pineal. qPCR to detect ddc mRNA in the same samples showed that this transcript is significantly overexpressed in whole opn4 dko larvae (Fig 4K, ZT3: P = 0.0005, Fig EV4A), ophthalmectomised opn4 dko larvae (Fig 4L, U-test: P = 0.0045) and their eyes (Fig 4M, P = 0.0005). However, the higher ddc mRNA level

![Figure 4. opn4 dko larvae display attenuated locomotor activity in the wake state.](image)
Figure 4.
in ophthalmoctomised opn4 dko larvae does not give insight into the expression level in the pineal, as ddc is expressed in multiple brain areas. Thus, we performed an in situ hybridisation for ddc, and assayed the ddc transcript levels in the pineals of individual 6 dpf larvae by measuring the adjusted volume intensity. This experiment revealed significant higher ddc transcript levels in the opn4 dko than in the wild-type pineal (Fig 4N and O, P = 2.12 × 10⁻⁷, Appendix Fig S2). Tyrosine hydroxylases (th1, th2), which encode essential enzymes for dopamine synthesis, and the dopamine transporter (slc6a3) are not expressed in the pineal (Filippi et al., 2010), implying that dopamine is not produced by this gland. Thus, the overexpression of ddc in the pineal predicts a higher serotonin level. As aanat2 is only expressed in the pineal the transcript was measured in whole larvae, and similar aanat2 mRNA levels were detected in wild-type and opn4 dko (Fig EV4B, ZT21: P = 0.318). The overexpression of multiple genes that encode melatonin synthesis enzymes in the pineal of the opn4 dko larva suggests a higher melatonin level, which would provide a causal explanation for its attenuated locomotor activity during the wake state.

**Larvae adapt their locomotor activity to the light level**

Light has been demonstrated to suppress the rest state in diurnal vertebrates (Lowrey & Takahashi, 2000; Yokogawa et al., 2007). Thus, the intensity of light may also affect locomotor activity during the wake state. To test this, wild-type larvae exposed to medium light intensity were compared with wild-type exposed to more than an order of magnitude reduced light intensity (Fig EV3I), both under the same photoperiod and spectrum. Larvae under a low intensity LD regime were significantly less active in the light phase than under a medium intensity LD regime at 5 dpf (P = 0.001, d = −1.213), 6 dpf (P = 0.015, d = −0.933) and 7 dpf (P = 0.011, d = −0.887) (Fig 5A–D), and showed no difference in activity during the dark phase (Fig 5E). The significance and effect size independently indicate a very low probability that the observed differences in locomotor activity are the result of interindividual variability. To determine whether the light intensity also affects the expression of genes that encode melatonin synthesis enzymes, we compared ddc, aanat2 and asmt transcript levels at 6 dpf under the same conditions. A significant increase in the asmt transcript level was detected in the light phase of the low intensity LD regime (Fig 6l, U-test at ZT3: P = 0.00284), but ddc (Fig 6H, ZT21: P = 0.538, ZT3: P = 0.953) and aanat2 (Fig EV4D, U-test at ZT21: P = 0.068, ZT3: P = 0.553) mRNA levels did not show significant differences. Thus, solely reducing the light intensity without changing the photoperiod and spectrum induces asmt transcription and reduces locomotor activity during the wake state. In addition, a reduction in large-angle turns was observed under the low intensity LD regime (Fig 5F 6 dpf: P = 0.005, d = −1.024, 7 dpf: P = 0.002, d = −1.284, 6 dpf: P = 0.005, d = −1.024, 7 dpf: P = 0.001).

**Figure 5. Larvae adapt their locomotor activity to the ambient light level.**

A–C Actograms show mean locomotor activity in wild-type under a medium (dark blue line) and low (light blue line) intensity LD regime at (A) 5 dpf, (B) 6 dpf and (C) 7 dpf. Spectrum and photoperiod are the same under both conditions. The band on each side of the mean indicates the 95% confidence interval. The bar under the chart indicates the 12:12 h LD interval.

D Box plot shows a significant reduction in locomotor activity during the wake state under a low (light blue box) than a medium (dark blue box) intensity LD regime on the 5th, 6th and 7th dpf.

E As in (D) for the rest state (dark phase). No difference in locomotor activity was detected.

F Larvae show significant less displacement in large-angle turns at the onset of the dark interval under a low intensity LD regime than under a medium intensity LD regime on the 6th and 7th dpf (see also Fig EV3D–F and I).

Data information: In (D–F), boxplot divides the data in quartiles: the box indicates the interquartile range, with the horizontal line in the box denoting the median of the data set, the whiskers extend to the minimum and maximum, and meet the box at the median of the lower (quartile 1) and median of the upper (quartile 3) half of the dataset. Black dots indicate biological replicates (n = 18), the red dot indicates the mean, red error bars indicate the confidence interval (95%) and asterisks indicate significance (0.01 < P(*) < 0.05, 0.001 < P(**) < 0.01, P(***) < 0.001, ns = not significant).
Per2 functions as an intermediary between the light signal and the melatonin synthesis pathway, as light-dependent onset of *aurat2* transcription in the pineal requires Per2 (Ziv et al., 2005; Ziv & Gothilf, 2006) and as *per2* transcription has been shown to be directly light induced (Cermakian et al., 2002; Ziv et al., 2005; Dekens & Whitmore, 2008). Therefore, we measured the transcript levels of *per2* in 6 dpf larvae at ZT3, 9, 15 and 21 under the stated conditions, which revealed a significant reduction in the *per2* transcript level under the low intensity light phase (Fig 6A, ZT3:

**Light intensity and core clock gene transcription are correlated**

*P* = 0.004, *d* = −1.083), which further demonstrates that zebrafish can detect differences in the light level.

Figure 6. The light level is correlated with *per2* and *asmt* transcript levels.

A. *per2* is known to act as an intermediary between the light detector and the melatonin synthesis pathway. Box plot shows *per2* mRNA levels measured by qPCR in 6 dpf larvae placed under a low (light blue box) or medium intensity (dark blue box) LD regime. Under the low intensity light phase a significant reduction in the *per2* transcript levels is detected. Bar under the chart indicates the 12:12 h LD interval.

B. Box plot as in (A) shows mRNA levels of *amrt1b*, which is in part regulated by *per2*. Under low intensity light a significant reduction in the *amrt1b* transcript level is detected at ZT3 and an increase at ZT21.

C. Plot as in (A) shows mRNA levels of *per1a*, which is regulated by the Arntl-Clk heterodimer. Under low intensity light a significant reduction in the *per1a* transcript level is detected at ZT21 and an increase at ZT9.

D. Box plot shows similar *per2* mRNA levels between wild-type (blue box) and opn4 dko (grey box) under a medium intensity LD regime at 6th dpf.

E. Box plot as in (D), shows similar *amrt1b* mRNA levels.

F. Box plot as in (D), shows similar *clk1a* mRNA levels.

G. Box plot as in (D), shows similar *ddc* mRNA levels. Similar levels were detected between wild-type larvae under a low or medium intensity LD regime.

H. Box plot as in (A) shows *asmt* mRNA levels. Under a low intensity LD regime a significant increase in the *asmt* transcript level is detected at ZT3.

I. Box plot as in (A) shows mRNA levels of *opn4*.

Data information: boxplot divides the data in quartiles: the box indicates the interquartile range, with the horizontal line in the box denoting the median of the data set, the whiskers extend to the minimum and maximum, and meet the box at the median of the lower (quartile 1) and median of the upper (quartile 3) half of the dataset. Black dots indicate biological replicates (*n* = 12); the red dot indicates the mean, red error bars indicate the 95% confidence interval and asterisks indicate significance (0.01 < *P* < 0.05, 0.001 < *P* < 0.01, *P* < 0.001, ns = not significant).
P = 2.16 × 10⁻⁵, U-test at ZT9; P = 1.16 × 10⁻⁵). Per2 has been reported to regulate the core clock gene arrntl (Wang et al., 2015). Consistent with this report, we detected a significant reduction in the arrntl mRNA level (Fig 6B, U-test at ZT3: P = 0.0205, ZT21: P = 0.0065) under the low intensity LD regime. Next, we measured the expression of per1a, which is under the control of the Clk-Arrn heterodimer, and detected a significant difference in per1a mRNA levels between the low and medium intensity LD regimes (Fig 6C, ZT9: P = 0.0015, ZT21: P = 0.0095). As mouse OPN4 also functions in clock resetting, we compared the transcript levels of per2 (Fig 6D), arrntl (Fig 6E), per1a (Fig 6F) and clk1a (Fig 6G) on the 6th dpf at ZT3, 9, 15 and 21 between wild-type and opn4 dko larvae. We detected no significant differences in transcript levels, suggesting that during this larval stage opn4.1 and opn4xb do not play a role in clock resetting or are redundant in this process. Transcriptome sequencing on cDNA of adult posterior brains that were sampled in the light phase showed a significant reduction in the transcript level of per1a (P = 0.0153), roraα (P = 0.0365) and cdk5 (P = 0.0093), and a similar trend in per2 (Appendix Fig S1B,S–V) in the opn4 dko, implying that Opn4.1-Opn4xb input into the clock when the fish has matured. Furthermore, several MAPK pathway genes are differentially expressed, which may point to an altered clock amplitude and/or rhythm, as this pathway has been reported to input into the circadian clock (Cermakian et al., 2002).

Discussion

A comparison between mammals and zebrafish

The traditionally studied vertebrate genetic model systems in chronobiology are nocturnal rodents. The zebrafish offers insight into the diurnal chronotype, a perspective from a different class of vertebrates and suitability for genetic manipulation. Whereas the function of OPN4 in the rodent ipRGCs has been extensively studied, the function of Opn4 in the zebrafish pineal is unknown. In addition, the zebrafish is relevant for studies on the regulation of the melatonin pathway (Gandhi et al., 2015), while genes encoding melatonin synthesis enzymes are nonfunctional in most laboratory mouse strains (Ebihara et al., 1987; Roseboom et al., 1998).

A common function for Opn4/OPN4 in zebrafish and rodents is not evident, given the drastically different connection of the pineal with its light input and as the zebrafish expresses several distinct non-visual photoreceptors. For instance, Exorhodopsin (Exorh) has been proposed as the primary candidate for regulating the melatonin pathway in the zebrafish pineal, as it has been shown to induce aanaat2 during embryonic development (Pierce et al., 2008; BenMoshe et al., 2014). Furthermore, the transcriptome data presented here shows overexpression of paraparopsin (pp2) in the opn4 dko pineal, which could be due to a compensatory mechanism and therefore Pp2 may be involved in the same process (Koyanagi et al., 2015). The suppression of melatonin synthesis by red light in zebrafish (Ziv et al., 2007) is another indication that different photoreceptors input in the melatonin pathway. However, these data do not exclude that one or more of the zebrafish Opn4 photoreceptors have a similar function as reported in mammals.

We demonstrate that adult zebrafish opn4 dkos express in the light phase significantly higher levels of the transcripts: tph1, tph2, ddc, aanaat2 and aasmt than wild-type. These transcripts encode all the enzymes required for the conversion of tryptophan into melatonin. In addition, significantly higher transcript levels were detected of: cxr, otx2, otx5 and lhx4 transcripts (Appendix Fig S1A,H,L,J), which encode homeodomain transcription factors that have been shown to regulate genes that encode melatonin pathway enzymes. CRX (Rohde et al., 2014b, 2019) and OTX2 (Rohde et al., 2019) regulate Tph1, Aanat and Asmt in the rat pineal, and LHX4 (Hertz et al., 2020) regulates Aanat in rats. Otx5 has been demonstrated to regulate aanaat2 in the zebrafish pineal (Gamse et al., 2002). These reports are consistent with the data presented here, and suggest a common mechanism for the regulation of melatonin synthesis through homeodomain transcription factors. Furthermore, the Opn4-dependent transcriptome shows that creb3li (Appendix Fig S1A,R, P = 0.028), which product may also be involved in the regulation of the melatonin pathway, is differentially regulated in the opn4 dko. Its underexpression may predict a decrease in the transcription of CRE-regulated genes. However, transcriptome sequencing does not reveal all regulatory processes, such as post-translational modifications. adenylate cyclase (ady1 in Appendix Fig S1A, P = 0.012) is overexpressed in the opn4 dko anterior brain, implying an increase in pCrb and the expression of the putative genes under its control, consistent with the observed higher aanaat2 transcript level and the presence of CRE elements in the aanaat2 promoter (Falcon et al., 2007). Interestingly, aanaat2 mRNA levels show no difference between wild-type and opn4 dko larvae, suggesting that at this stage Opn4.1-Opn4xb are redundant or may not regulate aanaat2. The reported role for Exorh in the regulation of aanaat2 raises the question if distinct photoreceptors could directly or via the clock control different steps of the melatonin pathway and/or if distinct photoreceptors could perform the same function at different stages of development. The appearance of the opn4 dko locomotor activity phenotype on the 6th dpf, after larvae can already adapt their locomotor activity to the light level, suggests that a developmental aspect plays a role. In addition to the discussed analogy on the molecular level between zebrafish and rodents, opn4 dko larvae exhibit attenuated locomotor activity during the wake state in line with the mouse Opn4 knockout wake state phenotype (Mrosovsky & Hattar, 2003; Lupi et al., 2008; Tsai et al., 2009). The zebrafish opn4 dko phenotype also resembles the light-dependent wakefulness observed in humans (Cajochen et al., 2000; Phipps-Nelson et al., 2003; Lockley et al., 2006; Smolders et al., 2012; Smolders & de Kort, 2014; Knaier et al., 2016). Thus pointing to a similar function for Melanopsin in distant vertebrates.

Locomotor activity in the diurnal and nocturnal wake states

We assessed the effect of light on locomotor activity in the diurnal zebrafish by double knockout of melanopsins, and demonstrate that Opn4.1-Opn4xb elevate locomotor activity during the wake state. In nocturnal mice, OPN4 controls the light-induced sleep state, as exposure to light during the night inhibits activity in wild-type while Opn4 mutants remain active (Mrosovsky & Hattar, 2003; Altimus et al., 2008; Lupi et al., 2008). This phenotype is the opposite of that observed in the zebrafish opn4 dko, and is consistent with activity being induced by light in diurnal vertebrates (Shapiro & Hepburn, 1976; Tobler & Borbely, 1985; Yokogawa et al., 2007) and suppressed in nocturnal vertebrates (Campbell & Tobler, 1984; Mrosovsky & Hattar, 2003). Interestingly, mice with retinal
Together, these data indicate that the reduced locomotor activity in diurnal vertebrates (Shapiro & Hepburn, 1976; Campbell & Tobler, 1984; Yokogawa et al., 2007). Nevertheless, the higher level oscillators of diurnal and nocturnal vertebrates have a similar phase and function resulting in the melatonin level reaching its peak during the night (Challet, 2007). A common light signalling mechanism can be postulated as light represses, through Opn4, melatonin synthesis in both chronotypes. This repression brings about the opposite effect on the activity of diurnal and nocturnal vertebrates, suggesting that activity is inversely regulated downstream of the melatonin receptor. As the efficacy of melatonin receptors depends on receptor-associated partners, cell-dependent receptor expression and other context-dependent factors (Cecon et al., 2018), system bias could be at the root of the opposite light-dependent behaviour between diurnal and nocturnal animals.

Input from the pineal versus the eye

The opn4 dko displays a large-angle turns phenotype, while a role for Opn4a has been reported in the routine turns of the VMR (Fernandes et al., 2012). The defect in large-angle turns indicates a function for Opn4.1–Opn4xb in the adaptation to loss of illumination. Large-angle turns have been shown to be regulated by the eye (Fernandes et al., 2012), thus pointing to a defect in the opn4 dko eye. This can be explained by the expression of opn4.1 in horizontal cells and single cone photoreceptors and opn4xb in bipolar cells (Davies et al., 2011), and overexpression of ddc in opn4 dko eyes. The expression of opn4.1 and opn4xb in the eyes raises the question if the eyes may also contribute to the opn4 dko locomotor activity phenotype. However, the zebrafish pineal is not connected to the retinotectal projection (Robles et al., 2014) and so far sympathetic innervation could not be demonstrated. Importantly, zebrafish pineal function is not affected by catecholamine receptor agonists including NE (Cahill, 1997), thus excluding NE innervation as well as NE released in the circulation by chromaffin cells. Furthermore, the zebrafish pineal functions autonomously as shown by in vitro studies. Zebrafish pineals in vitro synthesise melatonin rhythmically in synchrony with the LD regime and continue rhythmic melatonin production under constant conditions (Cahill, 1996). The melatonin synthesised in the eyes has been shown in over 30 fish species to act as a paracrine signal and is not released in the blood (Zachmann et al., 1992a; ligo et al., 1997; Iuvone et al., 2005; Falcon et al., 2007). Together, these data indicate that the reduced locomotor activity in the opn4 dko wake state ought to be due to a defect in the pineal. It is plausible that the difference between rodents and zebrafish in indirect versus directly light regulation of the pineal is due to nocturnal versus diurnal niche adaptation.

Locomotor activity, light intensity and Opn4 function

As light represses the rest state in diurnal vertebrates (Campbell & Tobler, 1984; Yokogawa et al., 2007), we investigated whether the intensity of light alone could affect locomotor activity during the wake state. We reasoned that reducing the light intensity may partially lift the inhibition on the expression of genes encoding melatonin synthesis enzymes and thereby raise the melatonin level (Max & Menaker, 1992; Zachmann et al., 1992b; Bolliet et al., 1995). A reduction in locomotor activity was measured during the wake state under a low intensity LD regime when compared to medium intensity with the same photoperiod and spectrum. This result is in accordance with a significant higher asmt transcript level detected in the light phase of larvae exposed to a low intensity LD regime. Thus, locomotor activity depends on light intensity and is correlated with the asmt transcript level. These data suggest that sustained repression of the melatonin pathway by light is required to elevate locomotor activity during the diurnal wake state. Importantly, light intensity defines the amplitude of per2 transcription. The reduced per2 transcript level and locomotor activity under a low intensity light phase is supported by the reduced locomotor activity of the per2 mutant (Wang et al., 2015) and the role of per2 in regulating aanat2 transcription in the pineal (Ziv et al., 2005). While the circadian clock has been reported to control the melatonin pathway by regulating aanat2 transcription (Klein, 2007), aanat2 transcript levels between the medium and low intensity LD regimes did not reach a difference that is significant (Fig 4D, ZT21: U-test P = 0.068). However, qPCR does not reveal all regulatory processes, so that post-transcriptional regulation cannot be excluded. Experimenting with light intensities does demonstrate the strong effect of short wavelength light on behaviour, as even dim light induces considerable locomotor activity in wild-type. In contrast to the effect of light intensity on wild-type, the opn4 dko larvae do not show altered expression levels of the core clock genes: per2, arntl1b and per1, suggesting that at this stage Opn4.1–Opn4xb may not function in resetting the circadian clock or are redundant. However, opn4 dko larvae do not show a true block wave function as locomotion (Fig 4E) and the ddc mRNA level (Fig EV4A) and so far sympatric experiments suggests that in zebrafish multiple distinct photoreceptors have the property to regulate locomotor activity. We did not knock out all opn4 genes, which could potentially have a more profound effect on locomotor activity, as expression of the other opn4 genes in the pineal cannot be excluded. The data collected in this study may well support future research to further our understanding of how light affects several non-visual light-dependent processes.

Materials and Methods

Animal husbandry

Animal procedures were conducted according to Austrian (BMWFFW-66.006/0012-WF/II/3b/2014) and European (Directive 2010/63/EU) legislation covering the use of animals for scientific purposes. Zebrafish (Danio rerio) strains were cared for and bred as previously described (Mullins et al., 1994; Westerfield, 2007).

In situ hybridisation

Labelled antisense RNA probes were synthesised following standard protocol. The probe was diluted in hybridisation buffer and heated.
for 2 min at 90°C. Mature fish were anaesthetised with MS-222 (tricaine methane-sulphonate, pH7) and then euthanised by decapitation. After removal of the eyes and lifting the skullcap, the head was fixed overnight (O/N) in formaldehyde (4% FA, pH7.5). Following careful brain dissection, the brains were washed in Phosphate Buffered Saline with 0.1% Tween-20 (PBST, pH7.4) and permeabilised in 100% methanol (MeOH) O/N at –20°C. After removing MeOH through washes with successively lower percentage MeOH, the brains were permeabilised with proteinase K (10 µg/ml in PBST) for 30 min at room temperature (RT) followed by rinsing with glycine (10 mg/ml in PBST) to inactivate proteinase K, fixation and PBST washes. Brains were prehybridised for 2–4 h at 64°C, then hybridised with probe for ~40 h at 64°C, followed by multiple washes with successively lower formamide/SSC (Saline Sodium Citrate, pH7) to remove unbound probe. Next, brains were embedded in 3% agarose in PBS and cut with the Leica VT1000S vibrating blade microtome in 100 µm sagittal sections. Note that sagittal sections show labelling of the pineal clearer than coronal vibratome sections. Note that the observed effect can be solely attributed to the reduction of the local background subtraction method.

**Site-directed mutagenesis**

Knockouts were generated with Transcription Activator-Like Effectors fused to FokI Nuclease (TALEN), as previously described (Cermak et al, 2011; Bedell et al, 2012). The TALENs were designed with online TALEN targeter software (https://tale-nt.cac.cornell.edu/) to bind exclusively to the target genes. The following settings were applied: spacer length of 15–20bp and guanine bound by the Repeat Variable DiResidue (RVD) NN, cytosine by HD, thymine by NG and adenine by NI. The different RVD sequences were subcloned in their order into the pFUS-A and B plasmids with the GoldenGate method (TALEN assembly kit, Addgene). Next, TALE1 was subcloned into pCS2TAL3DD and TALE2 into the pCS2TAL3RR plasmid, each carrying the sequence of one of the two domains of the heterodimeric FokI nuclease (Miller et al, 2011). The intermediate and final constructs were transformed, screened by colony PCR, colonies grown O/N, plasmids isolated and sequenced for quality control. The final sequences were checked against the sequence of the full RVD arrays generated with the TAL plasmids sequence assembly tool (http://bao.rice.edu/). Thereafter the plasmids were linearised with KpnI, in vitro transcribed (SP6 mMessage mMachine kit, Invitrogen), mRNA purified (RNeasy kit, Qiagen) and a range of transcript concentrations was microinjected in zygotes. A target site in the opn4 gene was selected with a unique restriction recognition sequence, which is lost when a mutation is introduced. For genotyping, the target site was amplified with PCR followed by a restriction digest on the amplicon (Table EV2). PCR was performed on a dilution (20x) of 10 µg/ml of proteinase K-treated (2 h at 60°C) tail fin clips or 2 dpf offspring with primers designed to bind in introns (or the 5’-UTR) to specifically amplify genomic DNA. When the restriction recognition site in the amplicon was absent, the type of mutation was identified by sequencing. TALENs were selected that generate premature STOP codons which are transmitted through the germ line. The DNA-binding domains of these TALENs are presented in Table 1 and their RVD sequences in Table EV3.

**Transcriptome sequencing**

Photobiology protocols were followed as previously described (Dekens et al, 2017). Animals were exposed to blue light (peak wavelength LED: ~470 nm, Fig EV31) covering the spectral sensitivity of Melanopsin. With monochromatic light we aim to reduce the number of activated photoreceptors. Even though zebrafish exhibits multiple blue light photoreceptors, we may to some extent minimise the effect of redundant photoreceptors on the analysis of the phototype (Ziv et al, 2007). In addition, applying monochromatic light when comparing different light intensities has the advantage that the observed effect can be solely attributed to the reduction of the light level instead of a change in the spectrum (Dekens et al, 2017). Mature wild-type and opn4 dko fish of the same strain and age were entrained to a blue LD 12:12 h regime for 14 days in a synchronisation instrument at constant temperature (28 ± 0.2°C), and subsequently anaesthetised between ZT4 and ZT6. After decapitation, the head was pinned on silicone in a Petri dish filled with PBS under blue light (LED connected to fibre optic of dissection scope). The eyes were removed by cutting the optic nerves with surgical scissors (Fine Science Tools, FST) and the brain was carefully removed after lifting the skullcap with surgical forceps (FST) and cutting the spinal cord. The brain was separated in an anterior and posterior part with a surgical blade. For each biological replicate, the same tissue parts of three individuals were pooled and frozen in liquid nitrogen to obtain sufficient mRNA. A total of three biological replicates were processed. RNA isolation for sequencing was executed according to a modified version of an existing protocol (Mortazavi et al, 2008; Schenk et al, 2019). Total RNA was isolated by adding RNAzol (Sigma-Aldrich) and a steal bead to the frozen sample, followed by disruption in a bead mill for 3 min at 30 Hz (TissueLyser II, Qiagen) and extraction of total RNA (Direct-zol RNA kit, Zymo Research). mRNA was isolated from total RNA by selective binding to oligo-dT coupled beads (Dynabeads mRNA purification kit, Invitrogen) followed by washes and elution. To increase purity the eluate was bound, washed and eluted for a second round, and thereafter quality checked (Bioanalyzer RNA 6000 Pico assay, Agilent). Next, mRNA was fragmented for 3 min at 75°C (fragmentation reagents, Invitrogen) and the fragment size distribution was quality checked (Bioanalyzer RNA 6000 Pico assay, Agilent). Subsequently, the mRNA was transcribed (SuperScript VILO cDNA synthesis Kit,
Invitrogen) and libraries were constructed (ultra II kit, NEB), followed by adding barcodes with PCR (multiplex sequencing). The samples were single read 100bp high throughput sequenced with the Illumina HiSeq 2500 v4 chemistry. Reads were mapped to the reference genome (assembly GRCz10.86) with NextGenMap software and the number of reads per transcript was counted with the featureCounts tool. To assign genes to pathways, the Ensembl IDs of the differentially expressed genes were converted to KEGG IDs (https://biodbnet-abcc.ncifcrf.gov/db/db2db.php) and input into KEGG software (https://www.genome.jp/kegg/tool/map_pathway2.html), and Ensembl gene IDs were input into FuncAssociate software (http://llama.mshri.on.ca/funcassociate) to reveal gene ontology attributes.

Quantitative PCR

For the first ~30 h of development, embryos were raised in an incubator under a white LD regime. Thereafter the larvae were divided over two synchronisation instruments (Dekens et al., 2017), one set at a medium and the other at a low intensity blue LD 12:12 h regime (Fig EV3I) and constant temperature (28 ± 0.2°C), or wild-type and opn4 dko were placed in the same synchronisation instrument under a medium blue LD regime. The ILT950 spectrometer (International Light Technologies) was applied for light measurements. On the 6th dpf at ZT3, 9, 15 and 21, 12 biological replicates were harvested at each time point for both conditions and placed in liquid nitrogen. Each replicate consists of 10 whole larvae. To determine transcript levels in eyes and ophthalmectomised larvae separately, the larvae were euthanised on the 6th dpf between ZT3 and ZT4 and pinned with 0.1 mm insect pins on silicone in a Petri dish filled with PBS. One pin was placed in the trunk and another between eye and brain, followed by removal of the eyes with surgical forceps (FST) under blue light. The eyes and ophthalmectomised larvae were pooled separately, and 10 larvae were sampled for each biological replicate. In all experiments, a total of 12 biological replicates were harvested for each wild-type and opn4 dko. qPCRs for eyes and ophthalmectomised larvae were performed using the same amount of cDNA transcribed from the same amount of total RNA. The transcript levels in the eyes were normalised by the ratio of the yield in total RNA between eye and ophthalmectomised larval extracts. For whole larvae, ophthalmectomised larvae, mature brain parts and eyes, total RNA was isolated as described for transcriptome sequencing. Next, genomic DNA was eliminated for 2 min at 42°C and 1 μg total RNA was transcribed for 30 min at 42°C using random hexamers (QuantiTect reverse transcriptase kit, Qiagen) followed by inactivation for 3 min at 95°C. ProbeFinder software from Roche was used to design primers (Table EV4) that bind to neighbouring exons spanning the exon–exon junction to avoid amplification of genomic DNA, and generate a short amplicon to increase the overall efficiency of amplification. Note that the applied aaat2 forward and reverse primers do not target aaat1. qPCR was performed with SYBR® Green PCR master mix (Applied Biosystems) and 500 nM final concentration of each primer in the StepOnePlus™ thermocycler from AB. Amplification was executed with the thermocycling protocol: 10 min at 95°C followed by 40 cycles of 15 sec denaturation/annealing at 95°C and 1 min extension at 60°C. All wild-type and mutant biological replicates that were harvested at the same ZT time were analysed together on the same 96 well plate. For each sample, a technical replicate was included. To allow comparison between data sets, the threshold to determine the quantification cycle (Cq) was set at the same relative fluorescence units (RFU) for all reactions detecting a particular transcript and all qPCR plates were run on the same machine. After each run, the specificity of the qPCR was determined by dissociation curve analysis. The relative transcript level in each sample was determined by normalising to the actb1 transcript level in that sample.

Behaviour assay

For the first ~30 h of development, embryos were raised in an incubator under a white LD regime. Thereafter, the larvae were placed in the DanioVision® automated observation chamber (Noldus) under a medium or low intensity blue LD 12:12 h regime with illumination from the top (Fig EV3I) and constant temperature (28°C). One larva was placed in each well (Ø35 mm wells, 8 ml of Aqua Condition Plus KH+ water) of a 6 well plate on the 1st dpf and entrained for 8 days. Note that small wells result in unreliable locomotor activity readout (Wolter & Svoboda, 2020). In each experiment, 3 wild-type and 3 opn4 dko larvae were placed next to each other. The larvae were recorded and tracked with EthoVision® software during the 5th, 6th and 7th dpf. Their activity or velocity was measured as the rate of change of a larva’s position in a unit time. The distance covered by a larva during 6 min was binned. Locomotor activity was measured of 18 larvae (biological replicates) from spawnings of different parents for each experiment. To determine if the activity of wild-type and mutant larvae in the light or dark phase on a particular day shows a difference that is significant, we pooled all activity bins in the respective phase of each larva separately and then applied the test statistics on the data. The aggregated 12 h activity data of each larva on one particular day is in this case one single observation in the test statistics. As the consecutive days were analysed and presented separately, the assumption of independent identical observations is fulfilled. For the analysis of large-angle turns, the distance covered in 6 sec was binned for each larva, and the mean distance covered of each larva over the last 6 min in the light phase was used as a baseline to calculate the displacement in the first 6 sec of that larva in darkness (Fig EV3A–F). For routine turns, the mean distance covered of each larva over the last 6 min in the light phase was used as a baseline to calculate the displacement of that larva above this level in the following 12 min of darkness (Fig EV3A–H).

Test statistics

Statistical analysis of the transcriptome data was implemented with edgeR software (version 3.9). For differential expression, edgeR applies an exact test analogous to Fisher’s exact (Fisher, 1922) with consideration of overdispersion and adjusts the P-values for the expected proportion of type I errors (false discovery rate) with the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). The cutoff for differential expression was set at a significance level of α = 0.05. Fisher’s exact test was applied to determine if the common differentially expressed genes among two data sets are shared due to coincidence or dependence (https://www.r-project.org/). For analysis of behaviour and qPCR data, the distribution in a data set was calculated with the Shapiro–Wilk test (Shapiro & Wilk, 1965). For normally distributed data, the unpaired Student t-test (Student, 1908) was applied. To determine if a significant difference
between the variances of two normally distributed data sets exists, the F-test was applied. When $H_0$ is accepted ($P > 0.05$), a t-test with equal variance was applied and when $H_0$ is rejected, a t-test with Welch’s correction was applied (unequal variance). In all cases we analysed if there is a difference between the data sets (two-tailed). Hypothetically one could apply for the locomotor activity a one-tailed test, as the aggregated velocity in the light phase of a diurnal vertebrate can be assumed to be less when less photons are detected. In this case, the $P$-values for locomotor activity in the wake state, which follows a normal distribution, are half of the stated $P$-values in this report. To determine if the difference between the wild-type and $opn4$ dko locomotor activity is due to interindividual variability or the result of the phenotype, we calculated Cohen’s effect size ($d$), which is a comparison of the difference between the means with the pooled standard deviation (Cohen, 1988). Note that this calculation of the effect size can only be applied on data that follows a normal distribution. Cohen defined descriptors for magnitude of effect size as medium: $0.5 < |d| < 0.8$, or large: $|d| > 0.8$. For non-normally distributed data, the nonparametric Wilcoxon rank-sum test/Mann-Whitney U-test (Mann & Whitney, 1947) was applied. The latter distribution is indicated with $U$-test in front of the stated $P$-value. The significance level was set in all cases at $\alpha = 0.05$, following standard practice in this field.

Data availability

RNA-Seq data: NCBI Gene Expression Omnibus GSE189906 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189906).

Expanded View for this article is available online.

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Author contributions

Marcus P S Dekens: Conceptualization; Resources; Data curation; Formal analysis; Supervision; Funding acquisition; Validation; Investigation; Visualization; Methodology; Writing—original draft; Project administration; Writing—review & editing. Bruno M Fontinha: Investigation. Miguel Gallach: Formal analysis. Sandra Pflügler: Investigation. Kristin Tessmar-Raible: Conceptualization; Resources; Supervision; Funding acquisition; Project administration; Writing—review & editing.

In addition to the CiRediT author contributions listed above, the contributions in detail are: MPSD and KT-R conceived and designed the study. MPSD generated the $opn4$ knockouts, conducted KEGG and GO analysis on the differentially expressed genes, executed the photobiology experiments and conducted quantitative and statistical analysis of gene expression and behaviour. MPSD and SP performed the in situ hybridisations on adult brains. MPSD and BMF executed the transcriptome sequencing in collaboration with the Vienna BioCenter Core Facilities (VBCF). MG processed the raw sequence data. MPSD wrote, and KT-R reviewed the manuscript. All authors approved the manuscript.

Disclosure and competing interests statement

The authors declare that there is no conflict of interest.

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