Circadian and Circalunar Clock Interactions in a Marine Annelid

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http://dx.doi.org/10.1016/j.celrep.2013.08.031

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SUMMARY

Life is controlled by multiple rhythms. Although the interaction of the daily (circadian) clock with environmental stimuli, such as light, is well documented, its relationship to endogenous clocks with other periods is little understood. We establish that the marine worm Platynereis dumerilii possesses endogenous circadian and circalunar (monthly) clocks and characterize their interactions. The RNAs of likely core circadian oscillator genes localize to a distinct nucleus of the worm’s forebrain. The worm’s forebrain also harbors a circalunar clock entrained by nocturnal light. This monthly clock regulates maturation and persists even when circadian clock oscillations are disrupted by the inhibition of casein kinase 1δ/ε. Both circadian and circalunar clocks converge on the regulation of transcript levels. Furthermore, the circalunar clock changes the period and power of circadian behavior, although the period length of the daily transcriptional oscillations remains unaltered. We conclude that a second endogenous noncircadian clock can influence circadian clock function.

INTRODUCTION

Most, if not all, organisms feed periodic changes in light conditions into molecular clockworks that allow them to anticipate rhythmic changes in their environment and to synchronize their behavior and physiology (Cold Spring Harbor Symposia on Quantitative Biology, 2007; Roenneberg and Merrow, 2005). Efforts to study the underlying molecular mechanisms have focused almost exclusively on circadian clocks (i.e., clocks anticipating daily cycles). One of the critical mechanisms driving animal circadian clocks are transcriptional/translational feedback loops formed by a set of regulatory genes. These genes are partially shared between insect and mammalian models, arguing for a common origin of animal circadian clocks. The feedback loops continue to run under constant conditions and are coordinated with the animal’s environment by entrainment (Cold Spring Harbor Symposia on Quantitative Biology, 2007; Roenneberg and Merrow, 2005).

However, many organisms also exhibit rhythms of longer and shorter period lengths (Aschoff, 1981; Naylor, 2010). In order to maximize the chance of finding mature mates, to avoid predators, and to have favorable environmental conditions, organisms ranging from brown algae and corals to worms and vertebrates synchronize their maturation and spawning to a particular moon phase, to particular times of the day, and/or to specific seasons within a year (Fox, 1924; Harrison et al., 1984; Korringa, 1947; Tessmar-Raible et al., 2011). As with circadian rhythms, such noncircadian (e.g., annual and monthly) rhythms are often driven by internal oscillators (circannual and circalunar clocks, respectively), which use light cues (photoperiod and moonlight, respectively) for the adjustment with the outer environmental conditions (Dupré and Loudon, 2007; Franke, 1985; Lincoln et al., 2006; Naylor, 2010; Hazlerigg and Lincoln, 2011; Kaiser et al., 2011).

Numerous studies have assessed the influence of additional light cues on the molecules and function of the circadian clock. Photoperiod influences circadian clock gene oscillations in insects (Kostál, 2011) and the waveform of circadian oscillations in mice (Clareggio et al., 2011), resulting in activity differences between animals raised under different photoperiods. Likewise, dim nocturnal light at moonlight intensity has been shown to influence circadian clock gene expression levels and timing in Drosophila, resulting in elevated nocturnal activity under laboratory conditions (Bachleitner et al., 2007). However, no elevated nocturnal activity was observed in corresponding moon phases under natural conditions, and the level of Period was differently altered (Vanin et al., 2012). Lunar light influences the levels of the putative light receptor and/or core clock gene cryptochrome in the circalunar spawning coral Acropora and the rabbit fish.
Siganus guttatus (Fukushiro et al., 2011; Levy et al., 2007). In Siganus, moonlight has also been shown to elevate levels of the circadian clock gene per2 (Sugama et al., 2008). However, a key issue that has remained obscure is if and how circadian and noncircadian internal oscillators interact molecularly to influence the behavior of an organism, independent of illumination effects.

A suitable model system to assess this question has to be a molecularly accessible, extant animal that at the same time possesses circadian and noncircadian timing mechanisms. The bristle worm Platynereis dumerilii offers this dual advantage. Platynereis was among the first species for which a circalunar spawning rhythm was scientifically documented (Fage and Legendre, 1927; Ranzi, 1931a, 1931b). In addition, Platynereis has emerged as a highly suitable model for molecular neurobiology (Arendt et al., 2004; Backfisch et al., 2013; Tessmar-Raible et al., 2007; Tomer et al., 2010).

Here, we establish that Platynereis dumerilii possesses both a circadian and a circalunar clock. Whereas the circalunar-clock-controlled reproductive timing rhythms are insensitive to the functional disruption of circadian clock gene oscillations, the circalunar clock affects the circadian clock on at least two levels. First, the period length and power of circadian-clock-controlled locomotor behaviors are significantly different between different phases of the circalunar clock, while the period length of the presumptive core circadian clock molecular oscillations remains unaffected. Second, clock, period, pdp1, and timeless transcript levels oscillate in specific brain nuclei of the worm’s forebrain not only over 24 hr, but also across different phases of the lunar month. This establishes changes in RNA levels as a direct or indirect output of the circalunar clock.

RESULTS

Platynereis Possesses a Light-Entrained Circalunar Clock

The circalunar reproductive periodicity of Platynereis dumerilii (Figures 1A and 1B) has been extensively documented (Figure S1). Reproductive state, as measured by the number of animals reaching sexual maturity, is maximal shortly after new moon (NM) and minimal during periods of full moon (FM) (Figure S1).

We first assessed if our Platynereis dumerilii culture also possesses a nocturnal-light-adjusted circalunar spawning cycle. Following the conditions used in classical experiments (Hauenschild, 1954, 1955, 1960), we subjected the culture to a circadian light regimen of 16 hr light and 8 hr darkness (Figure 1C). For eight consecutive nights of a lunar month, we exposed the worms to dim nocturnal light (termed “full moon period” [FM]). We refer to the middle week of the remaining period as NM. This monthly light cycle in the lab can be in phase (Figure 1D) or out of phase (Figure 1E) with the natural moon. In accordance with classical observations (Figure S1), the daily number of mature animals peaked at the time between the FM stimuli (Figures 1D and 1E). Irrespective of the natural moon phase, these peaks of maturing animals remained in phase with respect to the week of the nocturnal light stimulus (Figures 1D and 1E). This establishes that nocturnal light stimuli alone are sufficient to synchronize circalunar reproductive cycles in our Platynereis lab culture.

Next, we tested if the observed circalunar spawning rhythm was controlled by an endogenous circalunar clock. As this point was debated previously (Hauenschild, 1960; Palmer, 1974), we performed lunar free-running experiments. After entrainment of animals for more than 2 months in the described circadian and circalunar light regimens, the FM stimulus was omitted, whereas the circadian light cycle remained unchanged (termed “free-running full moon” [FR-FM] in Figure 1C). The NM after this FR-FM is termed “free-running new moon” (FR-NM in Figure 1C). Worms continued to exhibit a monthly reproductive periodicity under these conditions (Figure 1F), with a period of 30 days (Figure 1G). Worms under constant light or raised without any nocturnal illumination did not show reproductive rhythms (Figure 1H). This establishes that circalunar reproductive periodicity in our culture is governed by an endogenous circalunar clock.

Platynereis Possesses the Full Complement of Drosophila and Mouse Core Circadian Oscillator Gene Orthologs

After we established that our worms possessed an endogenous circalunar clock, we tested for the presence of an endogenous circadian clock. For this, we determined the worms’ complement of core circadian clock genes and their expression dynamics. Bmal, period, and clock are present in the core circadian oscillator in vertebrates and flies (Young and Kay, 2001); cryptochrome (cry) acts as core clock component in vertebrates and nondrosophilid invertebrates (Chaves et al., 2011; Zhan et al., 2011; Zhu et al., 2005); timeless is crucial for the insect circadian clock (Myers et al., 1995), but the gene is absent from vertebrates (Göttler, 2006). Orthologs of timeout (also termed tim2) and cry are important for circadian clock entrainment in insects (Benna et al., 2010; Emery et al., 2000). Moreover, cry is part of the circadian oscillator in the fly peripheral clock (Vanchenko et al., 2001; Krishnan et al., 2001; Levine et al., 2002). Pdp1 acts together with vrille in a modulatory feedback loop on the core transcription/translational feedback loop in insects (Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003). Of these genes, only bmal had been identified in larval Platynereis (Arendt et al., 2004). By a combination of degenerated PCR and massive transcript sequencing, we identified Platynereis orthologs of period, clock, timeless, timeout, pdp1, and vrille, as well as two cry genes that we name L-cry (“L” indicating orthology to light-receptive Crys) and tr-cry (“tr” indicating orthology to Crys acting as transcriptional repressors) (Figure S2A).

As transcriptional oscillations are important for circadian clock function (Kadener et al., 2008; Padmanabhan et al., 2012), we next investigated messenger RNA (mRNA) dynamics of these genes. In addition, we focused our analyses on premature adult heads (Figure 1A). It had previously been shown that the maturation of Platynereis, which is the major event known to be synchronized by circadian and circalunar clocks, is controlled by the brain (Hauenschild, 1964, 1966; Hofmann, 1975). In order to ensure that any observed changes were due to the experimental conditions, but not due to developmental stage differences of the worms, we carefully staged the worms based on segment numbers, appendage shape, pigment appearance, and eye and body size.
We first investigated the temporal expression profiles of *bmal*, *period*, *clock*, *tr-cry*, *timeless*, *vrille*, *pdp1*, and *timeout* using quantitative PCR (qPCR). This would also allow us to obtain an understanding of how the different circadian clock genes might relate to each other in terms of their regulation. We sampled heads during different circadian points at the...
NM phase under light-dark (LD) conditions. In these experiments, bmal, period, clock, tr-cry, and timeless (Figures 2A–2E) and vrille, pdp1, and timeout (Figures S2B–S2D) exhibited robust circadian cycles. With the exception of timeless and timeout, this cycling was maintained during constant darkness (DD) (Figures 2F–2J; Figures S2E–S2G), consistent with the notion that bmal, period, clock, pdp1, vrille, and tr-cry are components of a core circadian oscillator in Platynereis heads. Clock and bmal transcripts cycled in phase with each other (Figures 2A, 2C, 2F, and 2H), consistent with a possible heterodimer formation known from flies to mammals (Darlington et al., 1998; Gekakis et al., 1998). Period, pdp1, and timeout transcript oscillations (Figures 2B and 2G; Figures S2C, S2D, S2F, and S2G) were in antiphase with bmal/clock expression. In contrast to Drosophila, where vrille RNA levels peak prior to pdp1 levels (Cyran et al., 2003), vrille RNA peaks followed those of pdp1 in Platynereis (Figures S2B, S2C, S2E, and S2F).

Figure 2. Platynereis Circadian Clock Gene Orthologs Show Circadian Oscillations on the RNA Level

(A–J) Temporal profiles of clock gene RNA expression in Platynereis heads sampled under NM (A–E) circadian light regimen and constant darkness (F–J) are shown. Values are means ± SEM, n = 5–16 (A–E), n = 6 (F–J); four to five heads/n. The p value was determined by one-way ANOVA. See Figures S2B–S2G for additional circadian clock genes.

(K) Platynereis L-cry transcript levels fluctuate, but do not show regular cycling patterns over 4 days (n = 2).

(L) Light decreases Pdu-L-Cry, but not Pdu-tr-Cry, levels in S2 cells. Dp-Cry1 and Dp-Cry2 serve as positive and negative controls, respectively. V5 epitope-tagged Pdu-L-Cry, Pdu-tr-Cry, Dp-Cry1, and Dp-Cry2 was coexpressed with GFP. After a 6 hr light pulse (gray bars) or constant darkness (black bars), cell extracts were collected, western blotted, and probed with anti-V5 and anti-GFP (see Figure S2H). CRY levels were quantified by densitometry of antibody staining after normalization with GFP. The dark value for each CRY was plotted as 100%. Data are means ± SEM; n = 3 independent transfections. Significant differences were assessed by Student’s t test (**p < 0.01; ****p < 0.0001).

(M) Platynereis tr-Cry, but not the closely related Pdu-L-Cry or Pdu-6-4-photolyase, strongly inhibit Pdu-CLK/Pdu-BMAL-mediated transcription in a luciferase reporter gene assays. The monarch butterfly per E-box-containing enhancer (DpPer4Ep-Luc) was used in the absence (control) or presence of Pdu-clock/Pdu-bmal plasmids (350 ng each). Dp-cry1 and Dp-cry2 serve as positive and negative controls, respectively. Data are means ± SEM; n = 4–8 independent transfections. Significant differences were determined by Student’s t test (****p < 0.0001).
Pdu-L-Cryptochrome and Pdu-tr-Cryptochrome Can Function as a Light Receptor and Transcriptional Repressor, Respectively

In order to test if the investigated Platynereis circadian clock genes can indeed function in the conventional clock mechanism, we employed two assays previously used to validate the activity of presumptive core circadian clock genes of the monarch butterfly (Zhu et al., 2005, 2008). Cryptochromes functioning as light receptors undergo a light-dependent reduction in protein levels in S2 cells because of proteasome-mediated degradation (Lin et al., 2001; Zhu et al., 2005). If Pdu-L-CRY can indeed function as light receptor, we should be able to observe such a reduction. We assessed the effect of a 6 hr light pulse to promote Pdu-L-CRY, as well as Pdu-tr-CRY degradation, and compared the responses to those of the two monarch butterfly Cryptochrome proteins, sense controls and expression of noncircadian genes, see Figures S3A–S3F. Arrowheads indicate expression in eyes. Scale bar represents 50 μm, and an asterisk indicates the position of major brain neuropil.

(E) Scheme of worm head indicating area is shown. Circadian clock gene expressing brain nuclei are indicated as blue ovals. e, adult eyes.

Platynereis Core Circadian Clock Gene Orthologs Are Confined to Specific Domains in the Medial Forebrain

In order to determine if the uncovered circadian clock gene orthologs localize to a centralized structure or are broadly expressed, we performed whole-mount in situ hybridizations (WMISH).

All genes tested were specifically expressed in the posterior medial brain (Figures 3A–3E; Figures S3A–S3C), particularly in paired oval-shaped structures (arrows and magnifications in Figures 3A–3D; Figure S2H). These brain regions were already noted by Retzius as distinct nuclei in the brain of Nereis, a close relative of Platynereis (Retzius, 1895), hence representing nuclei conserved among nereidid worms. The brain morphology of Platynereis changes little during development from larvae to premature adults (Tomer et al., 2010). By position and relation to the axonal scaffold and the prominent cilia of the ciliary photoreceptor cells (arrows in Figures S3G–S3J), these distinct nuclei arise from the area demarcated by bmal in the 2-day-old larval cells led to a strong activation of luciferase activity (Figure 2M). Additional transfection of Pdu-tr-cry strongly and highly significantly reduced this activation, similar to our positive control, the monarch butterfly’s tr-cry ortholog, cry2 (Figure 2M). Addition of Pdu-L-cry or its monarch ortholog cry1 did not reduce Pdu-Bmal/Pdu-Clock-mediated luciferase expression in comparable levels (Figure 2M). Similarly, Pdu-6-4photo-lyase, a gene most closely related to Pdu-tr-cry, but whose orthologs function in UV-induced DNA repair (Sancar, 2008), did not show obvious transcriptional repressor activity (Figure 2M). We thus conclude that bmal, clock, and tr-cry likely function in a core circadian clock positive/negative transcriptional loop in Platynereis dumerilli, like their orthologs in other species.
Figure 4. The Circalunar Clock Affects Circadian-Clock-Controlled Activity Rhythms

(A) Mean locomotor activity (hourly average ± SEM) shows higher nocturnal activity in Platynereis in NM under 16:8LD circadian illumination over the course of 3 days (N = 12 rhythmic animals). Active behaviors were counted as 1, inactive as 0. See Figures S4A–S4C for details on active versus inactive behaviors and recording setup.

(B) Quantification of average locomotor activity per hour of day hours (yellow bar) versus night hours (black bar) of 3 consecutive days is shown. Error bars represent ±SEM. Significant differences were determined by Student’s t test (****p < 0.0001).

(C) Percentage of present period length of individual worms under NM/LD conditions is shown. See individual periodograms in Figure S4J.

(D) Average periodogram (N = 12) for NM/LD conditions shows a dominant period of 24 hr and an additional 12 hr peak. The red line indicates the significant p level = 0.05.

(F) Platynereis locomotor activity cycles continue in NM under complete darkness (DD) over at least 3 consecutive days (N = 10 rhythmic animals) showing a higher nocturnal activity. NM/DD: worms were entrained normally with circadian and circalunar illumination conditions. Recordings were performed during NM in complete darkness. See (A) for scoring details and Figure S4E for activity cycles including arrhythmic animals and Figures S4H and S4K for periodogram analysis.

(G) Mean locomotor activity cycles continue in FR-FM under normal light/dark (LD) conditions showing an increase in daily locomotor activity (N = 18 rhythmic animals). See (A) for scoring details and Figure 1C for details on illumination. See Figures S4F and S4L for activity cycles including arrhythmic animals and periodogram analysis, respectively.

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medial forebrain (Arendt et al., 2004). Our findings of a medial forebrain nucleus harboring the core circadian clock genes are hence also consistent with our previous analyses in Platynereis larvae (Arendt et al., 2004).

The observed coexpression of the Platynereis clock gene orthologs is consistent with them acting together in a positive-negative feedback loop, as typical for the core circadian oscillators of all animals analyzed to date. Likewise, the expression of L-cry in the same oval-shaped posterior medial forebrain domains (Figure S3B; compare to Figures 3A–3E), along with the presented functional data, are consistent with L-Cry serving as a possible light sensor for the Platynereis circadian clock. This is also coherent with the fact that light should be able to reach these cells, as the worm’s brain is relatively small and the cuticle largely transparent.

In addition to these nuclei, we also noted circadian clock gene expression in the area of the eyes (arrowhead in Figures 3A and 3B). Again, this staining was not present in sense controls, nor was it typically present when other genes were stained (Figures S3D–S3F). In order to analyze the exact position and extent of this expression, we performed WMISH on a Platynereis eye pigment mutant (Fischer, 1969). As in Drosophila (Hunter-Ensor et al., 1996), cells in the eyes also exhibited circadian clock gene expression (Figure S3K), albeit in general less than in the posterior medial forebrain domain.

We next analyzed if our WMISH confirms the daily transcriptional oscillations observed by qPCR. For this, we focused on the two most strongly expressed clock gene orthologs, Pdu-bmal and Pdu-period. When analyzed at different circadian time points, the expression of both genes showed circadian fluctuations within the described two medial brain nuclei (Figure 3A; Figure S3A), suggesting that these are the major circadian clock centers of Platynereis.

**Platynereis Locomotor Activity Is under Circadian Clock Control**

Given that Platynereis exhibits molecular circadian oscillations in paired medial forebrain nuclei, we next asked if the worms also displayed circadian behavior. We therefore recorded worms in a box over several days using an infrared camera and categorized their behavior into active (searching, fighting) and inactive (no movements, undulatory fanning movements) types (Figures S4A–S4C). We first analyzed if the worms showed any consistent activity patterns over multiples of 24 hr during NM/LD. The activity data were analyzed using ActogramJ for chronobiological analyses for rhythmicity and period lengths (Schmid et al., 2011). Under NM/LD conditions, the worms displayed primarily nocturnal activity (Figures 4A and 4B) with an average period length of 24.2 hr (±0.2) (Figures 4C–4E and 4J; Figures S4D and S4J). These data are consistent with the fact that the nuptial dance of Platynereis only occurs during few hours of the night (Korringa, 1947), and with previous observations in the related nereidid Nereis (Last, 2003; Last and Olive, 2004).

In addition, a dominant ∼12 hr period was observed in 8% of the analyzed individuals (n = 14, Figures 4C and 4E). This 12 hr activity rhythm does not appear to be crepuscular, possibly rather resembling a circatidal rhythm (see example worm 3 in Figure 4E). Under NM/DD conditions, the worms continued to show a circadian periodicity (23.6 ± 1.5 hr) over at least 3 days (Figures 4F, 4J, and 4L; Figures S4E, S4H, and S4K), evidencing that the worm’s locomotor activity is under circadian clock control. The generally still relatively high level of variability in the period lengths of our periodogram analyses might be due to the representative, yet still relatively short analyses timeframe and small sample size.

**The Circalunar Clock Impacts Circadian Behavior**

Having established that both circadian and circalunar clocks exist in Platynereis, we next investigated how these two clocks interact with each other.

We started by comparing the Platynereis circadian locomotor activity cycles between two different phases of the circalunar clock (NM versus FR-FM; see Figure 1C). Compared to NM, the worms were less rhythmic in their locomotor behavior under FR-FM in both circadian LD and DD conditions (Figures 4F–4H; Figures S4M and S4N; Figures S4L and S4M). Occasionally, worms already showed rhythms of shorter period lengths during NM (LD and DD) (Figures 4C and 4L; Figures S4J and S4K). However, the
The number of worms exhibiting behavioral rhythms with periods clearly different from 24 hr was strongly increased in FR-FM (LD and DD) compared with NM (LD and DD) (Figures 4 C and 4L–4N). This provides strong evidence that the circalunar clock affects circadian behavior.

The Circalunar Clock Impacts Transcript Levels of clock, period, pdp1, and timeless
Changes in circadian behavior have been directly connected to changes in circadian clock gene levels in Drosophila and mice (Antoch et al., 1997; Benito et al., 2007; Kadener et al., 2008). We therefore next investigated if the oscillation and levels of circadian clock gene orthologs were also affected by the circalunar clock by comparing RNA levels between NM and FR-FM (cf. Figure 1C). For the genes pdp1, clock, period, and timeless, the circadian expression dynamics (period lengths and phase, represented as shape of the graphs) under FR-FM were not detectably different to NM conditions (Figures 5A–5C; Figure 5A0–5C0; Figures S5A and S5A0). However, their overall transcript levels were significantly elevated at FR-FM compared to NM (Figures 5A–5C and 5A0–5C0; Figures S5A and S5A0). In contrast, expression levels and circadian dynamics of bmal (Figures 5D and 5D0), tr-cry, vrille, and timeout (Figures S5B–S5D and S5B0–S5D0) did not differ between FR-FM and NM conditions (pink versus blue graphs).

We hence conclude that the overall transcript levels of clock, period, pdp1, and timeless are directly or indirectly modulated by the circalunar clock.

If this is indeed the case, the transcript levels at the next NM under circalunar free-running conditions (FR-NM; see Figure 1C) should return to the levels observed under normal NM. This is indeed the case for the three genes tested representatively. Circadian oscillations and transcript levels of clock, period,
and bmal in FR-NM resembled that of NM (Figures S5F–SSH and S5I–SSH).

Analyses of premature adult brains using WMISH revealed that the elevation of clock, period, pdp1, and timeless transcripts during FR-FM was not due to additional brain domains expressing these genes, but that the same cells in the two core circadian brain nuclei now express at higher levels (Figure 5E; Figure S5E).

These results predict that at least one of the circadian clock genes clock, period, pdp1, or timeless function either downstream of the circalunar oscillator, or as part of it, and establish the regulation of mRNA levels as an output of the circalunar clock.

**Circadian Clock Gene Oscillations Are Not Required for Circalunar Clock Function**

We next asked if the circadian clock affects, or is part of, the worm’s circalunar clock. Different hypotheses have been put forward to explain rhythms with a semilunar or lunar period length. Many of these models involve circadian oscillators. One model relies on the interaction of the circadian oscillator with an oscillator running with a circalunidian or tidal period (i.e., 24.8 hr or 12.4 hr) so that both only coincide once per lunar or semilunar month (Figure 6A; Soong and Chang, 2012). Alternatively, the counting of circadian cycles has been proposed in the frequency demultiplication hypothesis to lead to a circalunar rhythmic output (Soong and Chang, 2012).

We thus next tested if circadian clock gene oscillations were required for circalunar clock function in Platynereis. For this, we interfered with the Platynereis circadian clock and assessed the effects of this interference on circalunar spawning peaks. Mammalian casein kinase 1ε and its Drosophila ortholog Double time (DBT) are crucial for normal circadian clock function (Lee et al., 2009). Their best-documented function is Period phosphorylation, which serves to enhance Period degradation in both systems (Gallego and Virshup, 2007). PF-670462 and other CK1ε inhibitors severely affect the circadian period in mammalian cells (Eide et al., 2005; Walton et al., 2009).

The Platynereis ck1ε ortholog is widely expressed, including in areas of the medial forebrain and the oval- shaped core circadian clock brain nuclei (Figure S3L). Upon PF-670462 treatment, the amplitudes of bmal, clock, tr-cry, timeout, timeless, and pdp1 transcriptional cycling were flattened to a level that no clear oscillations were observable anymore in Platynereis (Figures 6B and 6C; Figures S6A–S6D), while period transcription showed irregular fluctuations (Figure S6E).

Consistent with the abolished molecular circadian clock oscillations, we also found that 70% of PF-670462-treated worms were arrhythmic in their daily activity when tested under NM(LD) conditions (Figures 6D–6G). The remaining 30% showed weak rhythmicity, but their period length was severely altered to about 17 hr. Despite their severely disrupted circadian rhythmicity, PF-670462-treated worms were still capable of displaying all types of normal behaviors (Figure 6D). This is apparent from the mean analysis (Figure 6E), but also from individual worms (Figures 6D–6G), attesting to the notion that PF-670462 treatment leads to a disruption of the circadian core clock in Platynereis in the majority of the population. Despite these significant changes in circadian clock gene dynamics, however, PF-670462 treatment did not affect the circalunar spawning periodicity of Platynereis when compared to controls in free-running experiments (Figures 6H and 6I; compare to Figure 1H for arrhythmic spawning).

We tested several concentrations of PF-670462 and performed the circalunar spawning assays with the lowest concentration still exhibiting robust effects on circadian clock molecular oscillations. Whereas we cannot exclude that PF-670462 also affects other targets at the given concentration, we can conclude that none of these effects, including the one on the circadian clock, shows an obvious impact on the circalunar clock.

Based on these results, we conclude that the circalunar clock in Platynereis is independent of the oscillations of the circadian transcriptional clock (Figure 7).

**DISCUSSION**

**Life with More Than One Type of Clock**

Here, we show that the bristle worm Platynereis dumerilii harbors two endogenous clocks, with a circadian and a circalunar period length, respectively. The coexistence of multiple clocks in one organism is likely a rather common phenomenon, yet most clearly displayed outside of the group of the conventional molecular animal model species (Naylor, 2010; Tessmar-Raible et al., 2011). Consequently, the interactions of such clocks have only been investigated to a very limited extent (Takekata et al., 2012). We provide evidence that the oscillatory mechanisms of both clocks are distinct, but that they both converge on the regulation of transcript levels and behavior.

Whereas our behavioral analyses focused on premature adult Platynereis worms, we propose that the observed modulation of circadian behavior by the circalunar clock also underlies the regulation of other behaviors, such as the nuptial dance of mature animals. This mating behavior is known to be synchronized both to particular days of the month and to specific hours of the night (Korringa, 1947). Synchronized mating likely increases the reproductive success of externally fertilizing animals, especially when they occur in large populations, as for instance in reef corals (Harrison et al., 1984).

The biological implication of the changes in behavioral period length of the premature adult worms might only be understandable when we will know more about the natural conditions the worms have to adapt to outside of the time of mating.

Parallel to our study, work on Eurydice pulchra revealed the coexistence of molecularly independent circatidal and circadian clocks in this crustacean (Zhang et al., 2013). A possible coordination of these two clocks might occur by their coregulation by CK1ε, as PF-670462 incubation led to an increased period length of both circadian and circatidal clocks (Zhang et al., 2013). An effect of PF-670462 on the period length of the Platynereis circalunar clock is possible, but as Platynereis only spawns once, our current analyses rely on scoring large populations, making this question technically very difficult to test. Live readouts of the circalunar clock in individual worms will be helpful to answer such and further questions on circalunar and circadian clock interactions in the future.
The Effect of the Circalunar Clock on Circadian Period Length

Our results show that on the behavioral level, the period length and strength of the circadian rhythm are significantly modulated by the circalunar clock. The change in behavioral period length contrasts with the seemingly unaltered period length of the molecular oscillations of the core circadian clock genes. We currently see two possibilities to explain this discrepancy.

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On the one hand, the period length of the worm’s locomotor rhythms could be modulated independently of the core circadian clock, albeit still also under circadian clock control. In such a model, the circadian clock would directly target genes (downstream or independently of the circadian clock) that can regulate behavior. It is for instance conceivable that the circadian clock affects the levels of hormone precursors, processing enzymes or neurotransmitters. By changing thresholds, these changes (in combination with the circadian clock control) could subsequently result in the observed behavioral phenotype. To exemplify, if lowering the overall levels of a suppressor, a transmitter affecting behavioral activity could reach critical levels high enough to elicit activity more often (e.g., twice per day instead of once per day).

On the other hand, it could be possible that the elevation of clock, period, pdp1, and timeless mRNA levels during FR-FM causes (at least partly) the behavioral changes. A possible scenario how this could be the case is outlined below.

It is well-established that 12 hr rhythms occur in the expression of approximately 1% of all genes in mouse liver, although the circadian clock is unaltered (Hughes et al., 2009, 2012; Vollmers et al., 2009). In addition, 8 hr rhythms in gene expression also occur naturally (Hughes et al., 2009). It seems plausible that what happens in the liver might also happen to cells in other tissues, such as neurons in the brain. In addition, it is also plausible that changes in locomotor activity rhythms can be controlled by changes in gene activity of genes affecting behavior, such as hormonal precursors, processing enzymes, or neurotransmitters. Thus, gene activity cycling with 12 hr or 8 hr rhythms could generate 12 hr or 8 hr behavioral activity cycles in the background of a normally functioning circadian clock.

A recent theoretical work provides a mathematical model explaining the generation of such naturally occurring 12 hr gene expression cycles based on changes in the binding of circadian transcription factors to separate (noncompetitive) binding sites (Westmark and Herzel, 2013). More specifically, two points of the mathematical model might help to explain the findings described in our work. (1) The oscillation amplitudes of the core circadian transcription factors have an impact on the circadian term of the equation (i.e., if they are equal, the circadian term will vanish). In other words, 12 hr cycles can occur based on changes in the amplitude of the core circadian transcription factors that themselves still cycle with a 24 hr periodicity. This could explain, how the changes in transcript levels we observe for some core circadian transcription factors could finally lead to changes in locomotor activity cycles. (2) A combination of less 24 hr and more 12 hr periods in transcription factor rhythms can produce 8 hr fluctuations. In both FR-FM (DD and LD) conditions, we observe such a decrease of 24 hr behavioral periods, combined with an increase in 12 hr periods. Thus, our observed combination might “automatically” lead to the appearance of ~8 hr rhythms, which is what we indeed observed.

Furthermore, besides the mathematical-model-based considerations, there is also functional evidence that slight changes in gene levels can influence the period length of locomotor activity. The introduction of one or more additional copies of the clk genomic region significantly alters the circadian locomotor activity period in Drosophila (Kadener et al., 2008). This effect is thought to be caused by the increased transcriptional levels of clk’s direct target genes per, pdp1, and tim (Kadener et al., 2008). Remarkably, we see the same genes upregulated by the circalunar clock in Platynereis, raising the possibility that, in analogy to Drosophila, an increase in RNA levels of Platynereis clock can account for the upregulation of period, pdp1, and timeless transcript levels, and in consequence for the significant shortening of the circadian behavioral period length of the worm. One additional piece of evidence that changes in circadian clock gene mRNA levels can manifest themselves in changes in locomotor output rhythms stems from a study of the pdp1 gene (Bennito et al., 2007).

Finally, it should also be taken into consideration that the changes in locomotor period length are differently prominent in different individual animals. While we can observe individual difference on behavioral levels, the observation of gene activity in individual animal heads (and not in pools of animal heads) over time is currently technically not feasible. This could blur smaller changes in the period length of the molecular oscillations.

Possible Circalunar Clock Models in Platynereis dumerilii

Our study shows that circalunar clock function is not affected even when the transcriptional oscillations of putative circadian clock genes are severely impaired, arguing against any circalunar clock model involving the classical circadian clock. It is,
however, conceivable that the maintained daily light-dark cycle is sufficient to drive circalunar rhythms, in absence of circadian clock oscillations. Finally, our data do not test if the classical circadian clock might still be required for the entrainment of the circalunar oscillator.

**EXPERIMENTAL PROCEDURES**

**Worm Culture and Light Conditions**

Worms were maintained as described previously (Hauenschild and Fischer, 1969). See the Extended Experimental Procedures for further detail. Worms of the following inbred strains were used: PIN-mix, VIO-mix, and ORA-mix. All animal work was conducted according to Austrian and European guidelines for animal research.

**Gene Identification**

Fragments of Platynereis sequences described in this study were identified by high-throughput sequencing of normalized complementary DNA (cDNA) using 454 technology. These fragments were subsequently expanded by rapid amplification of cDNA end (RACE) PCR, using Clontech’s Smart RACE cDNA amplification kit. Primers and program are listed in the Extended Experimental Procedures.

**Phylogenetic Analyses**

Sequences were aligned using the MAFFT alignment algorithm. (http://www.ebi.ac.uk/Tools/mafft/index.html). The resulting alignments were subsequently used to generate NJ and ML trees. See the Extended Experimental Procedures for further detail.

**Total RNA Extraction and RT-PCR**

Total RNA was extracted from heads of premature adult worms using the Total RNA Extraction and RT-PCR Procedures for primers and program. cDNA amplification kit. Primers and program are listed in the Extended Experimental Procedures for animal research.

**Statistical Analysis**

Statistical analysis of real-time data was performed using the nonparametric Wilcoxon signed rank test using R Software: A Language and Environment for Statistical Computing, providing a conservative test for significant differences between two sample types (http://www.R-project.org) (Hollander and Wolfe, 1973).

For the Wilcoxon signed rank test, a paired, one-tailed significance interval of 0.05 was used (*p < 0.05, **p < 0.005, ***p < 0.001). One-way ANOVA test and Student’s t-test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software Inc., La Jolla, CA).

**Treatment of Worms with PF-670462**

Premature adult worms of mixed ages were incubated in 800 μm PF-670462 (Tocris, #3316) and grown as the rest of the worm culture. Water was changed and new drug added every week. After 2 weeks of continuous treatment, worms were incubated repeatedly for 5 days in 800 μm PF-670462 and in fresh seawater for 2 days to avoid possible side effects. Treatment was always continuous during the FR-FM phase. PF-670462 is dissolved in water. Thus,
control animals were cultured under the same conditions (same room, light cycle, moon cycle, water change, feeding), but not incubated in PF-670462.

Whole-Mount In Situ Hybridization
Platynereis WMISH was performed according to Tessmar-Raible et al. (2005), with the modifications for adult heads outlined in Backfisch et al. (2013).

Immunocytochemistry
Monoclonal anti-mouse anti-acetylated α-tubulin (clone no. 6-11B-1; Sigma-Aldrich, T6793) was used in a 1:200 dilution as previously described (Arendt et al., 2004).

Mounting and Microscopy
Platynereis adult heads were mounted in 90% glycerol. See Extended Experimental Procedures for details.

ACCESSION NUMBERS
Gene and genomic sequences have been deposited into the NCBI Genbank under the accession numbers GU322428, GU322429, GU322430, GU322431, KF316921, KF316922, KF316923, KF316924, KF316925, KF316926, KF316927, KF316928, KF316929, KF316930, and KF316931.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.031.

ACKNOWLEDGMENTS
The authors wish to thank Barbara Helm, Rodolfo Costa, and four anonymous reviewers for constructive feedback on the manuscript as well as Steven Ruppert and Michael Rosbash for constructive feedback on its initial stage. Barbara Helm also gave very valuable input for the interpretation of the locomotor activity data analyses. The authors thank Florian Raible, Stephanie Bannister, Ruth Fischer, and Sven Schenk for discussions on the work and manuscript and Gerald Nyakatura and Berthold Fartmann (LGC Genomics) for expert activity data analyses. The authors thank Florian Raible, Stephanie Bannister, Ruth Fischer, and Sven Schenk for discussions on the work and manuscript and Gerald Nyakatura and Berthold Fartmann (LGC Genomics) for expert technical assistance. Steven Ruppert kindly provided the plasmids encoding VS-tagged dpcry1 and dpcry2. This work was supported by funds from the Max F. Perutz Laboratories and the University of Vienna, the research platform “Marine Rhythms of Life” of the University of Vienna, an FWF START award (#Y0041321; to K.T.-R.), funds from the IMP (to A.D.S.), and an HFSP research grant (RGP0082/2010; to K.T.-R. and T.I.-F.).

Received: February 6, 2013
Revised: July 3, 2013
Accepted: August 28, 2013
Published: September 26, 2013

REFERENCES


