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Endocrine regulation of non-circadian behavior of circadian genes in insect gut

Adam Bajgar [a,b], David Dolezel [a,b], Magdalena Hodkova [a,*]

[a] Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic
[b] Department of Molecular Biology, Faculty of Science, University of South Bohemia, 37005 Ceske Budejovice, Czech Republic

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ABSTRACT

The linden bug *Pyrrhocoris apterus* exhibits a robust diapause response to photoperiod. Photoperiod strongly affected basal levels of circadian gene transcripts in the gut, via the neuroendocrine system. *Cryptochrome 2* (*cry2*) mRNA level was much higher in diapause promoting short days (SD) than in reproduction promoting long days (LD), while *Par Domain Protein 1* (*Pdp1*) mRNA level was higher in LD than in SD. The effect of photoperiod on gene expression was mediated by the neurosecretory cells of the pars intercerebralis (PI) and the juvenile hormone (JH) producing corpus allatum (CA). In LD-females, CA ablation resulted in SD-like levels of gene transcripts, while PI ablation had little effect. Conversely, in SD-females, CA ablation had only a little effect, while PI ablation resulted in LD-like levels of gene transcripts. Thus, the CA is responsible for LD-like characteristics of gene expression in reproducing females and the PI is responsible for SD-like characteristics of gene expression in diapausing females. A simultaneous ablation of both PI and CA revealed two roles of PI in SD-females: (1) inhibition of CA, and (2) weak CA-independent stimulation of *cry2* mRNA. Overall, our results indicate that peripheral circadian gene expression in the gut reflects the physiological state of females (with respect to diapause or reproduction) rather than the external light–dark cycle.

1. Introduction

Molecular components of circadian oscillators have been identified in both central nervous system and peripheral tissues. In mammals, the central oscillator in the suprachiasmatic nucleus is entrained by light, while peripheral oscillators are sensitive to food intake (Reppert and Weaver, 2002; Kobayashi et al., 2004). Central and peripheral oscillators are thought to be synchronized by unidentified hormonal and/or nervous signals (Schibler and Sassone-Corsi, 2002). Several observations indicate that glucocorticoid signaling is one of these pathways (Balsalobre et al., 2000; Le Minh et al., 2001; Dickmeis et al., 2007). In contrast, both central and peripheral oscillators of insects are directly responsive to light (Plautz et al., 1997; Giebultowicz, 1999; Tanoue et al., 2004; Kostal, 2011), while feeding does not seem to be a potent cue (Oishi et al., 2004). Circadian oscillators have generally been thought to provide timing information for various behavioral (central clock) or metabolic (peripheral clock) activities but not to otherwise participate in the regulation of these states. However, there is increasing evidence for involvement of circadian oscillators in fundamental physiology (Kondratov, 2007).

Photoperiodic responses, mediated by the endocrine system, switch activities of insects to distinct developmental alternatives, such as diapause or nondiapause (Saunders, 2002). Photoperiodic time is measured by the central clock with possible involvement of circadian clock genes, but it is not clear whether they act as a core photoperiodic timer or play a pleiotropic role, downstream of the mechanism (Kostal, 2011; Saunders and Bertosa, 2011; Schiesari et al., 2011). Interestingly, the knockdown of three circadian genes, *period* (*per*), *cycle* (*cyc*) and mammalian-type *cryptochrome* (*m-cry*) affect photoperiodic response in the bean bug *Riptortus pedestris*. Injection of *per* or *m-cry* dsRNA promotes ovarian development whereas injection of *cyc* dsRNA promotes diapause regardless of the daylength. The authors proposed that the three genes are components of the photoperiodic clock (Ikeno et al., 2010, 2011). However, the fact that the knockdown of *per*, *cyc* or *m-cry* rendered circadian clock dysfunctional led to an alternative explanation that phenotypic differences between treatments were caused by action of circadian genes downstream of a still undefined photoperiodic clock (Emerson et al., 2009; Bradshaw and Holzapfel, 2010a, b). In the linden bug, *Pyrrhocoris apterus*, about one third of the females kept in a diapause promoting photoperiod oviposited after knockdown of *per* (unpublished data), but knockdown of *cyc* or *cry* had no effect on reproduction (Bajgar et al., 2013).

* Corresponding author.
E-mail address: magda@entu.cas.cz (M. Hodkova).

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Endocrine signaling controls the physiological function of peripheral organs, but the question of whether hormonal output alters expression of the circadian clock genes in insect peripheral tissue has been ignored. The reason for this lacuna may be the very low intensity of reproductive diapause in *Drosophila melanogaster*, which makes this powerful genetic model not very suitable for the study of photoperiodism. The linden bug, *P. apterus*, exhibits a robust diapause response to photoperiod. Hallmarks of diapause in this species include cessation of reproduction (Hodek, 1971) and changes in the physiology of the digestive system (Socha et al., 1997) and fat body (Socha et al., 1991). Photoperiod acts via the endocrine pathway, particularly neurosecretory cells of the pars intercerebralis (PI) in the brain and the corpus allatum (CA), the glands producing juvenile hormone (Hodkova, 1976, 2008). Expression of two circadian genes *period* (*per*), and *Par Domain Protein 1* (*Pdp1*) in the fat body are inversely affected by contrasting photoperiods via the CA (Dolezel et al., 2008). This was the first experimental evidence for the effect of an endocrine gland on circadian clock gene expression.

The present paper focuses on photoperiodic regulation of circadian genes in another peripheral organ of *P. apterus*, the gut. The gut is the major organ of nutrient uptake that is a prerequisite to either oogenesis or storage of energy reserves needed for successful overwintering. The expression levels of two circadian genes, *cry2* (mammalian-type) and $Pdp1_{iso1}$ in the gut of *P. apterus* were inversely affected by contrasting photoperiods, but did not show circadian rhythms (Bajgar et al., 2013). Here, we ask whether the photoperiodic effect on expression levels of *cry2* and $Pdp1_{iso1}$ in the gut of *P. apterus* is mediated by the endocrine system, particularly by the PI and the CA, or represents a direct response to the external light–dark cycle.

2. Material and methods

Animals: Colonies of *P. apterus* (L.) (Heteroptera) were reared at 25 ± 2 °C under either a reproduction-promoting LD photoperiod of 18 h light/6 h darkness or diapause-promoting SD photoperiod of 12 h light/12 h darkness and supplied *ad libitum* with linden seeds and water. Adult females were used for all analyses. Females destined for surgical manipulations were deprived of food for 12 h after adult ecdysis. CA and neurosecretory cells of the PI were removed through a neck membrane incision under Ringer insect saline. Neurosecretory cells of the PI were visible on surface area of the middle part of brain (whitish color) and were removed through a small cut in brain envelope. The neck membrane was cut in control females. It was shown earlier that the implantation of SD–neuroendocrine complex (brain–suboesophageal ganglion–corpora cardiaca–CA) with extirpated PI stimulates reproduction, while the removal of suboesophageal ganglion, associated with brain injury, has no effect (Hodkova, 1979). This indicates that the inhibition of the CA originates from the PI and not from a general damage in the brain. Females were shifted from LD to SD and vice versa on the day of operation. Food was given immediately after operation. Guts (without stomodeum) were dissected 10 days after operation under Ringer insect saline and immediately placed on dry ice, and stored at −85 °C until analysis.

2.1. JH analogue application

Five microliters of JH analogue Methoprene (Research Institute of Organic Syntheses, Rybitvi, Czech Republic) was dissolved in acetone (concentration 0.3 mM) and applied on the dorsal surface of CO_2 anesthetized animals. Acetone was applied on control animals. Animals were sacrificed 4 days after administration of the JH mimic.

2.2. RNA extraction and cDNA synthesis

Gut tissues were dissected from females in RNAse-free Ringer solution. Samples from each treatment were collected from eight individuals, and the states of the gonad was determined. Total RNA was isolated with Trizol reagent (Invitrogen), and its quality was established by gel electrophoresis. Concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific). After Turbo DNAse treatment (Ambion), 1 μg of total RNA was used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen).

2.3. Quantitative PCR-primers

Relative transcript levels were measured by qRT-PCR, using iQ SYBR Green Supermix kit and a C1000 Thermal Cycler (Bio-Rad). All measured transcripts were normalized to relative levels of the *ribosomal protein 49* (*rp49*) mRNA as described previously (Dolezel et al., 2007). Primer sequences are listed in Table 1.

2.4. RNA interference

dsRNA was synthesized using T3 and T7 MEGAscript kit (Ambion) from plasmids containing the appropriate gene fragment (Table 2 shows sequences of primers used for cloning). The identity of fragments was verified by sequencing. Experimental individuals were injected with 4 μl of a 4 μg/μl concentrated dsRNA in Ringer solution. Control group was injected with plain Ringer solution. The efficiency of knockdown was verified by qRT-PCR (data in Fig. 2).

2.5. Statistical analysis

We evaluated differences in expression levels using the software Statistica10 (StatSoft). For comparison of multiple samples, we used OneWay Anova test and Tukey post hoc comparison analysis.

3. Results

3.1. Circadian genes respond to endocrine conditions

To determine whether the effect of photoperiod on $Pdp1_{iso1}$ and *cry2* expression is mediated by the CA, we compared levels of clock gene transcripts in guts of LD- and SD-females deprived of this gland (Fig. 1). Ablation of the CA from LD-females resulted in a clear change in abundance of both transcripts, a decrease of $Pdp1_{iso1}$ mRNA and an increase of *cry2* mRNA (Fig. 1A). However, the amount of *cry2* mRNA in LD-allatectomized females was slightly, but significantly ($p < 0.001$), lower compared to SD-allatectomized females, indicating that, in addition to JH, a factor independent of JH is involved in the regulatory mechanism (Fig. 1A and B). On the other hand, ablation of the CA from SD-females had only a slight effect on *cry2* mRNA and no effect on *Pdp1* mRNA (Fig. 1B). As the CA is active at LD but not at SD (Hodkova, 1976; Hodkova et al., 2001), this is not surprising. When the JH analogue methoprene was given to LD-allatectomized females, expression of both *cry2* and *Pdp1* was rescued. A similar effect was obtained after application of JH analogue to diapause females (Fig. 2). Thus, in LD-allatectomized females, *cry2* and $Pdp1_{iso1}$ mRNA assumed SD-like characteristics, reflecting an SD-like hormonal milieu (absence of CA hormones) rather than the ambient (LD) photoperiod.

Effects of ablation of the PI on *cry2* and $Pdp1_{iso1}$ mRNA in the gut were opposite to the ablation of CA (Fig. 1). Ablation of the PI from LD-females resulted in a small decrease of *Pdp1* mRNA, but the