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Protein Phosphatase 2A in the circadian clock of the linden bug *Pyrrhocoris apterus*

Bachelor's thesis

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Annotation:

The aim of this thesis was to identify putative genes encoding Pp2A phosphatase in the linden bug *Pyrrhocoris apterus*, clone fragments of those genes and use the RNA interference approach to test the knockdown on *P. apterus* regarding changes in the activity and their free running period.

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Abstract

Circadian rhythms are generated by a system of self-regulated positive and negative feedback loops of the main clock genes (*Clock*, *cycle*, *period*, *timeless*) in the brain and the peripheral tissues. This feedback loops are regulated by post-translational modifications such as phosphorylation and ubiquitination, which have a big impact on the clock protein stability and the pace of the clock. We examined the role of the Pp2A phosphatase and its various subunits in regulating the circadian clock and the impact on the free running period of the linden bug *Pyrrhocoris apterus* by monitoring the locomotor activity after specific RNAi treatment.

Content:

1. Introduction

1.1 Pyrrhocoris apterus

In the chronobiology laboratory the linden bug *Pyrrhocoris apterus* is used as a model organism to study the molecular mechanism of the circadian clock. *P. apterus* is a hemimetabolan phytophagous true bug (Heteroptera: Pentatomomorpha: Pyrrhocoridae) that feeds on the host plants from the order Malvales. The preferred food source in the Czech republic are nut-like seeds of the linden tree (*Tilia cordata*). It has a western Palaearctic distribution, spanning from Spain to south of western Siberia and from Sweden to Israel. The species was also recorded in parts of North Africa, USA, India and Central America (Socha 1993). The outer appearance of the linden bug is characteristic with a typical aposematic red and black colour pattern at the thorax, the abdomen and the wings. The wings are functionally reduced and the bug is not able to fly. Generation time at 25-26 °C is one month and the animals overwinter (diapause) as adults. The linden bug is easy to work with and has a steady and rhythmic free running period of the locomotor activity (Socha, 1993; Pivarciova et al., 2016).

1.2 Circadian Rhythms

In all three domains – bacteria, archaea and eukaryotes – organisms developed different mechanisms that enable them to detect changes in the environment and react appropriately (Edgar et al., 2012; Bhadra et al., 2017). Two major mechanisms that occur in all domains are the photoperiodic timer and the circadian clock. The photoperiodic timer allows long-term adaption to seasonal changes in the environment by using day-to-night length ratio for initiating diapause in insects (Nunes and Saunders, 1999), regulation of the proper flowering timing in plants (Song et al., 2015) or impact the start of seasonal migration in birds (Sharp, 2005). The circadian clock enables to anticipate day-night cycles and is therefore a powerful tool for the organism to regulate its metabolic processes and its activity (Pittendrigh, 1981). A circadian clock usually consists of a light sensing molecule, a pacemaker and an output reaction (Dolezel, 2015; Kostal, 2011). The light sensing molecule collects light signals from the environment and sends them via signalling pathway(s) to the pacemaker. The pacemaker oscillates with an endogenous rhythm of about 24 hours that is maintained even under constant, non-periodical conditions. Oscillation and rhythmicity are created by a molecular mechanism of transcription-translation feedback loops (TTFL; Cermakian and Sassone-Corsi, 2000). The clock is restarted every morning by the incoming light signals to enable the

organism to anticipate environmental fluctuations and react accordingly to the regular changes in day-night rhythm. This process is called entrainment (Pittendrigh and Minis, 1964; Hardin, 2004).

There was plenty of research done on circadian rhythms in model organisms such as *Drosophila melanogaster* (Dubowy and Sehgal, 2017*)*, *Arabidopsis thaliana* (Sanchez and Kay, 2016) and in mammals (Partch et al., 2014). Due to the evolutional distance the exact mechanism of the circadian clock differs slightly in different organisms. In *D. melanogaster* high levels of circadian genes are expressed in six neuron clusters in the brain, photoreceptors of the eye and in the peripheral tissues. (Hermann-Luibl and Helfrich-Förster, 2014). The expression of the core clock genes is rhythmical. This rhythmicity is created by two highly regulated interconnected TTFLs (Hardin, 2009).

1.3 Molecular mechanism of the circadian clock

The core of the circadian clock mechanism in *Drosophila* is based on interlocked TTFLs. The primary TTFL involves two basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) transcription factors encoded by genes *Clock* (*Clk*) and *cycle* (*cyc*) that activate the transcription of two transcription repressors, bHLH-PAS gene *period* (*per*) and *timeless* (*tim*).

The cycle starts with binding of CLK-CYC heterodimer to E-box (CACGTG) sequence of the *period* (*per*) and *timeless* (*tim*) gene promoters and triggering the transcription of *per* and *tim* (Darlington et al., 1998; Wang et al., 2001; Figure 1). *per* and *tim* mRNA get translated in the cytoplasm. PER proteins bind kinase DOUBLETIME (DBT; Kloss et al., 2001) and get phosphorylated by DBT, CASEIN KINASE 2 (CK2) and NEMO kinase (Lin et al., 2005; Chiu et al., 2011; Yu et al., 2011). Although the complete pattern of PER phosphorylation is not clear, extensive PER phosphorylation destabilizes PER, thus PER proteins are targeted to the proteasome and degraded. PER is stabilized by heterodimerization with TIM and the DBT-PER-TIM complexes get phosphorylated by Shaggy (Sgg) and CK2 and translocate to the nucleus (Martinek et al., 2001). DBT-PER-TIM bind to CLK and the complex promotes the phosphorylation of CLK and PER (Hardin, 2009; Yu et al., 2011) and lowers CLK-CYC binding affinity to *per* and *tim* E-boxes (Zhou et al., 2016). The hyperphosphorylated CLK and PER are degraded in the proteasome. Concurrently, CLK-CYC heterodimers at *per* and *tim* E-boxes are replaced by the transcription repressor basic-helix-loop-helix-Orange transcription factor CLOCKWORK ORANGE (CWO). CWO protein enhances the removal of CLK-CYC from the E-boxes to maintain the transcription repression (Zhou et al., 2016). Light-dependent degradation of TIM is critical for *Drosophila* circadian clock entrainment by

light in the morning. Blue light-dependent photolyase-related CRY1, encoded by *cryptochrome 1* (*cry1*) gene, physically interacts with TIM in a light-dependent manner and promotes phosphorylation of TIM by a tyrosine kinase (Naidoo et al., 1999). Phosphorylated TIM is committed to be degraded in the proteasome. Fast light-dependent TIM degradation permits restart of the cycle. CRY1 itself is later degraded in a light-dependent manner.

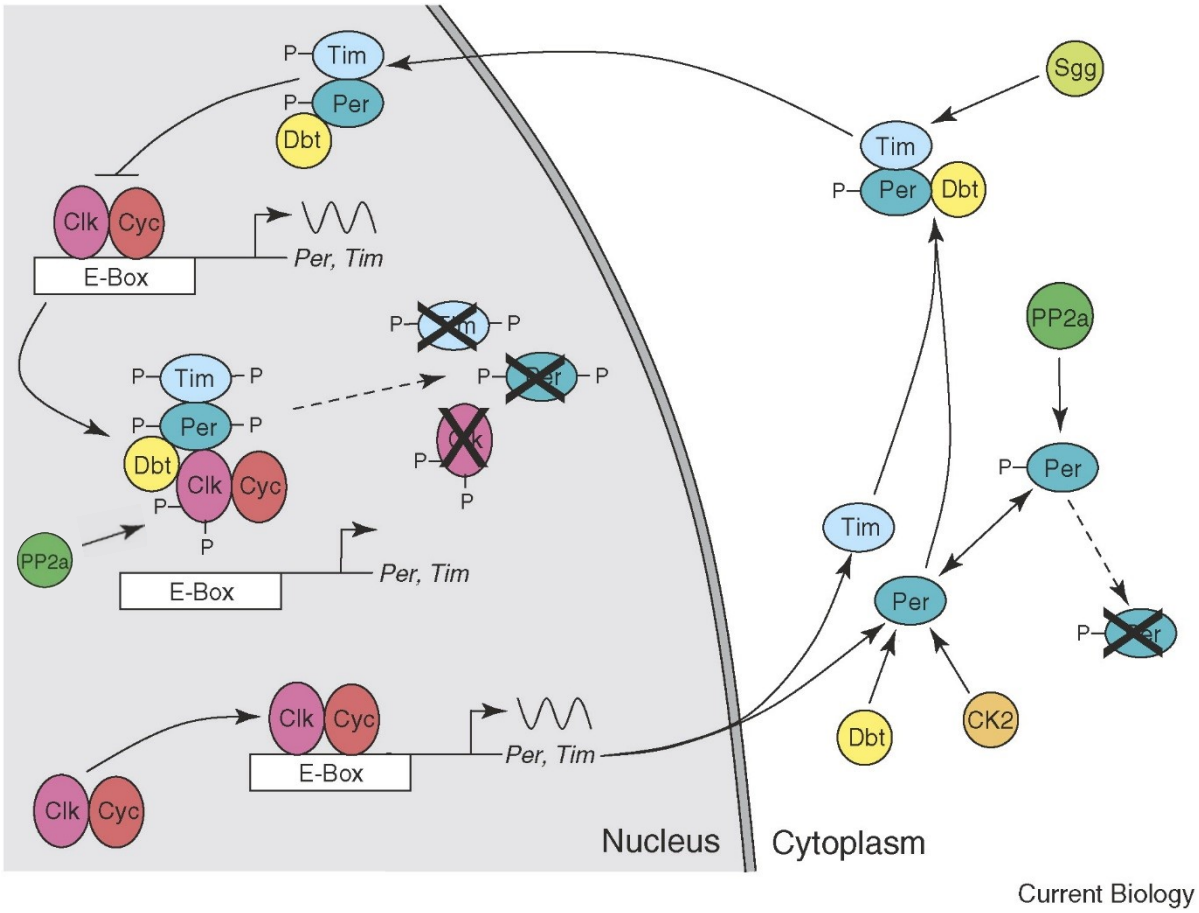

Figure 1. Schematic drawing of the circadian clock negative feedback loop in *Drosophila melanogaster*. The CLOCK (Clk) and CYCLE (Cyc) transcription factors, which are expressed periodically through a positive feedback loop in the brain neurons, enter the nucleus and form a CLK-CYC heterodimer. Hypophosphorylated CLK-CYC heterodimer binds to E-box sequence of the *period* (*per*) and *timeless* (*tim*) gene promoters and triggers the expression of PER and TIM proteins (Darlington et al., 1998; Wang et al., 2001). During the day PER protein gets phosphorylated by several kinases such as DOUBLETIME (Dbt), CASEIN KINASE 2 (CK2) and gets degraded. Phosphorylated PER with bound DBT gets stabilized by forming a complex with TIM protein. TIM and PER within the DBT-PER-TIM complex get phosphorylated by Shaggy (Sgg) and CK2 and translocate to the nucleus (Martinek et al., 2001). PER-TIM as a part of a bigger complex promote the phosphorylation of CLK and PER (Hardin, 2009). The hyperphosphorylated CLK and PER are degraded, followed by hyperphosphorylated TIM several hours later, and the transcription of *per* and *tim* is repressed. Another cycle will start after continuously expressed CYC and hypophosphorylated CLK accumulate and activate *tim* and *per* transcription. The Protein Phosphatase 2A dephosphorylates PER and CLK and thus stabilizes them. Adapted from Hardin, 2009.

Interestingly, *P. apterus* do not possess light-sensing *cry1* gene but mammalian-type *cry2* (Bajgar et al., 2013) and *P. apterus* CRY2 might participate on the CLK-CYC repression as its silencing results in arrhythmicity (Chodakova, 2019).

Another cycle will start after continuously expressed CYC and hypophosphorylated CLK accumulate and activate *tim* and *per* transcription. Another TTFL tunes the expression of *Drosophila Clock*. CLK-CYC heterodimer binds E-boxes in the promoters of two basic leucine zipper domain (bZIP) transcription factors, *vrille* (*vri*) and *PAR-domain protein 1 epsilon* (*Pdp1ε*). VRI protein accumulates during the midday to early evening, binds VRI/PDP1ε box (V/P-box) and represses *Clk* transcription. Transcription activator PDP1ε accumulates from middle evening to late evening and compete with VRI at the V/P-box and drives *Clk* expression (Hardin, 2009).

1.4 Posttranslational modifications

After the translation of proteins in the ribosomes, post-translational modifications (PTMs) can increase the functional diversity of proteins by covalent addition or removal of functional groups. The therefore altered structural conformation of proteins can cause changes in protein activity, protein localisation and can change interaction dynamics with other proteins (Seo and Lee, 2004). The most common modifications involve phosphorylation, acetylation, glycosylation, amidation, hydroxylation, methylation and ubiquitination (Khoury et al., 2011). Especially in eukaryotic cells, phosphorylation and dephosphorylation processes play a crucial role in regulating cellular processes. The addition of the phosphate group is initiated by enzymes so called kinases and the removal of this group is then regulated by phosphatase enzymes. Kinases and phosphatases are often simultaneously present thus enable a reversible (de)phosphorylation with a high turnover rate. The balance of the phosphorylation level is therefore highly dependent on the presence of the two counter players (Hunter, 1995). Number of phosphatases in eukaryotes is in general lower than number of kinases, 102 phosphatases and 261 kinases were found in *Drosophila* genome (Smoly et al., 2002). Although outnumbered by the kinases, phosphatases form number of specific complexes due to spatial and temporal expression of several isoforms (often encoded by several paralogs in mammals, Janssens et a., 2008) and their various combinations. The functional phosphatase diversity is further multiplied by their PTMs and interactions with different regulatory proteins (Hunter, 1995; Seshacharyulu et al., 2013; Wlodarchak and Xing, 2016).

1.5 Posttranslational modifications regulate circadian clock

Circadian clock function is highly regulated by PTMs such as phosphorylation and ubiquitination. Protein interactions within the TTLFs such as PER-TIM heterodimer formation

and PER, TIM and CLK degradation are highly affected by kinases, phosphatases and ubiquitin ligases (Mehra et al, 2009).

For example, the protein PER is degraded after the phosphorylation in the cytoplasm (Price et al., 1998) but PER-stabilizing formation of the PER-TIM heterodimer only occurs in PER phosphorylated, and therefore unstable, state (Zeng et al., 1996). Responsible for PER phosphorylation are DBT, CK2 and NEMO kinases, which are opposed by the actions of PP1 and PP2A phosphatases to keep a pool of hypophosphorylated PER at a proper time of the cycle (Sathyanarayanan, 2004; Kim and Edery, 2006; Fang et al., 2007). Additionally, relocation of the TIM and PER proteins from the cytoplasm to the nucleus is again phosphorylation-dependent (Martinek et al., 2001; Lin et al., 2002).

Similar processes can be observed for the CLK-CYC activity regulation. Degradation of the CLK protein is triggered again after phosphorylation by DBT and other kinases (Yu et al., 2011). The PP2A phosphatase stabilizes CLK and keeps it in a hypophosphorylated state. Regulation of PER, TIM and CLK degradation is heavily dependent on PTMs. Beside the phosphorylation, ubiquitination mediated by CUL-3 and SCF^{SLMB}, JETLAG and CTRIP proteins of ubiquitination complexes is crucial for PER, TIM and CLK timely degradation (Fang et al., 2007; Chiu et al., 2008; Chiu et al., 2011; Szabó et al., 2018).

Therefore, the balanced expression and activity of PTM-mediating proteins and their complexes have a great influence on core clock proteins stability and activity. PP2A complex, we decided to focus on, is one of the cardinal regulators of the circadian clock machinery.

1.6 Phosphatases

Nine different amino acids (AA) tyrosine, serine, threonine, cysteine, arginine, lysine, aspartate, glutamate and histidine can be phosphorylated but predominantly tyrosine, serine and threonine AA are phosphorylated in eukaryotic cells. Although phosphatases can be classified based on different criteria, protein phosphatases are typically classified into three major groups by their catalytic signatures and domain sequences and by their substrate specificity: protein serine/threonine phosphatases (PSTPs), tyrosine phosphatases (PTPs) and aspartate-based protein phosphatases. Each of this group has multiple subgroups as seen in Table 1 (Moorehead et al., 2007; Moorhead et al., 2009; Hatzihristidis et al., 2015).

Table 1. Classification of protein phosphatases. Three major groups Protein serine/threonine phosphatases (PSTP), Protein tyrosine phosphatases (PTP) superfamily and Aspartate-based protein phosphatases are distinguished by their catalytic signatures/domain sequences and target specificity. PP2A belongs to PSTPs phosphoprotein phosphatases (PPP) family.

Abbreviations: PP1 to PP7 = Phosphoprotein Phosphatase 1 – 7, MKP = MAP(mitogen-activated protein)-kinase phosphatase, PRL = phosphatase of regenerating liver, CDC14 = cell division cycle 14, PTEN = phosphatase and tensin homologue deleted on chromosome 10), MTMs = myotubularins, LMWP = low molecular weight phosphatases, CDC25 = cell division cycle 25, FCP/SCP = TFIIF (transcription initiation factor IIF)-associating component of CTD (C-terminal domain) phosphatase/small CTD phosphatase, HAD = haloacid dehalogenase, EyA = Eyes Absent

Group	Family		Members
Protein serine/threonine phosphatases (PSTP)	Phosphoprotein phosphatases (PPP)		PP1, PP2A, PP2B, PP4, PP5, PP6, PP7
	Metal dependent phosphatases (PPM)		PP2C
Protein tyrosine phosphatases (PTP) superfamily	Class I	Classical PTPs	Receptor and Non-receptor PTPs
		Dual-specificity PTPs	MAPKP, Atypical, Slingshots, PRL, CDC14, PTEN, Myotubularins
	Class II	LMWP	LMWP
	Class III	CDC25	CDC25
Asp-based protein phosphatases	FCP/SCP family		FCP1, SCP, FCP/SCP-like
	HAD family		Eya

PSTPs are further divided into phosphoprotein phosphatases (PPP) and metal-dependent phosphatases (PPM). The PPP protein family members have a tendency to form a functional enzymes by combinatorial interactions of subunits whereas PPMs, PTPs and aspartate-based families by rather fusing with additional domains. PPP family is characterized by a conserved active site configuration and its members PP1 (Phosphoprotein Phosphatase 1), PP2A, PP2B (calcineurin), PP4, PP5, PP6, PP7 are present in all eukaryotes and play a central role in the regulation of many cellular processes (Wera and Hemmingst, 1995; Wlodarchak and Xing 2016).

1.7 Phosphoprotein phosphatase PP2A

The PP2A protein complex obligatory consists of two subunits, the scaffold subunit PP2A-29B (A) and the catalytic subunit MTS (C), together forming a heterodimeric holoenzyme However, PP2A typically involves one of the regulatory (B) subunits and forms as a heterotrimeric complex. There are four biochemically distinguishable subclasses of regulatory subunits B, B’, B’’, B’’’ (Wera and Hemmingst, 1995; Figure 2.A). Each of them regulates different cellular processes and is supposed to ensure target specificity and PP2A complex

subcellular localization (Wlodarchak and Xing, 2016). The scaffold subunit of PP2A phosphatase holoenzyme is in *Drosophila* encoded by gene *Pp2A-29B* and the catalytic subunit is encoded by gene *microtubule star* (*mts*). Regulatory subunits are represented by 5 genes belonging to all four classes. B class regulatory subunit is encoded by gene *twins* (*tws*), B' class by genes *well-rounded* (*wrd*) and *widerborst* (*wdb*), B'' class by gene *CG4735* and the B''' class by gene *Connector of kinase to AP-1* (*Cka*).

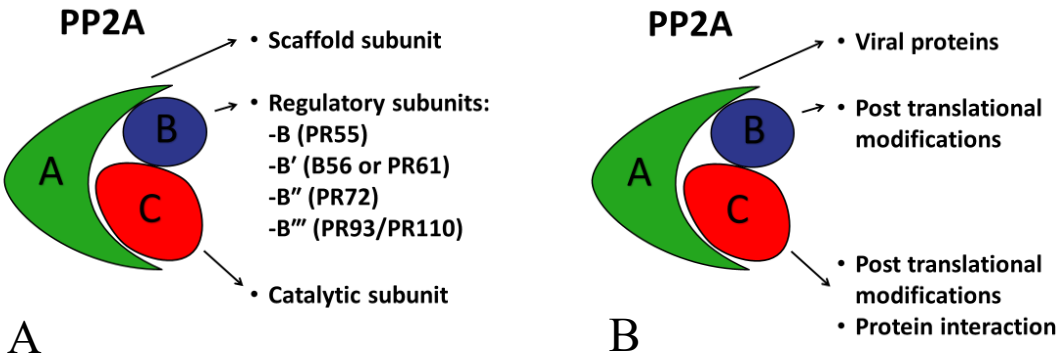

Figure 2. A) PP2A complex with its subunits. The catalytic subunit (C) interacts with the structural subunit (A). One of the four different types of regulatory subunit (B) (B-B′′′) then attaches to the structural subunit (A) and the catalytic subunit (C). B) Regulation of the complete assemble of the holoenzyme can be regulated by multiple factors. Adapted from (Thompson and Williams, 2018)

Figure 2. B) The structural subunit (A) can be deactivated via binding of viral proteins. The different regulatory subunits and the catalytic subunit are targets for different post translational modifications. The function of the catalytic subunit is also modulated by interacting proteins. (Virshup and Shenolikar, 2009). Adapted from (Martin et al., 2010).

PP2A is a major player in regulating cellular processes, it can represent roughly 0.2 % of total cell protein in the mammalian cell (Ruediger et al., 1991). PP2A together with PP1 account for more than 90 % of the protein phosphatase activity in eukaryotes (Moorhead et al., 2007; Virshup and Shenolikar, 2009). PP2A is involved in cell signalling pathways such as the Wnt- (Zhang et al., 2009), mTOR- (Nakashima et al., 2013) and MAP kinase pathway (Ugi et al., 2002; Letourneux et al., 2006), the cell cycle G1→S transition (Kolupaeva and Janssens, 2012), DNA synthesis (Wlodarchak et al., 2013) and mitotic initiation (Mochida et al., 2009). These processes are vital for proper cell survival and proliferation and its deregulation is often correlated with cancer and other diseases. Due to its various possibilities of PTMs and other regulation interaction of its subunits (Virshup and Shenolikar, 2009, Figure 2.B) PP2A can dephosphorylate more than 300 different substrates (Janssens and Goris, 2001; Wlodarchak and Xing 2016). Due to its importance is highly conserved among various organisms from yeast to humans (Shi et al., 2009).

1.8 PP2A in *Drosophila* circadian clock

Expression alternation of some genes encoding PP2A subunits in *Drosophila* resulted in various circadian phenotypes. The circadian role was established for *Pp2A-29B*, *mts*, *tws*, *wdb* and *Cka* using tissue specific RNA interference, overexpression of wild-type or mutant forms of the proteins and by using mutants (Hannus et al., 2002; Sathyanarayanan et al., 2004; Andreazza et al., 2015; Agrawal and Hardin, 2016). Tissue- and cell-specific gene expression in *Drosophila* is possible by employing yeast Gal4/UAS system. Gal4 transcription factor, under control of a particular *Drosophila* gene promoter or enhancer, binds Upstream Activating Sequence (UAS) and the gene under control of UAS is expressed in a pattern following Gal4 expression (Duffy, 2002; Chintapalli et al., 2007).

Pp2A-29B and *mts* RNAi constructs driven by *tim*-Gal4 (*timeless*) or *pdf*-Gal4 (*Pigment-dispersing factor*) drivers, both expressed in *Drosophila* brain clock cells, prolonged the period of the clock (Agrawal and Hardin, 2014). Knockout MTS and PP2A-29B mutants are developmentally lethal (Snaith et al., 1996) but Andreazza et al. (2015) used N-terminal truncated dominant-negative form of MTS (mts^{DN}; Hannus et al., 2002), still capable of binding to the wild-type scaffold (A) and regulatory (B) subunits and competing with the wild-type MTS, to study the effect on the circadian clock. mts^{DN} expressed in the clock neurons under *tim*-Gal4 driver dramatically reduced the rhythmicity to 10 % and the small portion of mts^{DN} rhythmic flies showed longer FRP (Sathyanarayanan et al., 2004; Andreazza et al., 2015) consistent with the RNAi screen performed by Agrawal and Hardin (2016). However, the phenotype might be caused by a defective clock neurons expressing mts^{DN} (Andreazza et al., 2015). Flies expressing mts^{DN} under *pdf*-Gal4 driver displayed multiple periodicities (a complex phenotype) and a longer period (Sathyanarayanan et al., 2004). MTS overexpression in the *Drosophila* clock neurons also caused arrhythmicity (Sathyanarayanan et al., 2004; Agrawal and Hardin, 2016).

Overexpression of *tws* also led to arrhythmicity (or was lethal) but *tws* RNAi did not change the pace of the clock (Sathyanarayanan et al., 2004; Agrawal and Hardin, 2016).

WDB overexpression lengthened the period of the clock (Sathyanarayanan et al., 2004) and *wdb* RNAi led also to a longer period of the clock (Andreazza et al., 2015; Agrawal and Hardin, 2016). Knockdown of another B' class gene *wrd* had no effect on the circadian clock (Agrawal and Hardin, 2016).

CG4733 doesn't seem to have a function in the *Drosophila* circadian clock (Agrawal and Hardin, 2016).

Cka knockout mutants are embryonic lethal but developmentally-rescued *Cka*^{2}-null mutants became arrhythmic after several days in constant dark. *Cka* RNAi flies prolonged the period of the circadian clock (Andreazza et al., 2015). CKA, as a member of B''' class, interacts with MTS, protein STRIP (*striatin-interacting protein*) and kinases to form a STRIPAK (STRiatin-Interacting Phosphatase And Kinase) complex. CKA together with STRIP and its STRIPAK complex is promoting the dephosphorylation of CLK in the nucleus during the day and therefore stabilizing it. Interestingly, WDB also stabilizes the CLK protein but through a different mechanism than CKA, without affecting its phosphorylation state (Andreazza et al., 2015).

Summing up, catalytic and scaffold PP2A subunits together with TWS and WDB regulatory subunits are irreplaceable for precise function of the clock and are needed for proper PER and CLK phosphorylation state and level of PER expression (Sathyanarayanan et al., 2004; Andreazza et al., 2015; Agrawal and Hardin, 2016). CKA as a part of PP2A and STRIPAK complex mainly affects CLK phosphorylation state (Andreazza et al., 2015) and plays an important role in the *Drosophila* circadian clock. On the other hand, WRD and CG4733 don't seem to have a function in the *Drosophila* circadian clock.

As seen in Table 2 we just start to unveil PP2A function of the PP2A encoding genes in *Drosophila* circadian clock. Especially, for a general understanding of the PP2A function in the circadian clock in insects, different insect species have to be examined as well. *Drosophila* molecular toolkit, including Gal4/UAS system, balancer chromosomes, generation and availability of gene-specific mutants and non-systemic RNAi interference (Perrimon and Perkins, 2010), enables tissue- and cell-specific gene studies.

The toolkit in *P. apterus* is much more limited and out of the previously mentioned methods only RNAi is broadly in use. However, CRISPR/Cas9 gene-editing was established in *P. apterus* (Kotwica-Rolinska et al., 2019) although mutant generation is laborious and time consuming. Nevertheless, systemic RNAi is very efficient in *P. apterus* (Konopova et al., 2011; Bajgar et al., 2013; Urbanova et al., 2016) and was used in our work as a molecular tool to study the effect of PP2A encoding genes to the circadian clock of *P. apterus*.

Table 2. Summary of the targeted genes and their circadian effect in *Drosophila melanogaster*

Gene name	Gene symbol	Subunit role in the PP2A complex	Annotation symbol	Circadian effect	Mechanism
Protein phosphatase 2A at 29B	Pp2A-29B	Scaffold	CG17291	Arrhythmicity	Stabilizes PER and CLK
microtubule star	mts	Catalytic	CG7109	Arrhythmicity	Stabilizes PER and CLK
twins	tws	Regulatory, class B	CG6235	Prolonged clock	stabilizes PER-TIM dimer in cytoplasm
widerborst	wdb	Regulatory, class B’	CG5643	Arrhythmicity and prolonged clock	Stabilizes CLK in the nucleus
well-rounded	wrd	Regulatory, class B’	CG7913	No effect	-
CG4733	CG4733	Regulatory, class B’’	CG4733	No effect	-
Connector of kinase to AP-1	Cka	Regulatory, class B’’’	CG7392	Prolonged clock	Stabilizes CLK in the nucleus

2. Goals of the work:

I) Identify PP2A genes in the *Pyrrhocoris apterus* transcriptome using *Drosophila melanogas*ter orthologs as a query.

II) Design primers for 2 fragments of each gene and clone the fragments in to the plasmid.

III) Produce double-stranded RNA and knockdown the PP2A genes by RNA interference.

IV) Determine survival, rhythmicity and measure the free running period of the treated RNAi bugs and compare the results to the known *Drosophila* data.

3. Materials and methods:

3.1 Keeping Pyrrhocoris apterus

The Lyon-red (Socha and Hodkova, 1994) and Roana (Pivarciova et al., 2016) strains of the bug *Pyrrhocoris apterus* were kept in glass jars in incubators (25°C, 16/8 LD, 16 hours light and 8 hours darkness). Linden seeds were provided as a food resource and folded filter paper was used as a hiding place. For water supplement, water filled flasks with a cotton stopper that enables the bug to drink *ad libitum* were given into the jars.

The Lyon strain has a highly rhythmic locomotor activity and thus was used for dsRNA injections and locomotor activity measurements, although it has slightly shorter free running period than 24 hours (Pivarciova 2016).

3.2 *P. apterus* PP2A candidate genes search and identification

Based on literature, protein sequences encoding Pp2A candidate genes *Pp2A-29B, mts, tws, wrd, wdb, Cka* and *CG4733* were downloaded from the website FlyBase (http://flybase.org/). We employed Basic Local Alignment Search Tool (BLAST) to search for Pp2A *P. apterus* candidate genes orthologs in Oldřichovec, Lyon and Roana strain transcriptomes. This was done in software Geneious 11.0.3 (Biomatters Ltd., New Zealand). The identity of found candidate *P. apterus* proteins was further supported by blasting those as a query against *Drosophila melanogaster* and other insect species protein databases in NCBI. *P. apterus* protein sequences with confirmed identity were then used to search for *P. apterus* DNA sequence counterparts. Common regions (not isoform specific) of the genes' open reading frames were taken for primer design.

3.3 RNAi

3.3.1 Cloning primer design

The primers were designed with the website tools Primer3 (http://primers3.com), OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer) and the Geneious program. Two pairs of primers were designed for each gene and used for cloning of two non-overlapping fragments, later employed as *in vitro* dsRNA templates. Forward and reverse primer sequences for each gene fragment are shown in Table 3 including the length of the amplified gene fragment. Primers M13 forward and reverse were used for the colony PCR (see section 3.4.10),

and M13 forward and pGEM RNAi reverse primers were used for *in vitro* double-strand RNA template amplification (see section 3.4.10).

Table 3. List of Primers

Gene	Forward primer 5' - 3'	Reverse primer 5' - 3'	Fragment length (bp)
Pp2a-29B f. 1	ATCGCATCACTCCTTCAGC	AACCTAACTCGCCACTTTGC	572
Pp2a-29B f. 2	GTCAGTTCACTCCGCTTGTC	CGATGCTAACACACGCTTC	474
mts f. 1	AGTTACAGTTTGTGGGGATGTC	GGTCCTTCGTGGGGAAC	429
mts f. 2	GAGGCGGTTGGGGTATATCA	TCTGGAGTCCTTCGTGTGAC	301
tws f. 1	GCTGTGCGATATGAGGC	GTCTTTGGTTTGGCTATTTC	414
tws f. 2	GTTATGGAAAGTGTCTGAA	GAACTATAAACAAAAACATTACA	378
wdb f. 1	ATTTCAATGGAGTGGGCG	TCTTTGCTTATTCGGTATAGAG	490
wdb f. 2	CGATAGAATTGACCCTTTTG	ATACTAGAGTTAAATGCGGC	422
wrd f. 1	TCTCTTTCTGTTTATCATCCG	CCCATGAATAGTTTTGTTCCA	390
wrd f. 2	ACTTCGTCAGTGTTGTGTTCT	AGTCTCTTTCTCTCGGGTCT	407
CG4733 f. 1	CGGGAGGAACGCTACAGA	GGAATCGGGAGGGAAGGT	507
CG4733 f. 2	GAAGCCGTCCGTAACAAAA	TCACAGGTCCAGGTTTCC	445
Cka f. 1	CGGGATTTTACACTTCATACAAC	GTCAGCATCAACATCATCTG	766
Cka f. 2	ACCTTCTCTCATCGTCTGCT	TCGTCAAACTTCTTTCGGTGT	508
M13	GTAAAACGACGGCCAGT	GGAAACAGCTATGACCATG	-
pGEM RNAi	TAATACGACTCACTATAGGGGACACTATAGAATACT		-

3.3.2 Dissection

Various tissues for RNA isolation were dissected from male bugs of the Roana strain. We collected four different tissue samples: abdominal epidermis of 4th instar larvae, adult fat bodies, heads and brains. To increase the RNA yield, we always collected, 10 brains, 6 heads, 2 fat bodies epidermis from 3 larvae and transferred the sample into 1.5 ml test tube with 400µl TRIzol Reagent (Ambion). The tubes were stored at -80 °C.

3.3.3 RNA isolation

Total RNA was isolated using TRIzol Reagent (pH = 5.5) according manufacturer's protocol. Briefly, the tissues were grinded with plastic pestle in 0.4 ml of TRIzol then 0.6 ml of fresh TRIzol was added and the mixture was incubated at room temperature (RT) for 5 min. Then 200 µl of chloroform was added, the tubes were hand-shaken for 15 s and incubated at RT for 3 min. The mixture was centrifuged at 12,000 g for 15 min at 4 °C. Afterwards, the aqueous phase was transferred to a new tube. 2 µl of LPA (Linear PolyAcrylamide; ThermoFisher Scientific) was added to the "brain" sample as a carrier to increase the yield of RNA. Additionally, 1 volume of isopropanol was added to precipitate RNA. The mixtures were incubated at RT for 10 min, except the sample with added LPA was incubated at -20 °C. The RNA was then pelleted by centrifugation at 12,000 g for 10 min at 4 °C and the supernatant

was discarded. The RNA was washed with 1 ml of 75 % EtOH by vortexing and then was centrifuged at 7,500 g for 5 min at 4 °C. Afterwards, the supernatant was discarded, and the pellet was air-dried for 5-10 min. The RNA was dissolved in 50 µl of DNase-/ RNase-free water (Gibco) and the concentration was measured with NanoDrop measurement (λ = 260 nm) and the RNA was stored at -80 °C.

3.3.4 DNase Treatment

Contaminating genomic DNA was removed by DNase treatment done with TURBO DNA-free Kit (Ambion/Thermo Fisher Scientific). 0.1 volume of 10X Turbo DNase Buffer and 1 µl Turbo DNase was added to 10 µg of RNA. The samples were incubated at 37 °C for 30 min and 5 µl of DNase Inactivation Reagent was added to the 50 µl total reagent volume. Then the samples were incubated again at RT for 5 min and mixed occasionally by tapping the tubes. Afterwards, the samples were centrifuged at 10,000 g for 90 s and then supernatant was carefully transferred into a fresh tube. The DNased RNA was stored at -80 °C.

3.3.5 cDNA synthesis

cDNA synthesis was performed with the SuperScript III Reverse Transcriptase (Invitrogen). All steps were done according to the protocol except we did not add the RNaseOUT reagent. Each 20 µl reaction contained: 1 µl (50 µM) oligo (dT) primer, 2 µg of total DNased RNA, 1 µl (10 mM each) dNTP MIX (Invitrogen), and up to 13 µl water. This mixture was heated up to 65 °C for 5 min, then cooled on ice for 1 min and briefly centrifuged. After that 4 µl of 5X First Strand buffer, 1 µl of 0.1 M DTT and 1 µl of SuperScript III Reverse Transcriptase was added and the reaction was gently mixed. At last the mixture was incubated at 50 °C for 60 min and the reaction was inactivated by heating up at 70 °C for 15 min. The cDNA was stored at 4 °C.

3.3.6 Determination of primer annealing temperature by gradient PCR

Gradient PCR was performed to find optimal annealing temperature of cloning primers. Dream Taq Hot Start DNA Polymerase (Thermo Scientific) was used according to the manufacturer's protocol: 95 °C for 2 min, 33 cycles (95 °C/ 30 s, 47-63 °C, 72 °C/ 1 min), 72 °C/ 15 min. Only four temperatures out of the 12 possible in the gradient (47-63 °C) were chosen for a particular primer pair and the temperature of the primer with the lower melting temperature (Tm) was increased by 2 °C and this temperature was used as the lowest annealing

temperature. Additionally, the next three higher annealing temperatures in the gradient were tested as well. For 50 µl PCR mixture 39 µl water, 5 µl 10X Dream Taq Buffer, 1 µl dNTP mix (10 mM each), 2 µl of gene-specific primer forward (10 µM), 2 µl of gene-specific primer reverse (10 µM), 1 µl of cDNA and 0.25 µl of Dream Taq Hot Start DNA Polymerase (1.25 U) were used. PCR products were analysed on a 1 % agarose TAE gel with 0.5 µl of DNA gel stain GelRed per 10 ml volume at 85 V. Out of 4 annealing temperatures tested for each pair of primers, temperature with only one band on the gel and with the highest yield was used for the large-scale PCR. The Dream Taq Hot Start DNA Polymerase generates PCR products with a 3'-dA overhang suitable for TA cloning.

3.3.7 Large-scale PCR and analysis of PCR products on agarose gel

PCR was carried out as it is mentioned in the previous paragraph except for some small changes: The final volume of the reaction was 100 µl and the best fitting annealing temperature determined in the gradient PCR was used (see Table 3 for annealing temperatures) PCR clean-up was done using QIAquick PCR Purification Kit according manufacturer's protocol. 1 volume of 100 % isopropanol was mixed with 1 volume of the PCR and then loaded on the column and spun at 12,000 g for 1 min at 18 °C. The flow-through was discarded and 750 µl of PE washing buffer was added and incubated for 5 min at RT. The column was spun twice at 12,000 g for 1 min at 18 °C and the flow through was discarded. The column was then transferred into a new empty tube and 30 µl of Elution buffer (10 mM Tris-Cl, pH = 8.5) was added and incubated for 3 min at RT. Then the column was spun down, the DNA concentration was measured on NanoDrop and the DNA was stored at -80 °C. To confirm a successful PCR, an agarose gel (1 % agarose TAE gel with 0.5 µl of DNA gel stain GelRed per 10 ml volume) was performed at 85 V. Gel image was captured on Smart3 EZ gel documentation system (VWR).

3.3.8 Ligation into PGEM-T Easy vector

1.5 µl of the cleaned PCR product and 0.5 µl of T4 DNA Ligase (Promega) was mixed with 0.5 µl (25 ng) pGEM-T Easy Vector (Promega) and 2.5 µl of 2X Rapid Ligation Buffer. This mixture was kept for ligation at 4 °C overnight.

3.3.9 Plasmid transformation into chemically competent bacterial cells

66 µl of *E. coli* XL1 blue competent cells (Stratagene) were mixed with 2.5 µl of ligation reaction and then incubated 30 min on ice, heat-shocked for 45 s at 42 °C and cooled on ice 2 min. After this procedure 700 µl of SOC medium was added and the cells were incubated at 37 °C for 45 min with shaking (180 RPM). Then 50 µl of X-Gal (GERBU Biotechnik GmbH) and 100 µl of the transformation mixture, respectively, was spread on the preheated Luria-Bertani (LB) broth-agar plate with a selective Carbenicillin (ThermoFisher Scientific; 50 µg/ml) antibiotic. The rest of the transformation was concentrated by centrifugation (1,000 g for 2 min), resuspended in remaining 100 µl SOC medium and the bacterial suspension was plate on an additionally agar plate. The bacteria were grown in the incubator (37 °C) overnight.

3.3.10 Colony PCR

Transformants were screened for inserts by colony PCR. The white colonies were selected and colony PCR using PPP Master Mix (Top-Bio) was performed. We touched the white colony with 10 µl tip, transferred the bacteria to the back-up LB-agar/ Carbenicillin plate by streaking and submersed the tip into a master mix (5 µl 2x PPP Master Mix, 0.5 µl of each of 10 µM M13F and M13R primer and water up to 10 µl per reaction) and PCR was performed (95 °C for 2 min, 33 cycles of (95 °C/ 30 s, 58 °C/ 30 s, 72 °C/ 1 min), 72 °C/ 15 min). 35 µl of the PCR was analysed on a 1 % agarose gel to confirm the expected size of the insert.

3.3.11 Plasmid DNA isolation

Positive bacterial clones from colony PCR were inoculated into 3.5 ml of LB broth with 3.5µl of Carbenicillin (50 mg/ml) and incubated at 37 °C for 14-16 hours with shaking (180 RPM). The work was done under sterile conditions. 400 µl of bacterial suspension and 200 µl of 80 % glycerol were mixed by shaking and stored at -80 °C as a backup glycerol stock. Plasmid isolation was done with the High pure plasmid isolation kit (Roche). The binding buffer was cooled on ice. The bacteria cells were transferred into a 1.5 ml tube and then pelleted at 6,800 g for 3 min at 4 °C. The supernatant was discarded, and the process repeated until no bacteria culture was left. The pellet was resuspended in 250 µl Suspension Buffer and 250 µl Lysis Buffer was added. The mixture was gently mixed by inverting the tube 4-6 times and incubated at RT for 5 min. Then 350 µl of the chilled Binding Buffer was added, mixed gently by inverting and the tubes were placed on ice for 5 min incubation. The lysate was centrifuged for 10 min at 12,000 g to separate protein/genomic DNA precipitate from the plasmid. The supernatant was put into new tube and spun again. The supernatant was transferred to High

Pure filter column and spun down in the centrifuge at 12,000 g for 60 s. Then 700 µl of the washing buffer was added, incubated at RT for 5 min and centrifuged twice for 60 s at 12,000 g. The flow through was discarded, the tube air-dried for 1 min and 50 µl of Elution Buffer was added and incubated at RT for 3 min. Finally, the plasmids were eluted by centrifugation at 12,000 g for 1 min. The DNA concentration was measured, and the plasmid was stored at -80 °C. For confirmation of successful plasmid isolation 200 ng of the plasmid was analysed on an agarose gel.

3.3.12 Sequencing

Sequencing was done by two companies SEQme s.r.o (https://www.seqme.eu/cs/) and Eurofins Genomics (https://www.eurofinsgenomics.eu/en/home/). Two plasmids for each gene fragment were sequenced. The plasmids were sequenced from both directions (if necessary).

3.3.13 In vitro double-strand RNA synthesis

Template for the *in vitro* dsRNA transcription was made by PCR. Large scale PCR of total volume 200 µl was prepared for each gene fragment and for the bacterial *lacZ* β-galactosidase gene fragment (Bajgar et al., 2013). Plasmid template (5 ng), 100 µl 2X PP Master mix (Top-Bio), M13 F (10 µM) 10 µl, pGEM-RNAi R (10 µM) 10 µl, water 85 µl were mixed and PCR was ran under following conditions 94 °C/ 3 min, 33x (94 °C/ 30 s, 56 °C/ 30 s, 72 °C/ 60 s) and 72 °C/ 5 min. pGEM-T Easy vector has one T7 and one SP6 promoter for *in vitro* transcription. PGEM-RNAi R primer replaces SP6 promoter to T7 promoter in the PCR template.

The PCR was purified with the QIAquick PCR purification kit as described before. For the dsRNA synthesis the MEGAscript T7 kit (Invitrogen) was used. For each 20 µl reaction 8 µl of Nucleotide mix of ATP, CTP, GTP, and UTP (75 mM each), 2 µl of 10X T7 Enzyme Mix, 1.0 µg of PCR template DNA with T7 promoters on both sides, 2 µl of 10X preheated (37 °C) Reaction buffer and H_2O up to 20 µl were mixed together. Then the mixture was gently pipetted up and down and incubated at 37 °C for 6 hours. After that precipitation was carried out with 0.1 volume of 3 M sodium acetate (NaAc) pH = 5.2 and 2.5 volume of 100 % ethanol (EtOH). dsRNA was precipitated overnight at -20 °C. After overnight incubation the following steps were performed: 30 min centrifugation at 12,000 g at 4 °C and washing 3x with 1 ml of 70 % EtOH. Then the pellet was air-dried for up to 10 min and resuspended in 20 µl of water. Annealing of the dsRNA was done by moving the tube to 200 ml of just boiled water. The

water was cooled down naturally to RT and 5 µl of 5x Ringer solution was added. One litre of Ringer contains: NaCl 7.5 g, KCl 0.1 g, dihydrate $CaCl_2$ 0.2 g, hexahydrate $MgCl_2$ 0.4 g, $NaHCO_3$ 0.2 g dissolved into 1 litre of distilled water with pH = 7.4. The dsRNA concentration was measured on NanoDrop. The dsRNA was diluted to 2-3 µg/ µl by adding 1x Ringer solution and then checked on a gel.

3.3.14 dsRNA injection

For the injections 2-3 days-old adult male bugs of the Lyon red strain were taken. Into each bug 2 µl of 2.5-3.0 µg/ µl dsRNA was injected dorsolateral under one wing.

3.4 Locomotor activity measurement

Locomotor Activity Monitors (LAM25; TriKinetics Inc., Waltham, MA) recorded the locomotor activity. The bugs were kept in glass tubes with water on one side and a linden seed at the other side. Activity was measured by recording the movement from water to food and vice versa in 5 min bins. Overall the bugs were kept at 25 °C for 5 days under LD (18 hours light/ 6 hours dark) cycles followed by 9 DD (constant darkness) cycles. The measurements were done in several runs. Altogether, we tested 768 Lyon red males in 9 runs.

3.5 Analysis of data and statistics

For analysing the data imageJ (NIH, Bethesda, Maryland) including the ActogramJ plugin (Schmid, 2011) was used. The activity, free running period (FRP) of the insects was averaged for each RNAi injection individually, and outliers were excluded by box plotting the data. Insect were considered as rhythmic if the PN≥15 (Lomb-Scargle periodogram analysis in ActogramJ). PN value was determined by ActogramJ plugin using default settings (Schmid et al., 2011; see also ActoJ manual https://imagej.net/ActogramJ) Significance was tested by t.test analysis. This was done in the program R (R Development Core Team, 2008).

4. Results

The main aim of this thesis was to examine the effect of Protein Phosphatase 2A knockdown on the linden bug *Pyrrhocoris apterus* circadian clock as it was described for the insect model *Drosophila melanogaster*. PP2A is a heterotrimeric complex, composed of one scaffold A subunit, one catalytic C subunit and one of five B subunits belonging to 4 subclasses (B to B'''). We decided to knockdown 7 genes in total belonging to all beforementioned subunits.

4.1 Pp2A gene candidates are conserved between *P. apterus* and *Drosophila*

We identified all 7 candidate Pp2A genes in *P. apterus* transcriptome. Searching in *P. apterus* transcriptome yielded in several open reading frames (ORF) for each *Pp2a* candidate gene and the sequence with the longest ORF was picked to be compared with the *Drosophila* ortholog (Table 4). PP2A-29B, MTS, TWS, WDB and WRD proteins were well conserved with over 69 % of amino acids identity and over 82 % protein similarity compared to the *Drosophila* orthologs. In contrast, CKA and CG4733 proteins varied more from *Drosophila*, with 54 % and 39 % AA identity, respectively (Table 4). The overall AA identity of CKA and CG4733 orthologous proteins was lower than for the other candidate genes, the conserved protein domains AA identities were high, and the genes were unequivocally determined as *P. apterus* orthologs. Although WDB and WRD proteins belong to the same B subunit subclass (B') their overall AA identity is only 64 %.

All ORF sequences encoding a particular Pp2a candidate gene were aligned and the regions common to the all sequences were chosen for cloning primers design to avoid isoform-specific knockdown. The length of all 7 *Pp2a* genes was sufficient to accommodate two pairs of primers per gene (Figure 3) resulting in amplification of 2 non-overlapping gene fragments. DNA sequence chosen for both *wdb* and *wrd* gene fragments is different enough to avoid concurrent knockdown.

Table 4. Pairwise amino acid identity and pairwise protein similarity between *D. melanogaster* and *P. apterus* PP2A candidate proteins

	PP2A-29B	MTS	TWS	WDB	WRD	CG4733	CKA	WDB vs. WRD
Pairwise Identity (%)	76.0%	94.5%	86.5%	86.5%	69.9%	39.2%	54.1%	64.4%
Pairwise Positive similarity (BLSM62) (%)	86.0%	98.7%	92.6%	92.2%	82.7%	56.1%	67.4%	76.8%

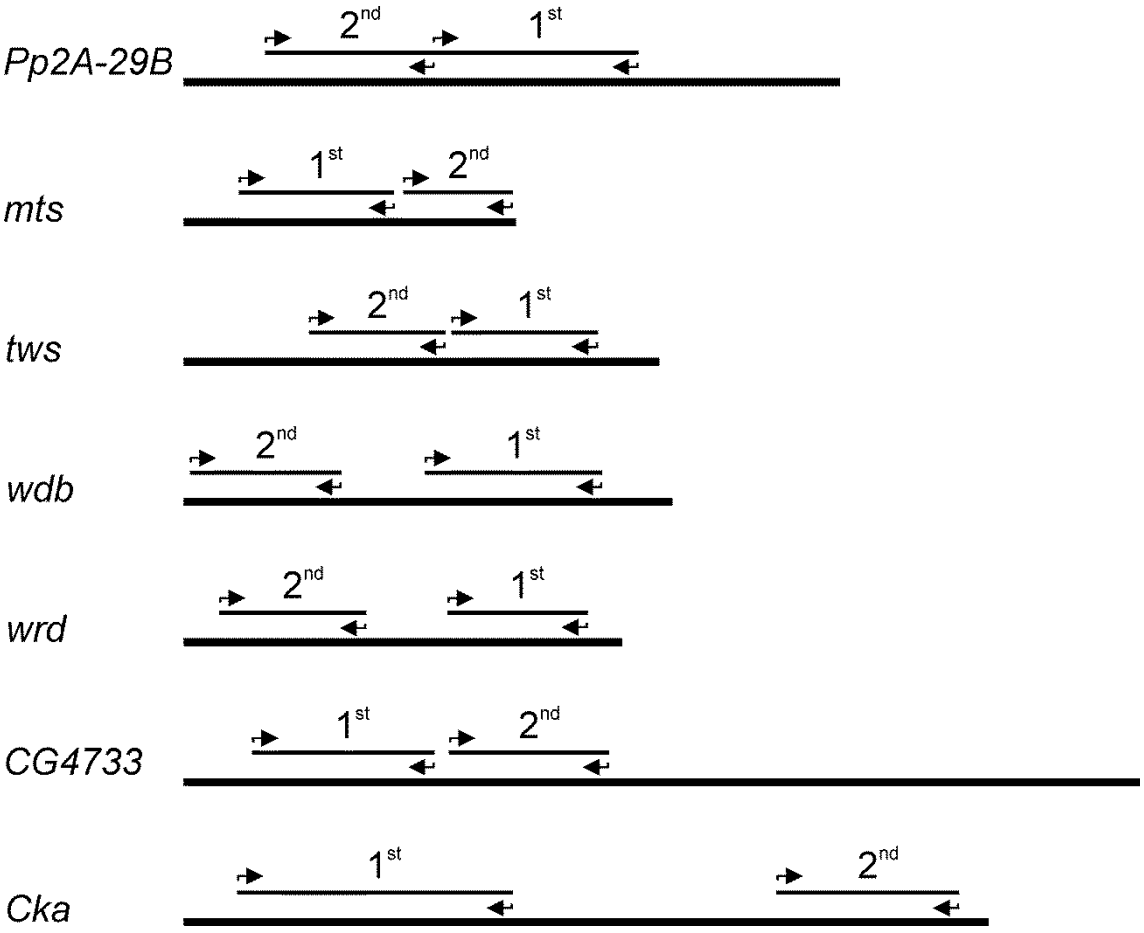

Figure 1. Position of the fragments and their cloning primers in the studied genes. Schematic graph representing the protein coding sequences of the studied genes and positions of the RNAi fragments and their respective cloning primers.

4.2 Locomotor activity measurements

DsRNA of the designed gene fragments were injected into *P. apterus* Lyon red 2-3 days old males.

4.2.1 1st fragment dsRNA injections

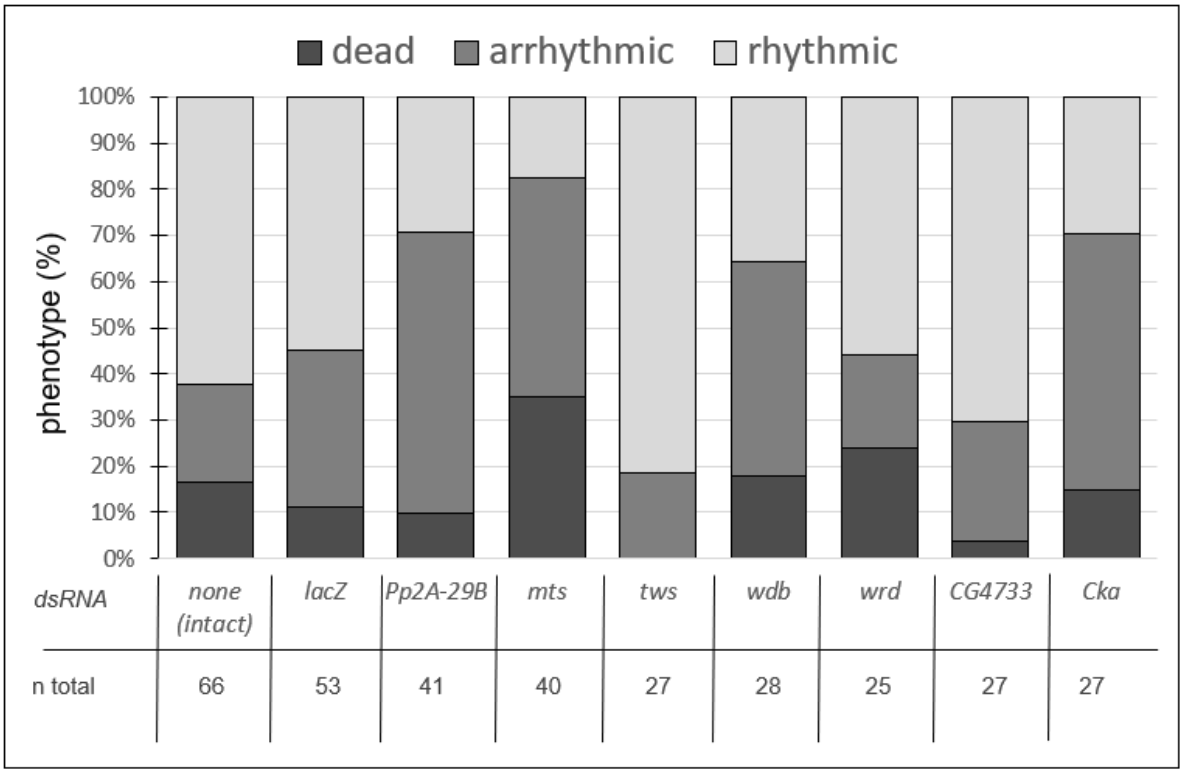

Figure 2. Percentage of phenotypes after 1st fragment dsRNA injection. The percentage of males with rhythmicity with one clear stable period (rhythmic) and males whose behaviour did not show a significant period (arrhythmic). Males were classified as dead when they died during the locomotor activity measurement assay, either within 5 days of entrainment or 9 days of constant dark. The total number of tested animals is given as n total for each treatment. Pp2A-29B, mts, wdb and Cka RNAi treatment caused higher than 50 % arrhythmicity of the survivors.

Gene specific dsRNA of 1st fragments was injected in several runs due to lack of sufficient number of animals at the same time. In all cases *lacZ* dsRNA served as a negative control and intact (non-injected) males served as a second (survival) control. The results representing the same gene specific fragment from different injection runs were pooled for further analysis. The actograms we obtained from locomotor activity measurements showed rhythmicity of survived males below 50 % for *Pp2A-29B*, *mts*, *wdb* and *Cka* RNAi (Figure 4). *Pp2A-29B* and *mts* RNAi were performed on more animals than other fragments because high arrhythmicity (and mortality, see below) resulted in lower number of animals for which the free running period (FRP) could be calculated (Figure 5 and Table 5). The FRP of negative *lacZ* dsRNA injected controls fluctuated between 22.65 and 23.00 hrs, which is common for the Lyon red strain (Table 5 and Pivarciova et al., 2016). The FRP calculated for the rhythmic animals of mostly arrhythmic *mts*, *wdb* and *Cka* RNAi animals were not significantly different when compared to *lacZ* dsRNA injected controls. In contrast, *Pp2A-29B* RNAi significantly decreased the FRP to 21.85 hours (Figure 6), so the circadian clock was ticking faster. Knockdowns of *tws*, *wrd* and *CG4733* showed arrhythmicity of 27 % or lower and thus can

be considered as a not affecting the rhythmicity. However, the FRP of the *tws* RNAi animals was significantly longer than FRP of the *lacZ* dsRNA injected animals, 23.82 hours vs. 23.00 hours, respectively (Figure 5 and Figure 6).

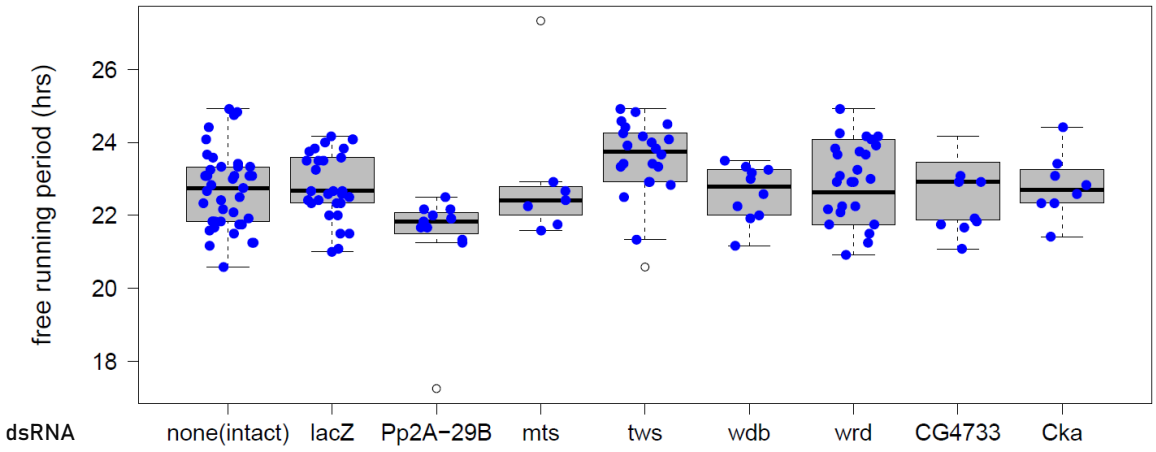

Figure 3. Locomotor activity of *P. apterus* males after 1st gene fragment RNAi. Boxplot showing free running period (FRP) of rhythmic *P. apterus* males injected with dsRNA fragment 1 for each PP2A gene and measured at constant dark conditions. The FRP of each individual male is represented by one dot. Open circle dots were considered as outliers and were excluded from the statistical evaluation.

Table 5. Summary of the locomotor activity for the gene-specific 1st fragment RNAi. The mean free running period (mean FRP) and the standard error of the mean (SEM) was calculated for gene-specific and control *lacZ* dsRNA injected animals. Statistical significance was evaluated by student's t-test and statistically significant ($p < 0.05$) differences are highlighted in red. Number of rhythmic males (n rhythmic) for which the mean FRP was calculated. Total number of males (n total) represents measured males pooled from the corresponding experimental runs (last column).

dsRNA	mean FRP	SEM	p-value	n rhythmic	n total	run
lacZ	22.65	0.89		26	43	1+3+5
Pp2A-29B fr.1	21.85	0.39	<0.001	10	41	
lacZ	22.83	0.95		21	33	1+3+4
mts fr.1	22.26	0.52	0.074	6	40	
lacZ	23.00	0.87		16	26	2+4
tws fr.1	23.82	0.72	<0.001	17	27	
CG4733 fr.1	22.75	0.92	0.407	19	26	
lacZ	22.86	0.98		16	25	1+4
wdb fr.1	22.62	0.76	0.478	10	23	
lacZ	22.74	0.93		11	27	1+3
wrd fr.1	22.78	1.35	0.942	12	19	
lacZ	22.94	0.78		8	28	2+3
Cka fr.1	22.8	0.88	0.731	11	23	

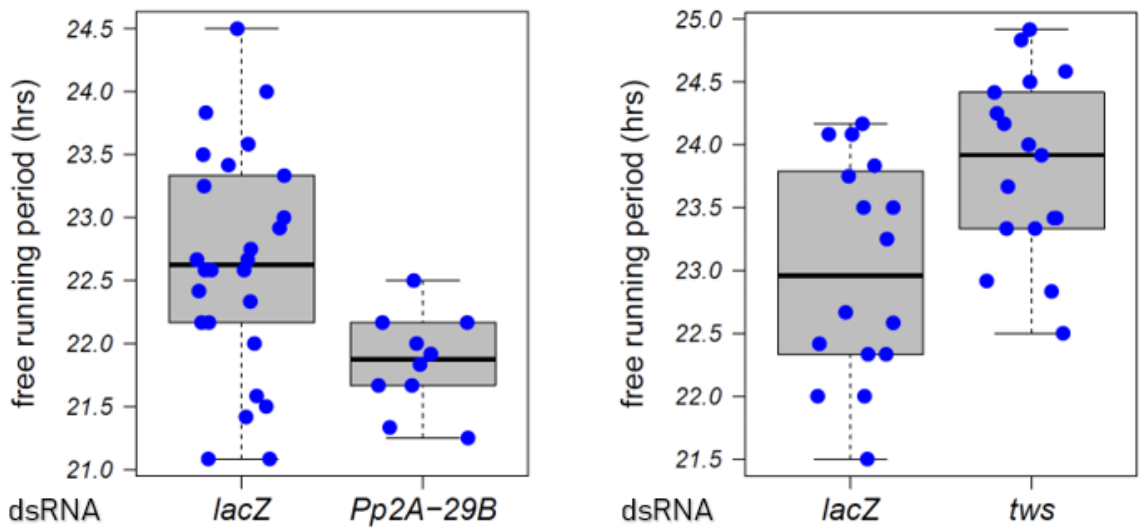

Figure 4. *Pp2A-29B* and *tws* 1st fragment RNAi males locomotor activity differ significantly from the controls. Locomotor activity of *lacZ* controls from only corresponding runs (see Table 5) was analysed. The FRP of each individual male is represented by one dot and the outliers were not included to the FRP analysis.

However, the mortality of the animals was relatively high and reached 17 % and 11 % for intact and *lacZ* dsRNA injected animals, respectively (Figure 4). The mortality reached 35 % for *mts* dsRNA injections, 24 % for *wrd*, 18 % for *wdb* and was ≥ 10 % for *Cka* and *Pp2a-29B* RNAi. High mortality of the controls suggested lower fitness of the animals used for the experiment. We decided to repeat the whole experiment (see section 1st fragment dsRNA injections repeated below) with a different cohort of animals to exclude the possible effect of the low fitness on the results.

4.2.2 1st fragment dsRNA injections repeated

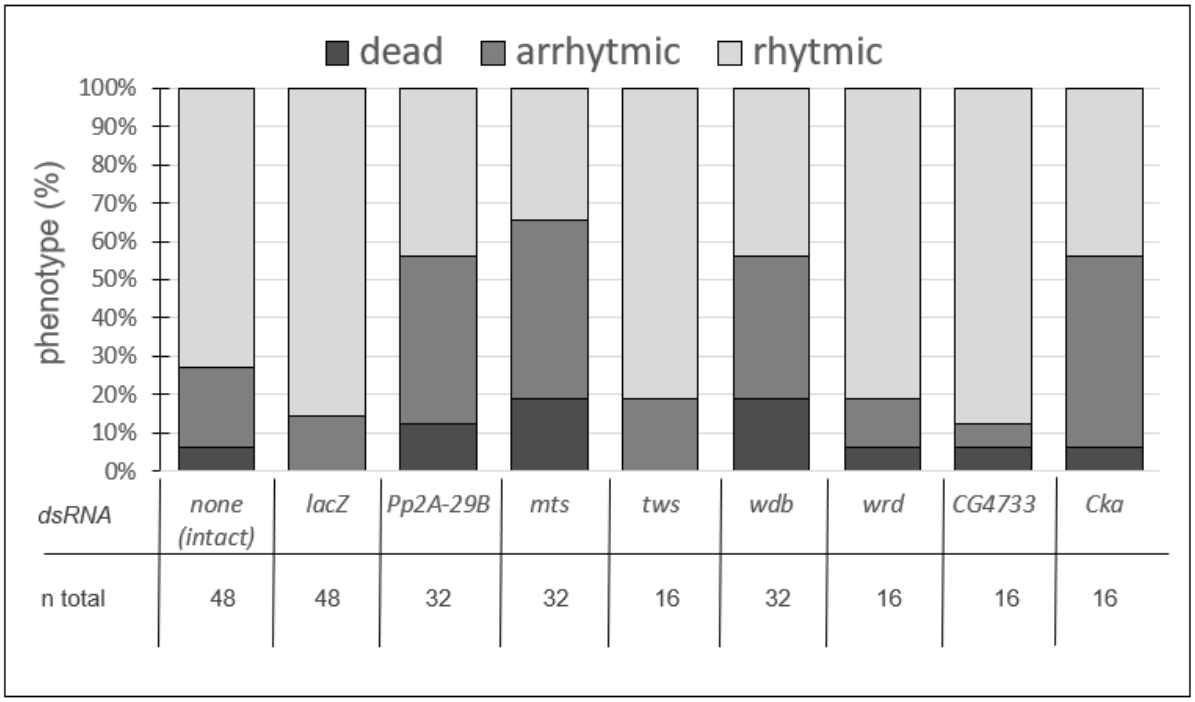

Figure 5. Percentage of phenotypes after 1*st* fragment dsRNA repeated injections. The percentage of males with rhythmicity with one clear stable period (rhythmic) and males whose behaviour did not show a significant period (arrhythmic). Males were classified as dead when they died during the locomotor activity measurement assay, either within 5 days of entrainment or 9 days of constant dark. The total number of tested animals is given as n total for each treatment. *Pp2A-29B*, *mts*, *wdb* and *Cka* RNAi treatment caused higher than 50 % arrhythmicity of the survivors.

Repetition of 1st fragment dsRNA injection was performed under similar condition as the injections before. Both *lacZ* dsRNA injected and intact animals manifested much lower mortality (Figure 7) than in the earlier 1st fragment injections (Figure 4). Mortality of the non-injected animals decreased from 16.66 % to 6.25 % and mortality of the *lacZ* dsRNA injected animals dropped to 0 %. Mortality also decreased in *mts*, *wrd* and *Cka* RNAi animals and only *Pp2A-29B* knockdown showed higher mortality compared to the earlier 1st fragment injections. We also measured higher rhythmicity of both control groups compared to the earlier 1st fragment injections, suggesting higher fitness of the animal cohort used in the 1st fragment repeated dsRNA injections. Due to the experience with high arrhythmicity (and lethality) in the earlier injections, *Pp2A-29B*, *mts* and *wdb* RNAi was performed on more animals than for the other fragments. The FRP of the negative *lacZ* control varied between 22.58 and 23.08 hrs (Figure 8). *Pp2A-29B*, *mts*, *wdb* and *Cka* RNAi resulted in a rhythmicity of the males between 34.4 and 43.8 % and can be considered as arrhythmic. *tws*, *wrd* and *CG4733* knockdown males were highly rhythmic, with the percentage of the rhythmicity between 87.5 and 81.2 %.

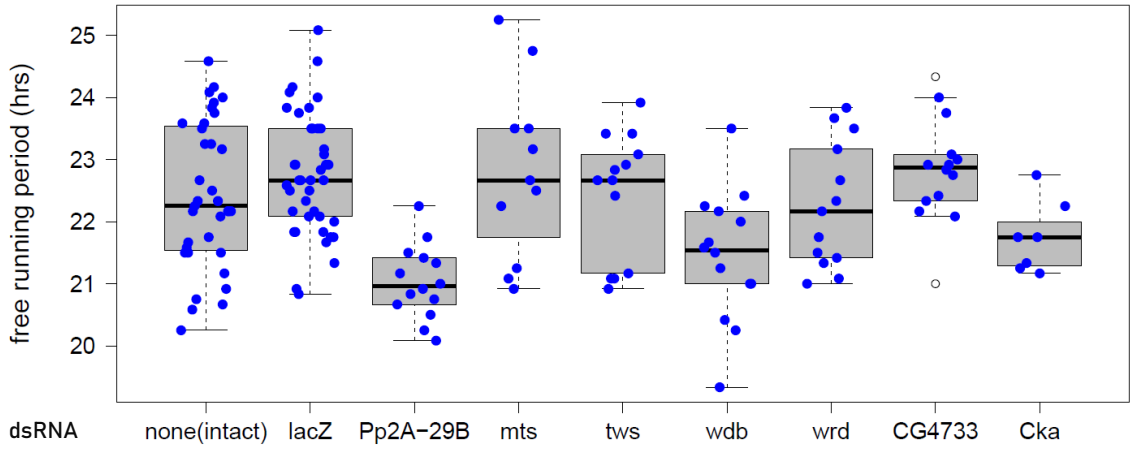

Figure 6. Locomotor activity of *P. apterus* males after the 1st gene fragment RNAi repeated. Boxplot showing free running period (FRP) of rhythmic *P. apterus* males injected with dsRNA fragment 1 for each PP2A gene and measured at constant dark conditions. The FRP of each individual male is represented by one dot. Open circle dots were considered as outliers and were excluded from the FRP analysis.

No significant difference between the FRP of the *mts*-, *tws*-, *wrd*- and *CG4733*-knockdowned bugs was detected when compared to the *lacZ* controls (Table 6). *Pp2A-29B* RNAi shortened the free running period to 21.03 hours. Decline of FRP could also be observed in the *wdb* and *Cka* RNAi males, with a period of 21.45 hrs and 21.75 hrs, respectively (Figure 8 and Figure 9).

Table 6. Summary of the locomotor activity for the gene-specific 1st fragment repeated RNAi. The mean free running period (mean FRP) and the standard error of the mean (sem) was calculated for gene-specific and control *lacZ* dsRNA injected animals. Statistical significance was evaluated by student's t-test and statistically significant ($p < 0.05$) differences are highlighted in red. Number of rhythmic males (n rhythmic) for which the mean FRP was calculated. Total number of animals males (n total) represents measured males pooled from the corresponding experimental runs (last column). Only *Pp2A-29B*, *wdb* and *Cka* RNAi males showed significant difference in FRP compared to the control *lacZ* dsRNA injected males.

dsRNA	mean FRP	SEM	p-value	n rhythmic	n total	run
lacZ	22.56	0.88		27	32	8+9
Pp2A-29B fr.1	21.03	0.59	<0.001	14	32	
lacZ	22.61	1.00		12	16	9
mts fr.1	22.80	1.42	0.720	11	32	
lacZ	22.90	0.87		15	16	7
tws fr.1	22.43	1.03	0.224	13	16	
wrd fr.1	22.26	1.02	0.102	13	16	
CG4733 fr.1	22.85	0.58	0.879	14	16	
lacZ	22.78	0.98		27	32	7+9
wdb fr.1	21.45	1.04	<0.001	14	32	
lacZ	22.52	0.82		15	16	8
Cka fr.1	21.75	0.58	0.023	7	16	

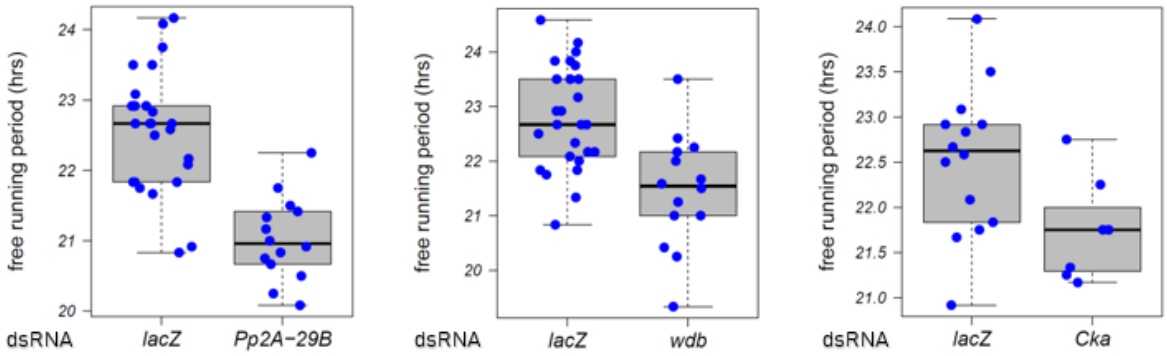

Figure 7. *Pp2a-29B*, *wdb* and *Cka* 1st fragment repeated RNAi males locomotor activity differ significantly from the controls. Locomotor activity of *lacZ* controls from only corresponding runs (see Table 6) was analysed. The FRP of each individual male is represented by one dot and the outliers were not included to the FRP analysis.

4.2.3 2nd fragment dsRNA injections

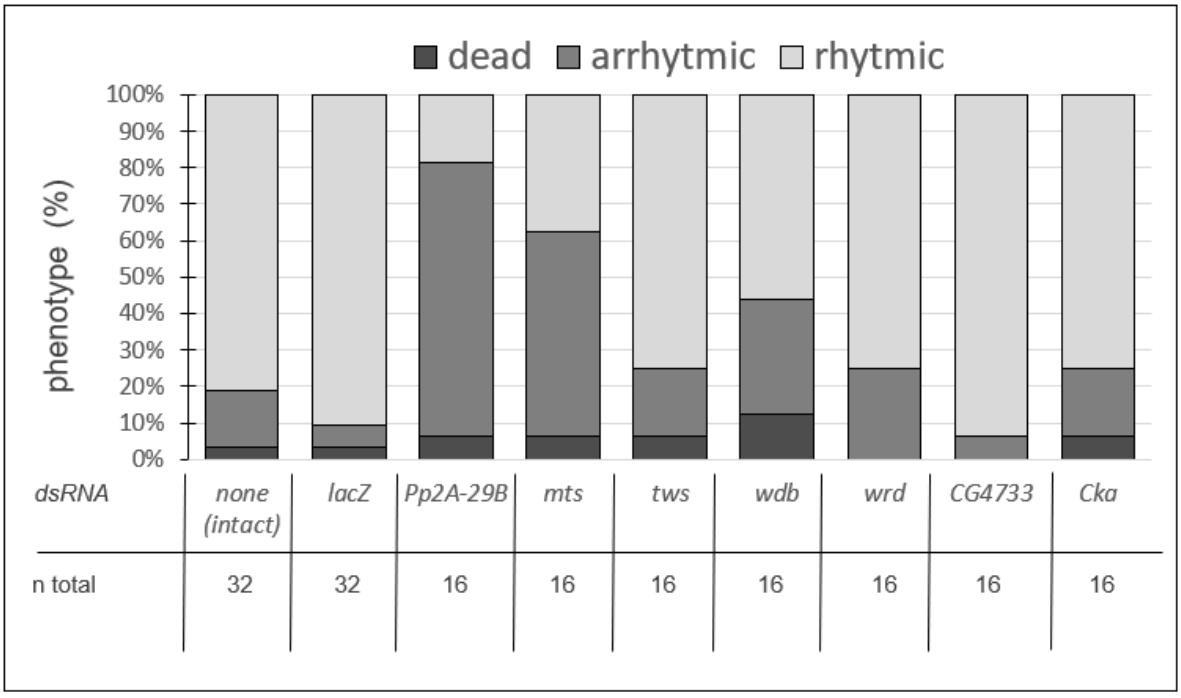

Figure 8. Percentage of different phenotypes after 2n*d* fragment dsRNA injections. The percentage of males with rhythmicity with one clear stable period (rhythmic) and males whose behaviour did not show a significant period (arrhythmic). Males were classified as dead when they died during the locomotor activity measurement assay, either within 5 days of entrainment or 9 days of constant dark. The total number of tested animals is given as n total for each treatment. Only *Pp2A-29B* and *mts* RNAi treatment caused higher than 50 % arrhythmicity of the survivors.

We injected 2nd dsRNA fragment for each studied gene to further confirm our results obtained by the 1st dsRNA fragment injections. Since the 2nd dsRNA fragment targets different, non-overlapping part of the corresponding PP2A gene transcript, the same or similar results obtained for the 2nd fragment RNAi would highly raise the reliability of the 1st fragment RNAi

results. We injected 16 males per treatment and surprisingly, mortality in all examined groups was not higher than 13 %, found for *wdb* RNAi (Figure 10). Arrhythmicity over 50 % was observed only in the *Pp2A-29B* and *mts* RNAi animals, reaching 80 and 60 %, respectively. Such a result correlates with the data for *Pp2A-29B* and *mts* 1st fragment repeated RNAi where the arrhythmicity was also higher than 50 % (Figure 7). Out of the other tested genes, only *wdb* RNAi showed substantial percentage (36 %) of arrhythmicity. FRP of the *lacZ* control fluctuated between 22.58 and 23.08 hours, which represent normal behaviour in the Lyon red strain (Pivarciova et al., 2016). High arrhythmicity and rather low number of injected animals (16) led to a low number of rhythmic *Pp2A-29B* and *mts* RNAi males suitable for FRP calculation (Figure 11). From 7 tested 2nd fragments only *wdb* and *Cka* RNAi significantly affected the FRP (Table 7). Clocks of *wdb* RNAi animals were faster with the mean FRP 21.96 hrs (Figure 11 and Figure 12). This mirrored the result observed for the 1st *wdb* dsRNA fragment injections. Contrary to the previous result, *Cka* 2nd fragment RNAi showed longer FRP, although the statistical support was rather low for both dsRNA fragment *Cka* injections (Figure 11 and Figure 12). Knockdown of *wrd* and *CG4733* resulted in unaffected FRP compared to the *lacZ* dsRNA injected control animals. Interestingly, 2nd fragment *tws* RNAi did not affect the clock period as it did in the 1st fragment RNAi suggesting the necessity of RNAi efficacy measurements (discussed below).

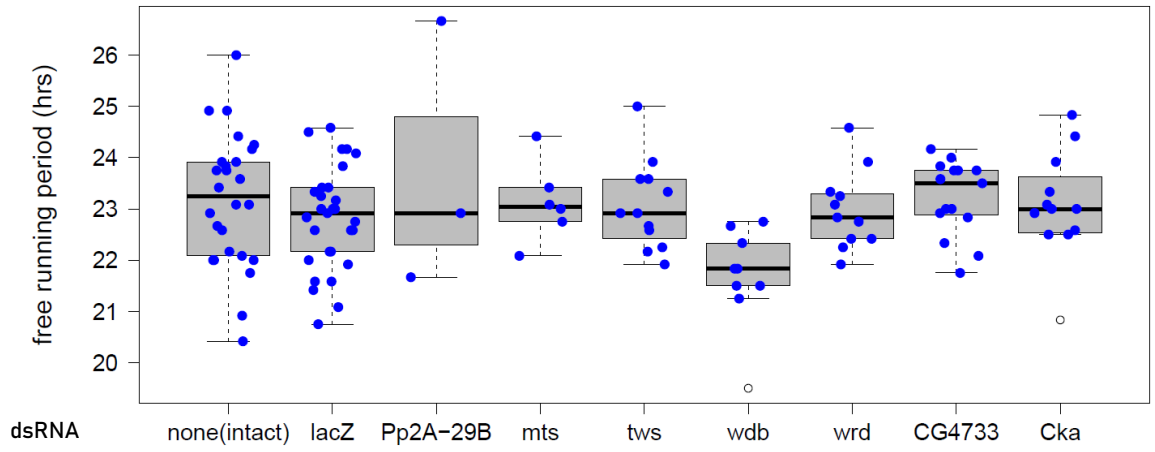

Figure 9. Locomotor activity of *P. apterus* males after 2nd gene fragment RNAi. Boxplot showing free running period (FRP) of rhythmic *P. apterus* males injected with dsRNA fragment 2 for each PP2A gene and measured at constant dark conditions. The FRP of each individual male is represented by one dot. Open circle dots were considered as outliers and were excluded from the FRP analysis.

Table 7. Summary of the locomotor activity for the gene-specific 2nd fragment RNAi. The mean free running period (mean FRP) and the standard error of the mean (sem) was calculated for gene-specific and control *lacZ* dsRNA injected animals. Statistical significance was evaluated by student's t-test and statistically significant ($p < 0.05$) differences are highlighted in red. Number of rhythmic males (n rhythmic) for which the mean FRP was calculated. Total number of animals males (n total) represents measured males pooled from the corresponding experimental runs (last column). Only *Cka* and *wdb* RNAi males showed significant difference in FRP compared to the control *lacZ* dsRNA injected males.

dsRNA	mean FRP	SEM	p-value	n rhythmic	n total	run
lacZ	22.58	0.89		15	16	5
Pp2A-29B fr.2	23.75	2.6	0.520	3	16	
tws fr.2	23.07	0.87	0.165	12	16	
Cka fr.2	23.28	0.78	0.045	12	16	
lacZ	23.08	1.10		14	16	6
mts fr.2	23.13	0.77	0.914	6	16	
wdb fr.2	21.96	0.56	0.050	9	16	
wrd fr.2	22.98	0.78	0.792	12	16	
CG4733 fr.2	23.22	0.73	0.694	15	16	

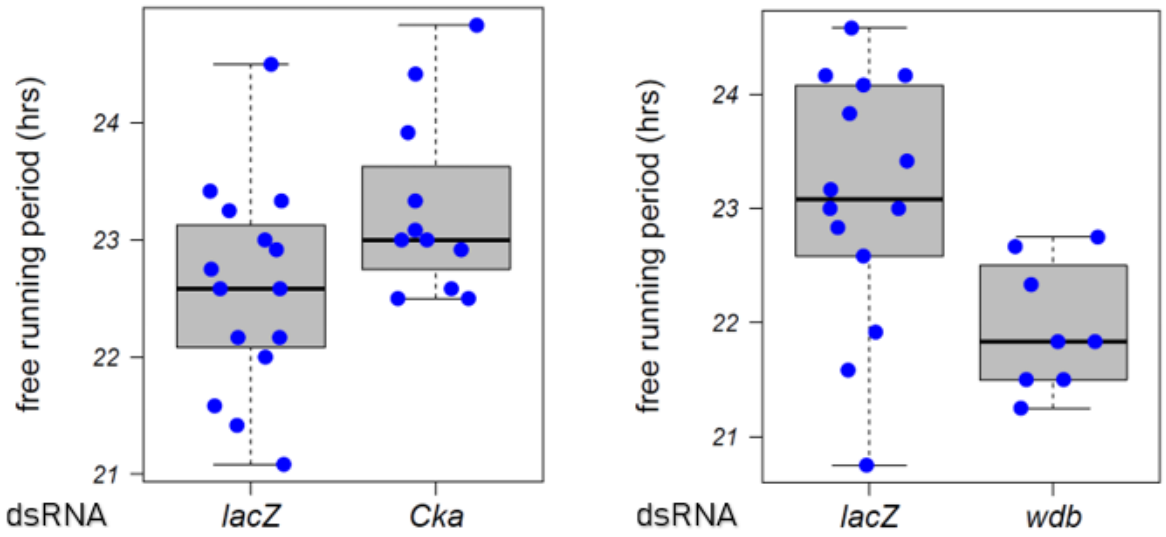

Figure 10. *wdb* and *Cka* 2nd fragment RNAi males locomotor activity differ significantly from the controls. Locomotor activity of *lacZ* controls from only corresponding runs (see Table 7) was analysed. The FRP of each individual male is represented by one dot and the outliers were not included to the FRP analysis.

5. Discussion

RNAi screening was used by Agrawal and Hardin (2016) to identify protein phosphatases that function in the *Drosophila* circadian clock. They employed the Gal4/UAS expression system, to perform RNAi in the clock cells in the brain of the fruit fly. They knocked down all annotated protein phosphatases and protein phosphatase regulators including the members of the Protein phosphatase 2A, except for the *Cka* gene that was not annotated as encoding a PP2A subunit. We decided to perform similar screen focused on PP2A in *Pyrrhocoris apterus*.

Gal4/UAS system is not established in *Pyrrhocoris apterus* so the gene knockdown is achieved by the injection of the *in vitro* synthetized dsRNA into the *P. apterus* body. RNA interference in the *P. apterus* has a systemic effect and efficiently silences the gene expression in all the tissues (Kotwica-Rolinska et al., 2017; Smykal et al., 2014). However, systemic RNAi can be potentially disadvantageous when pleiotropic genes with broad range of functions are targeted. Components of PP2A are expressed in many tissues in *Drosophila* (Chintapalli et al., 2007) and the variability of PP2A heterotrimeric complex enables mediation of substantial number of intracellular processes such as cell cycle progression (Kolupaeva and Janssens, 2012) stem cell self-renewal and tumorigenesis (Wang et al., 2009), planar cell polarity (Hannus et al, 2002) and circadian clock regulation (Sathyanarayanan et al., 2004; Andreazza et al., 2015; Agrawal and Hardin, 2016). Also the RNA interference mechanism can cause an off-target effect if gene only partially similar to the targeted gene fragment is knocked down. We decided to design two non-overlapping dsRNA fragments for each of the studied gene to overcome the possible off-target effect.

The first fragment injections yielded a general increase in mortality and arrhythmicity that was not only limited to the gene-specific dsRNA injections but also affected the intact and negative *lacZ* dsRNA controls (Figure 4). This might have been caused by some unspecified infection in the *P. apterus* males picked for the 1st fragment injections. We decided to repeat the 1st dsRNA fragment injections to assess the possible effect of the infection on the survival and the rhythmicity. After repeating the first fragment injections, control animal (*lacZ* and intact) mortality decreased and also the overall high arrhythmicity decreased to normal levels observed in Lyon red strain (compared Figure 4 and Figure 7; Pivarciova et al., 2016).

Nevertheless the higher mortality and arrhythmicity in the earlier 1st dsRNA injections, the trend of higher arrhythmicity could be observed in both earlier and later 1st fragment *Pp2A-29B*, *mts*, *wdb* and *Cka* RNAi injections (Figure 4 and Figure 7, summarized in Table 8). High level of arrhythmicity was further supported by 2nd dsRNA fragment injections, where *Pp2A-*

29B and *mts* RNAi animals manifested arrhythmicity over 50 % (Figure 10). Rhythmic *Pp2A-29B* RNAi males in both 1st dsRNA fragment injections showed shorter free running period (FRP). The effect of 1st fragment RNAi was not supported by the 2nd *Pp2A-29B* fragment RNAi and arrhythmicity for both *Pp2A-29B* and *mts* seems to be the prevailing phenotype. We can conclude that knockdown of both scaffold and catalytic subunits, encoded by *Pp2A-29B* and *mts* genes, respectively, disrupted *P. apterus* circadian clock. The RNAi phenotypes of *Pp2A-29B* and *mts* in *P. apterus* seem to be more severe than RNAi phenotypes in *D. melanogaster* where the *Pp2A-29B* and *mts* RNAi in the clock cells in the fly brain caused longer period (FRP) rather than arrhythmicity (Agrawal and Hardin, 2016; Table 6). However, expression of N-terminal truncated dominant-negative form of MTS (mts^{DN}), capable of binding to the wild-type scaffold (A) and regulatory (B) subunits, in the clock neurons dramatically reduced the rhythmicity to 10 % (Hannus et al., 2002). The small portion of mts^{DN} rhythmic flies showed longer FRP (Sathyanarayanan et al., 2004; Andreazza et al., 2015)., although the phenotype might be caused by the defect in the clock neurons (Andreazza et al., 2015). Interestingly, MTS overexpression in the *Drosophila* clock cells also caused arrhythmicity (Agrawal and Hardin, 2016; Sathyanarayanan et al., 2004) showing the importance of the balanced dosage of MTS expression.

Table 8. Summary of the results and comparison with *Drosophila*. Statistically significant ($p<0.05$) differences in FRP are marked red. ar=arrhythmic (over 50 % animals were not rhythmic); <=FRP shorter than control; >=FRP longer than control; ()=parentheses denote weak or uncertain result. Data for Drosophila summarized from Santhyanarayanan et al., 2004; Andreazza et al., 2015; Agrawal and Hardin, 2016.

dsRNA	1st frag	FRP	1st frag rep	FRP	2nd frag	FRP	*Drosophila*	FRP
Pp2A-29B	ar	<	ar	<	ar	(-)	ar	>
mts	ar	>	ar	-	ar	-	ar	>
tws	-	<	-	-	-	>	(ar)	-
wdb	ar	-	ar	<	-	<	-	>
wrd	-	-	-	-	-	-	-	(<)
CG4733	-	-	-	-	-	-	-	-
Cka	ar	-	ar	<	-	>	(ar)	>

PP2A regulates the circadian clock at several levels (Sathyanarayanan et al., 2004; Andreazza et al., 2015; Agrawal and Hardin, 2016) so unsurprisingly the manipulation of the core PP2A components in both *Pyrrhocoris* and *Drosophila* caused severe clock phenotypes. PP2A B subunits facilitate the PP2A substrate and intracellular localisation specificity and thus the knockdown or other manipulation of individual B subunit class members would suggest less profound phenotypes on the circadian clock. We silenced 5 genes belonging to all four known

B subunit subclasses. As mentioned before, *wdb* and *Cka* 1st fragment repeated RNAi in *Pyrrhocoris* led to arrhythmicity over 50 %, which was only partially supported by the *wdb* 2nd fragment RNAi with 36 % arrhythmicity of the survivors. The FRP in both RNAi experiments was shorter for *wdb*, which is contrary to *Drosophila*, where both *wdb* RNAi and overexpression in the clock cells caused longer FRP with unaffected rhythmicity (Sathyanarayanan et al., 2004; Andreazza et al., 2015; Agrawal and Hardin, 2016). Both *Cka* dsRNA injections showed contradictory FRPs with weak statistical support for both. *Cka* RNAi in *Drosophila* clock cells induces long FRP that corresponded more to the 2nd dsRNA fragment injections in *Pyrrhocoris*. *Drosophila* Cka^2-null mutants die during embryos but can be rescued by daily heat-shock driven CKA expression. Those developmentally rescued adult Cka^2-null mutants became arrhythmic in several days in the constant dark (Chen et al., 2002; Andreazza et al., 2015). We can conclude that WDB and CKA play a role in the *Pyrrhocoris* and *Drosophila* circadian clock, however, additional pieces of evidence are needed for reliable comparison with *Drosophila*. *Pyrrhocoris tws* 1st fragment RNAi lengthened the FRP but the phenotype was not supported by any of the successive dsRNA injection experiments. *tws* RNAi in *Drosophila* clock cells did not alter the FRP (Andreazza et al., 2015; Agrawal and Hardin, 2016) but the TWS overexpression in the clock cells led to arrhythmicity (Sathyanarayanan et al., 2004). We can only speculate that TWS overexpression in the *Drosophila* clock cells might have led to the PP2A subunit disproportion and thus impairment of the clock.

wrd and *CG4733* RNAi in *Pyrrhocoris* showed no clock phenotype. *CG4733* silencing in *Drosophila* clock cells has also no effect on the circadian clock. *wrd* RNAi in *Drosophila* displayed enhanced arrhythmicity and shorter FRP but only when driven by more wide-spread *tim*-Gal4 driver (Agrawal and Hardin, 2016) so its role in the circadian clock is still unclear.

Our trial, low-scale RNAi screening of PP2A components in *Pyrrhocoris* showed its conserved and irreplaceable role in the circadian clock. However, the *in vivo* function of PP2A in *P. apterus* is far from being well understand. We need to assess the RNAi efficacy as our inability to get the same circadian phenotype for both dsRNA fragments might have been due to inefficient RNAi knockdown. We also need to expand the number of tested animals mainly for the genes with prevailing arrhythmic phenotype, where the systemic nature of RNAi in *Pyrrhocoris* might have hindered the full range of phenotypes. Another option, although not applicable to all the genes because of the limited gene length, is to design and employ a 3rd dsRNA fragment. The fundamental proof of PP2A function would require a study of

mechanism of PP2A action on the phosphorylation status of the circadian clock proteins, which is far behind our capabilities.

The scope and the mechanism of PP2A mediatory role(s) on the circadian clock is poorly understood even in the *Drosophila* model, which provide an extensive set of molecular and genetic methods. Thus, studies in non-model insect can substantially improve our understanding of molecular principles governing insect circadian clock.

6. Conclusion

1) *P. apterus* genes encoding PP2A scaffold, catalytic and five regulatory subunits were cloned and the sequence analysis confirmed that all genes are true orthologs of *Drosophila Pp2A-29B*, *mts*, *tws*, *wdb*, *wrd*, *CG4733* and *Cka* genes.

2) *Pp2A-29B* and *mts* RNAi destroyed the circadian clock in *P. apterus* males with high ratio of arrhythmicity, which phenocopied the results from the *Drosophila* and proved PP2A to be a crucial circadian clock regulator in a non-model insect.

3) RNAi of regulatory B subunits suggested that WDB and CKA play a role in *Pyrrhocoris* circadian clock although the phenotypes do not fully match those found in *Drosophila*.

4) *wrd* and *CG4733* knockdowns did not affect the circadian clock in *Pyrrhocoris*, which comply with non-circadian or weak circadian function in *Drosophila*, respectively. The function of TWS in *Pyrrhocoris* is unclear and requires additional experiments.

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8. Index of abbreviations

AA	amino acid
ar	arrhythmic
bHLH-PAS	basic helix-loop-helix PER-ARNT-SIM
bZIP	basic leucine zipper
CDC14	cell division cycle 14
CDC25	cell division cycle 25
cDNA	complementary DNA
CK2	casein kinase 2
Cka	connector of kinase to AP-1 (gene, mRNA)
CKA	connector of kinase to AP-1 (protein)
Clk	clock (gene, mRNA)
CLK	clock (protein)
cry1	cryptochrome 1 (gene, mRNA)
CRY1	cryptochrome 1 (protein)
cry2	cryptochrome 2 (gene, mRNA)
CRY2	cryptochrome 2 (protein)
CTRIP	circadian trip (protein)
CUL-3	cullin 3 (protein)
CWO	clockwork orange (protein)
cyc	cycle (gene, mRNA)
CYC	cycle (protein)
DBT	doubletime (kinase)
DD	Constant darkness
DN	dominant negative
dNTP	deoxyribose nucleoside triphosphate
Drosophila	*Drosophila melanogaster*
dsRNA	double stranded RNA
e-box	enhancer box
EyA	Eyes Absent protein
FCP	TFIIF (transcription initiation factor IIF)-associating component of CTD (C-terminal domain) phosphatase
fr.	Fragment
FRP	free running period
g	centrifugal force
HAD	haloacid dehalogenase
hrs	hours
lacZ	lacZ (gene, mRNA)
LB	Lysogeny broth
LD	Light dark
LMWP	low molecular weight phosphatases
LPA	Linear PolyAcrylamide
MAP	Mitogen-activated protein
min	minutes
mM	millimol
ml	millilitre
MKP	mitogen-activated protein-kinase phosphatase
MTM	myotubularins
mTOR	mammalian target of rapamycin
mts	microtubule star (gene, mRNA)
MTS	microtubule star (protein)
mts^{DN}	mts dominant negative
n	number
NCBI	National Center for Biotechnology Information
ORF	open reading frame
P. apterus	*Pyrrhocoris apterus*
PCR	polymerase chain reaction
pdf	Pigment-dispersing factor

Pdp1ε	PAR-domain protein 1 epsilon (transcription factor)
per	period (gene, mRNA)
PER	period (protein)
PN	probability values
PPM	metal-dependent phosphatases
PPP	phosphoprotein phosphatases
PP1	Phosphoprotein Phosphatase 1
Pp2A-29B	Protein phosphatase 2A at 29B
PP2A	Protein Phosphatase 2A
PP2B	calcineurin
PP4	Phosphoprotein Phosphatase 4
PP5	Phosphoprotein Phosphatase 5
PP6	Phosphoprotein Phosphatase 6
PP7	Phosphoprotein Phosphatase 7
PRL	phosphatase of regenerating liver
PSTP	protein serine/threonine phosphatases
PTEN	phosphatase and tensin homologue
PTM	posttranslational modification
PTP	protein tyrosine phosphatases
RNAi	RNA interference
RPM	revolutions per minute
RT	room temperature
s	seconds
SCP	Small CTD phosphatase
Sgg	Shaggy (gene, mRNA)
SOC medium	Super Optimal broth with Catabolite repression
STRIPAK	striatin interacting phosphatase and kinase
tim	*timeless* (gene, mRNA)
TIM	timeless (protein)
Tm	melting temperature
TTFL	transcription-translation feedback loops
tws	twins (gene, mRNA)
UAS	Upstream Activating Sequence
V	Volt
vri	vrille (transcription factor)
VRI	vrille (protein)
vs.	Versus
wdb	widerborst (gene, mRNA)
WDB	widerborst (protein)
wnt pathway	wingless (Int-1) signalling
wrd	well-rounded (gene, mRNA)
X	X-times
µg	microgram
µl	microliter