Rhythmic behavior is controlled by the SRm160 splicing factor in

*Drosophila melanogaster*

Esteban J. Beckwith¹,⁴, Carlos E. Hernando¹, Sofía Polcowñuk², Agustina P. Bertolin³, Estefania Mancini¹, M. Fernanda Ceriani²* and Marcelo J. Yanovsky¹*.

¹ Laboratorio de Genómica Comparativa del Desarrollo Vegetal, Fundación Instituto Leloir, IIBBA-CONICET, Buenos Aires, Argentina.
² Laboratorio de Genética del Comportamiento, Fundación Instituto Leloir, IIBBA-CONICET, Buenos Aires, Argentina.
³ Laboratorio de Ciclo Celular y Estabilidad Genómica, Fundación Instituto Leloir, IIBBA-CONICET, Buenos Aires, Argentina.
⁴ Current Address: Department of Life Science, Imperial College London, London, UK.

*Corresponding authors

E-mails: fceriani@leloir.org.ar (MFC); myanovsky@leloir.org.ar (MJY)
Abstract

Circadian clocks organize the metabolism, physiology, and behavior of organisms throughout the day-night cycle by controlling daily rhythms in gene expression at the transcriptional and post-transcriptional levels. While many transcription factors underlying circadian oscillations are known, the splicing factors that modulate these rhythms remain largely unexplored. A genome-wide assessment of the alterations of gene expression in a null mutant of the alternative splicing regulator SR-related matrix protein of 160 kD (SRm160) revealed the extent to which alternative splicing impacts on behavior-related genes. We show that SRm160 affects gene expression in pacemaker neurons of the Drosophila brain to ensure proper oscillations of the molecular clock. A reduced level of SRm160 in adult pacemaker neurons impairs circadian rhythms in locomotor behavior, and this phenotype is caused, at least in part, by a marked reduction in period (per) levels. Moreover, rhythmic accumulation of the neuropeptide PIGMENT DISPERSING FACTOR (PDF) in the dorsal projections of these neurons is abolished after SRm160 depletion. The lack of rhythmicity in SRm160 downregulated flies is reversed by a fully spliced per construct, but not by an extra copy of the endogenous locus, showing that SRm160 positively regulates per levels in a splicing-dependent manner. Our findings highlight the significant effect of alternative splicing on the nervous system and particularly on brain function in an in vivo model.
Introduction

Networks of neurons that contain molecular clocks allow animals to withstand daily environmental and ecological changes. These circadian timing mechanisms are classically described as transcriptional-translational negative feedback loops that operate at the cellular level. However, the emerging picture is that multiple regulatory layers control the circadian oscillations in gene expression [1-3]. Examples from distantly related organisms show that, in addition to transcriptional and post-translational modifications, molecular mechanisms controlling the chromatin landscape [4,5], alternative splicing (AS) [6,7], RNA modifications [8], 3′-end processing and polyadenylation [9], mRNA nuclear export [10], and translation [11,12] are in place to support circadian rhythms in gene expression.

AS of immature pre-mRNAs has a profound role in the development and function of the nervous system across phyla, and the underlying mechanisms and key players have recently started to be uncovered [13]. This post-transcriptional mechanism is employed in clock regulation by distant species and finely tunes the circadian gene expression profile. For example, in Neurospora, AS and the use of two alternative promoters generates six isoforms of the core clock gene frequency, and the ratio of these isoforms is key to temperature compensation [14]. In Arabidopsis, several core clock genes undergo AS [15], and many of these genes also seem to be related to adjusting the clock in response to changes in temperature [16-18]. Interestingly, in mice, U2af26 alternative splicing is regulated by light and regulates PERIOD1 stability, affecting re-entrainment to new environmental conditions [19]. In humans, the central clock gene BMAL2 has four transcripts that encode proteins with various levels of transcriptional activity, although the exact role of this diversity is not fully understood [20]. Drosophila is no exception; to adjust behavior to different temperatures and seasons, per intron 8 is controlled by AS [6,21].

Splicing regulators enrich the potential and flexibility of the genome. Two families of RNA-binding proteins, the serine/arginine-rich (SR) and the heterogeneous nuclear
ribonucleoproteins (hnRNPs), are the most studied splicing regulators; however, their role in physiological contexts *in vivo* remains largely unexplored. Both families function as constitutive and AS modulators [22]. Importantly, SR and hnRNP proteins recognize and act upon exonic or intronic splicing enhancers (ESE or ISE) or silencers (ESS or ISS) [23,24], and tend to act coordinately with each other [25]. In addition to their known role in splicing regulation, SR proteins also participate in genome stability, chromatin binding, transcription elongation, mRNA stability, mRNA export, and mRNA translation [26], and thus are emerging as key regulators of gene expression.

In the fruit fly *Drosophila melanogaster*, the core molecular clock is comprised of two interconnected loops [27]. In the first loop, the transcription activators Clock (Clk) and Cycle (Cyc) heterodimerize and bind to the E-box DNA elements found on the evening genes, such as *period (per)* and *timeless (tim)*, and the levels of the proteins encoded by these genes peak in the late night. This first loop ends with the Per-Tim heterodimer repressing Clk-Cyc transcription activity at the *per* and *tim* promoters. In the second loop, Clk–Cyc dimers drive the expression of *vrille (vri)* and *Par domain protein 1e (Pdp1e)*, and the mRNA levels of these genes accumulate at the same rate during the early night phase. The protein product of *Pdp1e* is delayed by 3-6 hours [28]; thus, Vri accumulates faster and inhibits Clk expression through the V/P box DNA element. In the late night, Pdp1e translation ensues and Pdp1e competes with Vri for V/P sites, promoting Clk expression and starting a new cycle. This molecular clock running in the pacemaker neurons ensures rhythmicity and sets the endogenous period. The latter is controlled by the accumulation and nuclear translocation of Per and Tim [29-32], and the abundance of Clk [33], regulated at the expression level by Pdp1e [28], Vri [34] and also by Mothers against DPP (Mad), the transcription factor within the BONE MORPHOGENETIC PROTEIN (BMP) pathway [35]. Through a meta-analysis of available transcriptomic data [36,37] we uncovered that genes co-expressed with the Clk regulator MAD are enriched in those related to RNA metabolism.
Then, we identified SRm160, a MAD co-expressed gene, as a necessary component of the *Drosophila* timekeeping system. SRm160 is the fly ortholog of mammalian SRRM1, originally named B1C8 [38], which was previously characterized as a co-activator of constitutive and exon enhancer-dependent splicing in mammals [39], worms [40,41], and flies [42,43]. However, the functions attributed to the SR and SR-related proteins are diverse. SRm160 has been described as part of the exon junction complex [44,45] and is also involved in 3'-end processing and mRNA nuclear export [46]. More importantly, it is physiologically relevant for processes such as tumor cell invasion [47] and chromatin regulation [48]. Thus, SRm160 is emerging as a coupling factor that links different steps in the control of gene expression.

In this work we characterized the effect of SRm160 knockout through a genome-wide assessment of the fly larval transcriptome. We found that behavior-related genes are specifically enriched among SRm160 splicing targets, suggesting that AS, and specifically SRm160, have broad roles in brain function. Then, we analyzed the impact of SRm160 on circadian control of locomotor behavior. We found that SRm160 contributes to the proper functioning of the core molecular clock in pacemaker neurons, controlling per function in a splicing-dependent manner. Our findings provide new evidence of the relevance of alternative splicing on the operations of the adult brain taking full advantage of an *in vivo* model.
**Materials and methods**

**Fly stocks**

Flies were reared under light cycles (12 h light:12 h darkness; referred to as LD 12:12) on *Drosophila* standard medium at 25°C. For expression in the circadian-relevant neuronal clusters, the drivers *pdf*Gal4 [56], *tim*Gal4 [89] and *pdf*GeneSwitch [59] were employed. The strains *SRm160*100751 (RNAia, Vienna Drosophila Resource Center, VDRC) and *SRm160*26578 (RNAib, Transgenic RNAi Project, TRiP) alone or in combination were used to downregulate *SRm160* expression. To maximize RNAi-mediated silencing, we overexpressed *dicer2* (VDRC transformant ID 25090) in all experiments. The *SRm160*18603 allele (stock 26938) and the fluorescent reporters RFPmyr, CD8GFP and GFPnls were obtained from the Bloomington Stock Center. The *SRm160*18603 strain was backcrossed to *w*1118 twice to eliminate unspecific mutations and several independent lines were established, all showing arrested development at the second larval stage. To manipulate core clock gene expression, *dClk* [33] and *dper* [90] were obtained from M. Rosbash (Brandeis University). These two fly strains allowed the addition of the entire genomic loci of each gene. UAS-*per*3.2 (referred as *per*, [64]) were obtained from A. Sehgal (University of Pennsylvania) and UAS-*Clk* (referred as *Clk* [91]) from R. Allada (Northwestern University). By means of these UAS construct we were able to express fully spliced version of the core clock components. To evaluate the effect of a genetically disrupted circadian clock on *SRm160* expression the *per*01 [92] null mutant and the *ClkJk* [93] dominant negative mutant were employed. *per*01 was also used as the genetic background to assess the levels of Per protein achieved through expression of Per rescue lines. All heterozygote controls were generated by crossing the corresponding strain to the *w*1118 stock.
**High-Throughput sequencing**

The 6 libraries from control (w1118) and SRm16018603 36h AEL larvae were prepared following the TruSeq RNA Sample Preparation Guide (Illumina). To validate libraries, size and purity were assessed with the Agilent 2100 Bioanalyzer and the Agilent DNA1000 kit (Agilent Technologies). Samples were double-end sequenced with an Illumina HiSeq 1500 at INDEAR, Argentina. The analysis of the data sets was conducted as previously described [55]. To score changes in gene expression we employed a FDR threshold of 0.01 and a LogFC threshold of 1 and -1. In the case of the AS events the thresholds were 0.1 for FDR and 0.6 or -0.6 for LogFC. The criterion employed in each case is associated to the amount of reads available for the corresponding analysis (i.e. measurement of gene expression levels is based on reads of the entire gene while the analysis of alternative events is based on smaller regions, and thus, a smaller number of reads).

The FlyBase converter tool was employed to assign FB gene numbers to hit lists. GO term enrichment within the hit lists was determined using DADIV [94,95]. To eliminate redundant GO terms the REVIGO algorithm was employed [96].

**Analysis of alternative splicing events**

The TRIzol reagent (Life Technologies) was used for RNA isolation. cDNA was generated by standard procedures employing 1 µg of total RNA, RQ1-DNasa (Promega) and M-MLV retrotranscriptase (Invitrogen). The relative position of the primers in each locus is depicted in the Figure S3. Sequences are:

- CG14642_F:TATGTGGAGCGCATCTTTCC,
- CG14642_R:GCTATCGTAGTGGGCAGCTC,
- CG6206_F:GATCAGCGAATTTGGGAGAG,
- CG6206_A_R:TCTTGGCGAAATCCAAAAC,
Locomotor activity

For circadian locomotor activity recordings, flies were placed in Drosophila Activity Monitors (DAM, Trikinetics) and entrained to 12:12 LD cycles for 3 complete days before transferring to constant darkness (DD). Data were collected every 5 minutes for 9 entire days and were analyzed by ClockLab. Period length was determined using the Chi square algorithm with $\alpha=0.05$, rhythmic power was calculated as the height of the peak in the periodogram minus the corresponding significant level [97] and the percentage of rhythmicity was calculated as previously described [35].

To analyze behavior under entrainment, each 5-minute activity bin was normalized to the total activity of the corresponding animal per day. The mean value for each time point was obtained, averaged data from 3 consecutive days for each fly, and the mean for all the flies of a given genotype was calculated. Data showed is the average of 3-5 independent experiments together with the standard error of the mean. The anticipatory indexes of morning and evening activity were calculated as previously described [98].

To induce an adult specific knockdown of SRm160, the respective genotypes were reared under regular conditions and food. Three to four day old animals were loaded in the behavioral tubes containing food supplemented with RU486 (mifepristone, Sigma, USA).
those experiments, food was mixed with RU486 in 80% ethanol to a final concentration of 200 μg/ml (+RU) or with the same amount of ethanol (vehicle) in control treatments.

**SRm160 reporter strain**

A 3650bp fragment of the *SRm160* promoter was amplified by PCR using Phusion DNA Polymerase (NEB) from *w^1118* genomic DNA. The sequence of the primers employed were:

`fw`: GTGCAGCGATTATTCTCAACAG and `rev`: GTCCTGCTGCTGATTGGTGCC. The product was cloned in the pCasperDest6 vector. Random transgenesis was performed by BestGene Inc. using the *w^1118* strain. Seven independent transgenic lines were obtained displaying similar results (data not shown). Since expression levels were low, two strains were combined to increase GAL4 expression.

**Immunostaining**

Brains were dissected in PT (PBS supplemented with 0.1% Triton X-100) and fixed in 4% paraformaldehyde in PB (100 mM KH$_2$PO$_4$/Na$_2$HPO$_4$). After fixation brains were rinsed three times in PT and then blocked in 7% goat serum in PT for 1 h at room temperature (RT). Tissue was incubated with primary antibodies ON at 8°C. The primary antibodies employed were rat anti-PDF (1/500) [59], rabbit anti-RFP (1/1000, Rockland, USA), rabbit anti-Per (1/250 Alpha Diagnostics Inc.). The secondary antibodies used were Cy2-conjugated, Cy3-conjugated and Cy5-conjugated (Jacksons Immunoresearch) diluted to a final concentration of 1/250 and incubated for 2h at RT. After staining, brains were washed three times for 15 min and mounted in 80% glycerol (in PT).

For quantitation of Per levels, single plane images describing 2-4 sLNvs per brain were obtained and only one hemisphere was measured. To quantitate PDF levels a gallery of sequential images was acquired and a maximum intensity projection was performed prior to measuring PDF immunoreactivity. To define the area of interest the membrane bound RFP
signal was used to create an ImageJ ROI, and PDF signal inside this area was quantified. In all cases 9-10 brains were averaged in each experiment and the reporter values are the mean of three independent experiments. Identical settings were employed to acquire images from all the brains in each experiment and normalization to the mean intensity of each experiment was performed to allow further comparisons.

A Zeiss LSM510 microscope (Carl Zeiss, Thornwood, NJ) was employed for Per and PDF measurements and to evaluate the integrity of the PDF positive neurons in the *SRm160* downregulated brains. *SRm160* expression pattern was assessed using a Zeiss LSM 710 NLO microscope. All confocal images were analyzed with the ImageJ software (NIH).

### Statistical analysis

Statistical analyses employed were conducted with the InfoStat version 2009 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

### Larval collection

Eggs were collected during a 2h window in an agar plate supplemented with sugar; prior to and during the egg collection adults were stimulated with fresh yeast paste. Around 150 larvae of each genotype were collected 24h after egg laying (AEL) and transferred to a plate with standard food. For RNAseq experiments 3 groups of 100 larvae from each genotype were collected 12h after transferring to standard food. Larvae of each group were collected, rinsed in PBS to wash the excess of food, and transferred to TRIzol. For larval growth curves (Figure S2), in each time point 10 larvae were removed from the food, transferred to an agar plate, photographed and discarded. Larval area was measured with the ImageJ software.
The TRIzol reagent (Life Technologies) was employed for RNA isolation. cDNA was generated by standard procedures employing 1 µg of total RNA, RQ1-DNasa (Promega) and M-MLV retrotranscriptase (Invitrogen). qPCR was conducted with the Fast SYBR® Green Master Mix (ROCHE) in a Mx3005P (Stratagene) device. Relative mRNA abundances were estimated employing internal standard curves for each gene in each experiment. The $SRm160$ primers are SRmPF1: CGACGACAGAACGCATTAGA, SRmPR1: AAATATGTAACCCGGCACCA; SRmPF3: GGCAGGTGGACGGCAACAG and SRmPR3: GCGGGACAGACTGGCATAAGG. The relative localization of these primers in the $SRm160$ locus is indicated in Figure S1.

To validate observation in the RNAseq dataset (Figure S3) the employed primers were:

- Cyp9b2_2_F: TGATGTGCAACAAGCTCTCC,
- Cyp9b2_2_R: ACGTCGAGATGTTAAAGCCAG,
- CG14691_2_F: ATCACGGTAGCTGGAATTGG,
- CG14691_2_R: CATCAGTGAGCAAAGCCAGA,
- CG10924_3_F: CAACTGCATTAGCTGCCAAG,
- CG10924_3_R: TGATGGTTCCTCTTCTTCAGC,
- Cyp6a17_2_F: GCTGGGTTTGAGACAAGCTC,
- Cyp6a17_2_R: CGATTTCCTCGTGTAAGCAG,
- lip3_2_F: GCCCAGCAATAAGTTCAAGC,
- lip3_2_R: AAGTTCTGGTTCACCGATGC,
- mur89F_1_F: TCTACCAGTGCAAGCGAAAGTG,
- mur89F_1_R: TGGCTACGTCAAGTTCAAGC,
- 578-mas_3_R: TATGCACTCCGTATCGCTCA,
- CG3397_2_F: GAAAGCTGCTGCGGATTAAC,
- CG3397_2_R: CAAGTGGTCCGCTTGAAAG,
- CG10081_2_F: TCGGTGCTAAGCTCCAGTGG,
- CG10081_2_R: CCACTCCGCTAAGCTCCAGTGG,
minature_2_F: TGCCGATCTCGATGTTATCC,
minature_2_R: CCAAATTTCATCGGACAGGTT.

Data Availability Statements
We declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. The RNAseq data files and the supplementary files S1, S2 and S3 are available from GEO database (Accession number GSE102361).
Results

SRm160 is a splicing regulator co-expressed with MAD

In a previous attempt to identify novel components of the molecular clock we described that the BMP pathway, and in particular MAD nuclear translocation, impacts on the pace of the molecular clock regulating Clk transcription [35]. Its unexpected link to the molecular clockwork prompted us to explore the role of associated genes, with the expectation of identifying novel clock components. Here, we took advantage of genome-wide transcriptomic data already available [36,37] to analyze genes that share expression pattern with MAD (Table S1), which is usually taken as an indication of involvement in similar biological processes [49]. Surprisingly, a GO term analysis of the genes co-regulated with MAD (Table S1) showed a clear enrichment in genes related to RNA metabolism (Table S2). Comparison of this list with spliceosome components [50] retrieved a list of 22 elements (Table S3). Because the SR proteins are key regulators of splicing, we then focused our attention on the SR-like protein co-expressed with MAD, SRm160.

Lack of SRm160 affects the expression and splicing of a restricted subset of genes

The initial characterization of SRm160 as a splicing regulator was based on an in vitro approach and focused on the splicing of a particular gene, doublesex [42]. In addition, SRm160 controls the AS of the transmembrane glycoprotein CD44 in mammalian cells [47]. However, information regarding the breadth of SRm160 activity on the fly transcriptome in vivo, as well as at which level this gene exerts its effect, is clearly missing. For this high-throughput approach we employed the previously characterized SRm160^{18603} null mutant allele [51] (Fig S1). In this way, we were able to comprehensively assess the global effect of SRm160 loss of function in an animal that reaches post-embryonic development. The insertion of the P-element in the SRm160^{18603} allele leads to arrested development 48 h after egg laying (AEL) and eventually results in lethality (Figure S2).
We used RNA-sequencing (RNA-seq) to analyze the transcriptome of wild-type and $SRm160^{18603}$ flies at 36 h AEL, focusing on gene expression and AS [52,53] (Fig 1 and Files S1 and S2). Interestingly, regarding global expression levels, the absence of $SRm160$ either directly or indirectly impacted about 10% of the expressed genes, while alterations in the inclusion of constitutive exons or introns (constitutive splicing) was smaller, affecting 3% and 5.5% of the expressed exons or introns, respectively (Fig 1A). Not surprisingly, $SRm160$ depletion had a larger effect on AS, influencing about 7% of AS events measured (Fig 1A).

Loss of $SRm160$ impacted all types of alternative splicing events alike, ruling out the possibility that SRm160 regulatory function is restricted to a specific type of splicing event (Fig 1B and 1C). To validate the data obtained by this high-throughput technique, we independently evaluated several of the identified miss-regulated genes and splicing events with an alternative technique. We evaluated 5 up-regulated and 5 down-regulated genes by RT-PCR confirming the results from the transcriptomic analysis (Figure S3A). In addition, we validated 5 alternative splicing events spanning different type of events by PCR (Figure S3B). These analyses confirmed and validated our initial observations.

Although several genes in $SRm160^{18603}$ showed altered expression levels or deficits in constitutive and alternative splicing, this fly strain survives through embryogenesis and lives for several days as larvae (Figure S2). This means that, despite SRm160 activity being necessary to complete development, the machinery for constitutive splicing is not significantly affected in this null mutant. These results are similar to the ones reported for mutants affecting other splicing factors, such as U1C in zebrafish [54] and LSm4 in Arabidopsis thaliana [55].

Interestingly, clock genes were not highlighted as $SRm160$ targets in the 36h AEL larval sample, since the expression of most of these genes, including $per$, was not detectable at this developmental stage (Files S1 and S2).
To further characterize the affected genes, we assigned GO terms to the list of genes that were differentially expressed or spliced in SRm160 mutant, and analyzed the enrichment of each term within each list (File S3). We performed a dendrogram analysis on the top 10
enriched terms from all of the categories (Figure 1D). As a result, a single cluster spanning
the six categories associated to splicing was uncovered (marked in red in Figure 1D). This
cluster comprised GO terms related to brain function and behavior (GO:0007610: behavior,
GO:0007611: learning or memory, and GO:0042048: olfactory behavior; Fig 1D and File
S3). Interestingly, this cluster did not include genes with altered expression levels; on the
contrary, altered expression was mostly observed within genes associated to metabolism,
probably underscoring a pleiotropic and potentially indirect effect derived from the loss of
SRm160 function. None of these terms were related to nervous system function (Fig 1D),
arguing for a prevalence of SRm160 on AS regulation in the brain.

Thus, SRm160 has an important role in regulating a subset of pre-mRNA splicing events,
which probably shapes the use of alternative variants of genes associated with brain function
and behavior.

SRm160 sustains overt rhythms in pacemaker neurons

Circadian regulation of locomotor activity is one of the best characterized behaviors at the
molecular and circuital level. To study the impact of SRm160 on behavior we knockdown
SRm160 expression specifically in pacemaker neurons of the fly brain. We directed the
knockdown to the subset of clock neurons known as the Lateral Neurons Ventral (LNvs) by
means of the promoter of the neuropeptide PIGMENT DISPERSING FACTOR (PDF), which
is expressed exclusively in this neuronal cluster [56]. In this way, we avoided the deleterious
effects of broader genetic manipulations and focused our search on a key cluster involved
Fig 2. SRm160 supports a functional clock.
(A) Representative locomotor activity profiles of the indicated genotypes showing 3 days in LD 12:12 and 10 days in constant darkness. Grey shading indicates darkness. White bars indicate light, dark bars indicate dark, and grey bars indicate subjective day. (B) Percentage of rhythmic flies for each genotype. Statistical analysis included one-way ANOVA (p<0.0001, F(9,27)=47.26). (C) Quantitation of rhythmic power for the indicated genotypes, calculated as the amplitude of the peak over significance in a periodogram analysis. Statistical analysis included one-way ANOVA (p<0.0001, F(9,27)=29.23). Error bars represent SEM and averages of at least three independent experiments; different letters indicate significant differences according to Tukey’s comparisons, α=0.05.

in behavioral control. Thus, we employed a paradigm that allowed us to evaluate the role of a spliceosome component in the function of the nervous system, while restricting the manipulation to a small group of cells in an otherwise intact animal. SRm160 knockdown in PDF expressing (PDF+) neurons significantly impacted the locomotor activity profile when flies were deprived of environmental cues (Fig 2A). There was a clear reduction in the...
percentage of rhythmic flies in the population (Fig 2B) and in the rhythm strength (Fig 2C; see Table 1 for details, sample size, and replicates). A similar phenotype was observed when the knockdown was directed to the entire circadian network through the *tim* promoter [57] (Fig 2A, 2B and 2C). To overcome potential unspecific effects of the knockdown strategy, we employed a second RNAi from a different library that showed similar results (Fig 2). Interestingly, the combination of both RNAis led to more severe phenotypes, suggesting that *SRm160* has a specific role in sustaining rhythmic locomotor behavior (Fig 2).

**Table 1.** *SRm160* is necessary for a coherent locomotor activity pattern, but has no effect on the endogenous circadian period.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>N</th>
<th>Period</th>
<th>% Rhythmicity</th>
<th>Rhythmic power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mean</td>
<td>SE</td>
<td>S</td>
</tr>
<tr>
<td><em>pdf&gt;dicer2,GFP&lt;sub&gt;nls&lt;/sub&gt;:RFP&lt;sub&gt;myr&lt;/sub&gt;</em></td>
<td>129</td>
<td>5</td>
<td>24.0</td>
<td>0.1</td>
<td>a</td>
</tr>
<tr>
<td><em>tim&gt;dicer2,GFP&lt;sub&gt;nls&lt;/sub&gt;:RFP&lt;sub&gt;myr&lt;/sub&gt;</em></td>
<td>85</td>
<td>3</td>
<td>23.8</td>
<td>0.0</td>
<td>a</td>
</tr>
<tr>
<td><em>SRm160&lt;sup&gt;RNAia&lt;/sup&gt;/+</em></td>
<td>90</td>
<td>3</td>
<td>23.9</td>
<td>0.2</td>
<td>a</td>
</tr>
<tr>
<td><em>SRm160&lt;sup&gt;RNAib&lt;/sup&gt;/+</em></td>
<td>111</td>
<td>4</td>
<td>23.6</td>
<td>0.1</td>
<td>a</td>
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<td><em>SRm160&lt;sup&gt;RNAia&lt;/sup&gt;;SRm160&lt;sup&gt;RNAib&lt;/sup&gt;/+</em></td>
<td>111</td>
<td>4</td>
<td>23.6</td>
<td>0.1</td>
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<td><em>pdf&gt;dicer2;SRm160&lt;sup&gt;RNAia&lt;/sup&gt;</em></td>
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<td>83</td>
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<td>a</td>
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<td><em>tim&gt;dicer2,SRm160&lt;sup&gt;RNAia&lt;/sup&gt;;SRm160&lt;sup&gt;RNAib&lt;/sup&gt;</em></td>
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<td>3</td>
<td>23.8</td>
<td>0.1</td>
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The analyzed data correspond to the results shown in Figure 2. N: Total number of analyzed animals. n: Number of analyzed experiments. SE: Standard error of the mean. S: Statistical analysis; different letters indicate significant differences according to Tukey’s comparisons, α = 0.05.

Behavioral analysis showed that under driven conditions (12h light and 12h dark cycles), the impact of SRm160 reduction was somewhat diminished, although a slight reduction in the anticipatory behavior was observed when *SRm160<sup>RNAia</sup>* was expressed in PDF+ neurons (Figure S5). Importantly, knockdown of *SRm160* by means of the GAL4/UAS system did not
impose structural defects to the LNv projections (Figure S4), challenging the idea that the
circadian alterations were the consequence of potential developmental defects resulting
from these manipulations. To further rule out this possibility we examined the consequences
of SRm160 knockdown in PDF neurons exclusively during adult stages. Employing the
inducible GeneSwitch system under the control of the pdf promoter we observed a clear
reduction in the percentage of rhythmic flies in the induced group (pdfGS>SRm160RNAia;
dicer2 +RU) compare to the respective controls (Fig 3A and 3B; see Table 2 for details,
sample size, and replicates). Altogether, these results show that SRm160 is necessary in
adult pacemaker neurons to sustain a normal organization of rhythmic locomotor behavior.

Fig 3. SRm160 expression in the adult sLNvs is necessary for a wild type circadian
behavior.
(A) Representative locomotor activity profiles of the indicated genotypes showing 3 days in LD
12:12 and 10 days in constant darkness. Grey shading indicates darkness. White bars indicate
light, dark bars indicate dark, and grey bars indicate subjective day. (B) Percentage of rhythmic
flies for each genotype. Error bars represent SEM and averages of at least three independent
experiments; different letters indicate significant differences according to Tukey’s comparisons,
α=0.05.
Table 2. SRm160 has an adult specific function in circadian timekeeping system.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>n</th>
<th>Period mean</th>
<th>Period SE</th>
<th>% Rhythmicity mean</th>
<th>% Rhythmicity SE</th>
<th>% Rhythmicity S</th>
<th>Rhythmic power mean</th>
<th>Rhythmic power SE</th>
<th>Rhythmic power S</th>
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The analyzed data correspond to the results shown in Figure 3. N: Total number of analyzed animals. n: Number of analyzed experiments. SE: Standard error of the mean. S: Statistical analysis; different letters indicate significant differences according to Tukey’s comparisons, α = 0.05.

To further characterize the role of SRm160, we monitored the expression pattern of SRm160 in the adult brain. For this purpose we generated a reporter strain in which Gal4 is driven by a 3.6-kb promoter fragment of SRm160 (SRm160-Gal4). When this reporter line was combined with a UAS-GFP reporter it revealed a wide and dim expression pattern that included most brain regions. Interestingly there were a few intense areas between the central brain and the optic lobe (Figure 4A), the region where the PDF positive somas are found. We then crossed this strain to a UAS-RFP<sub>myr</sub> reporter line to visualize SRm160 expression pattern in combination with the PDF profile. As shown in Figure 4B, immunoreactivity was broad in the accessory medulla and, importantly, there was clear expression of the reporter line in the PDF+ small LNvs (sLNvs), supporting a role for this gene in these pacemaker neurons.
Taken together, these results show that SRm160 is expressed in circadian-relevant neurons and fulfills a critical role in the ability of the main pacemaker to control overt rhythms in adult flies.

SRm160 sustains PDF oscillations

The main circadian output of the LNvs is the rhythmic accumulation of PDF in their dorsal projections [58], where PDF levels are high during the start of the subjective day (CT02) and low at the beginning of the subjective night (CT14), even after several days in constant conditions [59]. We examined PDF levels in this area during the second day in constant darkness in control flies and in those with SRm160-depleted PDF+ neurons (Fig 5A). Consistent with the behavioral data, we found no PDF oscillations in the dorsal projections of the sLNvs (Fig 5B). Interestingly, the standard deviation in PDF levels was significantly increased when SRm160 levels were decreased, a result that supports the behavioral phenotype displayed by the SRm160 knockdown flies [60-62].
Thus, reduced levels of this posttranscriptional regulator halt the oscillation of the principal output of the pacemaker neurons in *Drosophila*.

**Fig 5. SRm160 sustains Per oscillations in the central pacemaker**

(A) Control (left) or *SRm160*-interfered (right) brains were dissected during the second day of constant darkness at CT02 and CT14. Brains were stained with anti-RFP (red) and anti-PDF (black) antibodies and images from the dorsal projections of small LNvs were acquired with the same settings. The image depicts representative confocal images. (B) Quantitation of PDF intensity at the sLNv dorsal projections for the indicated genotypes and time points. The different genotypes show different variances (Levene's test, p=0.0162, F(3,8)=6.38), precluding parametric comparisons. PDF levels oscillate in control flies (*p*=0.0011, Student’s *t*-test T=8.49), but the oscillation is lost in the RNAi-treated flies (p=0.9363 Student’s *t*-test T=0.09). (C) Whole-mount brain immunofluorescence was performed to monitor PDF (black) and Per (red) accumulation on the third day of exposure to constant darkness. Representative single-plane confocal images of small LNvs at the indicated time points and genotypes are shown. Images were taken using the same confocal settings throughout the time course. (D) Quantitation of Per nuclear intensity. Between 9 and 10 brains were analyzed per time point; the average of 2–4 neurons was used for each determination. Three independent experiments were analyzed by two-way ANOVA (genotype p=0.1239, F(1,16)=2.64, CT p>0.0001, F(3,16)=35.00, interaction p=0.0009 F(2,16)=9.29). A simple effect comparison was used to analyze differences between genotypes at different circadian times (CTs). CT05 *p*=0.0029 F(1,16)=12.33, CT11 p=0.0672 F(1,16)=3.85, CT17 p=0.1233 F(1,16)=2.65, CT23 *p*=0.0047 F(1,16)=10.73.

**SRm160 modulates Per levels**

Since the organization of overt rhythms and the oscillation of PDF immunoreactivity (the main outputs of pacemaker neurons) were impaired by *SRm160* knockdown, we wondered
whether the molecular clock within the pacemaker neurons was running normally in the
SRm160-depleted animals. Per oscillations and changes in Per subcellular distribution are
hallmarks of the molecular clock and are necessary for the temporal organization of
locomotor behavior [63]. Therefore, we assessed Per levels and subcellular localization by
immunostaining during the third day in constant darkness in the sLNvs. In control brains,
Per peaked at CT05, was least abundant at CT11 and CT17, and was again detected in the
nucleus by CT23 (Fig 5C). By contrast, SRm160-interfered neurons showed reduced Per
levels and slower degradation, resulting in a dampened oscillation (Fig 5C and 5D).
This result suggests that SRm160 is necessary for the correct function of the molecular clock
in the main pacemaker of Drosophila. Interestingly, SRm160 does not appear to be a clock-
controlled gene, since its mRNA levels were not affected in a per null mutant (per^01) or a
dClk dominant negative mutant (dClk^{Jrk}, Figure S6). However, the possibility that SRm160 is
differentially regulated in pacemaker neurons cannot be ruled out.

Bypass per splicing can rescue the lack of SRm160

Knockdown of SRm160 impacted Per levels ensuing miss-regulation of clock outputs, PDF
oscillations and rhythmic locomotor behavior. This could stem from a general effect of the
SR protein on basic cellular functions or it may point to a more specific targeting of clock
components. To distinguish between these possibilities, we attempted behavioral rescues
of the SRm160 downregulation phenotype. To this end, we used two strategies, (1)
overexpression of a fully spliced version of per or Clk, and (2) addition of an extra copy of
the per or Clk genomic locus (dper and dClk, respectively). Both of these strategies rescued
phenotypic defects associated with a null mutation in their corresponding locus [33,64-
66] and yield similar levels of Per protein in the sLNvs (Fig S7). We anticipated that, if the
arrhythmic phenotype caused by the SRm160 knockdown was the result of a deficit in
general cellular function, the sole addition of a clock component (such as Per or Clk) would
be insufficient to improve rhythmicity. In contrast, we found that the addition of extra Per rescued the behavioral phenotype (Fig 6A and 6B; see Table 3 for details, sample size, and replicates), pointing to a more specific deficit in the clock rather than a general disfunction of PDF+ neurons. Interestingly, only the fully spliced version of per significantly improved rhythmicity and the strength of the behavioral oscillations (Fig 6A and 6B, notice the difference between the blue and red columns), probably because overexpression of the fully spliced per bypassed the need for SRm160 function. Importantly, Clk, the main transcription factor responsible for per expression, was unable to rescue the behavioral phenotype (Fig
6B). Neither the extra Clk locus nor the fully spliced version of the gene product was able to improve the rhythmicity in the SRm160 knockdown. This finding reinforces the idea that transcriptional activation of per is not sufficient, and restoring the wild-type phenotype is only achieved by circumventing per splicing.

In summary, SRm160 is recruited by the molecular clock in pacemaker neurons of Drosophila and acts, at least in part, by modulating per at the posttranscriptional level, an effect that ultimately impacts on PDF oscillations and overt behavior.

Table 3. A fully spliced version of per rescues SRm160 knockdown.

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The analyzed data correspond to the results shown in Figure 6. N: Total number of analyzed animals. n: Number of analyzed experiments. SE: Standard error of the mean. S: Statistical analysis; different letters indicate significant differences according to Tukey’s comparisons, α = 0.05.
Discussion

Regulation of transcript levels and protein phosphorylation are key processes employed by biochemical clocks to ensure precise circadian oscillations. In particular, these processes impact the timing of Per accumulation and its translocation from the cytoplasm to the nucleus, which are central aspects of the timekeeping mechanism in animals. Here, coupling high-throughput transcriptomics with genetics and behavioral approaches we identified a splicing regulator that affects the circadian clock in pacemaker neurons of Drosophila.

SRm160 functions

A unified nomenclature system was proposed for SR protein splicing factors. According to this system, SR proteins contain a modular structure consisting of one or two N-terminal RNA-Binding Domains (RBDs) and a downstream RS domain consisting of at least 50 amino acids with >40% RS content, characterized by consecutive RS or SR repeats [67]. As SRm160 lacks a classical RBD motif [39], it is not a canonical member of the family and is defined as an SR-related protein; however, it functionally fulfils the definition of a SR protein because it binds nucleic acids directly through a “PWI” motif [68].

SRm160 proteins have several described biochemical functions that affect multiple steps that control gene expression. However, little is known about their role as regulators of specific biological processes. During early embryonic development in the fly, SRm160 is distributed ubiquitously and at high levels, showing early zygotic gene transcription by 2-3 h after fertilization [51]. After 10 h of development, SRm160 mRNAs are enriched in the central nervous system and completely restricted to it by 16 h after fertilization [51]. Then, SRm160 mRNA levels decrease, reaching a minimum at 20 - 24 h.

We found that pleiotropic alterations of SRm160 levels lead to arrested development and lethality during the larval stage. This correlates with previous data showing that SRm160 levels are recovered during larval stages [51], probably reflecting a second wave of
expression that is essential for development. In addition, during adult development, SRm160 enhances female-to-male somatic sex transformations and also regulates apoptosis in the adult eye [51]. Thus, our results showing that SRm160 is an adult-specific regulator of the circadian clock represent, to our knowledge, the first well-defined example of a specific biological process controlled by SRm160 beyond embryonic development and metamorphosis.

**Fine regulation of Per levels**

An emerging picture in the *Drosophila* molecular clock is that *per* constitutes a multistep regulatory node. At the transcriptional level, Clk-Cyc heterodimers are the main activators of *per* transcription and the Per-Tim heterodimer together with Clockwork Orange (CWO) are the main repressors [69,70]. At the posttranscriptional level, AS of *per* mRNA has previously been documented; the *per* 3’ terminal intron, dmpi8, is either spliced out or retained [21]. The abundance of the resulting two different mRNAs is regulated by the circadian clock, but also by temperature and photoperiod. *per* translation is also tightly controlled to ensure proper functioning of the molecular clock. Twenty-Four (TYF) physically interacts with Ataxin-2 (Atx2) [71,72] in a protein complex formed by Atx2, LSM12 and ME31B [73]. This complex binds directly to *per* mRNA in the LNvs, and acts with PolyA Binding Protein (PABP) and the translation initiation factor eIF4G [74] to activate *per* translation. In addition, the atypical translation factor NAT1 ensures *per* translation in a cap-independent mechanism [75]. Her we show that a reduction in the cyclic turnover of *per* transcript/protein in the sLNvs and a marked loss of behavioral rhythmicity are common hallmarks of the knockdown of SRm160 (Figs 2, 3 and 5) and other *per* translational regulators mentioned [71-73,75]. Hence, the findings presented herein contribute to the idea that neuron-specific post-transcriptional control systems impacting Per levels are particularly important for behavior. Interestingly, in mammals, PERIOD1 is also tightly regulated [19].
Finally, posttranslational modification also impacts Per regulation in both flies and mammals. In particular, the role of phosphorylation in Per regulation is well established, with several kinases and phosphatases acting on this protein at distinct and mutually regulated sites [32,76-79].

**Splicing and the brain**

AS is especially prevalent in neuronal tissue, and many AS events are specific to neural cell types [13]. In the last years it has become clear that neuronal development is highly influenced by alternative splicing, both in mammals [80] and flies [81,82], even at the single cell level [82,83]. More importantly, a growing body of evidence shows that behavioral traits are fine-tuned by alternative splicing in many species [84-86]. Importantly, the use of RNA-seq and other high-throughput technologies has identified widespread clock control of AS in the *Drosophila* brain [87], but the mechanisms underlying this regulation are unknown. Our meta-analysis of transcriptomic data combined with a genetic approach helped us identifying a splicing regulator that affects clock function, in an attempt to fill this gap in our knowledge. In addition to the reported effect of the *per* null mutation on AS [87], our results suggest that SRm160 regulates *per* levels, at least in part, through a splicing-regulated process (Figure 6 and Figure S7). However, considering the diversity of regulatory roles attributed to SRm160 in posttranscriptional regulation, other steps in RNA metabolism could also be involved in the modulation of *per* expression by SRm160. Unfortunately, a direct assessment of *per* splicing in pacemaker neurons of SRm160 knockdown flies is not possible, because the effect on *per* or other clock genes would be overshadowed by the contribution of other clock and non-clock neurons. In addition, clock genes are poorly expressed in the larval stages reached by the null SRm160 mutant, preventing the analysis of this gene at this developmental stage. However, the finding that the circadian phenotype caused by the knockdown of SRm160 specifically in
PDF+ neurons can be rescued by a fully spliced per version, but not by the genomic per locus (Fig 6), supports the notion that SRm160 directly or indirectly impacts per splicing, which in turn affects the oscillation of protein levels and clock function. Despite the limitations of the techniques employed to assess the SRm160 expression pattern in the adult brain, it appears that it extends beyond circadian relevant neurons (Fig 4). This suggests that this protein has a wide variety of functions and targets, Thus, SRm160 could fulfill a housekeeping or constitutive role in the sLNvs as well. In agreement with this observation, nearly 10% of the expressed genes exhibit altered expression in the RNA-seq dataset. Interestingly, SRm160 impacts a large number of splicing events, particularly among genes related to CNS function and behavior (Fig 1). This data set is in agreement with previous work showing that post-transcriptional control in the fly brain is particularly relevant for behavior-associated genes [84,88]. Our results reported here support the growing body of evidence that brain functions, and particularly behavioral patterns, are exquisite physiological outputs that require the maximum expansion of the coding capacities of the metazoan genome.
Acknowledgements

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References


Supplementary figures

S1 Fig. Characterization of **SRm160** mutant alleles.

(A) Schematic representation of the **SRm160** locus depicting the insertion site of the transposable element carried by the 18603 strain and the position of primers used to measure **SRm160** expression. Boxes and lines represent exons and introns, respectively, and orange shading indicates DNA sequences, red shading indicate coding sequences, pink shading indicates non-coding sequences, and green shading indicates amino acidic sequences. (B) RT-PCR analysis of **SRm160** expression in control (**w^118^**) and mutant larvae (**SRm160^18603^**) employing primers F1 and R1. **Tubulin** expression was used as a loading control. Note that there was no detectable expression of the 5’ primer region of the **SRm160** transcript in the mutant. (C) qPCR analysis of **SRm160** expression in control and mutant larvae employing primers that span the 3’-most intron. Note that by analyzing this region we were able to detect transcripts (most certainly abnormal), showing that transcription resumes downstream of the insertion. Thus, the wild-type version of **SRm160** transcript is completely absent in the mutant, although an aberrant form is still detectable. These data are consistent with the results obtained in the RNA-seq experiment.
S2 Fig. SRm160 is an essential gene in Drosophila

(A) Quantitation of larval area (as a proxy for body size) at different developmental stages. Ten larvae were measured in each experiment; the values represent the average of three independent experiments. Statistical analysis includes two-way ANOVA (genotype p>0.0001, F(1,32)=1520.66, hours after egg laying p>0.0001, F(7,32)=371.74, interaction p>0.0001 F(7,32)=94.59) and a Tukey’s post-test; * indicates significant differences between genotypes at the indicated time point, α=0.05. (B) Representative photographs of control (left) or mutant (SRm16018603, right) larvae at different developmental stages (24, 48, 72, and 96 hours after egg laying).
S3 Fig. RNA-seq hits validation.

(A) Ten genes that were shown to be miss-regulated in the RNA-seq experiment exhibited the same kind of miss-regulation in an independent set of three samples and by qPCR. (B)
Five alternative splicing events miss-regulated in the \textit{SRm160} mutant were evaluated by RT-PCR in an independent set of three samples. For each gene, the read density map (\textit{w}118: blue, \textit{SRm160}: black) and the locus structure (exon: boxes, introns: lines) are displayed. The misregulated event is highlighted by a red rectangle and the relative position of the primers used is marked by red arrows. Note that in the case of CG6206, the forward primer spans the exon-exon boundary. A representative image of the agarose gel with the RT-PCR amplicons is shown. Black arrows indicate amplicons and the sizes in base pairs are indicated. +: retrotranscriptase added, −: retrotranscriptase not added, gDNA (genomic DNA control). Note that the amplicon in the gDNA is not always present in the image, due to the lengths of the expected products.

S4 Fig. \textit{SRm160} has no effect on pacemaker neuron development.

Control (A) and \textit{SRm160}-silenced (B) brains. A gallery of confocal images spanning the entire PDF positive area was acquired and a maximal intensity projection was obtained with ImageJ software. The images show a low magnification view comprising right brain hemispheres. The region subjected to analysis in Fig 3A is marked with a rectangle.
S5 Fig. Light:dark entrainment is not impaired by SRm160 downregulation.

(A) Mean activity profiles under light entrainment conditions for the indicated genotypes. Grey and white areas indicate dark and light, respectively. Red shading indicates the standard error of the mean at each point. (B) Morning and evening anticipatory indexes for
the indicated genotypes. Slight decreases (not reaching significance in a Tukey post-test) were observed in strains with reduced \textit{SRm160} expression.

S6 Fig. \textit{SRm160} is not a clock-controlled gene.

qPCR measurement of \textit{SRm160} mRNA levels in control (\textit{w}$^{118}$) or clock mutant strains (\textit{per}$^{01}$ or \textit{Clk}$^{jrk}$) employing SRmPF1 and SRmPR1 primers. Statistical analysis included one-way ANOVA (\textit{p}>0.82, \textit{F}(2,5)=0.21). Different letters indicate significant differences according to Tukey’s comparisons, \textit{a}=0.05.
S7 Fig. The perG and uasPer transgenes give rise to similar Per levels in a per null background.

To assess Per levels resulting from the transgenes employed in the rescue experiment shown in Figure 5 we measured its levels by immunohistochemistry in a per null genetic background. (A) The image depicts representative confocal images of Per staining in the sLNvs of the four genotypes employed. Brains were dissected at ZT05. Brains were stained with anti-Per (red) and anti-PDF (black), images of small LNvs were acquired with the same settings. (B) Quantitation of Per intensity at the soma of the sLNv for the indicated genotypes. Between 12 and 13 brains were measured for each genotype. Different letters indicate significant differences according to Tukey’s comparisons, α=0.05. Notice that both rescue strategies yield higher Per levels than the wild type control, but there is no significant difference between them.