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Rhythmic Behavior Is Controlled by the SRm160 Splicing Factor in *Drosophila melanogaster*

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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 kDa (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 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.

KEYWORDS *Drosophila melanogaster*; SRm160 Splicing Factor; circadian rhythms; alternative splicing; behavior; locomotor activity

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 (Lim and Allada 2013b; Beckwith and Yanovsky 2014; Hernando *et al.* 2017). Examples from distantly related organisms show that, in addition to transcriptional and post-translational modifications, molecular mechanisms controlling the chromatin landscape (Koike *et al.* 2012; Le Martelot *et al.* 2012), alternative splicing (AS) (Sanchez *et al.* 2010; McGlincy *et al.* 2012), RNA modifications (Fustin *et al.* 2013), 3′-end processing and polyadenylation (Kojima *et al.* 2012), mRNA nuclear export (MacGregor *et al.* 2013), and translation (Huang *et al.* 2013; Robles *et al.* 2014) 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 (Raj and Blencowe 2015). 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 (Colot *et al.* 2005). In *Arabidopsis*,

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several core clock genes undergo AS (Romanowski and Yanovsky 2015), and many of these genes also seem to be related to adjusting the clock in response to changes in temperature (Petrillo *et al.* 2011; James *et al.* 2012; Seo *et al.* 2012). Interestingly, in mice, U2af26 AS is regulated by light and regulates PERIOD1 stability, affecting reentrainment to new environmental conditions (Preussner *et al.* 2014). 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 (Schoenhard *et al.* 2002). *Drosophila* is no exception; to adjust behavior to different temperatures and seasons, *per* intron 8 is controlled by AS (Majercak *et al.* 2004; Sanchez *et al.* 2010).

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 (Busch and Hertel 2012). Importantly, SR and hnRNP proteins recognize and act upon exonic or intronic splicing enhancers or silencers (Risso *et al.* 2012; Bradley *et al.* 2015), and tend to act coordinately with each other (Brooks *et al.* 2015). 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 (Long and Caceres 2009), 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 (Ozkaya and Rosato 2012). 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 hr (Cyran *et al.* 2003); 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 (Meyer *et al.* 2006; Kim *et al.* 2007; Nawathean *et al.* 2007; Chiu *et al.* 2011), and the abundance of Clk (Kadener *et al.* 2008), regulated at the expression level by Pdp1e (Cyran *et al.* 2003), Vri (Blau and Young 1999), and also by Mothers against DPP (Mad), the transcription factor within the BONE MORPHOGENETIC PROTEIN (BMP) pathway (Beckwith *et al.* 2013).

Through a meta-analysis of available transcriptomic data (modENCODE Consortium *et al.* 2010; Graveley *et al.* 2011), we uncovered that genes coexpressed with the *Clk* regulator MAD are enriched in those related to RNA metabolism. Then, we identified *SRm160*, a MAD coexpressed gene, as a necessary component of the *Drosophila* timekeeping system. *SRm160* is the fly ortholog of mammalian SRRM1, originally named B1C8 (Wan *et al.* 1994), which was previously characterized as a coactivator of constitutive and exon enhancer-dependent splicing in mammals (Blencowe *et al.* 1998), worms (Longman *et al.* 2000, 2001), and flies (Eldridge *et al.* 1999; Roignant and Treisman 2010). However, the functions attributed to the SR and SR-related proteins are diverse. SRm160 has been described as part of the exon junction complex (Le Hir *et al.* 2000; Custodio *et al.* 2004) and is also involved in 3′-end processing and mRNA nuclear export (McCracken *et al.* 2002). More importantly, it is physiologically relevant for processes such as tumor cell invasion (Cheng and Sharp 2006) and chromatin regulation (McCracken *et al.* 2005). 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 AS 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 hr light:12 hr darkness; referred to as LD 12:12) on *Drosophila* standard medium at 25°. For expression in the circadian-relevant neuronal clusters, the drivers *pdf*Gal4 (Renn *et al.* 1999), *tim*Gal4 (Emery *et al.* 1998), and *pdf*GeneSwitch (Depetris-Chauvin *et al.* 2011) were employed. The strains $SRm160^{100751}$ [RNA interference (RNAi)a, Vienna *Drosophila* Resource Center, VDRC] and $SRm160^{36578}$ (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 red fluorescent protein (RFP)^{myr}, CD8GFP, and GFP^{nls} were obtained from the Bloomington *Drosophila* 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* (Kadener *et al.* 2008)

and *dper* (Zeng *et al.* 1994) were obtained from M. Rosbash (Brandeis University). These two fly strains allowed the addition of the entire genomic loci of each gene. Upstream activating sequence (UAS)-*per*3.2 [referred to as *per*, Yang and Sehgal (2001)] were obtained from A. Sehgal (University of Pennsylvania) and UAS-*Clk* [referred to as *Clk*, Zhao *et al.* (2003)] from R. Allada (Northwestern University). By means of these UAS constructs, 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} (Konopka and Benzer 1971) null mutant and the Clk^{jrk} (Allada *et al.* 1998) 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 six libraries from control (w^{1118}) and $SRm160^{18603}$ 36 hr after egg laying (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 HiSEquation 1500 at Instituto de Agrobiotecnología Rosario (INDEAR), Argentina. The analysis of the data sets was conducted as previously described (Perez-Santangelo *et al.* 2014). To score changes in gene expression, we employed a false discovery rate (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 FlyBase gene numbers to hit lists. Gene ontology (GO) term enrichment within the hit lists was determined using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da *et al.* 2009a,b). To eliminate redundant GO terms the REVIGO algorithm was employed (Supek *et al.* 2011).

Analysis of AS events

The TRIzol reagent (Life Technologies) was used for RNA isolation. cDNA was generated by standard procedures employing 1 μg of total RNA, RQ1-DNase (Promega, Madison, WI), and M-MLV retrotranscriptase (Invitrogen, Carlsbad, CA). The relative position of the primers in each locus is depicted in Supplemental Material, Figure S3 in File S4. Sequences are:

CG14642_F: 5′-TATGTGGAGCGCATCTTTCC-3′, CG14642_R: 5′-GCTATCGTAGTGGGCAGCTC-3′.
CG6206_F: 5′-GATCAGCGAATTTGGGAGAG-3′, CG6206_A_R: 5′-TCTTGGCGAAATCCAAAAAC-3′.
CG6206_B_R: 5′-CCATGGTCAGAATCACGTTG-3′, CG5708_F: 5′-CTGTTCCTCATGGTGTTGTCA-3′.
CG5708_R: 5′-ACAGCTGGAACCCACTTCTG-3′.
CG12194_F: 5′-TGATTGTGCCCGAATATCAA-3′.
CG12194_R: 5′-AGCAGAATGTGCTCCGAGTT-3′.
Aats-thr_F: 5′-CTAAATAACTTGGATTTGAACAATC-3′.
Aats-thr_R: 5′-TTGGAGATGACGGTGTTGTC-3′.

Locomotor activity

For circadian locomotor activity recordings, flies were placed in *Drosophila* Activity Monitors (Trikinetics) and entrained to 12:12 LD cycles for three complete days before transferring to constant darkness. Data were collected every 5 min for nine entire days and were analyzed by ClockLab. Period length was determined using the χ^2 algorithm with $\alpha = 0.05$, rhythmic power was calculated as the height of the peak in the periodogram minus the corresponding significant level (Yao and Shafer 2014), and the percentage of rhythmicity was calculated as previously described (Beckwith *et al.* 2013).

To analyze behavior under entrainment, each 5-min activity bin was normalized to the total activity of the corresponding animal per day. The mean value for each time point was obtained, data averaged from three consecutive days for each fly, and the mean for all the flies of a given genotype was calculated. Data shown is the average of three to five independent experiments together with the SEM. The anticipatory indices of morning and evening activity were calculated as previously described (Harrisingh *et al.* 2007).

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 [Sigma Chemical], St. Louis, MO). In 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 3650 bp fragment of the *SRm160* promoter was amplified by PCR using Phusion DNA Polymerase (New England BioLabs) from w^{1118} genomic DNA. The sequence of the primers employed were: fw: GTGCAGCGATTTTCTCAACAG 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 PBS supplemented with 0.1% Triton X-100 (PT) and fixed in 4% paraformaldehyde in PB (100 mM KH_2PO_4/Na_2HPO_4). After fixation, brains were rinsed three

times in PT and then blocked in 7% goat serum in PT for 1 hr at room temperature (RT). Tissue was incubated with primary antibodies overnight at 8°. The primary antibodies employed were rat anti-PIGMENT DISPERSING FACTOR (PDF) (1/500) (Depetris-Chauvin *et al.* 2011), rabbit anti-RFP (1/1000; Rockland), and rabbit anti-Per (1/250; Alpha Diagnostics). The secondary antibodies used were Cy2-conjugated, Cy3-conjugated, and Cy5-conjugated (Jackson ImmunoResearch) diluted to a final concentration of 1/250 and incubated for 2 hr 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 two to four sLNvs (small Lateral Neurons Ventral) 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 Region Of Interest (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, NY) 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 (National Institutes of Health).

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 2-hr window in an agar plate supplemented with sugar; before and during the egg collection, adults were stimulated with fresh yeast paste. Around 150 larvae of each genotype were collected 24 hr AEL and transferred to a plate with standard food. For RNA sequencing (RNA-seq) experiments, three groups of 100 larvae from each genotype were collected 12 hr after transfer to standard food. Larvae of each group were collected, rinsed in PBS to wash off the excess of food, and transferred to TRIzol. For larval growth curves (Figure S2 in File S4), 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.

Quantitative PCR (qPCR)

TRIzol reagent (Life Technologies) was employed for RNA isolation. cDNA was generated by standard procedures employing 1 μg of total RNA, RQ1-DNase (Promega), and M-MLV retrotranscriptase (Invitrogen). qPCR was conducted with the Fast SYBR Green Master Mix (Roche) in a Mx3005P (Stratagene, La Jolla, CA) device. Relative mRNA abundances were estimated employing internal standard curves for each gene in each experiment. The *SRm160* primers are: SRmPF1: 5′-CGACGACAGAACGCATTAGA-3′; SRmPR1: 5′-AAATATGTAACCCGGCACCA-3′; SRmPF3: 5′-GGCAGGTG GACGGCAACAG-3′, and SRmPR3: 5′-GCGGGACAGACTGG CATAGC-3′. The relative localization of these primers in the *SRm160* locus is indicated in Figure S1 in File S4.

To validate observation in the RNA-seq data set (Figure S3 in File S4), the employed primers were: Cyp9b2_2_F: 5′-TGATGTGCAACAAGCTCTCC-3′; Cyp9b2_2_R: 5′-ACGTCGG GATTGTAAAGCAG-3′; CG14691_2_F: 5′-ATCACGGTAGCTG GAATTGG-3′; CG14691_2_R: 5′-CATCAGTGAGCAAAGCCAGA-3′; CG10924_3_F: 5′-CAACTGCATTAGCTGCCAAG-3′; CG10924_3_R: 5′-TGATGGTTCCCTTCTTCAGC-3′; Cyp6a17_2_F: 5′-GCTGGGTTTGAGACAAGCTC-3′; Cyp6a17_2_R: 5′-CGATTTC CTCGTCGGTAAAG-3′; lip3_2_F: 5′-GCCCAGCAATAAGTTCA AGC-3′; lip3_2_R: 5′-AAGTTCTGGTTCACCGATGC-3′; mur89F_1_F: 5′-CTACCAGTGCAGCGAAAGTG-3′; mur89F_1_R: 5′-TCGG CTAACGTTCCAGTAGG-3′; mas_3_F: 5′-GAGCTGCTTTAATCG GAACC-3′; 578-mas_3_R: 5′-TATGCACTCCGTATCGCTCA-3′; CG3397_2_F: 5′-GAAAGCTGCTGCGGATTAAC-3′; CG3397_2_R: 5′-CAAGTGGTCGCTCATTTGAA-3′; CG10081_2_F: 5′-TCGGTC TATTGGCCGTAACT-3′; CG10081_2_R: 5′-CCTTGCTCACTGTTC CATCA-3′; minature_2_F: 5′-TGCCGATCTCGATGTTATCC-3′; and minature_2_R: 5′-CCAAATTCATCGGACAGGTT-3′.

Data availability

We declare that all data supporting the findings of this study are available within the article and its Supplemental Material files, or from the corresponding author upon reasonable request. The RNA-seq data files and the File S1, File S2, and File S3 are available from the Gene Expression Omnibus database (accession number GSE102361).

Results

SRm160 is a splicing regulator coexpressed 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 (Beckwith *et al.* 2013). 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 (modENCODE Consortium *et al.* 2010; Graveley *et al.* 2011) to analyze genes that share their expression pattern with MAD (Table S1), which is usually taken as an indication of involvement in similar biological processes (van Dam *et al.* 2015). Surprisingly, a GO term analysis of the genes coregulated with MAD (Table S1) showed a clear

enrichment in genes related to RNA metabolism (Table S2). Comparison of this list with spliceosome components (Herold *et al.* 2009) 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 coexpressed 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* (Eldridge *et al.* 1999). In addition, *SRm160* controls the AS of the transmembrane glycoprotein CD44 in mammalian cells (Cheng and Sharp 2006). 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 (Fan *et al.* 2014, Figure S1 in File S4). In this way, we were able to comprehensively assess the global effect of *SRm160* loss-of-function in an animal that reaches postembryonic development. The insertion of the *P*-element in the *SRm160*^{18603} allele leads to arrested development 48 hr AEL and eventually results in lethality (Figure S2 in File S4).

We used RNA-seq to analyze the transcriptome of wild-type and *SRm160*^{18603} flies at 36 hr AEL, focusing on gene expression and AS (Hernando *et al.* 2015; Schlaen *et al.* 2015, Figure 1, File S1, and File S2). Interestingly, regarding global expression levels, the absence of *SRm160* either directly or indirectly impacted ~10% of the expressed genes, while alterations in the inclusion of constitutive exons or introns (constitutive splicing) were smaller, affecting 3 and 5.5% of the expressed exons or introns, respectively (Figure 1A). Not surprisingly, *SRm160* depletion had a larger effect on AS, influencing ~7% of AS events measured (Figure 1A). Loss of *SRm160* impacted all types of AS events alike, ruling out the possibility that SRm160 regulatory function is restricted to a specific type of splicing event (Figure 1, B and C). To validate the data obtained by this high-throughput technique, we independently evaluated several of the identified mis-regulated genes and splicing events with an alternative technique. We evaluated five upregulated and five downregulated genes by RT-PCR, confirming the results from the transcriptomic analysis (Figure S3A in File S4). In addition, we validated five AS events spanning different types of events by PCR (Figure S3B in File S4). These analyses confirmed and validated our initial observations.

Although several genes in *SRm160*^{18603} showed altered expression levels or deficits in constitutive splicing and AS, this fly strain survives through embryogenesis and lives for several days as larvae (Figure S2 in File S4). 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 (Rosel *et al.* 2011) and LSm4 in *Arabidopsis thaliana* (Perez-Santangelo *et al.* 2014).

Interestingly, clock genes were not highlighted as *SRm160* targets in the 36 hr AEL larval sample, since the expression of most of these genes, including *per*, was not detectable at this developmental stage (File S1 and File S2).

To further characterize the affected genes, we assigned GO terms to the list of genes that were differentially expressed or spliced in *SRm160*^{18603}, 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; Figure 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 (Figure 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 LNvs by means of the promoter of the neuropeptide PDF, which is expressed exclusively in this neuronal cluster (Renn *et al.* 1999). In this way, we avoided the deleterious effects of broader genetic manipulations and focused our search on a key cluster involved 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 (Figure 2A). There was a clear reduction in the percentage of rhythmic flies in the population (Figure 2B) and in the rhythm strength (Figure 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 (Kaneko and Hall 2000, Figure 2). To overcome potential unspecific effects of the knockdown strategy, we employed

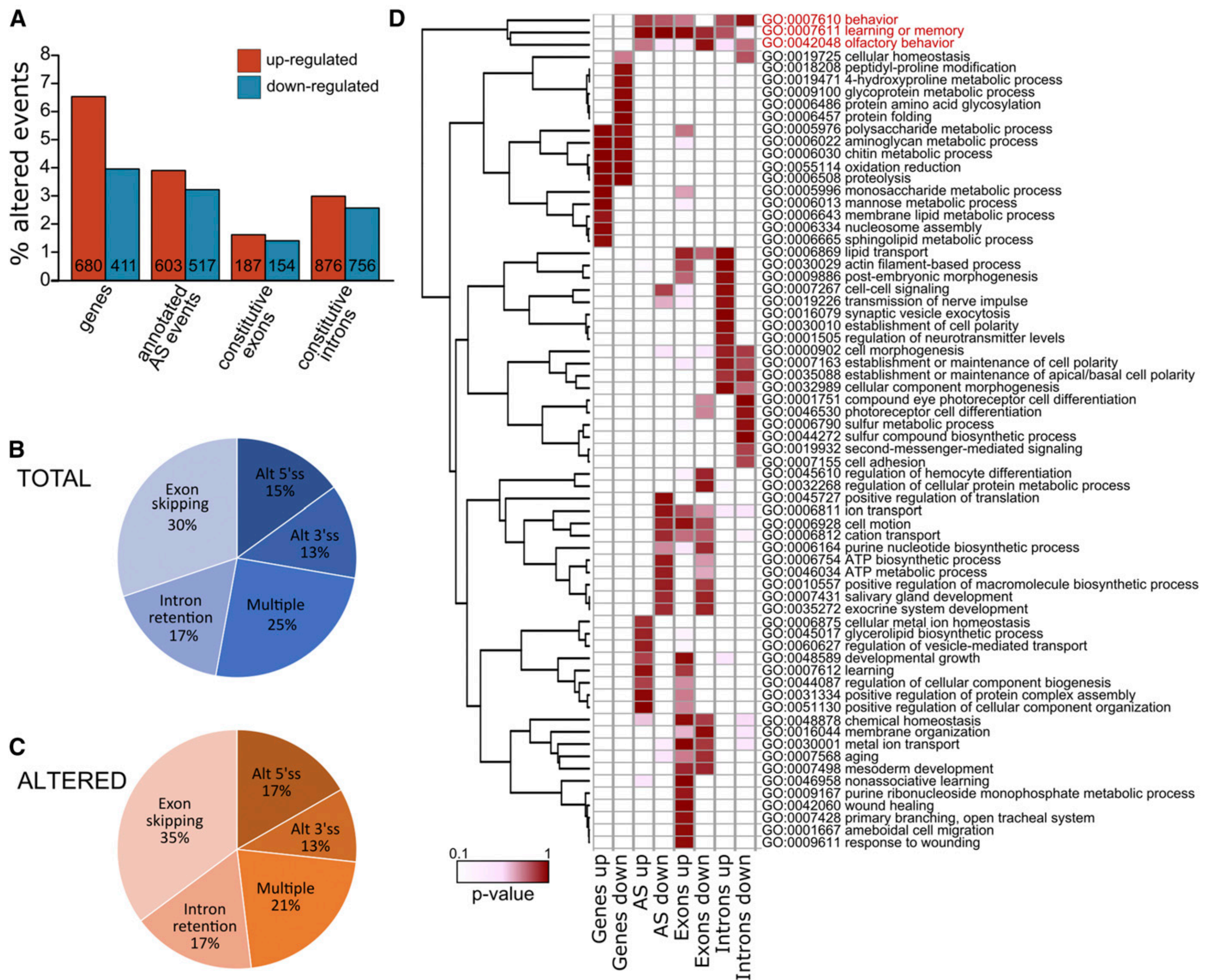

Figure 1 Genome-wide analysis of the impact of SRm160 on the transcriptome. (A) Percentage of genes and alternative splicing (AS) events identified as being up- or downregulated in the RNA sequencing (RNA-seq) data set. The total number for each category are: genes = 10,412, annotated AS events = 4791, evaluated exons = 54,046, and evaluated introns = 20,166. (B) Distribution of the different types of AS events measured in the RNA-seq data set. In 25% of the genomic regions analyzed, multiple AS events were simultaneously detected and it was not possible to reliably determine the type of events affected individually. 5′ splice site (5′ss), 3′splice site (3′ss). (C) Distribution of the different types of AS events altered in the *SRm160*^{18603} mutant. (D) The top 10 gene ontology (GO) terms enriched (*P*-value < 0.1) in each category were sorted by dendrogram analysis. This analysis illustrates the clustering of GO terms by their *P*-values between the different categories. One particular cluster of terms with enrichment in all the splicing categories is highlighted in red, note that this small cluster is exclusively integrated by terms specifically related to nervous system function.

a second RNAi from a different library that showed similar results (Figure 2). Interestingly, the combination of both RNAis led to more severe phenotypes, suggesting that *SRm160* has a specific role in sustaining rhythmic locomotor behavior (Figure 2). Behavioral analysis showed that under driven conditions (12 hr light and 12 hr dark cycles), the impact of SRm160 reduction was somewhat diminished, although a slight reduction in the anticipatory behavior was observed when *SRm160*^{RNAia} was expressed in PDF+ neurons (Figure S5 in File S4). Importantly, knockdown of *SRm160* by means of the GAL4/UAS system did not impose structural defects to the LNv projections (Figure S4 in File 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 (*pdf*GS > *SRm160*^{RNAia}; *dicer2* +RU) compared to the respective controls (Figure 3; 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.

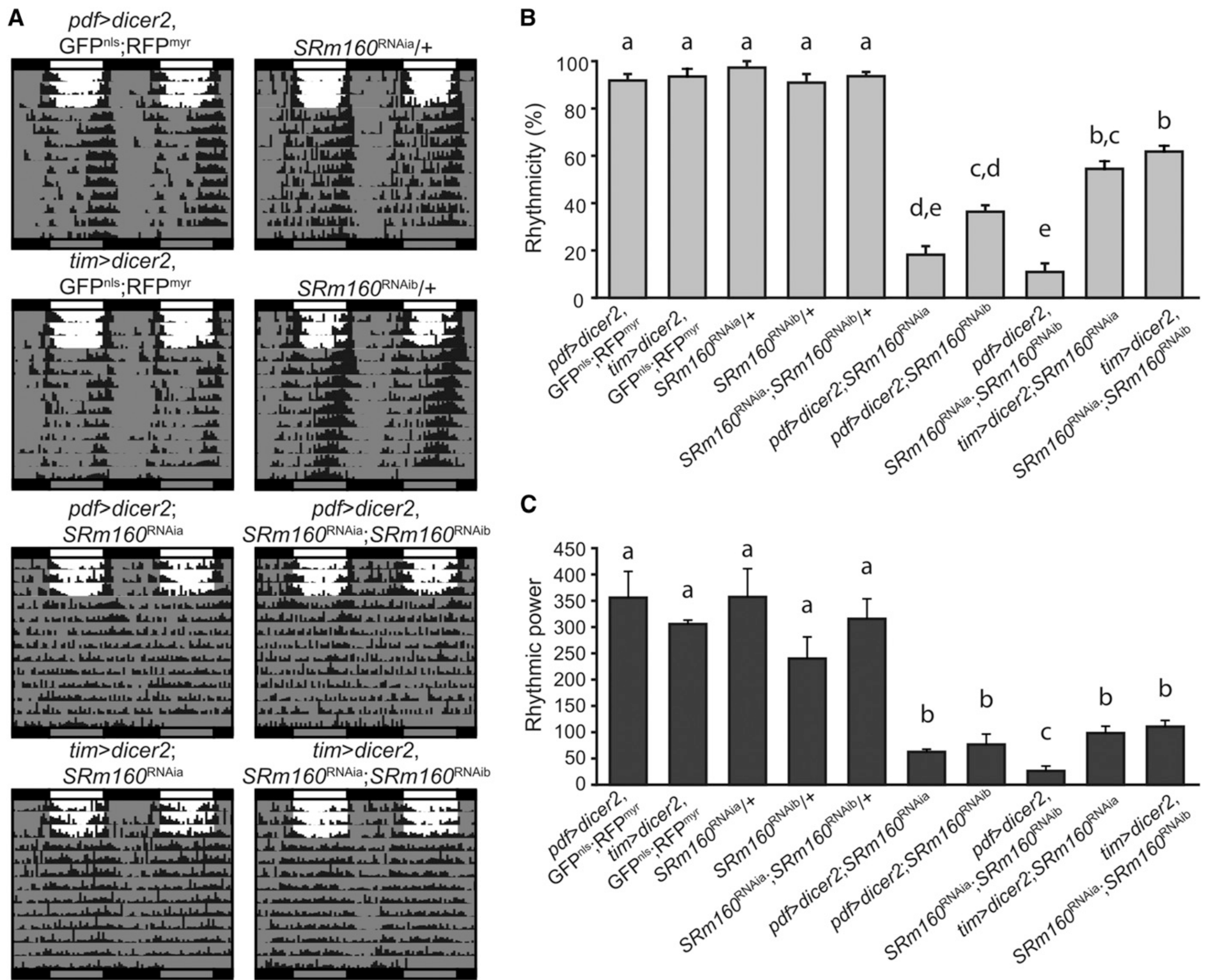

Figure 2 SRm160 supports a functional clock. (A) Representative locomotor activity profiles of the indicated genotypes showing 3 days in 12 hr light:12 hr darkness and 10 days in constant darkness. Gray shading indicates darkness. White bars indicate light, dark bars indicate dark, and gray bars indicate 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 comparisons, $\alpha = 0.05$. RFP, red fluorescent protein.

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^{myr} 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 (Park *et al.* 2000), 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

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

Genotype	N	n	Period			% Rhythmicity			Rhythmic Power		
			Mean	SE	S	Mean	SE	S	Mean	SE	S
pdf > *dicer2*,GFP^{nls};RFP^{myr}	129	5	24.0	0.1	a	91.6	3.4	a	356.6	50.2	a
tim > dicer2,GFP^{nls};RFP^{myr}	85	3	23.8	0.0	a	93.6	2.8	a	306.3	7.9	a
SRm160^{RNAia}/+	90	3	23.9	0.2	a	95.4	3.0	a	358.0	54.2	a
SRm160^{RNAib}/+	111	4	23.6	0.1	a	91.3	3.4	a	240.7	41.4	a
SRm160^{RNAia};*SRm160*^{RNAib}/+	111	4	23.6	0.1	a	93.5	2.4	a	316.5	37.8	a
pdf > dicer2;*SRm160*^{RNAia}	89	3	24.1	0.3	a	24.3	3.6	d,e	62.8	4.7	b
pdf > *dicer2*;*SRm160*^{RNAib}	90	4	23.9	0.1	a	36.3	3.2	c,d	76.9	19.8	b
pdf > *dicer2*,*SRm160*^{100751}; *SRm160*^{RNAib}	92	4	23.8	0.2	a	10.8	3.5	e	26.5	9.6	c
tim > *dicer2*;*SRm160*^{RNAia}	83	3	23.9	0.1	a	56.4	3.7	b,c	98.4	13.3	b
tim > *dicer2*,*SRm160*^{RNAia}; *SRm160*^{RNAib}	67	3	23.8	0.1	a	62.1	2.5	b	110.8	11.5	b

The analyzed data correspond to the results shown in Figure 2. *N*, total number of analyzed animals; *n*, number of analyzed experiments; S, statistical analysis; different letters indicate significant differences according to Tukey's comparisons, α = 0.05.

constant conditions (Depetris-Chauvin *et al.* 2011). 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 (Figure 5A). Consistent with the behavioral data, we found no PDF oscillations in the dorsal projections of the sLNvs (Figure 5B). Interestingly, the SD in PDF levels was significantly increased when *SRm160* levels were decreased, a result that supports the behavioral phenotype displayed by the *SRm160* knockdown flies (Depetris-Chauvin *et al.* 2014; Klose *et al.* 2016; Liang *et al.* 2016).

Thus, reduced levels of this post-transcriptional regulator halt the oscillation of the principal output of the pacemaker neurons in *Drosophila*.

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 (Curtin *et al.* 1995). 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 (Figure 5C). By contrast, *SRm160*-interfered neurons showed reduced Per levels and slower degradation, resulting in a dampened oscillation (Figure 5, C and D).

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 d*Clk* dominant negative mutant (d*Clk*^{jrk}, Figure S6 in File S4). However, the possibility that *SRm160* is differentially regulated in pacemaker neurons cannot be ruled out.

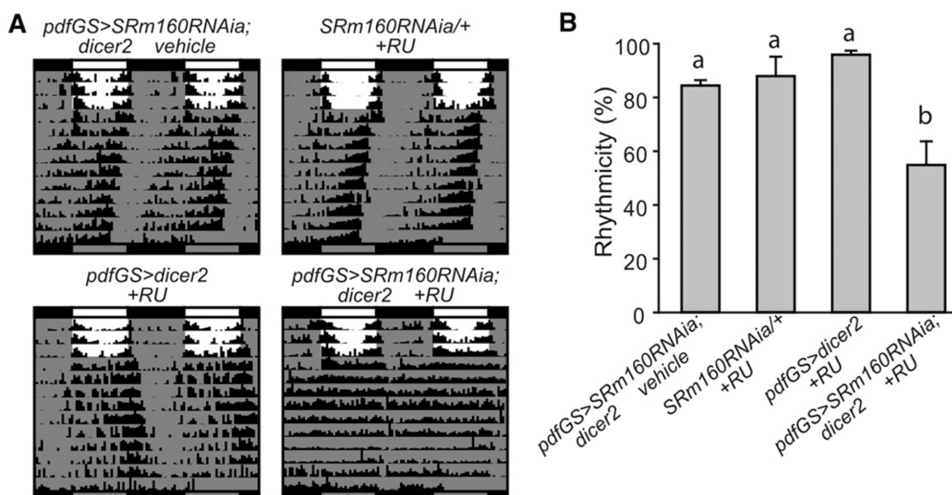

Figure 3 SRm160 expression in the adult sLNvs is necessary for a wild-type circadian behavior. (A) Representative locomotor activity profiles of the indicated genotypes showing 3 days in LD 12:12 and 10 days in constant darkness. Gray shading indicates darkness. White bars indicate light, dark bars indicate dark, and gray 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 comparisons, α = 0.05. LD 12:12, 12 hr light:12 hr darkness; RU, mifepristone; sLNvs, small Lateral Neurons Ventral.

Table 2 *SRm160* has an adult-specific function in circadian timekeeping system

Genotype	*N*	*n*	Period			% Rhythmicity			Rhythmic Power		
			Mean	SE	S	Mean	SE	S	Mean	SE	S
*pdf*GS > *SRm160*^{RNAia}; *dicer2* vehicle	52	3	23.65	0.05	a	84.41	2.40	a	652.21	82.31	a,b
SRm160^{RNAia}/+ +RU	50	3	23.69	0.06	a	87.78	7.78	a	885.04	77.81	b
*pdf*GS > *dicer2* +RU	46	3	24.13	0.07	a	95.54	2.25	a	524.01	50.89	a
*pdf*GS > *SRm160*^{RNAia}; *dicer2* +RU	53	3	23.78	0.08	a	54.36	9.56	b	426.41	62.85	a

The analyzed data correspond to the results shown in Figure 3. *N*, total number of analyzed animals; *n*, number of analyzed experiments; S, statistical analysis; different letters indicate significant differences according to Tukey's comparisons, α = 0.05; +RU, mifepristone.

Bypass of per splicing can rescue the lack of SRm160

Knockdown of *SRm160* impacted Per levels ensuing misregulation 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 (Smith and Konopka 1982; Baylies *et al.* 1987; Yang and Sehgal 2001; Kadener *et al.* 2008) and yielded similar levels of Per protein in the sLNvs (Figure S7 in File S4). 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 (Figure 6; 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 (Figure 6, 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 (Figure 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 post-transcriptional level, an effect that ultimately impacts on PDF oscillations and overt behavior.

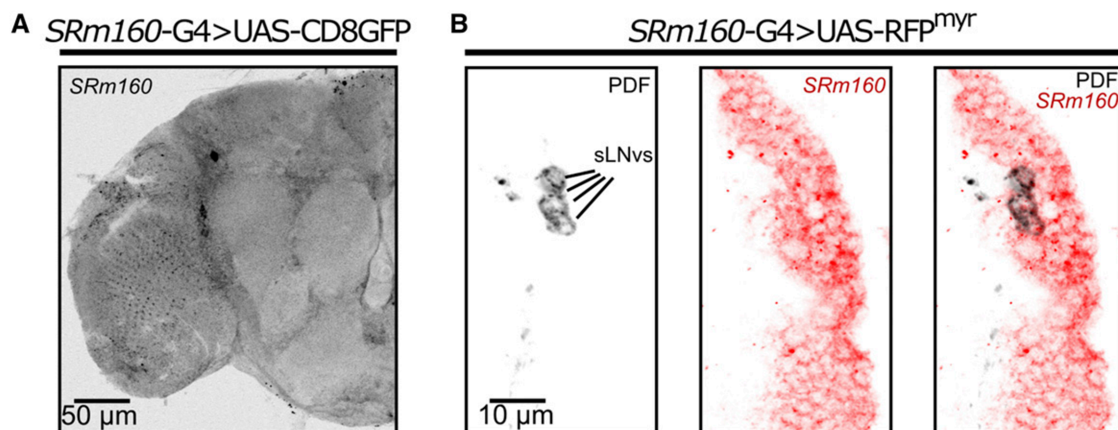

Figure 4 *SRm160* is expressed in central pacemaker neurons. (A) Low magnification of the expression pattern of SRm160 reported by a GAL construct. A dim signal is observed across the brain. It is important to note that GAL reporters do not necessarily represent a complete description of the endogenous expression pattern. (B) Spatial expression of *SRm160* in the accessory medulla was visualized using a RFP reporter (red), while pacemaker cells were identified by immunostaining with anti-PDF antibody (black). The image represents the maximal intensity projection of a gallery of single-plane images spanning (A) the entire brain or (B) all sLNvs somas. PDF, PIGMENT DISPERSING FACTOR; RFP, red fluorescent protein; sLNvs, small Lateral Neurons Ventral.

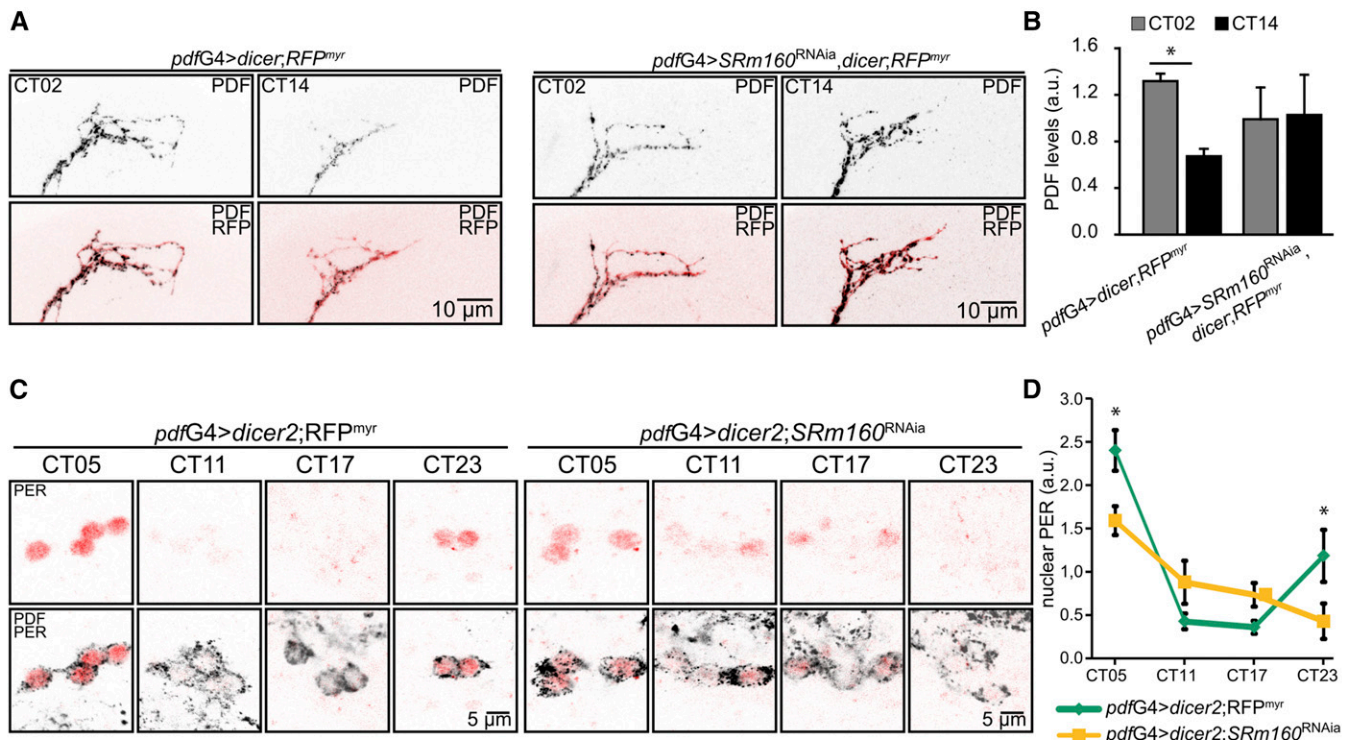

Figure 5 *SRm160* sustains Per oscillations in the central pacemaker. (A) Control (left) or *SRm160*-interfered (right) brains were dissected during the second day of constant darkness at CT02 and CT14. Brains were stained with anti-RFP (red) and anti-PDF (black) antibodies and images from the dorsal projections of sLNvs 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 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 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 sLNvs 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 two to four 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 $P > 0.0001$, $F_{(3,16)} = 35.00$; and interaction $P = 0.0009$, $F_{(2,16)} = 9.29$). A simple effect comparison was used to analyze differences between genotypes at different CT. CT05 * $P = 0.0029$, $F_{(1,16)} = 12.33$; CT11 $P = 0.0672$, $F_{(1,16)} = 3.85$; CT17 $P = 0.1233$, $F_{(1,16)} = 2.65$; and CT23 * $P = 0.0047$, $F_{(1,16)} = 10.73$. CT, circadian time; PDF, PIGMENT DISPERSING FACTOR; RNAi, RNA interference; RFP, red fluorescent protein; sLNvs, small Lateral Neurons Ventral.

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, by 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 and a downstream arginine-serine (RS) domain consisting of at least 50 amino acids with > 40% RS content, characterized by consecutive RS or SR repeats (Manley and Krainer 2010). As SRm160 lacks a classical RNA-binding domain motif (Blencowe *et al.* 1998), 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 (Blencowe and Ouzounis 1999).

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 hr after fertilization (Fan *et al.* 2014). After 10 hr of development, *SRm160* mRNAs are enriched in the central nervous system (CNS) and completely restricted to it by 16 hr after fertilization (Fan *et al.* 2014). Then, *SRm160* mRNA levels decrease, reaching a minimum at 20–24 hr.

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

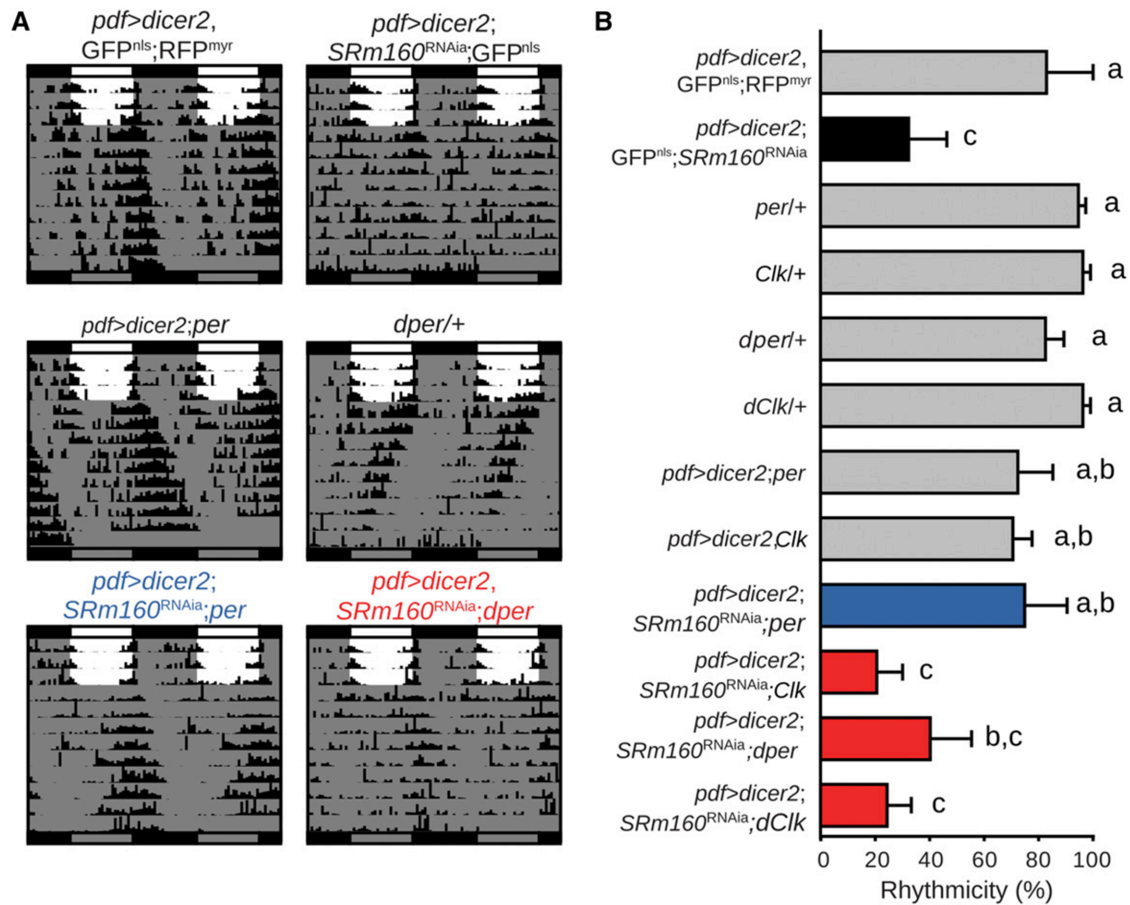

Figure 6 SRm160 impacts *per* splicing. (A) Representative locomotor activity profiles of the indicated genotypes after 3 days in LD 12:12 and 9 days in constant darkness. (B) Percentage of rhythmic flies for each genotype. Statistical analysis included one-way ANOVA ($P < 0.0001$, $F_{(11,38)} = 15$). Different letters indicate significant differences according to Tukey's comparisons, $\alpha = 0.05$. Gray, negative control; black, positive control; blue, rescue; and red, lack of rescue. LD 12:12, 12 hr light:12 hr darkness; RFP, red fluorescent protein.

SRm160 levels are recovered during larval stages (Fan *et al.* 2014), 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 (Fan *et al.* 2014). 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.

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

			Period			% Rhythmicity			Rhythmic Power		
Genotype	N	n	Mean	SE	S	Mean	SE	S	Mean	SE	S
pdf > *dicer2*,GFP^{nls};RFP^{myr}	136	5	24.1	0.2	a	84.4	17.5	a	417.9	49.4	a
pdf > dicer2,GFP^{nls};*SRm160*^{RNAia}	128	5	24.0	0.3	a	32.9	14.3	c	119.2	30.8	c,d
per/+	138	5	23.7	0.0	a	96.3	2.7	a	485.5	81.5	a
Clk/+	49	2	23.7	0.0	a	98.0	2.8	a	434.7	69.1	a
dper	51	2	22.9	0.0	b	84.0	6.9	a	213.1	7.4	b,c
dClk/+	53	2	23.7	0.1	a	98.0	2.8	a	399.6	19.5	a
pdf > dicer2;*per*	133	5	24.6	0.1	c	73.7	13.1	a,b	292.1	42.6	a,b
pdf > dicer2;*Clk*	50	2	24.1	0.1	a	71.8	7.3	a,b	225.3	77.9	b
pdf > dicer2,GFP^{nls};*SRm160*^{RNAia};*per*	147	5	24.1	0.1	a	76.3	15.8	a,b	246.6	54.9	b
pdf > dicer2,GFP^{nls};*SRm160*^{RNAia};*Clk*	56	2	24.0	0.0	a	21.0	9.6	c	78.0	52.8	d
pdf > dicer2,GFP^{nls};*SRm160*^{RNAia};*dper*	57	2	24.4	0.3	a,c	40.9	15.5	b,c	92.8	19.4	d
pdf > dicer2,GFP^{nls};*SRm160*^{RNAia};*dClk*	59	2	23.9	0.0	a	24.9	9.0	c	90.8	8.0	d

The analyzed data correspond to the results shown in Figure 6. *N*, total number of analyzed animals; *n*, number of analyzed experiments; S, Statistical analysis; different letters indicate significant differences according to Tukey's comparisons, $\alpha = 0.05$.

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 are the main repressors (Kadener *et al.* 2007; Matsumoto *et al.* 2007). At the post-transcriptional level, AS of *per* mRNA has previously been documented; the *per* 3′-terminal intron, *dmpi8*, is either spliced out or retained (Majercak *et al.* 2004). 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 physically interacts with Ataxin-2 (Atx2) (Lim and Allada 2013a; Zhang *et al.* 2013) in a protein complex formed by Atx2, LSM12, and ME31B (Lee *et al.* 2017). This complex binds directly to *per* mRNA in the LNvs, and acts with PolyA-Binding Protein and the translation initiation factor eIF4G (Lim *et al.* 2011) to activate *per* translation. In addition, the atypical translation factor NAT1 ensures *per* translation in a cap-independent mechanism (Bradley *et al.* 2012). Here, 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 (Figure 2, Figure 3, and Figure 5) and other *per* translational regulators mentioned (Bradley *et al.* 2012; Lim and Allada 2013a; Zhang *et al.* 2013; Lee *et al.* 2017). 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 (Preussner *et al.* 2014).

Finally, post-translational 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 (Bae and Edery 2006; Gallego and Virshup 2007; Ko *et al.* 2010; Chiu *et al.* 2011; Yu *et al.* 2011).

Splicing and the brain

AS is especially prevalent in neuronal tissue, and many AS events are specific to neural cell types (Raj and Blencowe 2015). Over recent years, it has become clear that neuronal development is highly influenced by AS, both in mammals (Vuong *et al.* 2016) and flies (Liu and Bossing 2016; Olesnicky *et al.* 2017), even at the single-cell level (Liu and Bossing 2016; Liu *et al.* 2017). More importantly, a growing body of evidence shows that behavioral traits are fine-tuned by AS in many species (Poplawski *et al.* 2016; Tomioka *et al.* 2016; Wang *et al.* 2016).

Importantly, the use of RNA-seq and other high-throughput technologies has identified widespread clock control of AS in the *Drosophila* brain (Hughes *et al.* 2012), but the mechanisms underlying this regulation are unknown. Our meta-analysis of transcriptomic data combined with a genetic approach helped us to identify 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 (Hughes *et al.* 2012), our results suggest that SRm160 regulates *per* levels, at least in part, through a splicing-regulated process (Figure 6 and Figure S7 in File S4). However, considering the diversity of regulatory roles attributed to SRm160 in post-transcriptional 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 nonclock 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 (Figure 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 (Figure 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 data set.

Interestingly, SRm160 impacts a large number of splicing events, particularly among genes related to CNS function and behavior (Figure 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 (Mezan *et al.* 2013; Wang *et al.* 2016).

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.

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