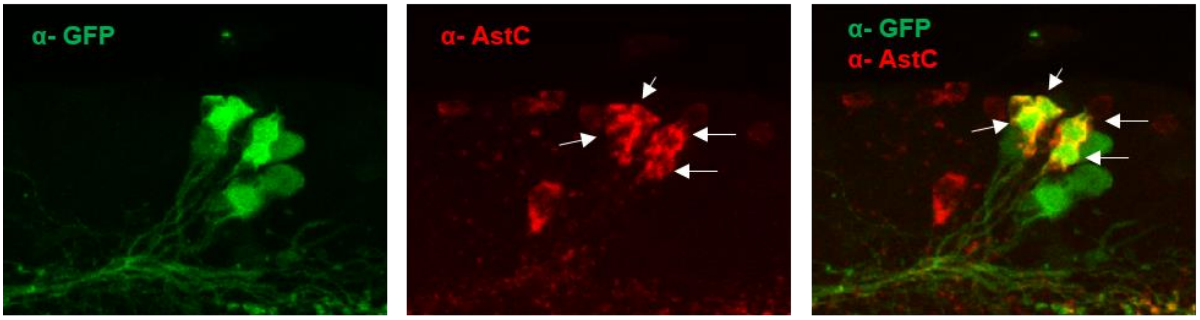
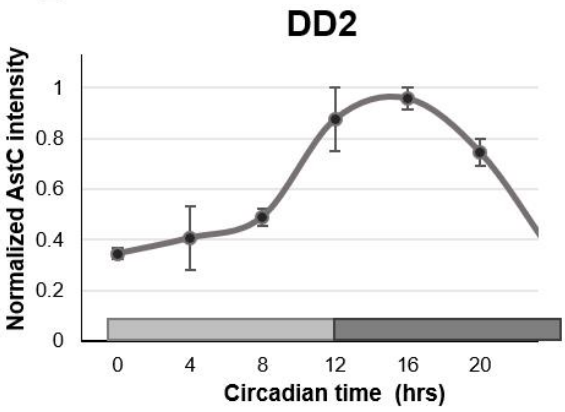
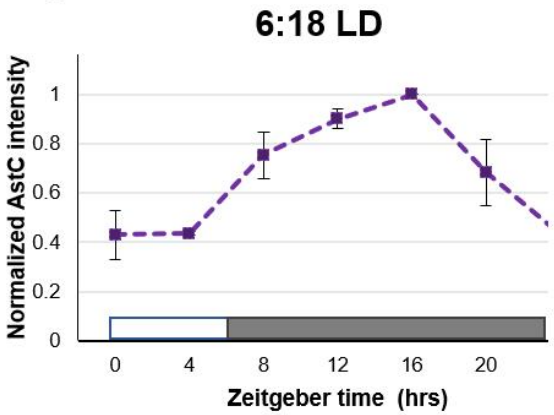
A


α- GFP
α- AstC
α- GFP
α- AstC

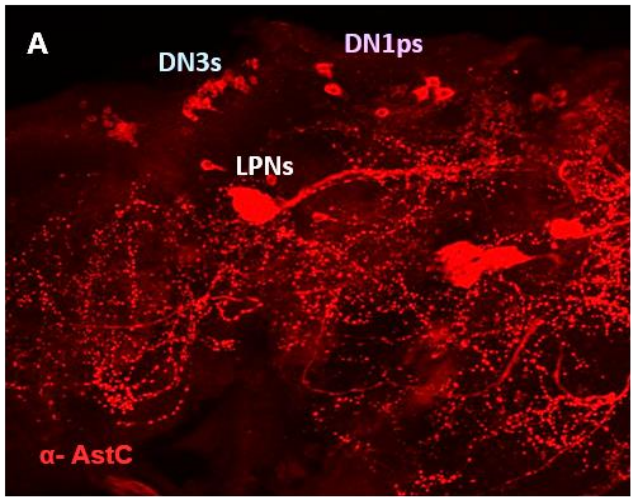
B

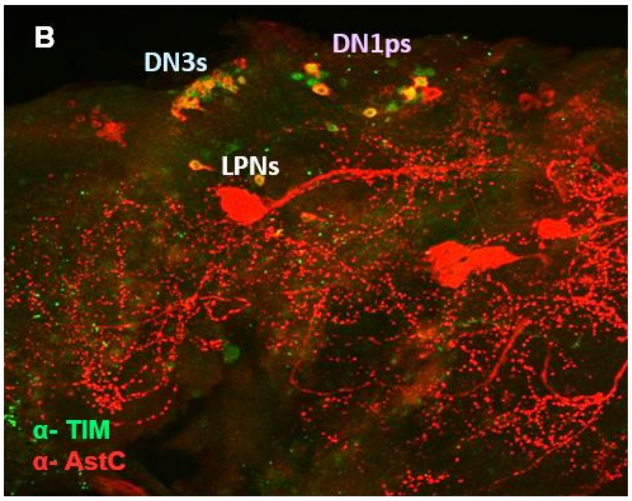

DD2
Normalized AstC intensity
0
0.2
0.4
0.6
0.8
1
0
4
8
12
16
20
Circadian time (hrs)

C

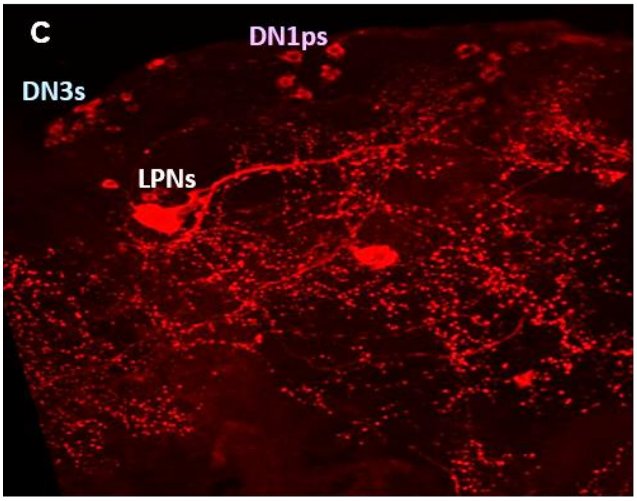

6:18 LD
Normalized AstC intensity
0
0.2
0.4
0.6
0.8
1
0
4
8
12
16
20
Zeitgeber time (hrs)

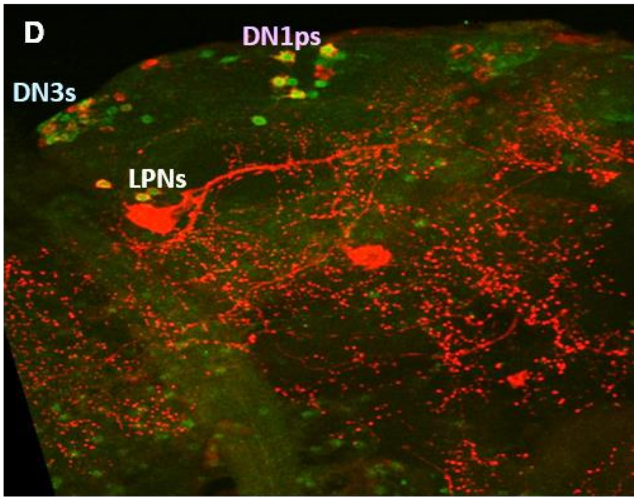
tim-GAL4 control



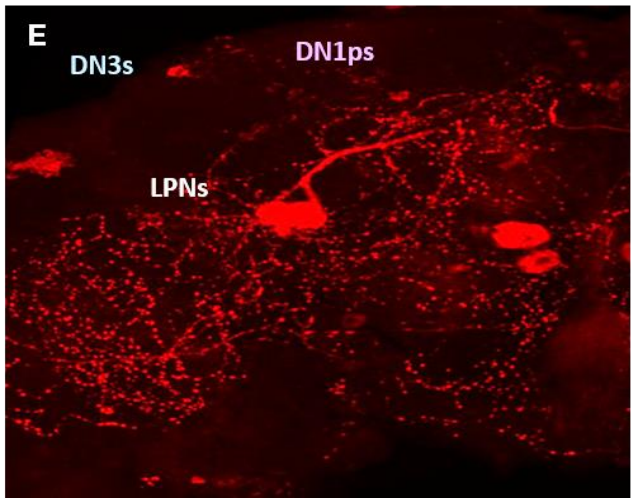


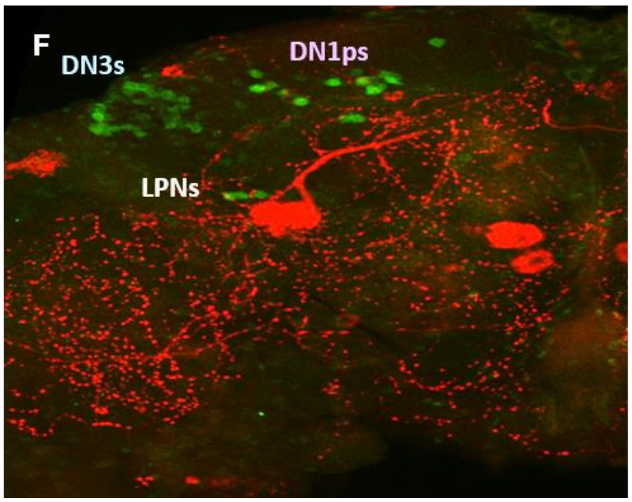
AstC-RNAi control

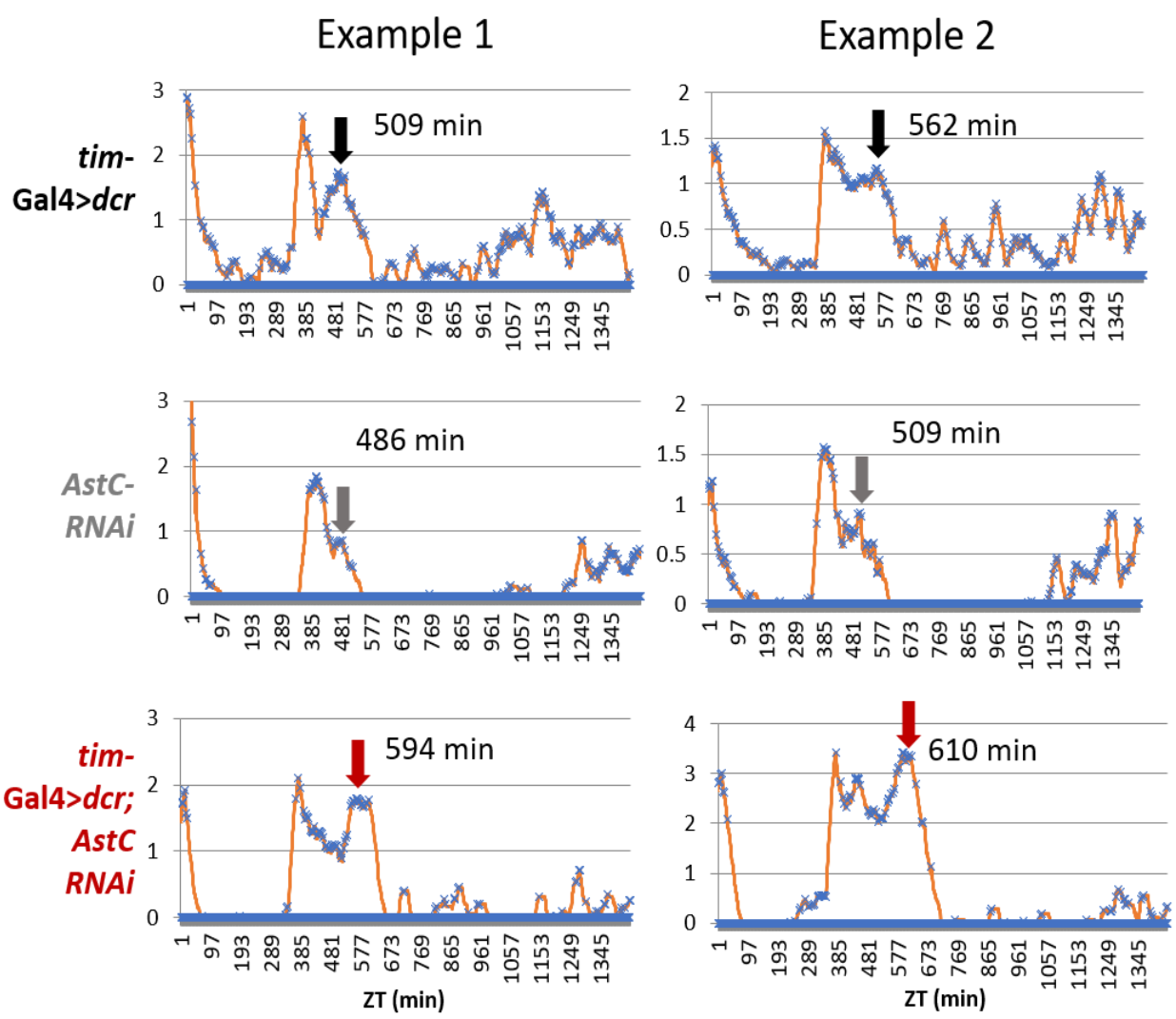


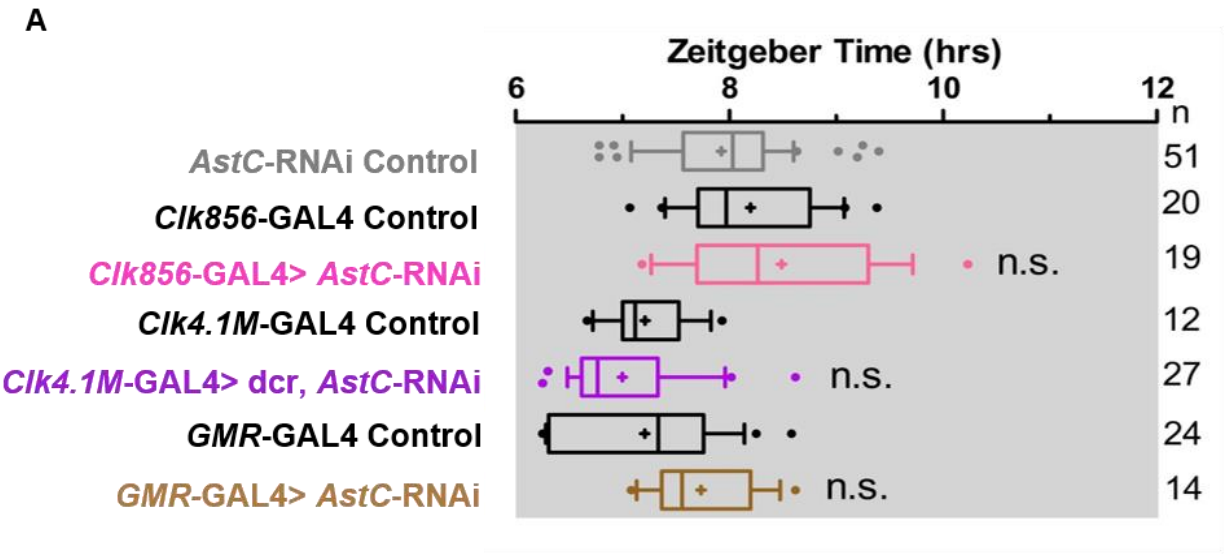


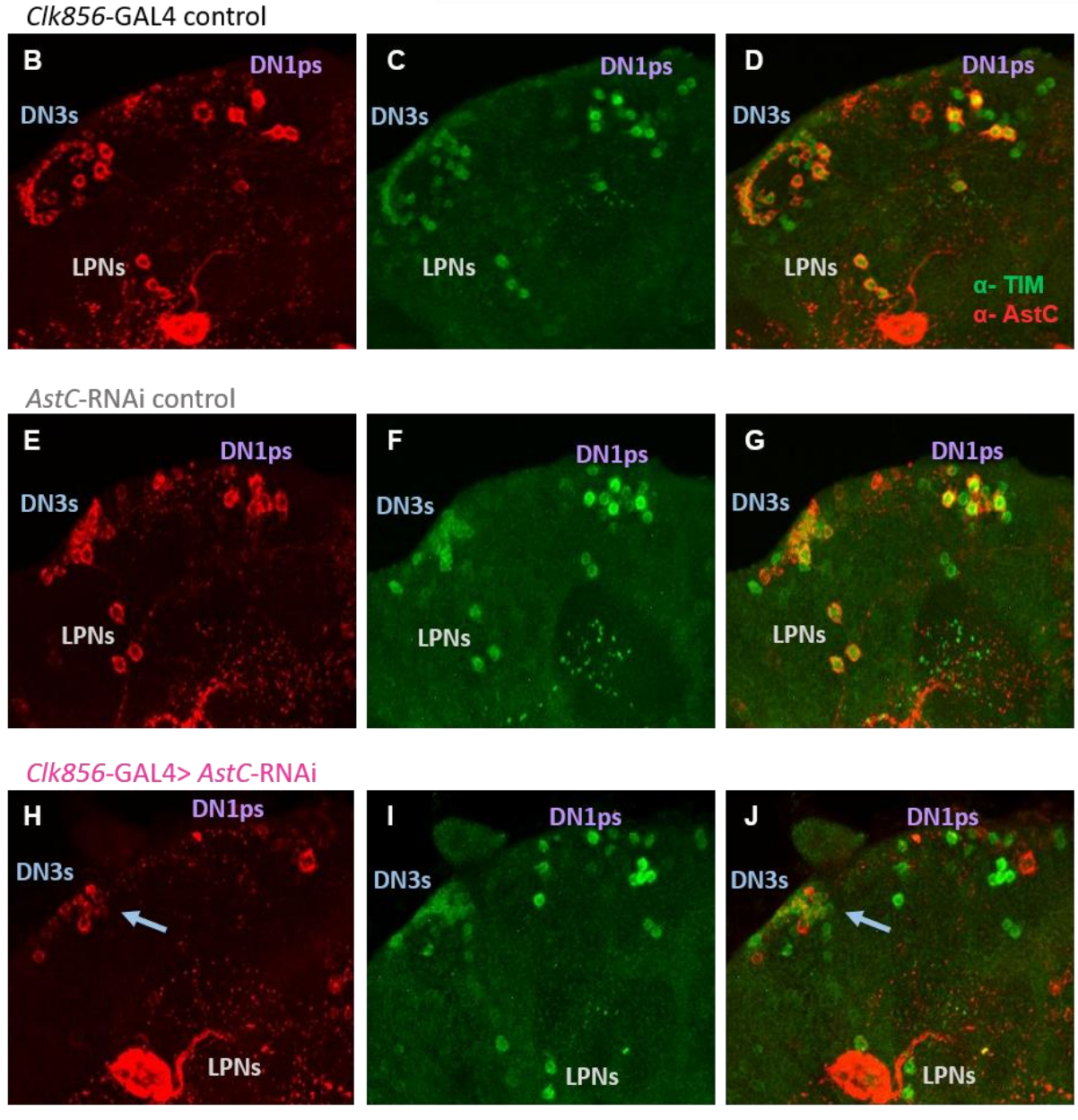
tim-GAL4> *dcr*, *AstC*-RNAi

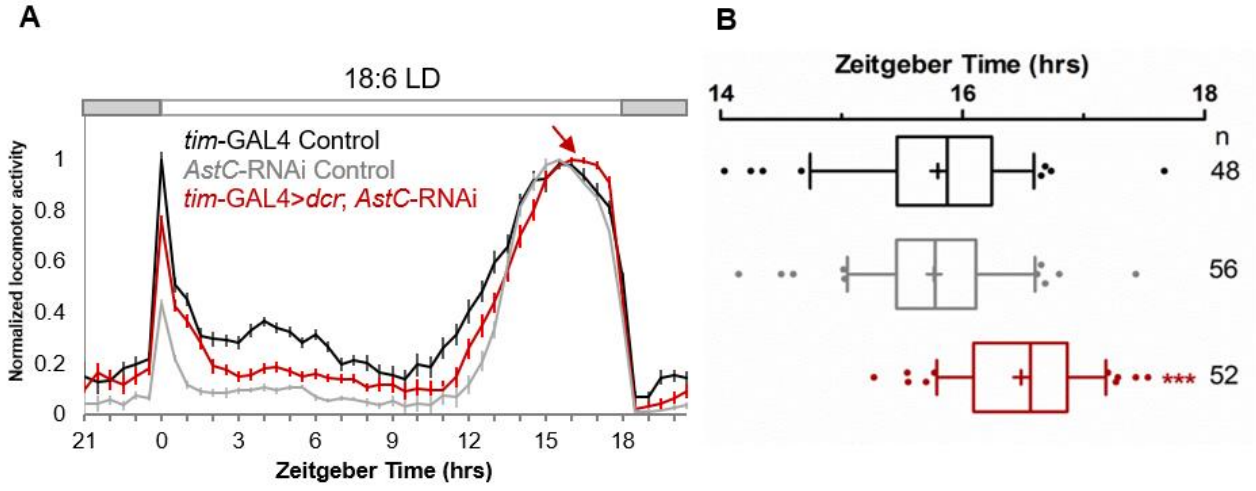





Example 1
Example 2
tim-Gal4>dcr
509 min
562 min
AstC-RNAi
486 min
509 min
tim-Gal4>dcr; AstC RNAi
594 min
610 min
ZT (min)
ZT (min)


A
Zeitgeber Time (hrs)
6
8
10
12
n
AstC-RNAi Control
51
Clk856-GAL4 Control
20
Clk856-GAL4> AstC-RNAi
n.s.
19
Clk4.1M-GAL4 Control
12
Clk4.1M-GAL4> dcr, AstC-RNAi
n.s.
27
GMR-GAL4 Control
24
GMR-GAL4> AstC-RNAi
n.s.
14


Clk856-GAL4 control
B
DN1ps
DN3s
LPNs
C
DN1ps
DN3s
LPNs
D
DN1ps
DN3s
LPNs
α- TIM
α- AstC
AstC-RNAi control
E
DN1ps
DN3s
LPNs
F
DN1ps
DN3s
LPNs
G
DN1ps
DN3s
LPNs
Clk856-GAL4> AstC-RNAi
H
DN1ps
DN3s
LPNs
I
DN1ps
DN3s
LPNs
J
DN1ps
DN3s
LPNs


A
18:6 LD
tim-GAL4 Control
AstC-RNAi Control
tim-GAL4>dcr; AstC-RNAi
Normalized locomotor activity
1
0.8
0.6
0.4
0.2
0
21
0
3
6
9
12
15
18
Zeitgeber Time (hrs)
B
Zeitgeber Time (hrs)
14
16
18
n
48
56
***
52

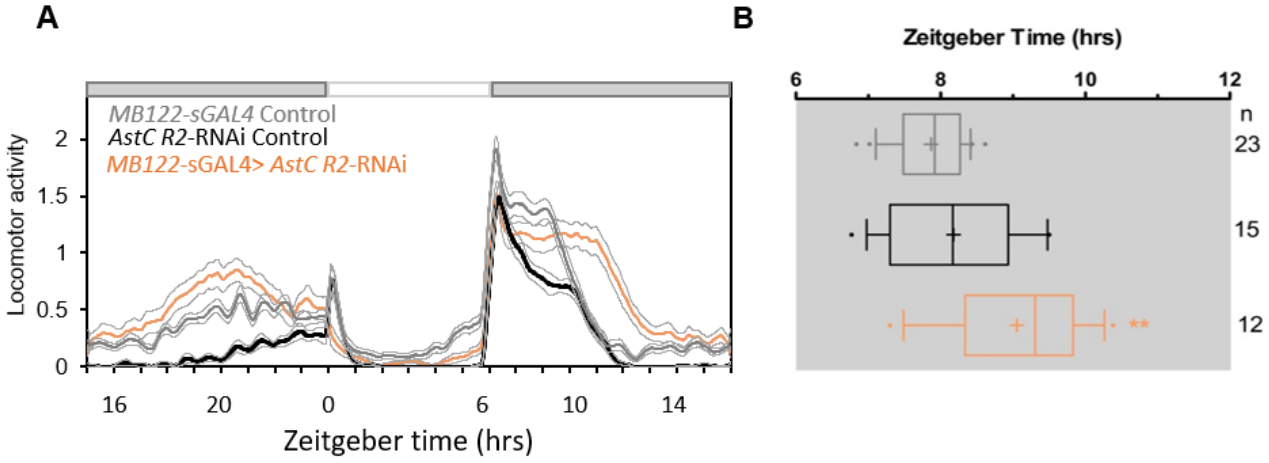

A
B
MB122-sGAL4 Control
AstC R2-RNAi Control
MB122-sGAL4> AstC R2-RNAi
Locomotor activity
0
0.5
1
1.5
2
16
20
0
6
10
14
Zeitgeber time (hrs)
Zeitgeber Time (hrs)
6
8
10
12
n
23
15
12
**

Figure S1. AstC in glutamatergic DN1ps continues to cycle in constant darkness and in short photoperiod. Related to Figure 3.

A, A subset of the DN1ps known to contain glutamate (*R51H05*-GAL4) was labeled with GFP and co-immunostained with AstC antibodies at ZT14. Co-localization of the two signals indicates that AstC is found in four glutamatergic DN1ps. Non-clock neurons also expressing AstC are found nearby. ***B***-***C***, Young males flies expressing GFP in a subset of the DN1ps (*Clk4.1M*-GAL4) were entrained to six timepoints and co-immunostained with AstC and GFP antibodies. Normalized quantification of AstC cycling in the DN1ps under the second day constant darkness (DD2) (**B**) or short photoperiod of 6:18 LD (**C**). AstC is more abundant during the dark phase in comparison to the light phase. Two biological replicates are averaged. Error bars are SEM. $n \geq 5$ brains per condition.

Figure S2. **Immunostaining validation of AstC RNAi knock-down in all clock cells. Related to Figure 4.**

AstC-RNAi was expressed with *tim*-GAL4>,*dcr* to efficiently knock-down AstC peptide in all clock cells. The GAL4 control (**A**,**B**), UAS control (**C**,**D**), and the experimental knock-down genotype (**E**,**F**) were stained with anti-AstC (red) and anti-TIM (green) at ZT20. ***A***-***D***, In both parental controls, AstC is expressed in four DN1ps, approximately half the DN3s, and the three LPNs. ***E***,***F***, AstC expression is abolished in all clock neurons in the experimental knock-down group. Examination of the whole brain shows that only the clock neurons showed reduced AstC expression. At least eight brains of each genotype were stained together under the same conditions. Confocal settings were maintained the same for all genotypes. One hemisphere is shown here.

Figure S3. Representative actograms in 6:18 LD. Related to Figure 4.

Averaged actograms from two individual flies are shown for the *tim*-GAL4 control (top, black), the *AstC*-RNAi control (middle, gray), and the experimental group with AstC knocked-down in all clock neurons (bottom, red). The arrows indicate the timing of the maximum E-peak considered for analysis. Actograms averaged from Days 3-6 in 6:18 LD. Y-axis measures total activity counts per minute.

Figure S4. AstC knock-down in subsets of clock neurons has no significant effects in E-peak under short photoperiod. Related to Figure 4.

A, Boxplot distribution showing the evening peak phase measured from individual flies. The *Clk856*-GAL4 mediated *AstC*-RNAi knock-down (pink) is not significantly different from both parental controls. AstC knock-down mediated by *Clk4.1M*-GAL4 (purple) and *GMR*-GAL4 (brown) also showed no significant differences compared to their respective parental controls. n.s. no significant difference ($p \geq 0.05$, one-way ANOVA). "+" indicates the mean and the whiskers denotes the 10^{th}/90^{th} percentiles. ***B-J***, Immunostaining of *CLK856*-GAL4 mediated AstC knock-down reveals ~11-13 DN3s are unaffected. The GAL4 control (B-D), UAS control (E-G), and the experimental knock-down genotype (H-J) were stained with anti-AstC (red) and anti-TIM (green) at ZT20. ***B-G***, AstC is expressed in four DN1ps, approximately half the DN3s, and the three LPNs in both parental controls. ***H-J***, Through RNAi-mediated knock-down, AstC expression is abolished in the four DN1ps, three LPNs, and some DN3s. Approximately 11-13 DN3s still express AstC, denoted by the arrow. At least eight brains of each genotype were stained together under the same conditions. Confocal settings were maintained the same for all genotypes.

Figure S5. AstC in clock neurons regulates evening phase in long photoperiod days. Related to Figure 4.

A, Normalized averaged actograms of the *tim*-GAL4 control (black, n=48), *AstC*-RNAi control (gray, n=56), and the AstC RNAi knock-down in all clock cells mediated by *tim*-GAL4 (red, n=52) under long photoperiod of 18:6 light:dark (LD) condition at 27°C. The red arrow denotes the delay of the evening peak when AstC is knocked-down compared to the two genetic controls. White and dark boxes indicate the respective light and dark phases. The error bars represent SEM. ***B***, Boxplot distribution showing the evening peak phase from individual flies under 18:6 LD. The *tim*-GAL4 mediated *AstC*-RNAi knock-down (red) is significantly delayed compared to both the *tim*-GAL4 control (black) and *AstC*-RNAi control (gray; $p < 0.0001$, one-way ANOVA). These data are combined from two independent biological repeats. *** $p < 0.0001$. "+" indicates the mean and the whiskers denotes the $10^{th}/90^{th}$ percentiles.

Figure S6. AstC-R2 in LNds is required to regulate evening phase in short photoperiod days. Related to Figure 5.

A, Averaged actograms of the *MB122*-sGAL4 control (gray, n=23), *AstC R2*-RNAi control (black, n=15), and the AstC R2-RNAi knock-down in the LNds mediated by *MB122*-sGAL4 (orange, n=12) under short photoperiod 6:18 LD at 27°C. White and dark boxes indicates the respective light and dark phases. Light gray lines represent SEM. ***B***, Boxplot distribution showing the evening peak phase from individual flies. The *MB122*-sGAL4 mediated *AstC R2*-RNAi knock-down (orange) is significantly delayed compared to both the *MB122*-sGAL4 control (gray) and *AstC R2*-RNAi control (black; $p < 0.001$, one-way ANOVA). ** $p < 0.001$.

n.s. no significant difference ($p \geq 0.05$). “+” indicates the mean and the whiskers denotes the $10^{th}/90^{th}$ percentiles.

Genotype	n	Period (hours) ± SEM	Rhythmic flies (%)
tim-GAL4>*dcr*	21	24.03 ± 0.06	80.77%
AstC-RNAi	18	24.00 ± 0.09	81.81%
tim-GAL4>*dcr*; *AstC*-RNAi	18	24.23 ± 0.06	100%

Table S1- AstC knock-down modestly lengthens the free-running period. Related to Figure 4.

Young male flies were entrained to 12:12: LD before shifting to constant darkness for at least five days. Each genotype showed normal rhythmicity. The free-running period of rhythmic flies was not statistically different among the three genotypes (*p*-value > 0.05, one-way ANOVA).

Probe number	Probe sequence (5’ to 3’)
1	agggaaattggtaactcgat
2	tctatcgatgtgaacgccaa
3	gcgttaactagttgctgttg
4	agtttggatctgatcctttg
5	tgatgtggagtttcgtttgg
6	atttgtgttagtttttcggc
7	cgtcaatcggtaatttgcga
8	tgagaaaaatctggcgccgg
9	cggcaatgaaattggcgtcg
10	cgtctggatgagattgggaa
11	aaacgcttctgcacgatact
12	tctgcacgaatttcatcatt
13	agtaggccgtagcacaataa
14	gagggcaaagaacagggtga
15	catcaagtccatcggagtcc
16	tattgggatagattgcctgg
17	aaagagcatctgcagccgat
18	tgtaactggtgggtctgtat
19	taggtgggactcctcaaata
20	gcagccgataaagttcattc
21	ctaacttgacgtttgctctc
22	gattaaagtagcactgccgg
23	acttacttcctaaagcagga
24	cggatattcttcccactgtc
25	agtttaaccaagagctctgg
26	tgcgtttatccattatgcta
27	gttgagtcttagctttagtt

28	tttcatacgtttcgtagaca
29	taccacaccatatgcaaagc
30	gcttattgaagcattgtatt
31	gcacacacattgaggaatgc
32	atacattagctgggacactt
33	tcctgtttgttgttctaatt
34	ttagatgacaccaagtttgc
35	gtatagttttgtgtaggagg
36	ggtcgcatttagtttattcg

Table S2- Sequences of AstC oligonucleotide probes for fluorescent *in situ* hybridization. Related to Figure 2.