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Supplemental information

Gap junction protein Innexin2 modulates the period of free-running rhythms in *Drosophila melanogaster*

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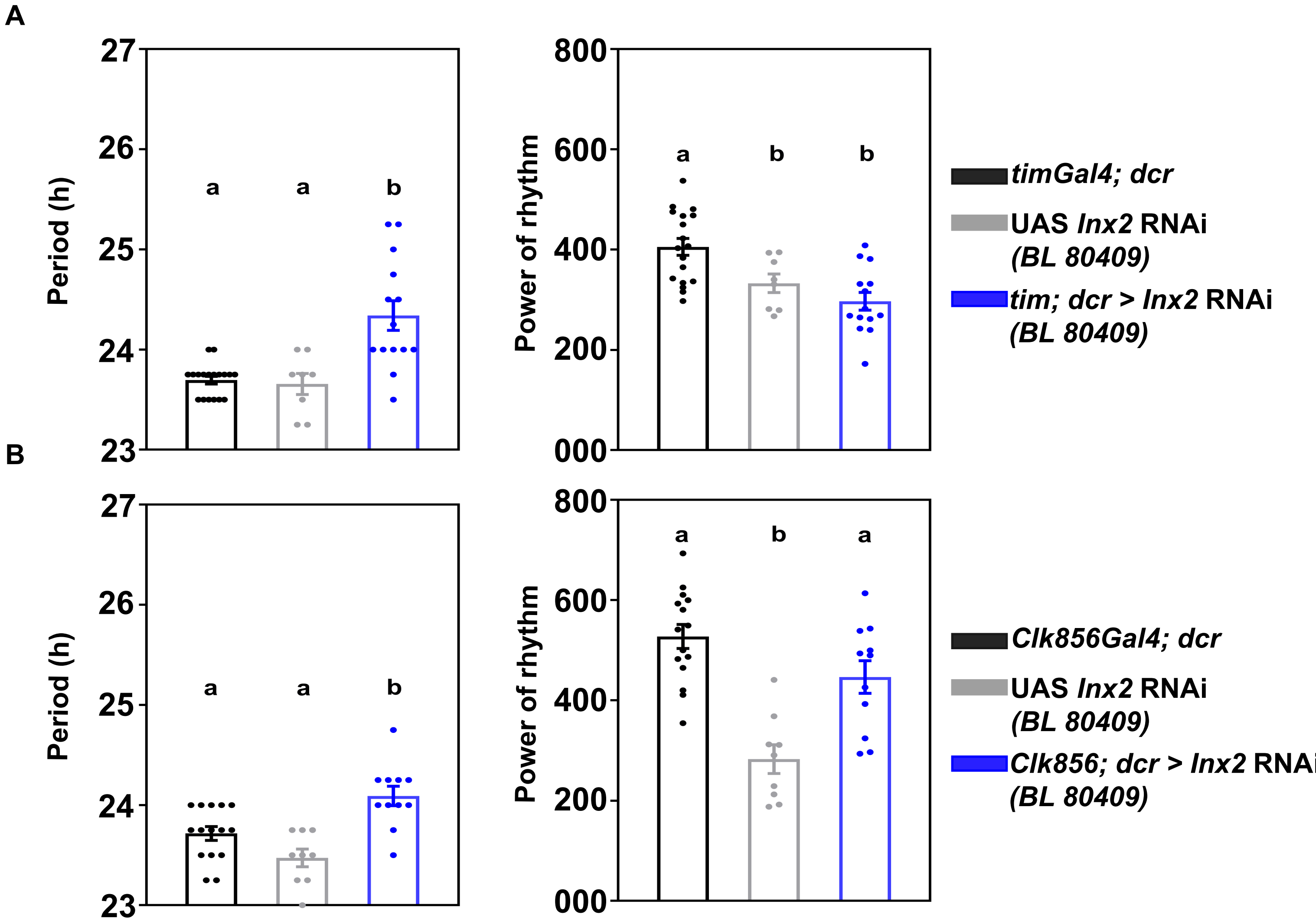
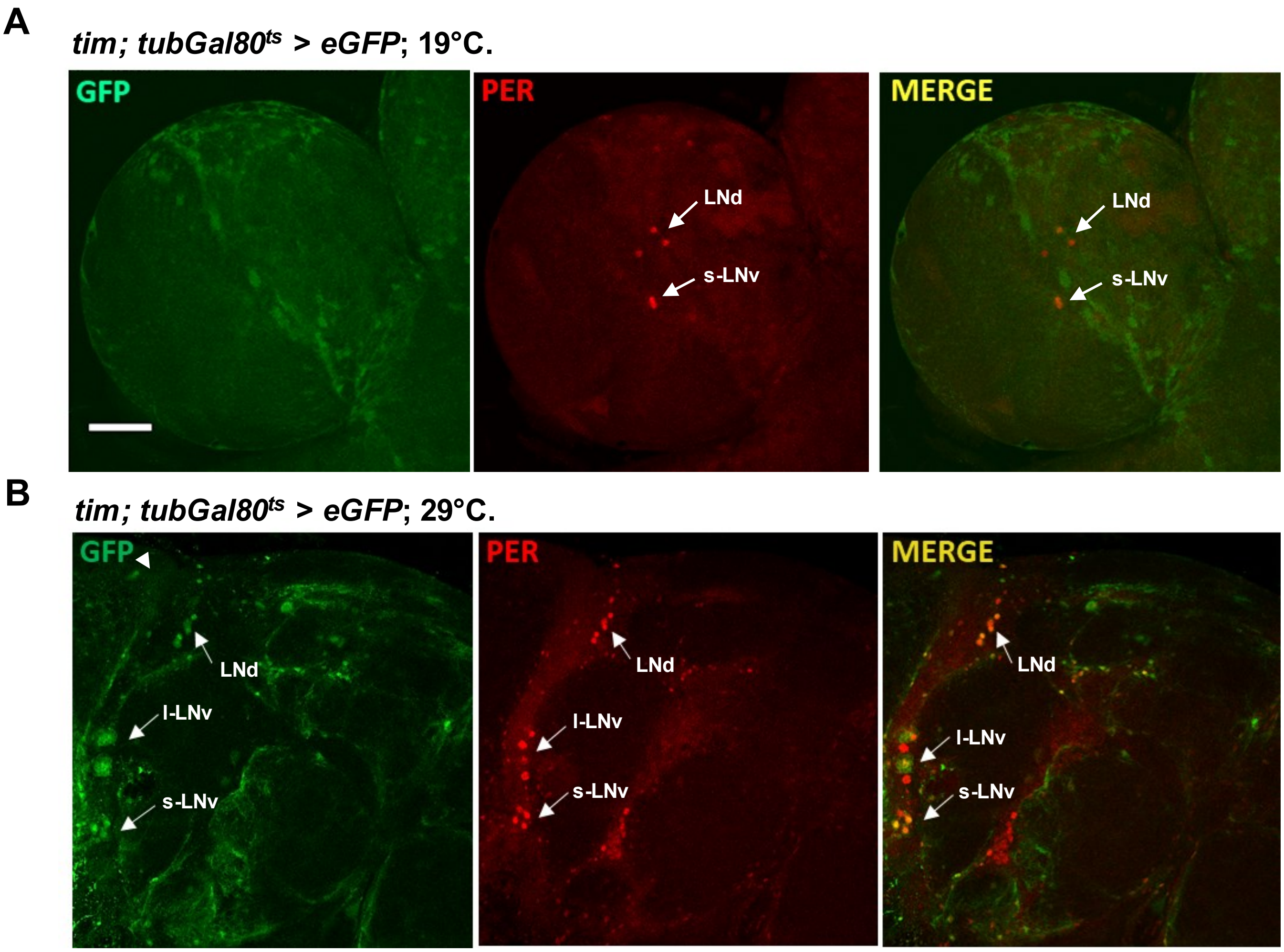
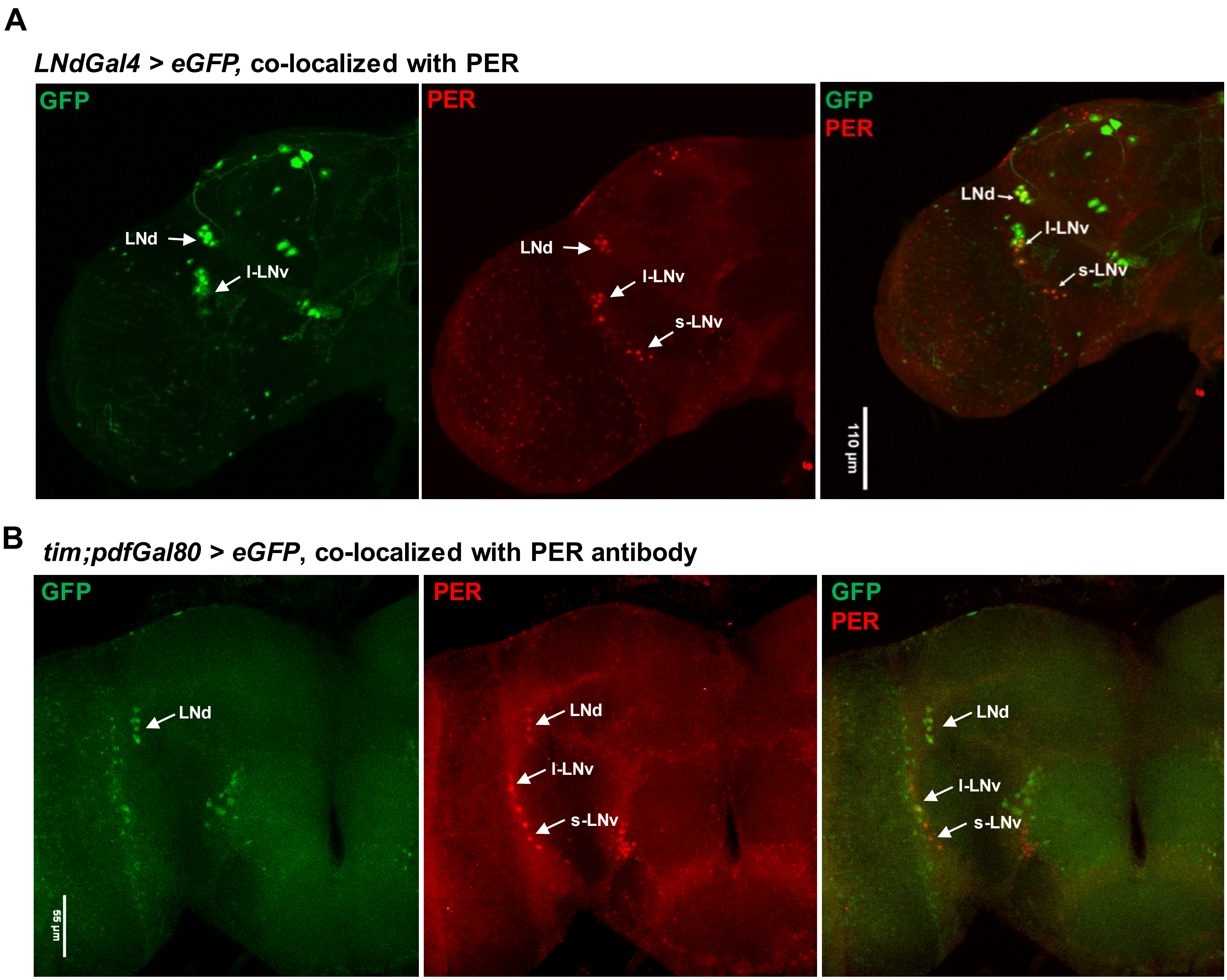


Supplementary fig. S1: Knockdown of *Innexin2* using a different construct (BL 80409) lengthens free-running period, related to Figure 2 (A) Mean free-running period (left) of flies with *Innexin2* downregulated in all clock neurons (*tim; dcr*> *Inx2* RNAi) (*n*=15) is significantly longer than both its Gal4 (*n*=18) and UAS control (*n*=8) genotypes, (one-way ANOVA, post-hoc Tukey's test, $p<0.01$) while the power of rhythm (right) is significantly lower from only the Gal4 control, (One-way ANOVA, post-hoc Tukey's test, $p<0.001$). **(B)** Mean free-running period (left) of flies with *Innexin2* downregulated in all clock neurons (*Clk856 > dcr; Inx2* RNAi) (*n*=11) is significantly longer than both its Gal4 (*n*=15) and UAS control (*n*=9) genotypes, (one-way ANOVA, post-hoc Tukey's test, $p<0.01$). Power of rhythm of experimental flies is only significantly different from the UAS control flies (One-way ANOVA, post-hoc Tukey's test, $p<0.001$).

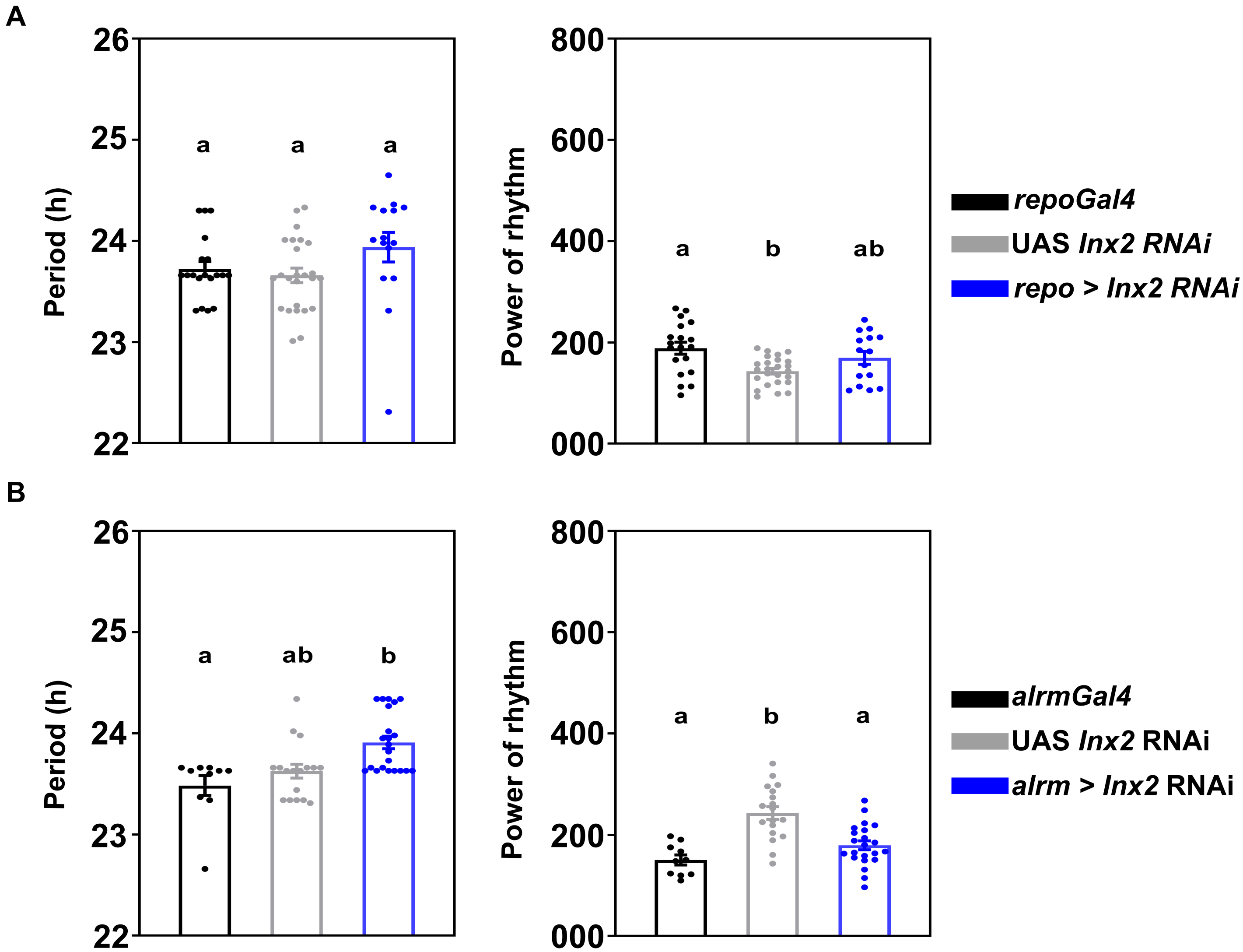
Error bars are SEM, period values are determined using Chi-square periodogram for a period of 8 days. All statistical comparisons were performed using one-way ANOVA with genotype as a fixed factor, followed by post-hoc analysis using Tukey's Honest Significant Difference (HSD) test.



Supplementary fig. S2: Verification of the efficiency of *tubGal80^{ts}* construct, related to Figure 3 (A) The efficiency of *tim; tubGal80^{ts}* construct was verified by crossing with *eGFP*. The flies were reared at a permissive temperature of 19°C. Larvae (L3 stage) from the progeny were dissected and stained with anti-GFP and anti-PER antibodies. s-LNv do not show presence of GFP in permissive temperatures (left), whereas strong PER staining was observed in these cells (middle panel). **(B)** Adult male flies were transferred to 29°C, 3 days after eclosion, dissected on day 7 and stained with antibodies against GFP and PER. s-LNv, l-LNv and LNds show GFP expression under a restrictive temperature of 29°C. *n=5* brain samples at each temperature. Scale bars represent 55µm.

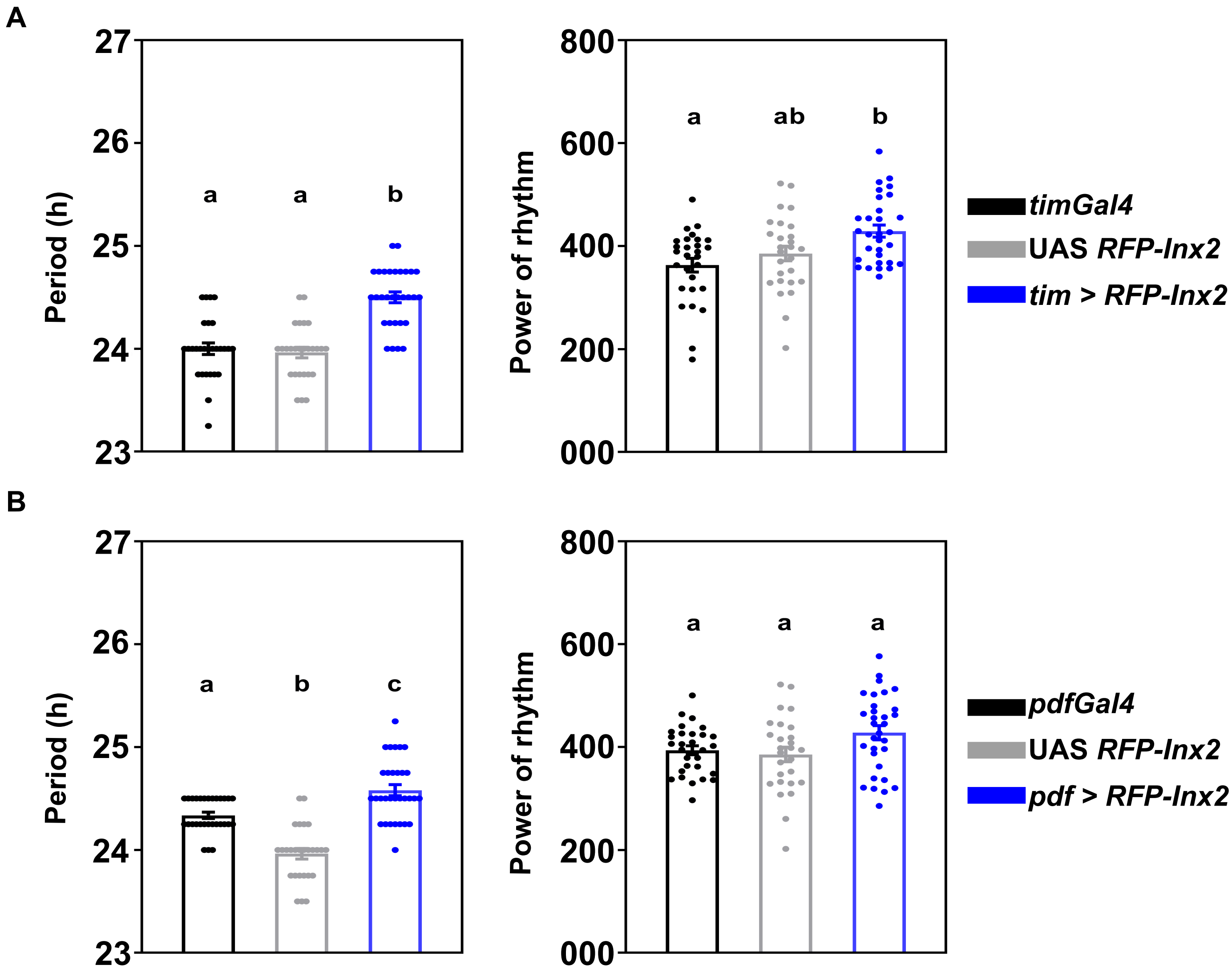


Supplementary fig.S3: Expression pattern of *LNdGal4* and verification of the efficiency of *pdfGal80* construct, related to Figure 4 (A) *LNdGal4 > e-GFP* co-stained with anti-PER antibody shows strong GFP expression in all 6 LNds and faint GFP expression in some l-LNvs as previously reported. No GFP expression was detected in s-LNvs, *n=5* brain samples. Scale bar represents 110µm. **(B)** The efficiency of *tim*; *pdfGal80* construct in suppressing *Gal4* driven UAS expression was verified by crossing *timGal4*; *pdfGal80* with GFP, dissecting the brains at ZT22 and staining with GFP and PER. Both s-LNv and l-LNv did not show any GFP staining (left), whereas PER staining was observed in all lateral clock neurons (middle). *n*=6 brain samples. Scale bars represent 55µm.



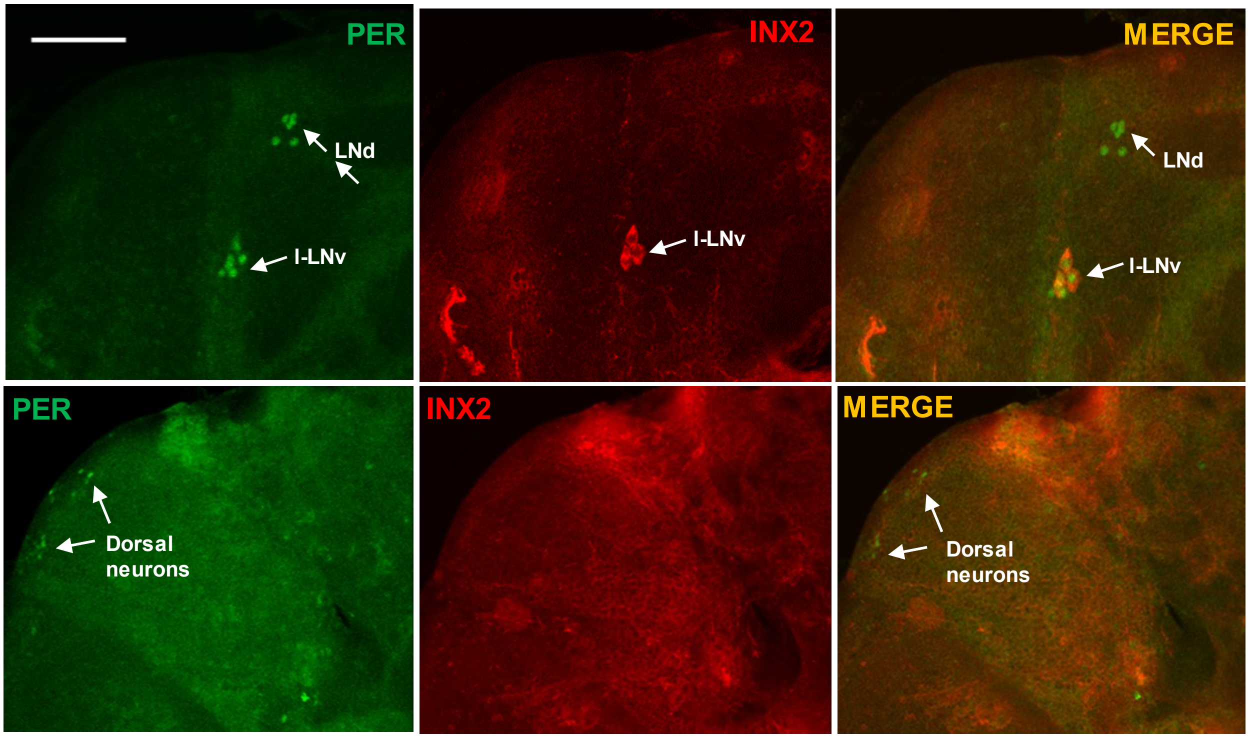
Supplementary fig. S4: Knockdown of *Innexin2* in glial cells does not affect the free-running period, related to Figure 4 (A) Free-running period of experimental flies (*repo* > *Inx2* RNAi) (*n*=15) is not significantly different from both its Gal4 (*n*=21) and UAS (*n*=25) parental control flies. Power of rhythm (right) of experimental flies is also not different from its parental control genotypes. **(B)** Free-running period of experimental flies (*alrm* > *Inx2* RNAi) (*n*=26) is only significantly different from its Gal4 control (*n*=15) and not from its UAS parental control (*n*=18) flies (one-way ANOVA followed by post-hoc Tukey's test, $p < 0.01$). Power of rhythm of experimental flies is only different from its UAS parental control (one-way ANOVA followed by post-hoc Tukey's test, $p < 0.001$).

Error bars are SEM, period and power values are determined using Chi-square periodogram for a period of 8 days. All statistical comparisons were performed using one-way ANOVA with genotype as a fixed factor, followed by post-hoc analysis using Tukey's Honest Significant Difference (HSD) test.

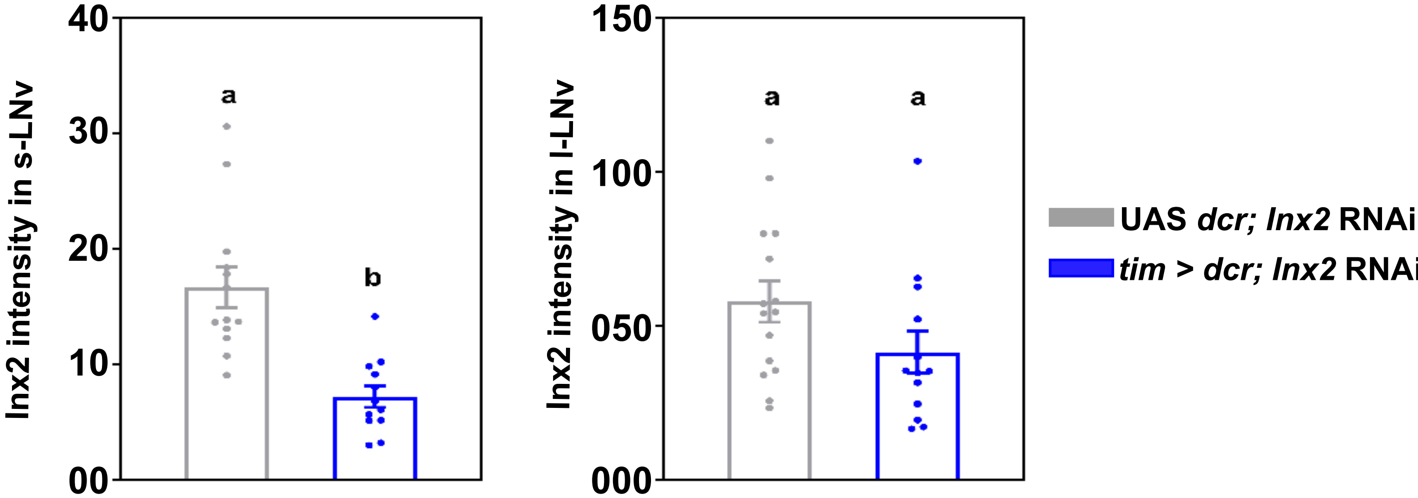


Supplementary fig. S5: Altering the gap junction forming domain of Innexin2 lengthens free-running period, related to Figure 4 (A) Free-running period of experimental flies (*tim* > *RFP-Inx2*) (*n*=30) is significantly longer than both its Gal4 (*n*=28) and UAS (*n*=27) parental control flies, (one-way ANOVA, post-hoc Tukey's test, $p < 0.001$). Power of rhythm (right) of experimental flies is only different from its Gal4 control genotype (one-way ANOVA, post-hoc Tukey's test, $p < 0.01$). **(B)** Free-running period of experimental flies (*pdf* > *RFP-Inx2*) (*n*=31) is significantly longer than both its Gal4 (*n*=29) and UAS (*n*=27) parental control flies, (one-way ANOVA, post-hoc Tukey's test, $p < 0.001$). Power of rhythm of experimental flies is not different from both its control genotypes.

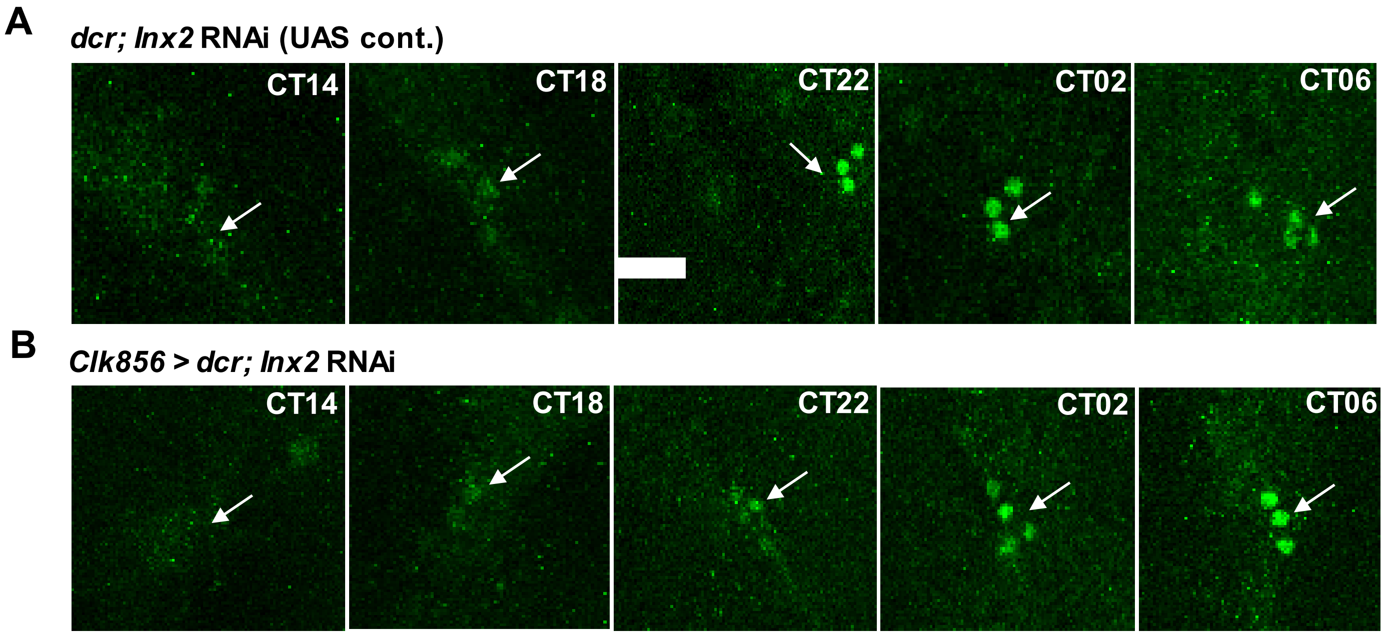
Error bars are SEM, period and power values are determined using Chi-square periodogram for a period of 8 days, All statistical comparisons were performed using one-way ANOVA with genotype as a fixed factor, followed by post-hoc analysis using Tukey's Honest Significant Difference (HSD) test.



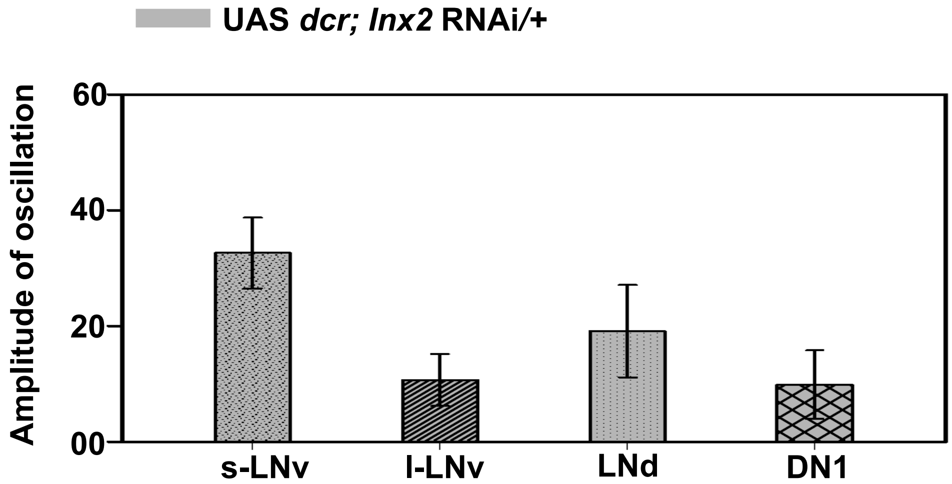
Supplementary fig. S6: Innexin2 is only expressed in the small and large ventral lateral neurons in the circadian pacemaker circuit, **related to Figure 5** Representative images from w^{1118} *Drosophila* adult brains showing the distribution of Inx2 protein among the clock cells. w^{1118} flies were dissected at ZT4 and stained with anti-INX2 antibody and co-stained with anti-PER for identification and co-localization with clock neurons. INX2 was not found to be co- localized with the LNds (top panel) or the DNs (bottom panel). Brightness and contrast of representative images were adjusted in Fiji to facilitate better visualization. Arrows are used to indicate l-LNvs, LNds and DNs. Scale-bar represents 55 µm, *n* = 8 brain samples.



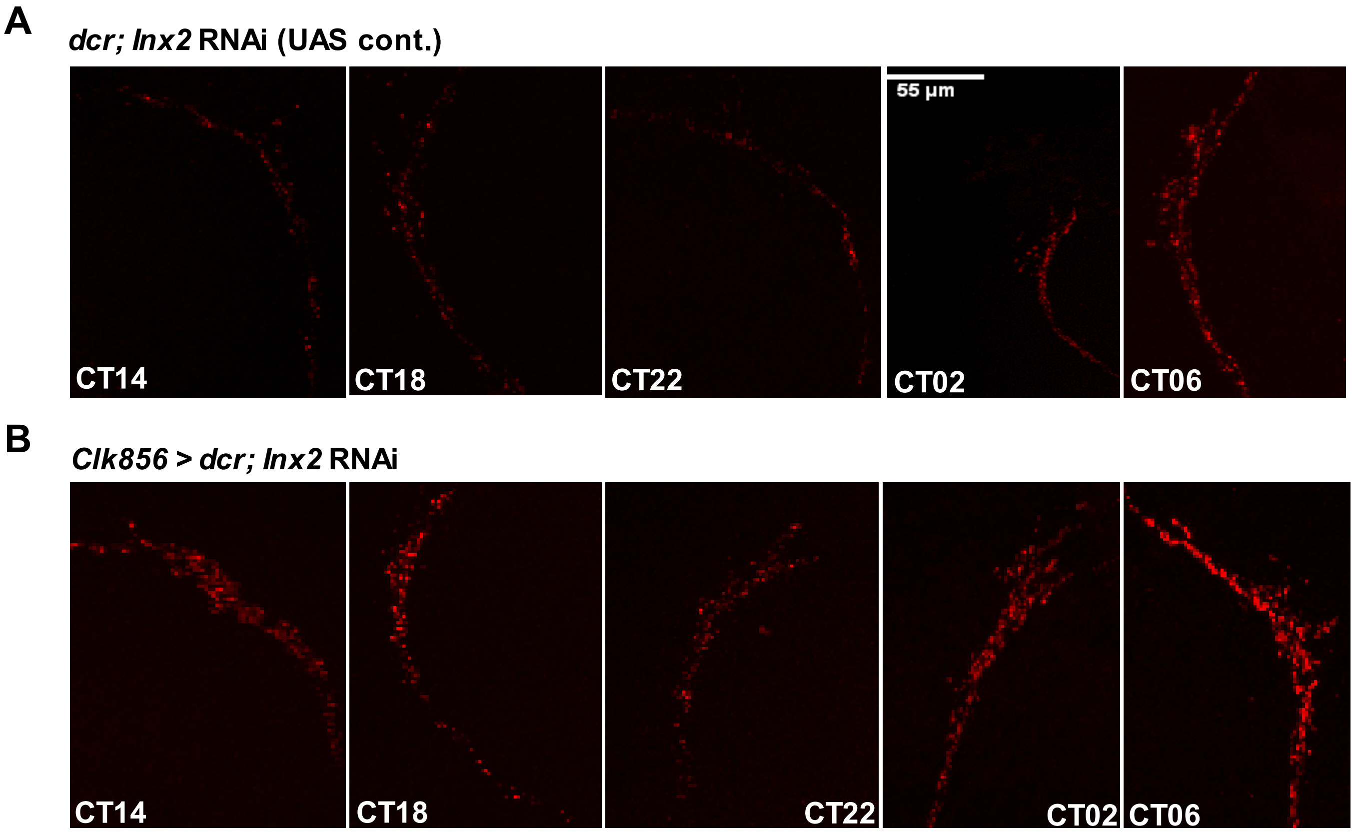
Supplementary fig. S7: Verification of *Innexin2* RNAi, related to Figures 2, 4 and 5 *Innexin2* RNAi construct was verified by dissecting adult brains of both control (*dcr; Inx2* RNAi) and experimental flies (*tim > dcr; Inx2* RNAi) at ZT12 and staining them with anti-INX2 antibody. Inx2 intensity was quantified in both s-LNvs and l-LNvs and compared between control and experimental flies. Inx2 levels were lower in s-LNvs (left) in case of experimental flies (n=12 brains, Mann Whitney U test, $p < 0.001$). In l-LNvs (right) there was only a decreasing trend observed in Inx2 levels in experimental flies as compared to control flies (n=13 brains, Mann Whitney U test, $p > 0.05$). Error bars are SEM. Each dot represents the mean Inx2 intensity value for each cell type averaged over both the hemispheres of one brain. The experimental and control genotypes were compared using the Mann-Whitney U test for each cell type.



Supplementary fig. S8: Knockdown of *Innexin2* delays the phase of oscillation of PER protein in s-LNv, related to Figure 6: Representative images of PER intensity in s-LNv at five different time points of a 24-h cycle on the third day of DD 25°C in both control (UAS *dcr Inx2* RNAi) **(A)** and experimental (*Clk856* > *dcr;Inx2* RNAi) **(B)** flies. Phase of PER oscillations is delayed in brain samples of experimental flies as compared to the control flies. Scale bar represents 55µm.



Supplementary fig. S9: Amplitude of PER oscillations are different in circadian neuronal subsets on third day of DD, related to Figure 6 Amplitude values of PER oscillation in circadian neuronal subsets, obtained from COSINOR curve fits are plotted for control (UAS *dcr; Inx2* RNAi) flies . Error bars are 95% CI values calculated from the standard error obtained from COSINOR analysis. Non-overlapping error bars in case of l-LNv, and DN1 compared to s-LNv indicate that these amplitude values are different from s-LNv. COSINOR analysis was implemented using the CATCosinor function from the CATkit package written for R (Lee Gierke and Cornelissen, 2016).



Supplementary fig. S10: Knockdown of *Innexin2* increases the amplitude of PDF cycling in dorsal projections, related to Figure 7 Representative images of PDF intensity in s-LNv dorsal projections at five different time points of a 24-h cycle on third day of DD 25°C in both control (UAS *dcr;Inx2* RNAi) **(A)** and experimental (*Clk856* > *dcr*;*Inx2* RNAi) **(B)** flies. Amplitude of PDF oscillation is increased in experimental genotype as compared to the controls. Scale bar represents 55µm.