Activity-dependent capture of neuropeptide vesicles prepares clock neuron synapses for daily release

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Short title: Rhythmic vesicle capture in clock neurons

12 pages

3 Figures

Funding: This research was supported by National Institutes of Health grant R01NS032385 to ESL.

***Drosophila* brain sLNv clock neurons release the neuropeptide PDF to control circadian rhythms. Strikingly, PDF content in sLNv terminals is rhythmic with a peak in the morning. Peak content drops because of activity-dependent release from dense-core vesicles (DCVs), but the mechanism for the daily increase in presynaptic PDF in the hours prior to release is unknown. Although transport from the soma was proposed to drive the daily increase in presynaptic PDF, live imaging in sLNv neurons shows that anterograde axonal DCV transport is constant throughout the day. Instead, capture of circulating DCVs, indicated by decreased retrograde axonal transport, rhythmically boosts presynaptic neuropeptide content. Genetic manipulations demonstrate that the late night increase in capture requires electrical activity but is independent of daily morphological changes. These results suggest that each day, during the hours of ongoing electrical activity, a toggle switches from inducing vesicle capture to triggering exocytosis, thereby maximizing daily rhythmic bursts of synaptic neuropeptide release by clock neurons.**

Introduction

Drosophila sLNv neurons release the neuropeptide Pigment Dispersing Factor (PDF) to synchronize molecular rhythms of individual neurons in the clock circuit in order to maintain daily rhythms in motor activity (Yoshii *et al.*, 2009; Ahmad *et al.*, 2021). Interestingly, PDF content in these neurons is rhythmic (Park *et al.*, 2000) with a drop in presynaptic PDF in the morning being due to activity-dependent dense-core vesicle (DCV) exocytosis (Klose *et al.*, 2021). Furthermore, rhythmic exocytosis is

preceded by an hours long surge in terminal neuropeptide content (Park *et al.*, 2000; Klose *et al.*, 2021) that promotes release because of the scaling of release with presynaptic neuropeptide stores (Bulgari et al., 2014; Cavolo et al., 2016). Daily increases in presynaptic PDF stores are not driven by PDF gene expression (Park *et al.*, 2000). Instead, transport from the soma was proposed to control sLNv terminal neuropeptide content (Fernandez-Chiappe *et al.*, 2021). However, because anterograde axonal transport of DCVs has not been examined directly, the mechanism underlying the rhythmic increase in presynaptic neuropeptide stores in sLNv clock neurons has remained unknown.

Recently, Klose, *et al.* (2021) found that rhythmic changes in PDF content observed in sLNv neurons (Park *et al.*, 2000) are recapitulated with the DCV marker Dilp2-GFP (GFP-tagged *Drosophila* proinsulin-like peptide 2) (Wong *et al*., 2012). Here, Dilp2-GFP imaging is used to determine how DCV traffic in sLNv axons produces daily increases in presynaptic neuropeptide in preparation for release. These experiments do not support a role for increased anterograde axonal transport, but instead show that rhythmic DCV capture increases presynaptic neuropeptide accumulation long before daily bouts of release. We then investigate how this capture relates to concurrent morphological plasticity (Fernandez *et al.*, 2008) and electrical activity, which triggers release following presynaptic neuropeptide accumulation (Klose *et al.*, 2021).

Results and Discussion

To test the hypothesis that increased delivery of DCVs upregulates neuropeptide stores in sLNv terminals in the morning, axonal transport of GFP-labeled DCVs was

imaged in brain explants isolated at different times of the day from PDF>Dilp2-GFP (UAS-Dilp2-GFP; PDF-GAL4) flies. Initially, the ratio of anterograde to retrograde DCV flux (A/R) was measured from kymographs (Fig. 1A). These experiments showed that the A/R ratio changed ($P < 0.001$, one-way ANOVA) with a peak late at night (i.e., at ZT23) (Fig. 1B,C), a time when *Drosophila* become active in anticipation of the morning and PDF is accumulating in terminals in preparation for its daily bout of release. Thus, prior to release there is a net increase in axonal transport of DCVs to upregulate presynaptic neuropeptide stores.

Follow-up focused on determining which component of the A/R ratio changed. Analysis of axonal transport showed that anterograde DCV flux (A) was constant at all times tested (Fig. 2A), thus ruling out an effect on A/R driven by changes in DCV biogenesis and delivery from the soma. Rather, retrograde axonal DCV flux (R) changed ($P < 0.01$, one-way ANOVA) with a minimum in the late night at ZT23 (Fig. 2B). Since the drop in retrograde transport was evident hours prior to rhythmic synaptic neuropeptide release (Klose *et al.*, 2021), it cannot be attributed to DCV depletion by exocytosis. Furthermore, decreased retrograde flux occurred without altering retrograde DCV velocity (Fig. 2C), thus excluding slowed motor function. Therefore, reduced retrograde transport while presynaptic neuropeptide levels are increasing is indicative of increased synaptic capture of circulating DCVs (Shakiryanova *et al.*, 2006; Wong *et al.*, 2012).

Rhythmic changes in presynaptic neuropeptide stores (Park *et al.*, 2000) overlap with the morphological expansion of the sLNv axonal arbor (Fernandez *et al.*, 2008). To probe whether presynaptic DCV capture and terminal morphology are linked, DCV

axonal transport was studied in a PDF receptor (PDFR) null mutant (*han*) that prevents daily changes in terminal morphology while preserving rhythmic changes in PDF (Herrero *et al.*, 2020). In PDFR null animals the rhythmic change in axonal anterograde to retrograde transport ratio persisted (Fig 3A). Therefore, rhythmic DCV capture occurs independently of morphological plasticity.

Genetic and cell biology experiments have demonstrated that capture of circulating DCVs at synaptic boutons occurs by independent mechanisms that operate either constitutively or in response to activity (Shakiryanova *et al.*, 2006; Wong *et al.*, 2012; Cavolo *et al.*, 2016). Activity-dependent capture was discovered at the neuromuscular junction based on drop in retrograde axonal DCV transport (Shakiryanova *et al.*, 2006). Furthermore, silencing electrical activity eliminates rhythmic changes in PDF in sLNv terminals (Depetris-Chauvin *et al.*, 2011). Therefore, we tested the hypothesis that electrical activity is required for the difference in DCV capture in sLNv neurons that is evident between ZT23 and ZT3. For this purpose, cell specific expression of the Kir2.1 channel was used to completely silence LNv neurons electrical activity (Nitabach *et al.*, 2002; Depetris-Chauvin *et al.*, 2011). Kir2.1 expression produced large A/R ratios (Fig. 3B), suggesting that there is a compensatory shift to constitutive capture with the disruption of the daily bout in activity-dependent capture. Moreover, with regard to the hypothesis, the change in A/R ratio was lost with electrical silencing. Thus, the daily increase in capture in preparation for synaptic neuropeptide release in the morning depends on activity.

DCV capture in the hours preceding the daily bout of exocytosis maximizes rhythmic activity-dependent synaptic neuropeptide release by sLNv clock neuron

terminals. However, this timing was unexpected because activity-evoked DCV exocytosis and capture occur in rapid succession at the neuromuscular junction (Shakiryanova *et al.*, 2006). It is also puzzling that rhythmic release by LNv neurons (Klose *et al.*, 2021) is more time delimited than electrical activity (Cao and Nitabach, 2008; Sheeba *et al.*, 2008; Muraro and Ceriani, 2015). These observations are consistent with sLNv neuron activity being a conditional trigger that toggles from inducing capture to hours later inducing exocytosis. Toggling could be controlled by ionotropic and neuromodulatory synaptic inputs to sLNv neurons (Muraro and Ceriani, 2015; Duhart *et al.*, 2020; Shafer *et al.*, 2022; Reinhard *et al.,* 2023) or the cell autonomous functioning of the clock so that activity-dependent capture mediated by kinases (Wong *et al.*, 2009; Bharat *et al.*, 2017) occurs long before activity-evoked exocytosis. Therefore, we propose that a toggle for activity effects explains the timing of capture and release that optimizes neuropeptide release by sLNv clock neurons.

Methods

Imaging: Flies were entrained for at least for 72 hrs in a 12 hour light: 12 hour dark schedule before dissection of 4-9 day old males to generate brain explants. Dissections during the dark phase were performed under a red light. Adult flies were immobilized with CO_2 gas and brains were dissected in 0 Ca^{2+} HL3 saline solution (70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$*$6H_2O$, 115 mM Sucrose, 5 mM Trehalose, 5 mM Hepes, and 10 mM $NaHCO_3$, pH 7.2) and then put into polylysine coated plastic dishes containing HL3 with 2 mM Ca^{2+} for imaging (Klose et al., 2016). Two regions were imaged on each hemisphere of the brain on an upright Olympus microscope equipped with a 60×1.1 NA

dipping water immersion objective, a Yokogawa spinning disk confocal head, a 488 nm laser for GFP illumination, and a Teledyne Photometrics Prime 95B sCMOS camera. Time-lapse experiments entailed acquiring 180 images with 150 ms exposure times at 3 Hz. From these movies, regions where dorsally projecting axons defasciculated from bundles were used for generating kymographs with the Kymograph plugin of Imagej. Statistical analysis of DCV flux and velocity data was performed with Graphpad Prism software.

Fly lines: A plasmid for UAS-Kir2.1 without a GFP tag provided by Sean Sweeney (University of York) was used by Bestgene Inc. to generate transgenic flies with insertion onto chromosome III. All flies used the PDF-Gal4 promoter on the third chromosome (provided by Paul Taghert, Washington University in St. Louis). UAS-Dilp2-GFP was reported previously (Wong *et al.*, 2012), Finally, other lines were from the Bloomington *Drosophila* stock center (*Bl#* 35785 and *Bl#* 33068, *han*).

Acknowledgements

We thank Sean Sweeney (University of York) for providing the UAS-Kir2.1 plasmid and Paul Taghert (Washington University in St. Louis) for the PDF-GAL4 (III) flies. We also thank David Deitcher (Cornell University) for his comments.

Legends

Figure 1. Dense-core vesicle flux in sLNv nerve terminal projections. Ai. sLNv projections containing DCVs filled with Dilp2-GFP at t=0.33 seconds of recording. White box reveals region of interest (ROI) for analysis. Aii. DCVs at t=0, 0.33, 0.66, and 1.0

seconds (red arrow indicates anterograde (AR) traffic and blue arrow indicates retrograde (RE) traffic, scale bar = 1 μm). Aiii. Kymograph of ROI (red arrow anterograde, blue arrow retrograde traffic). B. Kymographs reveal both anterograde (slope down, left to right) and retrograde (slope up, left to right) DCV traffic in sLNv projections of PDF>Dilp2-GFP flies at ZT 3 and ZT 23. C. Flux ratios (anterograde/retrograde) at different times of the day. n values for ZT 0, 3, 5, 8, 11, 14, 17, 20, 21, 22, 23 are 4, 10, 6, 6, 8, 6 ,8, 8,12, respectively. One-way ANOVA revealed significant difference ($P < 0.001$). Post-test analysis by Dunnett's multiple-comparison test is presented: ****$P < 0.0001$, ***$P < 0.001$, *$P < 0.05$.

Figure 2. Dense-core vesicle flux and velocity in sLNv nerve terminal projections. A. Anterograde flux and B. Retrograde flux (vesicles/min) in PDF>Dilp2-GFP brains. n= for ZT 2-4, 8-10, 14-16, and 23 are 11,12,10, and 9, respectively. $P < 0.01$, one-way ANOVA. **$P < 0.01$, Dunnett's multiple-comparison post-test. C. Retrograde velocity of dense-core vesicles at ZT 3 and 23 in PDF>Dilp2-GFP brains (left, average of each brain; ZT 3 n = 4, ZT 23 n = 4; right, average of all vesicles measured: ZT 3 n=49, ZT 23 n=51).

Figure 3. Dependence of rhythmic capture on PDFR and electrical activity. A. Ratios of anterograde to retrograde DCV transport (A/R) at ZT3 and ZT23 in *w*; UAS-Dilp2-GFP; PDF Gal-4 control (ctrl) and *han*; UAS-Dilp2-GFP; PDF-Gal4 PDFR null (*han*) sLNv axons. n $\geq$ 13 for ctrl; n > 20 for *han*. For both ctrl and *han*, ****$P < 0.0001$, unpaired t-test. B. Ratios of anterograde to retrograde sLNv axonal DCV transport

(A/R) at ZT3 and ZT23 in controls in which PDF>Dilp2-GFP and *Bl#35785* were crossed to take into account expression of two UAS constructs (left two columns) and in experimental animals in which PDF>Dilp2-GFP and UAS-Kir2.1 flies were crossed (Kir, right two columns). In controls, n was 15 at ZT3 and 6 at ZT23. In Kir2.1, n = 14 for each time point. **P < 0.01 for control by unpaired t-test, while there was no significant difference for Kir (p = 0.62).

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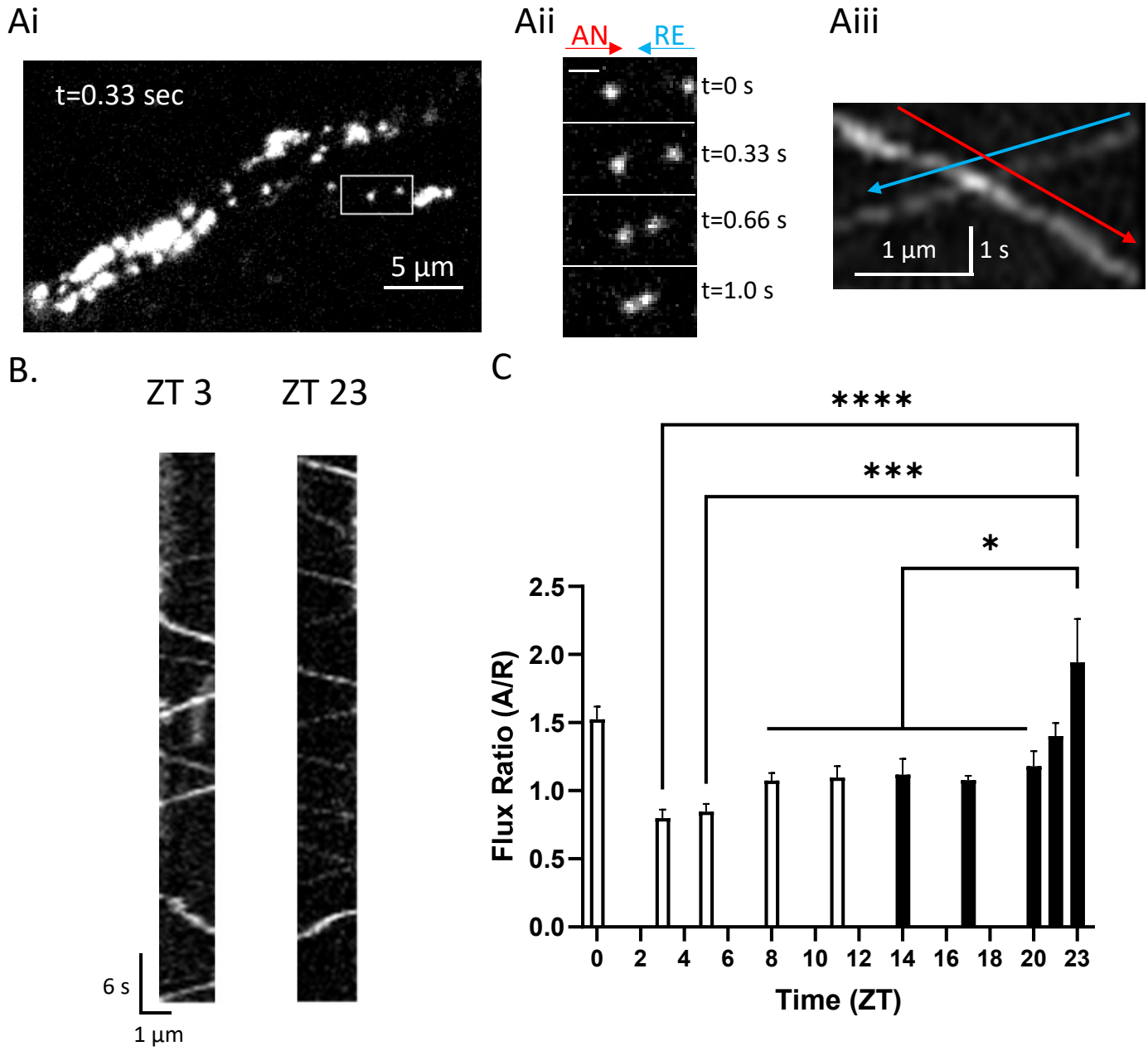
Ai
t=0.33 sec
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t=0.66 s
t=1.0 s
Aiii
1 µm
1 s
B.
ZT 3
ZT 23
6 s
1 µm
C
****
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*
Flux Ratio (A/R)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
23
Time (ZT)

Figure 1

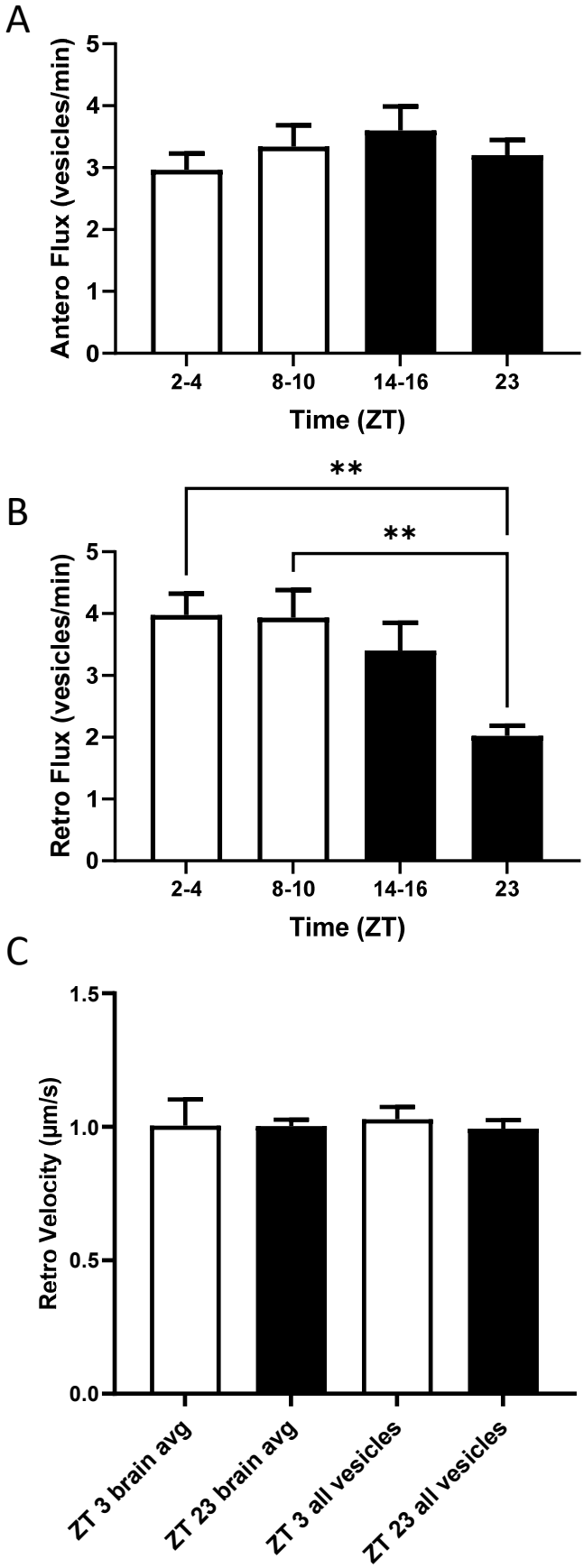
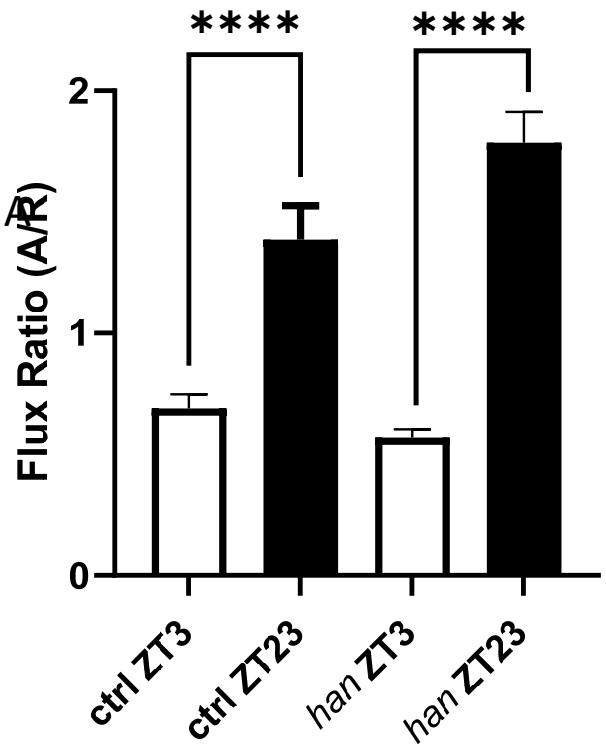

A
Antero Flux (vesicles/min)
0
1
2
3
4
5
2-4
8-10
14-16
23
Time (ZT)
B
**
**
Retro Flux (vesicles/min)
0
1
2
3
4
5
2-4
8-10
14-16
23
Time (ZT)
C
Retro Velocity (µm/s)
0.0
0.5
1.0
1.5
ZT 3 brain avg
ZT 23 brain avg
ZT 3 all vesicles
ZT 23 all vesicles

Figure 2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2
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Flux Ratio (A/R)
ctrl ZT3
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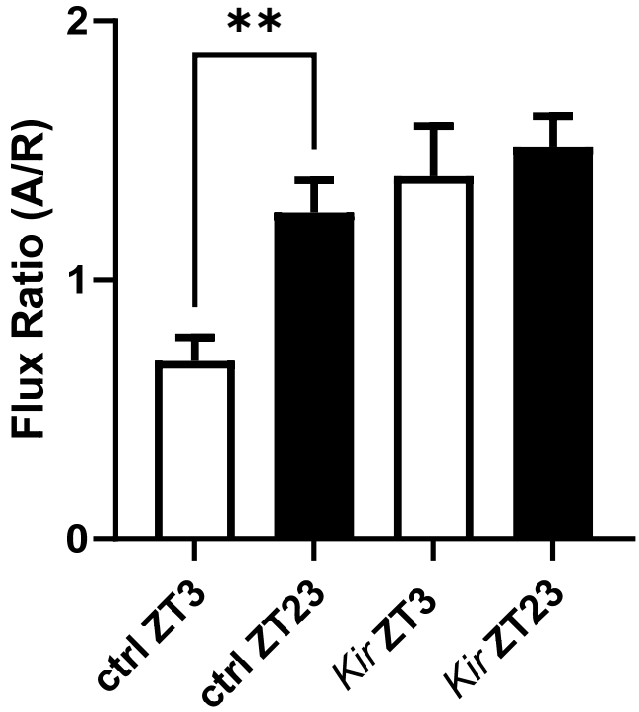
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Figure 3