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Circadian rhythms in visual responsiveness in the behaviourally arrhythmic *Drosophila* clock mutant *Clk^{Jrk}*

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Abstract:	<p>An organism's biological day is characterised by a pattern of anticipatory physiological and behavioural changes that are governed by circadian clocks to align with the 24-hour cycling environment. Here, we used flash electroretinograms (ERGs) and Steady State Visually Evoked Potentials (SSVEPs) to examine how visual responsiveness in wild-type <i>Drosophila melanogaster</i> and the circadian clock mutant <i>Clk^{Jrk}</i> varies over circadian time. We show that the ERG parameters of wild-type flies vary over the circadian day with a higher luminance response during the subjective night. The SSVEP response that assesses contrast sensitivity also showed a time of day dependence including two prominent peaks within a 24-hour period and a maximal response at the end of the subjective day, indicating a trade-off between luminance and contrast sensitivity. Moreover, the behaviourally arrhythmic <i>Clk^{Jrk}</i> mutants displayed a robust circadian profile in both luminance and contrast sensitivity but unlike the wild-types, which show bimodal profiles in their visual response, <i>Clk^{Jrk}</i> flies show a weakening of the bimodal character with visual responsiveness tending to peak once a day. We conclude that the <i>Clk^{Jrk}</i> mutation mainly affects one of two functionally coupled oscillators, and that the visual system is partially separated from the locomotor circadian circuits that drive bouts of morning and evening activity. As light is a major mechanism for entrainment, our work suggests that a detailed temporal analysis of electrophysiological responses is warranted to better identify the time window at which circadian rhythms are most receptive to light-induced phase shifting.</p>

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6 Circadian rhythms in visual responsiveness in the behaviourally
7 arrhythmic *Drosophila* clock mutant *Clk*^{Jrk}
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Abstract

An organism's biological day is characterised by a pattern of anticipatory physiological and behavioural changes that are governed by circadian clocks to align with the 24-hour cycling environment. Here, we used flash electroretinograms (ERGs) and Steady State Visually Evoked Potentials (SSVEPs) to examine how visual responsiveness in wild-type *Drosophila melanogaster* and the circadian clock mutant *Clk^{Jrk}* varies over circadian time. We show that the ERG parameters of wild-type flies vary over the circadian day with a higher luminance response during the subjective night. The SSVEP response that assesses contrast sensitivity also showed a time of day dependence including two prominent peaks within a 24-hour period and a maximal response at the end of the subjective day, indicating a trade-off between luminance and contrast sensitivity. Moreover, the behaviourally arrhythmic *Clk^{Jrk}* mutants maintained a circadian profile in both luminance and contrast sensitivity but unlike the wild-types, which show bimodal profiles in their visual response, *Clk^{Jrk}* flies show a weakening of the bimodal character with visual responsiveness tending to peak once a day. We conclude that the *Clk^{Jrk}* mutation mainly affects one of two functionally coupled oscillators, and that the visual system is partially separated from the locomotor circadian circuits that drive bouts of morning and evening activity. As light exposure is a major mechanism for entrainment, our work suggests that a detailed temporal analysis of electrophysiological responses is warranted to better identify the time window at which circadian rhythms are most receptive to light-induced phase shifting.

Keywords: electroretinogram, contrast sensitivity, *Clk^{Jrk}*, photoreceptor, SSVEP

Introduction

The ability of organisms to make anticipatory changes in behaviour and physiology in tune with daily environmental changes is attributed to the presence of cellular circadian clocks. The most robust and predictable environmental change that occurs during daily cycles is the intensity of light, which can change over 8 orders of magnitude within a 24-hour period. The visual system undergoes structural and physiological alterations to maintain optimal visual acuity over this large luminance range such that daily and circadian rhythms in visual sensitivity have been reported across species from mammals to invertebrates. In humans, time of day variations have been reported in visual psychomotor responses (Stolz et al., 1988) and in evoked electrophysiological responses of visual circuits (Hankins et al., 1988; Hankins et al., 2001; Stolz et al., 1987). Electroretinograms (ERGs), extracellular neuronal recordings at the eye that reflect the field potential changes in response to a flash of light, have been used to assess rhythms in the electrical activity of neurons in the mammalian visual system. An analysis of the ERG components indicates that both the excitation of photoreceptors and postsynaptic responses of second order neurons display a characteristic circadian profile in rodents (reviewed in Cameron et al., 2008).

The rhythms in mammalian visual sensitivity are mirrored in the genetically tractable model organism *Drosophila melanogaster*. Daily rhythms occur in ERGs (Chen et al., 1992), optomotor turning behaviour (Barth et al., 2010; Mazzotta et al., 2013) along with structural alterations in the size of the photoreceptor terminals (Barth et al., 2010) and the size and morphology of the second order lamina neurons (Pyza and Meinertzhagen, 1999; Gorska-Andrzejak et al., 2005; Weber et al., 2009). Once entrained, these patterns persist in constant darkness.

Circadian rhythms in *Drosophila* visual circuits are of particular interest because they not only have to ensure adaption of the eyes to the daily changes in light, but also because light is a key *zeitgeber* for the entrainment of the central clock neurons in *Drosophila* via visual and non-visual input pathways (Yoshii et al., 2016). The visual inputs convey light signals to the clock neurons via the compound eye photoreceptors, via the ocelli or via the specialised Hofbauer-Buchner eyelets (Rieger et al., 2003). Non-visual pathways for photoreception in clock neurons rely

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3 on the blue-sensitive cryptochrome pigment (Stanewsky et al., 1998; Emery et al.,
4 1998).
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8 All *Drosophila* cells including the central clock neurons are equipped with a genetic
9 time keeping mechanism that involves rhythmic transcription of genes whose protein
10 products feedback to inhibit their own transcription. This transcription-translation
11 feedback loop (TTFL) is conserved in *Drosophila* and mammals (Panda et al., 2002).
12 In *Drosophila*, *period* (*per*) and *timeless* (*tim*) are the two clock genes that auto-
13 regulate their transcription by inhibiting transcriptional activity of a heterodimer
14 comprised of CLOCK (CLK) and CYCLE (CYC). A second cellular timing apparatus,
15 a metabolic oscillator, generates rhythms in the oxidation state of peroxiredoxins
16 (Edgar et al., 2012; Rey et al., 2016), is conserved across species and can function
17 in the absence of the TTFL (O'Neill et al., 2011; O'Neill and Reddy, 2011). Circadian
18 rhythms in the morphological changes of lamina neurons are abolished in mutant
19 flies that are null for the *per* gene (*per*⁰¹; Weber et al., 2009; Barth et al., 2010) as
20 are the circadian changes in optomotor responses (Barth et al., 2010). In contrast,
21 visual sensitivity rhythms are unaffected in *per*⁰¹ mutants (Chen et al., 1992). Thus, it
22 is unclear whether visual rhythms require a functional TTFL and/or metabolic
23 oscillator.
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34 Here we examined visual sensitivity in the *Clk* gene mutant (*Clk*^{Jrk}), which is
35 behaviourally arrhythmic (Allada et al., 1998), to determine whether the TTFL is
36 dispensable for oscillations in visual function. To test this, we deployed the
37 conventional flash electroretinogram (fERG). ERGs performed on a dark background
38 measure the response to a light flash while the visual system is in a dark-adapted
39 state. The electrical response from the eye therefore gives a measure of the
40 luminance response of the eye. The contrast of a flash of light delivered in the ERG
41 assay is poorly defined: if it is expressed as a fraction of the mean background then it
42 is many hundreds or even thousands of a percentage change. We therefore
43 deployed a highly sensitive Steady State Visually Evoked Potential (SSVEP) assay
44 (Afsari et al., 2014) which measures the response to a flickering light. This assay
45 measures responses to modulations around a mean luminance, a situation that is
46 representative of natural scenes (Laughlin, 1981). By using different frequencies and
47 light levels the SSVEP can sweep out the entire contrast response profile of the
48 visual system (Norcia et al., 2015). Because the SSVEP measurements are based
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3 on a much larger number of events than a flash ERG and because the precise
4 modulation frequency of the SSVEP inputs allow us to ignore most broadband noise,
5 the signal to noise ratio of the SSVEP technique is much higher than that found in
6 single-trial ERG experiments. These properties make the SSVEP assay sensitive
7 and a reliable indicator of physiologically relevant visual function whilst also allowing
8 comparisons with human contrast sensitivity. Finally, a systems identification
9 approach to the SSVEP data distinguishes the response of three key components of
10 the fly visual system: photoreceptors, second order lamina neurons and third order
11 medulla neurons.
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17 18 **Materials and methods**

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21 *Fly stocks:* vials of *Drosophila melanogaster* were kept on a yeast-sucrose-agar food
22 medium (Carpenter, 1950). The $Clk^{rk} st^1$ mutant (Bloomington Stock 24515,
23 hereafter Clk^{rk}) was compared with its background st^1 (Stock 605) and with the
24 white-eyed standard $w^{1118} (w^-)$ (University of York stock). All vials were kept at 25°C
25 with a 12hr: 12hr light: dark schedule. Adult flies were collected within ~18 hours of
26 eclosion. They were photoentrained in 12hr: 12hr lights on: lights off (LD) cycles for
27 ~5/6 days in a constant temperature room (25°C), before being transferred to
28 constant darkness (DD) and constant temperature (again 25°C).
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35 *Electroretinograms:* flash ERGs and SSVEP were made as described by (Hindle et
36 al., 2013; Belusic, 2011) and (Afsari et al., 2014) respectively, with additional steps to
37 avoid disrupting the circadian rhythm. Flies were trapped in a shortened Gilson
38 pipette tip with the head and fore legs were exposed (Fig. 1A,B), and secured with a
39 small amount of nail polish (Creative Nail Design). Each fly was allowed to recover in
40 the dark for a period of ~20 minutes. Recordings were made with glass electrodes
41 filled with *Drosophila* saline, one resting on the eye, the other placed in the
42 mouthparts. In the case of flies that were currently experiencing subjective night or
43 were under constant conditions, this preparation process was performed under a red
44 light in order to minimize interference with the flies' current light cycle (Chiu et al.,
45 2010). fERGs were recorded using DasyLab (Measurement Computing Corporation,
46 2012), with analysis was made using custom Dasyview software
47 (<http://biolpc22.york.ac.uk/dasyview>), and the peak to peak (max to min) height,
48 receptor potential, and off-transients measured. SSVEP stimulation recording and
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analysis was achieved with Matlab. We presented 18 random contrast stimuli to each fly, with the light being flickered about the mean light intensity at 12 Hz (hereafter 1F1). This generates responses that the FFT (Fast Fourier Transform) analysis identifies at the input frequency (1F1) and at twice the input frequency (2F1). Genetic dissection shows that these two components are due to the photoreceptors and lamina neurons respectively (Afsari et al., 2014). In some stimuli, the 1F1 input was combined with a second input at 15 Hz (1F2, see Fig 1 B). This results in a combined 'beating' pattern in which the amplitude of the response changes at the sums and differences of the input frequencies (1F2-1F1, 1F1+1F2 and 2F1+2F2). This 'intermodulation' is the result of the activity of the medulla neurons, and, like Afsari et al., (2014) we chose to report the 2F1+2F2 term, which arises in the medulla (see Supplementary Figure S1). To remove any effects due to adaptation to the flickering light, only the last 9 responses were analysed.

Circadian periodicity in the dark was estimated by fitting the equation

$$SS = C + \alpha (\sin(\Omega t)) + \beta (\cos(\Omega t))$$

where SS is the response at time t , C is the overall mean, α and β are amplitudes, and Ω is the period. This equation has one non-linear unknown, Ω , and will have a number of good fits, with minimal residuals. We systematically supplied values of Ω from 0.4 to 1.6 days and, for each Ω determined the best linear fit of C, α and β using the R procedure 'lm'. The residual was plotted as a function of Ω (Fig. 4A). Once the approximate best fit Ω was determined, the values of C, α and β were determined using the R 'nls' non-linear fit procedure. All data acquisition and analysis code is available at <https://github.com/wadelab/flyCode>, using the 'Circadian' code set.

Locomotor activity rhythms: The *Drosophila* activity monitor system (Trikinetics Inc., Waltham, MA, USA) was used to record locomotor activity as described previously (Fogg et al., 2014). Male flies were collected within ~18 hours of eclosion, kept in a light and temperature controlled incubator (25°C) and were photoentrained to 12hr light: 12hr lights dark (LD) cycles for 3 days, and then monitored in constant darkness (DD) for a further 9 days. Locomotor activity was recorded in 2 minute bins. Actograms and a Lomb-Scargle periodograms for each individual fly were generated using the ActogramJ plugin for ImageJ program (Schmid et al., 2011).

Statistics: ANOVA was performed in R, using the Tukey post-hoc test where

required.

Results

We first compared the fly visual response at the end of subjective day (CT8) with that at the end of subjective night (CT20), as at these times ERG sensitivities have been previously reported to differ considerably (Chen et al., 1992). We entrained flies for 6 days and then moved them into darkness for 24 hours (DD1). We first tested white-eyed flies (w^-) since they give a larger fERG (flash electroretinogram) response than red-eyed flies and observed differences in their ERGs at the two time points. The ERG traces of wild-type w^- flies show marked differences at CT20 and CT8 (Fig 2Ai) in both the size of the receptor potential and the amplitude of the off transient. In contrast, the ERG traces of the scarlet-eyed Clk^{Jrk} flies differ less in their waveforms between the two time points. Quantitative analysis of the ERG peak to peak amplitude shows that wild-type flies have on average a larger response at CT20 than CT8 whereas the Clk^{Jrk} mutants respond similarly at CT20 and CT8. This might suggest a loss of rhythmicity in visual responses in the mutants. To investigate this further we also compared the genotypes in the SSVEP assay. Figure 2B shows that in the SSVEP assay the visual response of both wild-type flies and Clk^{Jrk} mutants has a higher amplitude at CT8 than CT20 suggesting that contrast sensitivity is higher at the end of the subjective day than at the end of the subjective night. This is true for all three parameters measured (1F1, 2F1 and 2F1+2F2), showing that there is increased response to changes in contrast by the photoreceptors, lamina neurons and medulla neurons at the end of subjective day.

Given the apparent loss of rhythmicity of Clk^{Jrk} mutants in fERGs but not in the SSVEP assay we extended the data set and sampled flies from free running constant darkness conditions (DD1) every four hours (Fig. 3). We also included the wild-type strain st^1 here to rule out genetic background as a cause for the different response of the Clk^{Jrk} mutants in fERGs and also analysed the photoreceptor potential and off-transients separately. Figure 3A shows that in the fERG responses the temporal profiles of the three genotypes are for the most part similar but diverge considerably at CT12. At CT12 the receptor potential of the wild-type strains (w^- and st^1) is maximum while for the Clk^{Jrk} mutants the photoreceptor response at CT12 is at its

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3 minimum. Overall, the fERG data suggests that all genotypes have a higher
4 luminance response in the subjective night.
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8 In the extended SSVEP assay (Fig. 3B): both genotypes show a circadian pattern,
9 but the response is dominated by a peak in the second half of the subjective day
10 (CT4-CT8). The photoreceptor response is stronger in the w^- than in the Clk^{Jrk}
11 mutants, but the neural signalling components (lamina neurons and medulla
12 neurons) are not separated by genotype. At CT4, there is a dip in the w^-
13 photoreceptor and lamina neuron SSVEP response, mirroring the photoreceptor
14 response peak in the fERG, but this is not seen in the Clk^{Jrk} data.
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20 To confirm our Clk^{Jrk} data, we next examined the periodicity in detail over LD6, DD1
21 and DD2. We compared the Clk^{Jrk} flies with a scarlet mutation (st^1), as the Clk^{Jrk}
22 mutation is in the st^1 background. For both genotypes the variation in 1F1 response
23 is larger in LD6 than in DD. We fitted a periodic cycle to the DD data, determined the
24 residuals (Fig. 4A), and found both genotypes showed a minimum in the residual at
25 ~14 hours. The Clk^{Jrk} (but not the st^1) showed a better fit for a period of 25 hours.
26 Plotting the curves shows a good fit between the data and the calculated lines (Fig.
27 4B), confirming that the visual sensitivity of st^1 flies have peaks approximately twice a
28 day, whereas the Clk^{Jrk} flies have a 'circadian' rhythm. The peak of the Clk^{Jrk} fitted
29 curve is at CT4, while the peak on the last LD day is at ZT4, suggesting there is no
30 phase shift over this time span.
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40 Finally, we confirmed the locomotor phenotype of the Clk^{Jrk} and st^1 flies. The scarlet-
41 eyed control flies st^1 exhibit 2 clear peaks in locomotor activity levels under LD
42 conditions, which center around light on- and offset or ZT0 and ZT12 (Fig. 5). Under
43 DD conditions 69% of the st^1 flies were rhythmic (Lomb-Scargle analysis), and these
44 had an average free running period length of 24.4 hours. The Clk^{Jrk} mutants have a
45 strong nocturnal rhythm under LD conditions (Kumar et al., 2012). They have
46 relatively constant activity levels during the day, which then increased by
47 approximately 60% 30 minutes after light offset and remained fairly constant until
48 ZT0. The sharp differences in activity that occur at the two light transitions indicate a
49 lack of light anticipatory behaviour in the Clk^{Jrk} mutant. Under constant darkness,
50 only 16.6% of the Clk^{Jrk} flies were rhythmic with mean DD period slightly lengthened
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3 at 25.2 hours.
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6 Discussion 7

8 Here we report that in both fERG assays and SSVEP responses visual sensitivity in
9 *Drosophila melanogaster* displays a notable time-of-day dependence. We have
10 further demonstrated that the *Clk^{Jrk}* mutation results in flies with a maintained
11 circadian rhythm in visual response in constant darkness. The *Clk^{Jrk}* rhythm largely
12 recapitulates that of the wild-type *w⁻* flies both showing a higher luminance response
13 in the subjective night and greater contrast sensitivity towards the end of the
14 subjective day. This is surprising given that *Clk^{Jrk}* flies are arrhythmic in their
15 locomotor activity rhythms. The *Clk^{Jrk}* mutants express a truncated CLK protein that
16 retains its DNA binding and dimerization domain but lacks its C-terminal
17 transactivation domain (Allada et al., 1998). This explains the *Clk^{Jrk}* mutant's
18 dominant phenotype in locomotor activity rhythms as it is likely able bind DNA and its
19 DNA binding partner CYC but unable to induce gene transcription.
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28 From our initial experiments it would seem that the genetic oscillator, the TTFL, is not
29 required for oscillations in visual responsiveness assessed by the ERG amplitude
30 and SSVEP assays. However, an extended time course comparing the SSVEPs of
31 *Clk^{Jrk}* with the genetically comparable *st¹* strain revealed notable differences in their
32 visual rhythms under DD conditions. The SSVEP photoreceptor response in *st¹*
33 displays an ultradian rhythm approximating to 14 hours while that of the *Clk^{Jrk}*
34 mutants oscillated with a circadian time course of 25 hours. Moreover, the
35 amplitude/duration of the *Clk^{Jrk}* circadian rhythm is more robust than that of the *st¹*
36 flies, even though the *Clk^{Jrk}* is in the *st¹* background.
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43 From a functional perspective, the twice a day contrast response in visual sensitivity
44 in wild-type flies could map on to the need for optimal visual acuity at morning (M)
45 and evening (E) peaks of locomotor activity in wild type flies (Helfrich-Förster, 2000).

46 A twice a day increase in the size of the L1 and L2 lamina neurons has been seen in
47 daily rhythms (Pyza and Meinertzhagen, 1999), which might be a potential correlate
48 of the physiological changes reported here. Similarly, a twin peak rhythm in a
49 synaptic protein, bruchpilot, is reported in LD cycles of wild-type flies (Górska-
50 Andrzejak et al., 2013).
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3 In *Clk^{Jrk}* mutants a robust circadian rhythm in contrast response is more apparent
4 due to suppression of one of the wild-type peaks in visual sensitivity suggesting that
5 they might be regulated separately similar to the morning and evening peaks in
6 locomotor activity that are controlled by different subsets of clock neurons (Grima et
7 al., 2004; Stoleru et al., 2004) In this context, we note that in DD, only L1 laminar
8 neurons oscillate in size in wild-type flies, being larger in the subjective night (Pyza
9 and Meinertzhagen, 1999). Interestingly, in assessing the contribution of different
10 neurons to contrast Joesch et al., (2010) note that L1 neurons mediate 'ON'
11 responses and L2 'OFF' responses so that circadian changes in the 'ON' response
12 pathway might explain our observation of a stronger SSVEP lamina response at the
13 end of the subjective day. Furthermore, in DD, the levels of bruchpilot seem to
14 display a unimodal rhythm (Górska-Andrzejak et al., 2013), though this was
15 measured at 9 hour intervals, which might miss an intervening peak. While a
16 differential effect of *Clk^{Jrk}* on the L1 and L2 lamina neurons is one possible
17 explanation for our results, we cannot discount effects on other neurons in the visual
18 circuit, nor can we exclude the possibility that this is the consequence of the aberrant
19 axonal organisation of the s-LNv neurons (Park et al., 2000).

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21 It is possible that the cyclical changes in visual sensitivity reported here are
22 controlled by the genetic clock oscillator as circadian expression of genes involved in
23 *Drosophila* visual processes have been reported (Claridge-Chang et al., 2001;
24 Ceriani et al., 2002). Claridge-Chang et al., (2001) observe circadian cycling of
25 mRNAs encoding the rhodopsins *Rh4*, *Rh5*, the *trpl* receptor involved in
26 phototransduction, the rhodopsin chaperone *ninaA* and *Pdh*, a photoreceptor
27 dehydrogenase that participates in chromophore recycling by retinoid isomerisation
28 (Wang et al., 2010). It is noteworthy that frequent sampling of gene expression in
29 mammalian systems has revealed mRNAs that oscillate with periods of 10-14 h
30 (Hughes et al., 2009) and mRNAs that peak twice in a 24 h period (Pembroke et al.,
31 2015). Alternatively, the maintained visual rhythms in the *Clk^{Jrk}* could be due to the
32 metabolic oscillator, which continues to generate robust oscillations in peroxiredoxin
33 oxidation state in *Clk^{Jrk}* flies, albeit with a different phase (Edgar et al., 2012). In this
34 regard it is interesting to note that a hypomorph CLK mutant, *Clk^{AR}*, accumulated
35 more reactive oxygen species with age than wild-type flies (Vacarro et al., 2017).

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37 Our findings also have implications for entraining the circadian system as light via the

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3 compound eyes can synchronize the *Drosophila* clock (Reiger et al., 2003). We
4 would like to suggest that rhythms in visual function reported here reveal critical time
5 windows when the *Drosophila* clock would be more receptive to light entrainment or
6 light-induced phase shifting.
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11 Finally we note from our experiments that, during the daily cycle luminance sensitivity
12 peaks in the subjective night, while the contrast response function is stronger in the
13 subjective day. Of note, a higher contrast sensitivity in the day has also been
14 reported in rodents (Hwang et al., 2013). Our work suggests a trade off between
15 luminance and contrast. In the dark, the gain control in the eyes is relaxed, allowing
16 photoreceptor sensitivity to be increased. A similar trade-off exists between visual
17 dynamic range, which was lowest at subjective night, and the optomotor response,
18 which was lowest in subjective day (Barth et al., 2010). Our data also shows faster
19 responses (shortened latency) in the subjective night, a phenomenon also seen in
20 the human daily visual rhythm (Hankins et al., 2001). These similarities suggest that
21 the mechanistic basis for circadian tuning of *Drosophila* visual function can
22 potentially provide insights into the mammalian system.
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32 References

33
34 Afsari F, Christensen KV, Smith GP, Hentzer M, Nippe OM, Elliott CJ, and Wade AR
35 (2014) Abnormal visual gain control in a Parkinson's disease model. *Hum Mol Genet*
36 23:4465-4478.

37
38 Allada R, White NE, So WV, Hall JC, and Rosbash M (1998) A mutant *Drosophila*
39 homolog of mammalian Clock disrupts circadian rhythms and transcription of period
40 and timeless. *Cell* 93:791-804.

41
42 Barth M, Schultze M, Schuster CM, and Strauss R (2010) Circadian plasticity in
43 photoreceptor cells controls visual coding efficiency in *Drosophila melanogaster*.
44 *PLoS One* 5(2):e9217.

45
46 Belušič G (2011) ERG in *Drosophila*. In *Electroretinograms*, G Belušič, ed, pp. 221–
47 238, InTech, Rijeka.

48
49 Carpenter JM (1950) A new semisynthetic food medium for *Drosophila*. *Dros Inf Serv*
50 24:96–97.

51
52 Cameron MA, Barnard AR, and Lucas RJ (2008) The electroretinogram as a method
53 for studying circadian rhythms in the mammalian retina. *J Genet* 87:459-466.

54
55 Ceriani MF, Hogenesch JB, Yanovsky M, Panda S, Straume M and Kay SA (2002)
56 Genome wide expression analysis in *Drosophila* reveals genes controlling circadian

1
2
3 behavior. *J Neurosci* 22:9305-9319.

4
5 Chen DM, Christianson JS, Sapp RJ, and Stark WS (1992) Visual receptor cycle in
6 normal and period mutant *Drosophila*: microspectrophotometry, electrophysiology,
7 and ultrastructural morphometry. *Vis Neurosci* 9:125-35.

8
9 Chiu JC, Low KH, Pike DH, Yildirim E, and Edery I (2010) Assaying locomotor
10 activity to study circadian rhythms and sleep parameters in *Drosophila*. *J Vis Exp*
11 43: e2157.

12
13 Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, and Young MW
14 (2001) Circadian regulation of gene expression systems in the *Drosophila* head.
15 *Neuron* 32:657-671.

16
17 Edgar RS, Green EW, Zhao Y, van Ooijen G, Olmedo M, Qin X, Xu Y, Pan M,
18 Valekunja UK, Feeney KA, Maywood ES, Hastings MH, Baliga NS, Mellow M, Millar
19 AJ, Johnson CH, Kyriacou CP, O'Neill JS, and Reddy AB (2012) Peroxiredoxins are
20 conserved markers of circadian rhythms. *Nature* 485:459-464.

21
22 Emery P, So WV, Kaneko M, Hall JC, and Rosbash M (1998) CRY, a *Drosophila*
23 clock and light-regulated cryptochrome, is a major contributor to circadian rhythm
24 resetting and photosensitivity. *Cell* 95:669-679.

25
26 Fogg PCM, O'Neill JS, Dobrzycki T, Calvert S, Lord E, Lord RL, Elliott CJH, Sweeney
27 ST, Hastings MH, and Chawla S (2014) Class IIa histone deacetylases are
28 conserved regulators of circadian function. *J Biol Chem* 289: 34341-34348

29
30 Górska-Andrzejak J, Makuch R, Stefan J, Görlich A, Semik D, and Pyza E (2013)
31 Circadian expression of the presynaptic active zone protein Bruchpilot in the lamina
32 of *Drosophila melanogaster*. *Dev Neurobiol* 73:14-26.

33
34 Górska-Andrzejak J, Keller A, Raabe T, Kilianek L, and Pyza E (2005) Structural
35 daily rhythms in GFP-labelled neurons in the visual system of *Drosophila*
36 *melanogaster*. *Photochem Photobiol Sci*. 4:721-76.

37
38 Grima B, Chelot E, Xia R, and Rouyer F (2004) Morning and evening peaks of
39 activity rely on different clock neurons of the *Drosophila* brain. *Nature* 431:869–873

40
41 Hankins MW, Jones RJ, and Ruddock KH (1998) Diurnal variation in the b-wave
42 implicit time of the human electroretinogram. *Vis Neurosci* 15:55-67.

43
44 Hankins MW, Jones SR, Jenkins A, and Morland AB (2001) Diurnal daylight phase
45 affects the temporal properties of both the b-wave and d-wave of the human
46 electroretinogram. *Brain Res* 889:339-343.

47
48 Hindle S, Afsari F, Stark M, Middleton CA, Evans GJO, Sweeney ST, and Elliott CJH
49 (2013) Dopaminergic expression of the Parkinsonian gene LRRK2-G2019S leads to
50 non-autonomous visual neurodegeneration, accelerated by increased neural
51 demands for energy. *Hum Mol Genet* 22: 2129–2140.

52
53 Helfrich-Förster C (2000) Differential control of morning and evening components in
54 the activity rhythm of *Drosophila melanogaster*—sex-specific differences suggest a
55 different quality of activity. *J Biol Rhythms* 15:135-154.

1
2
3
4 Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthy S, Baggs JE, Panda S,
5 and Hogenesch JB (2009) Harmonics of circadian gene transcription in mammals.
6 PLoS Genet 5(4):e1000442.
7

8 Hwang CK, Chaurasia SS, Jackson CR, Chan GC, Storm DR, and Iuvone PM (2013)
9 Circadian rhythm of contrast sensitivity is regulated by a dopamine-neuronal PAS-
10 domain protein 2-adenylyl cyclase 1 signaling pathway in retinal ganglion cells. J
11 Neurosci 33:14989-14997.
12

13 Joesch M, Schnell B, Raghu SV, Reiff DF, and Borst A (2010) ON and OFF
14 pathways in *Drosophila* motion vision. Nature 468:300-304.
15

16 Kumar S, Chen D, and Sehgal A (2012) Dopamine acts through Cryptochrome to
17 promote acute arousal in *Drosophila*. Genes Dev 26:1224-1234.
18

19 Laughlin S (1981) A simple coding procedure enhances a neuron's information
20 capacity. Z Naturforsch 36:910-912.
21

22 Mazzotta G, Rossi A, Leonardi E, Mason M, Bertolucci C, Caccin L, Spolaore B,
23 Martin AJ, Schlichting M, Grebler R, Helfrich-Förster C, Mammi S, Costa R, and
24 Tosatto SC (2013) Fly cryptochrome and the visual system. Proc Natl Acad Sci U S
25 A. 110:6163-6168.
26

27 Norcia AM, Appelbaum LG, Ales JM, Cottreau BR and Rossion B (2015) The
28 steady-state visual evoked potential in vision research: A review. J Vis 15:4.
29

30 O'Neill JS, van Ooijen G, Dixon LE, Troein C, Corellou F, Bouget FY, Reddy AB, and
31 Millar AJ (2011) Circadian rhythms persist without transcription in a eukaryote.
32 Nature 469:554-558.
33

34 O'Neill JS and Reddy AB (2011) Circadian clocks in human red blood cells. Nature
35 469:498-503.
36

37 Panda S, Hogenesch JB, and Kay SA (2002) Circadian rhythms from flies to human.
38 Nature 417:329-335.
39

40 Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, and Hall JC (2000) Differential
41 regulation of circadian pacemaker output by separate clock genes in *Drosophila*.
42 Proc Natl Acad Sci U S A 97:3608-3613.
43

44 Pembroke WG, Babbs A, Davies KE, Ponting CP, and Oliver PL (2015) Temporal
45 transcriptomics suggest that twin-peaking genes reset the clock. Elife. 2;4. pii:
46 e10518.
47

48 Pyza E and Meinertzhagen IA (1999) Daily rhythmic changes of cell size and shape
49 in the first optic neuropil in *Drosophila melanogaster*. J Neurobiol 40:77-88.
50

51 Rey G, Valekunja UK, Feeney KA, Wulund L, Milev NB, Stangherlin A, Ansel
52 Bollepalli L, Velagapudi V, O'Neill JS, and Reddy AB (2016). The Pentose Phosphate
53 Pathway Regulates the Circadian Clock. Cell Metab 24:462-473.
54

55 Rieger D, Stanewsky R, and Helfrich-Förster C (2003) Cryptochrome, compound
56
57

1
2
3 eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment
4 and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila*
5 *melanogaster*. *J Biol Rhythms* 18:377-391.

6
7 Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M,
8 and Hall JC (1998) The cryb mutation identifies cryptochrome as a circadian
9 photoreceptor in *Drosophila*. *Cell* 1998 95:681-92.

10
11 Schmid B, Helfrich-Förster C, and Yoshii T (2011) A new ImageJ plug-in
12 "ActogramJ" for chronobiological analyses. *J Biol Rhythms* 26:464–467.

13
14 Stoleru D, Peng Y, Agosto J, and Rosbash M (2004) Coupled oscillators control
15 morning and evening locomotor behaviour of *Drosophila*. *Nature* 431:862-868.

16
17 Stolz G, Aschoff JC, Born J, and Aschoff J (1988) VEP, physiological and
18 psychological circadian variations in humans. *J Neurol* 235:308-313.

19
20 Stolz G, Aschoff JC, Aschoff J, and Born J (1987) Circadian variation in the visual
21 evoked potential (VEP). *Electroencephalogr Clin Neurophysiol Suppl* 40:279-283.

22
23 Vaccaro A, Issa AR, Seugnet L, Birman S, and Klarsfeld A (2017) *Drosophila* Clock
24 Is Required in Brain Pacemaker Neurons to Prevent Premature Locomotor Aging
25 Independently of Its Circadian Function. *PLoS Genet* 13:e1006507.

26
27 Wang X, Wang T, Jiao Y, von Lintig J, and Montell C (2010) Requirement for an
28 enzymatic visual cycle in *Drosophila*. *Curr Biol* 20:93-102.

29
30 Weber P, Kula-Eversole E, and Pyza E (2009) Circadian control of dendrite
31 morphology in the visual system of *Drosophila melanogaster*. *PLoS One* 4:e4290.

32
33 Yoshii T, Hermann-Luibl C, and Helfrich-Förster C (2015) Circadian light-input
34 pathways in *Drosophila*. *Commun Integr Biol* 9:e1102805.

35 36 37 38 39 **Figure Legends**

40
41 **Figure 1.** Experimental setup for recording the visual neurophysiological response of
42 *Drosophila*. Flies were restrained with nail polish in a pipette tip. A recording
43 electrode placed on one eye and a second, indifferent earthed electrode placed in
44 the mouthparts. A. For the flash ERG (electroretinogram), which measures the
45 luminance response, a pulse of constant blue light from an LED (750 ms) was given,
46 and the recorded receptor potentials and off-transients were measured as indicated
47 by the dashed lines. B. For the SSVEP (steady state visual evoked potential)
48 stimulus, which measures the contrast sensitivity, a flickering blue light was applied.
49 The intensity of the light is the sum of two square waves: one at 12 Hz and the other
50 at 15 Hz. In each trial the amplitude of each component wave was determined
51 randomly. The amplitude of each frequency in the response was determined using
52 the Fourier transform, giving rise to harmonics (1F1, 2F1...) and intermodulation
53 terms (1F1+1F2, 1F2-1F1, 2F1+2F2...). These frequency components are related to
54 the anatomy of the fly eye (C), with the 1F1 component arising from the
55 photoreceptors, the 2F1 from the lamina, second order neurons and the
56 intermodulation terms (2F1+2F2) from the medulla.

Figure 2. Wild-type (w^-) and Clk^{Jrk} flies show different visual responses at CT8 and CT20 in DD1. A. Qualitative (i) and quantitative (ii) differences in the flash ERG response at CT8 and CT20. Bar chart plot of the ERG peak-peak amplitude shows significant difference in the w^- response between CT20 and CT8. Tukey Post-Hoc tests showing no overall difference between w^- and Clk^{Jrk} ($P=0.059$); a difference in the ERG of w^- between CT20 and CT8 ($P=0.33$), but no difference for Clk^{Jrk} between these timepoints ($P=0.71$). $N = 45$, at least 10 in each sample. B. SSVEP contrast response functions for the photoreceptor, lamina neurons and medulla neurons rise more steeply at CT8 than at CT20, indicating a stronger visual response to flickering light. The overall MANOVA indicates differences in genotype ($P < 10^{-6}$), timepoint ($P=0.0002155$) and the genotype*timepoint interaction ($P=0.0126175$). The subsequent ANOVA indicates differences in timepoint for each component of the SSVEP response (photoreceptors, lamina neurons and medulla neurons, See Supplementary Table 1). Only the photoreceptors show a difference due to genotype, while the lamina neurons show a genotype*timepoint interaction. Data from the same 45 flies in A. Exact genotypes: $w^- = w^{1118}$; $Clk^{Jrk} = Clk^{Jrk}, st^1$.

Figure 3. Circadian visual profile of wild-type (w^- , st^1) and Clk^{Jrk} flies on DD1. A. Flash ERGs show peak sensitivity in the subjective night (CT16-20) and minima at CT0 and CT8-12. For both photoreceptor response and off-transient, the two-way ANOVA shows significant effects of time of day and genotype (photoreceptor: $F_{5, 190df}=2.8$, $P = 0.019$ and $F_{1, 190df}=10.5$, $P < 10^{-4}$ respectively; off-transient: $F_{5, 190df}=2.4$, $P = 0.035$ and $F_{1, 190df}=38.4$, $P < 10^{-14}$ respectively), but no interaction. $N = 207$, at least 6 in each sample. B. SSVEP analysis shows peak sensitivity in the subjective day for the photoreceptors, lamina neurons and medulla neurons. The photoreceptor response is bigger for the Clk^{Jrk} flies than the w^- at all time points. The ANOVA shows significance for genotype and time, but not for their interaction (genotype: $F_{1, 131df} = 22$, $P < 10^{-5}$; time: $F_{5, 131df} = 9.8$, $P < 10^{-7}$). For the neuronal responses (lamina or medulla neurons) there is no difference between the Clk^{Jrk} and w^- flies. The sensitivity of the SSVEP assay is indicated in the 2F1+2F2 (medulla neuron) trace, where the response is $\sim 10x$ the noise level. The dotted line (sine) indicates a waveform with the maximum in the subjective night and minimum in the subjective day. Data from the same 135 Clk^{Jrk} and w^- flies in A, using the maximum response for each fly. Exact genotypes: $w^- = w^{1118}$; $Clk^{Jrk} = Clk^{Jrk}, st^1$.

Figure 4. Calculating the best fit of a sine wave to the photoreceptor component of the SSVEP data shows the Clk^{Jrk} flies maintain a DD rhythm with circadian periodicity, but the st^1 flies have a rhythm with a periodicity of ~ 2 cycles/day. A. Fitting successive values of Ω , the period, shows a good fit at ~ 14 hours for both genotypes. However, the Clk^{Jrk} have a better fit with a period of ~ 1.05 days. B. Plotting the best fit lines shows that the Clk^{Jrk} data is well explained by an equation with period 25.2 ± 3.1 hours, whereas the st^1 period is 14.6 ± 0.6 hours.

Figure 5. Nocturnal locomotor activity in LD for Clk^{Jrk}, st^1 but not st^1 flies. Average daily activity profiles of st^1 flies (left graphs) and Clk^{Jrk} mutants (right graphs) in 30 min bins during a 24-hour period in LD cycles (data are from LD3) and during free running constant darkness conditions (data shown from DD3). Note the elevated

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3 activity of the *Clk^{ms}*, mutants during the dark phase of LD and arrhythmic phenotype in
4 DD. N= 54 *st¹* and 21 *Clk^{rk},st¹* flies.
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For Peer Review

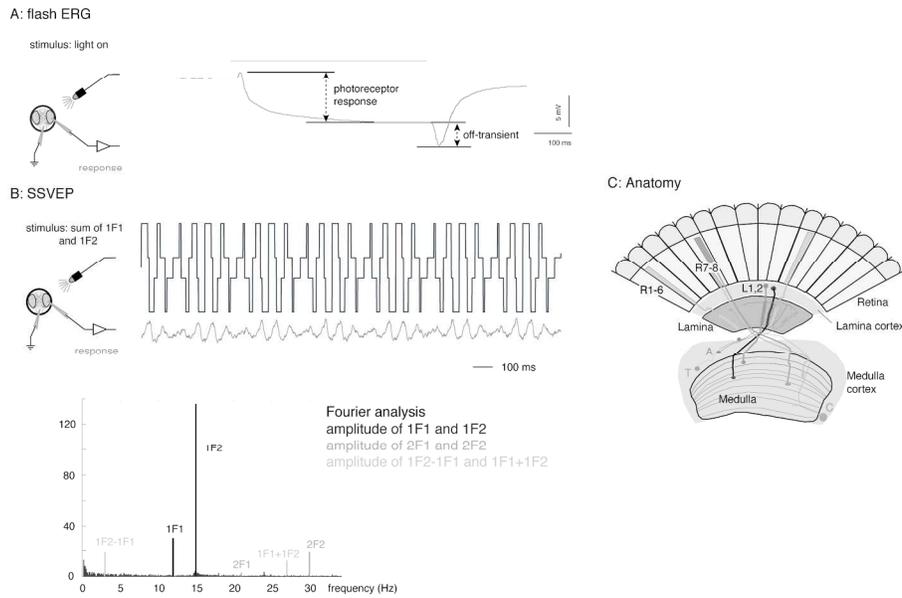


Figure 1 Black and White for print version

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Review

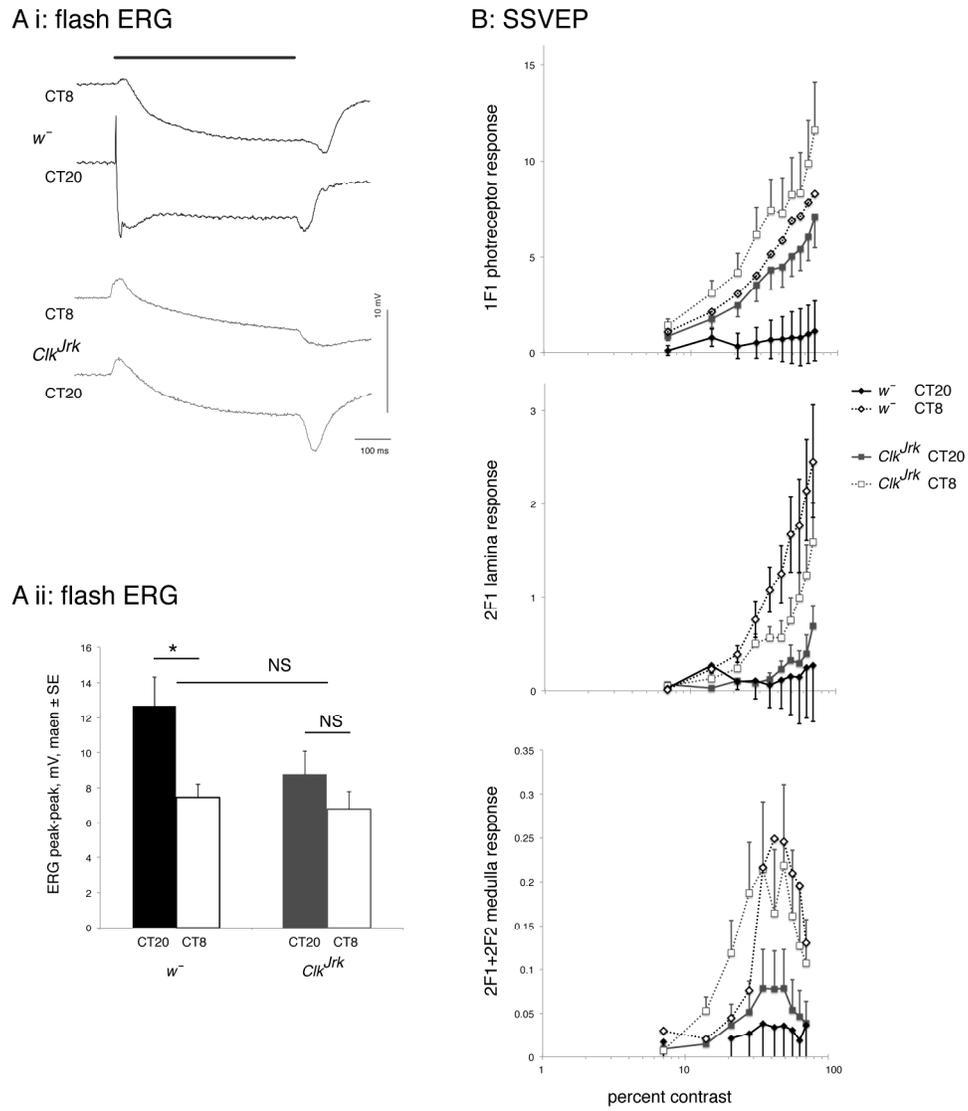
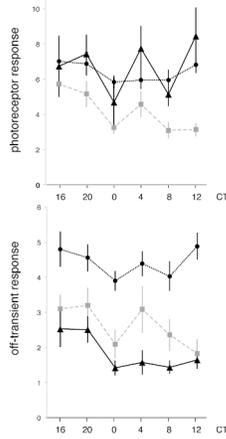


Figure 2 Black and White for print version

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A: flash ERG



B: SSVEP

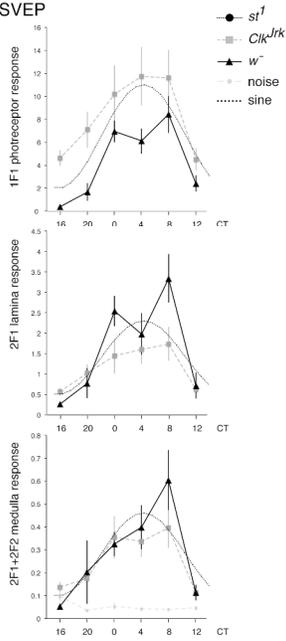


Figure 3 Black and White for print version

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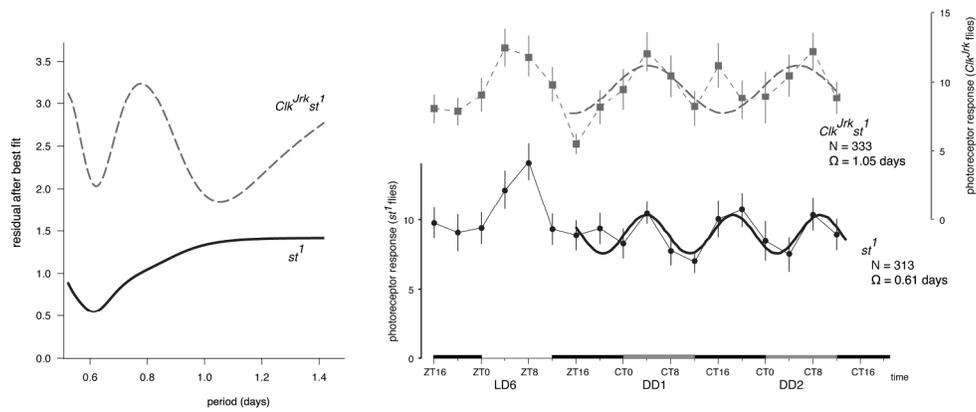


Figure 4 Black and White for print version

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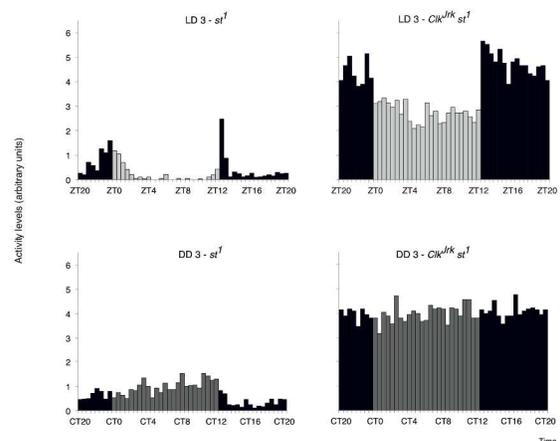


Figure 5

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3 Nippe et al Supplementary material
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5 Supplementary Figure S1 Legend. **Knockout of synaptic transmission**
6 **from second order neurons abolishes the 2F1+2F2 medulla neuron**
7 **response.** In the SSVEP (steady state visual evoked potential) analysis, we
8 compare the response to the mix of 12 Hz (1F1) and 15 Hz (1F2) blue light
9 stimuli with the response to a single input at 12 Hz. This scenario was
10 designed to mimic a human watching a flickering TV monitor, with horizontal
11 stripes at 12 Hz with or without vertical stripes at 15 Hz. Because the
12 additional vertical stripes reduce the response to the horizontal stripes, the
13 double stimulus is referred to as the 'masked' paradigm, the single 12 Hz
14 stimulus (horizontal stripes) as the 'unmasked' paradigm. A Fast Fourier
15 Transform (FFT) resolves a complex response from the fly eye into
16 components at the frequencies supplied (1F1, 1F2), but also at other
17 frequencies, notably a harmonic at 2F1 (24 Hz) and at the intermodulation
18 frequency, 2F1+2F2 (54 Hz). In the flickered steady-state illumination used
19 here, the photoreceptors follow the stimulus input and generate the 1F1
20 response. The lamina neurons encode transient signals, and generate a
21 response each time the light level goes up or down, so they are principally
22 responsible for the 2F1 response. Genetic dissection showed (Afsari et al.,
23 2014) that flies with no histamine receptors (*ort* null) showed no 2F1 response.
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28 Here we test the effect of knocking out synaptic transmission from the second
29 order neurons (lamina neurons, amacrine neurons) in the fly retina. All these
30 cells express the histamine receptor, ORT, and so we used an *ort*-GAL4
31 (Gengs et al., 2002) to express tetanus toxin (TNT, Sweeney et al., 1995) in
32 these cells.
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36 In the control fly with no transgene expressed (*TNT/+*) all three components of
37 the masked response (1F1, 2F1 and 2F1+2F2) are clearly seen (solid green
38 lines). The 1F1 and 2F1 components increase monotonically with the contrast,
39 but the 2F1+2F2 component shows a winner takes all scenario, peaking at
40 ~50 % contrast. In this respect, the fly response is similar to that of the
41 humans watching horizontal stripes and vertical stripes, where the response
42 of the 2F1+2F2 component shows the same peak when the contrasts are
43 equal.
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47 When tetanus toxin is expressed in all the second order neurons using the *ort*
48 histamine receptor GAL4 (*ort* > *TNT*), the masked paradigm 1F1 and 2F1
49 responses (magenta lines) are similar to the control flies, though shifted right
50 (or down) by the slightly darker eye colour. However, the 2F1+2F2 component
51 is markedly different, being now much reduced and monotonically increasing
52 with contrast.
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56 When only 12 Hz stimulation is applied ('unmasked paradigm' with no 1F2,
57 only 1F1), both control and *ort* knockout flies still show the 1F1 and 2F1
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3 responses (green dashed lines), but the level of the 2F1+2F2 component is
4 never above the noise recorded (0.0001) when no stimulation is applied.

5 Exact genotype: + is w^- . N = 10 for each cross.

6
7 [Note also that the solid and broken lines are much closer in the *ort* > *TNT* fly
8 than in the controls, particularly in the 2F1 response, also an expected
9 consequence of the masking paradigm, in which the third-order neurons feed
10 back to the lamina neurons and photoreceptors.]
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14 We conclude that the *ort* > *TNT* fly fails to show the full, normal pattern of
15 response particularly in the 2F1+2F2, intermodulation, component. Since the
16 major output of the second order neurons is in the medulla, this component
17 arises there. It remains possible that some of the 2F1+2F2 component arises
18 in more central parts of the visual system, but these are further from the
19 recording site and so less likely to be involved.
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23 References:

24 Afsari F, Christensen KV, Smith GP, Hentzer M, Nippe OM, Elliott CJ, and
25 Wade AR (2014) Abnormal visual gain control in a Parkinson's disease model.
26 Hum Mol Genet 23:4465-4478.
27

28 Gengs C, Leung HT, Skingsley DR, Iovchev MI, Yin Z, Semenov EP, Burg
29 MG, Hardie RC and Pak WL (2002) The target of *Drosophila* photoreceptor
30 synaptic transmission is a histamine-gated chloride channel encoded by *ort*
31 (*hclA*). J Biol Chem 277:42113-42120.
32

33 Sweeney ST, Broadie K, Keane J, Niemann H, and O'Kane CJ (1995)
34 Targeted expression of tetanus toxin light chain in *Drosophila* specifically
35 eliminates synaptic transmission and causes behavioral defects. Neuron
36 14:341-351.
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Supplementary Table 1. Statistical analysis of the SSVEP Contrast response function shown in Figure 2. Overall MANOVA followed by ANOVA of the three components in the SSVEP response

Overall	Pillai	F	P	
genotype	0.60489	18.3711	2.13E-07	***
timepoint	0.41412	8.482	0.0002155	***
genotype:timepoint	0.25708	4.1524	0.0126175	*

1F1 photoreceptor response

genotype		10.5611	0.0024205	**
timepoint		12.9101	0.0009248	***
genotype:timepoint		0.1414	0.7090138	NS

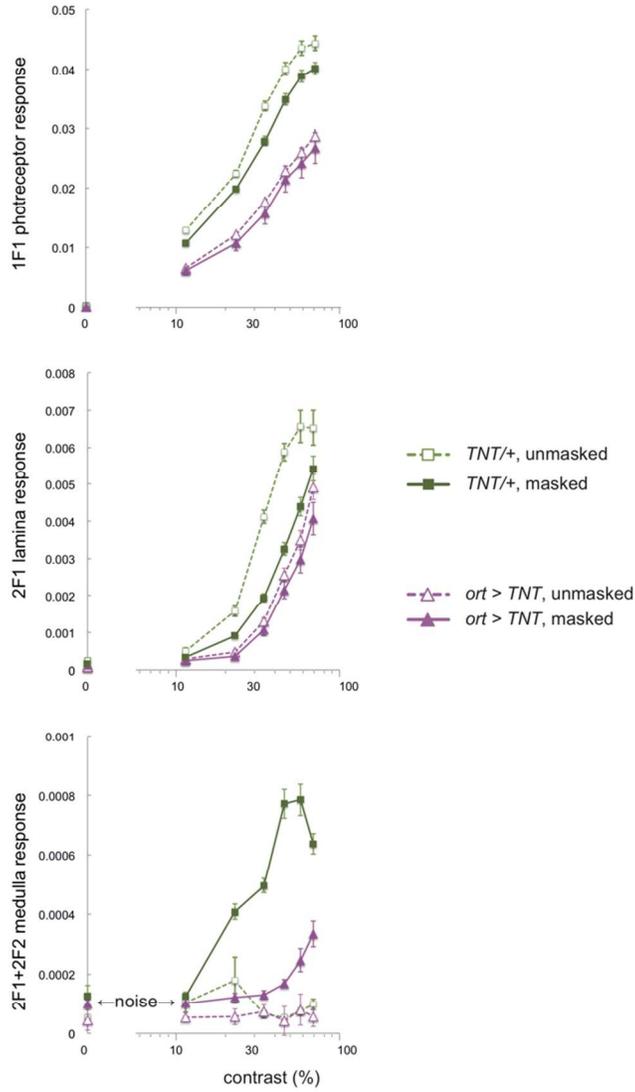
2F1 lamina neurons

genotype		0.5377	0.46789	NS
timepoint		19.335	8.55E-05	***
genotype:timepoint		4.7006	0.03648	*

2F1+2F2 medulla neurons

genotype		1.5958	0.214193	
timepoint		9.9119	0.003191	**
genotype:timepoint		0.5251	0.47313	

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Supplementary Figure S1

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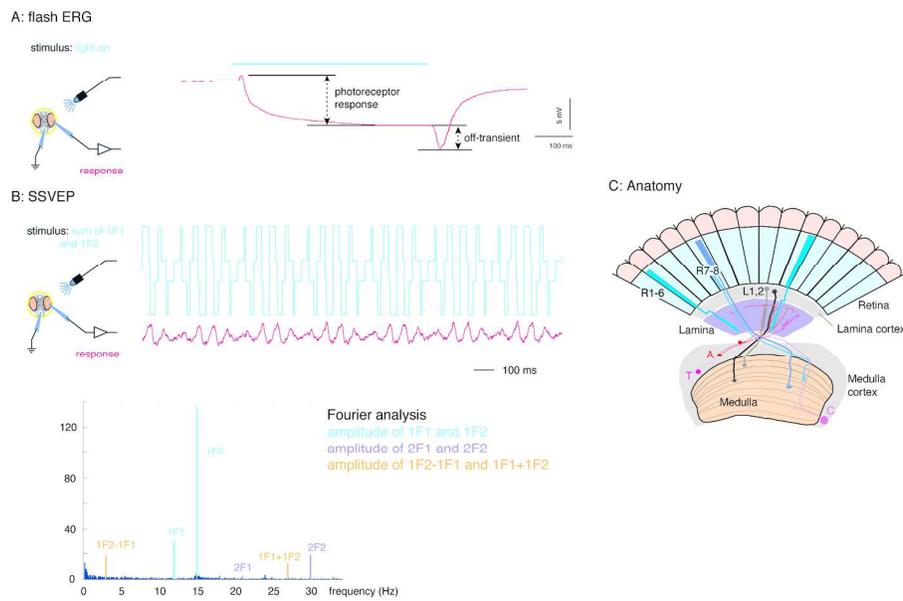


Figure 1 Colour version for online article

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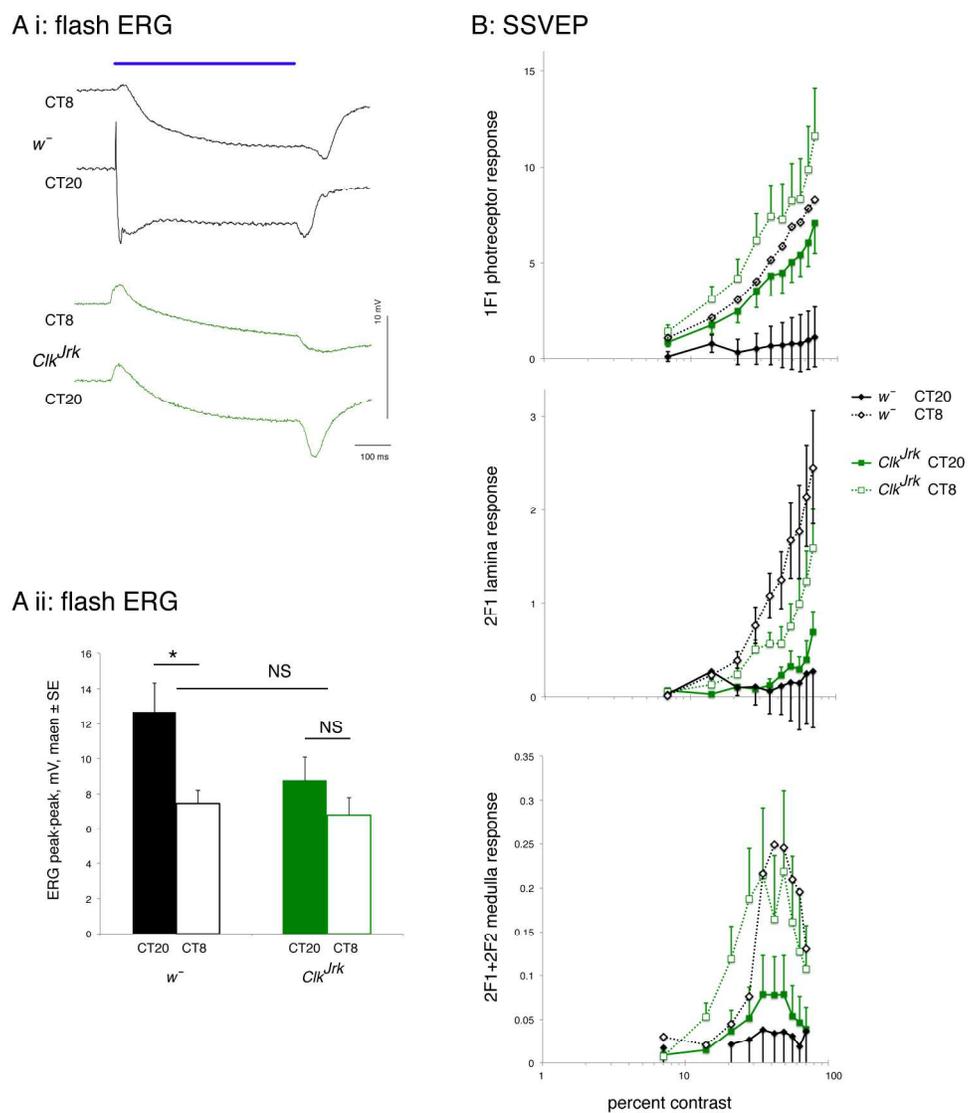
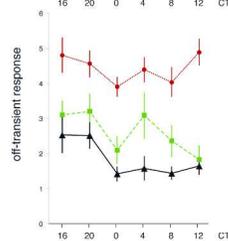
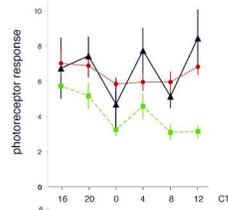


Figure 2 Colour version for online article

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A: flash ERG



B: SSVEP

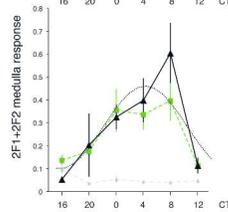
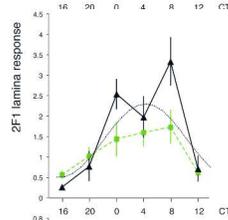
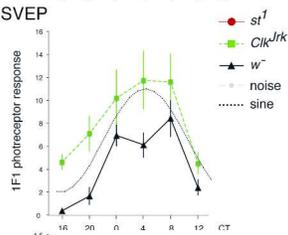


Figure 3 Colour version for online article

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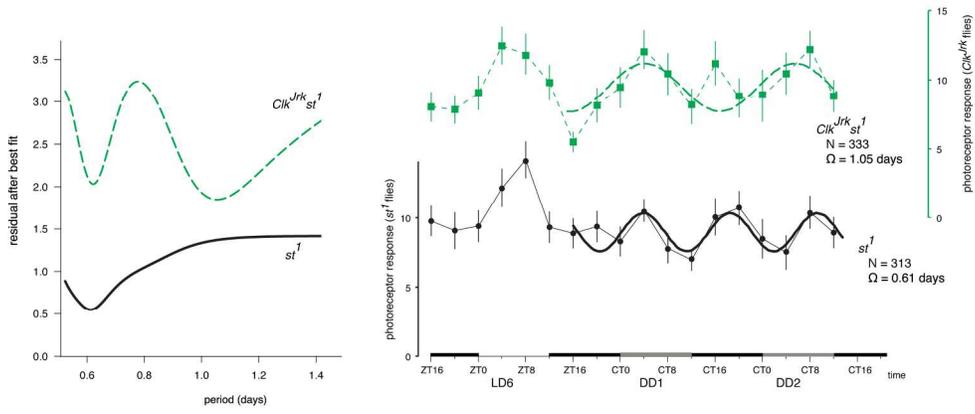


Figure 4 Colour version for online article

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