# Quasimodo mediates daily and acute light effects on *Drosophila* clock neuron excitability

Short title: **Quasimodo and clock neuron excitability**

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# ABSTRACT

We have characterized a novel light-input pathway regulating *Drosophila* clock neuron excitability. The molecular clock drives rhythmic electrical excitability of clock neurons and we show that the recently discovered light input factor Quasimodo (Qsm) regulates this variation presumably via a $Na^+$, $K^+$, $Cl^-$ co-transporter (NKCC) and the Shaw $K^+$ channel ($dK_V3.1$). Due to light-dependent degradation of the clock protein Timeless (Tim), constant illumination (LL) leads to a breakdown of molecular and behavioral rhythms. Both over-expression ($^{OX}$) and knock-down ($^{RNAi}$) of *qsm*, *NKCC* or *Shaw* led to robust LL-rhythmicity. Whole-cell recordings of the large ventral lateral neurons (l-LNv) showed that altering Qsm levels reduced the daily variation in neuronal activity: *qsm$^{OX}$* led to a constitutive less active, night-like state, and *qsm$^{RNAi}$* to a more active, day-like state. Qsm also affected daily changes in $K^+$ currents and the GABA reversal potential, suggesting a role in modifying membrane currents and GABA responses in a daily fashion, potentially modulating light arousal and input to the clock. When directly challenged with blue light, wild-type l-LNvs responded with an increase in firing at night and no net-response during the day, while altering Qsm, NKKC or Shaw levels abolished these day/night differences. Finally, co-expression of *Shaw$^{OX}$* and *NKCC$^{RNAi}$* in a *qsm* mutant background restored LL-induced behavioral arrhythmicity and wild-type neuronal activity patterns, suggesting that the three genes operate in the same pathway. We propose that Qsm affects both daily and acute light effects in l-LNvs probably acting on Shaw and NKCC.

## SIGNIFICANCE STATEMENT

Most organisms developed a circadian clock in order to adapt their behavior to daily changes of light and temperature. The molecular clock is remarkably conserved across species with much of our current understanding coming from *Drosophila* studies. In order to generate circadian behavior, appropriate levels of neuronal electrical activity are crucial but the regulators of this activity have remained largely elusive. Here we identify three membrane proteins that interact to set the clock neurons to 'day' or 'night', forming a novel circadian clock light-input pathway. The membrane-anchored extracellular protein Quasimodo affects both the daily changes in physiological properties and light resetting of brain clock neurons, possibly acting upstream of the potassium channel Shaw and the ion transporter NKCC.

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## INTRODUCTION

All organisms are subject to predictable but drastic daily environmental changes caused by the earth's rotation around the sun. It is critical for the fitness and wellbeing of an individual to anticipate these changes and this is done by circadian timekeeping systems (clocks). These regulate changes in behavior, physiology and metabolism to ensure they occur at certain times during the day thereby adapting the organism to its environment (1). The circadian system consists of three elements: the circadian clock to keep time, inputs that allow entrainment and outputs to influence physiology and behavior (2). Like a normal clock, circadian clocks run at a steady pace (24 h) and can be reset. In nature this environmental synchronization is done via daily light and temperature cycles, food and social interactions (3).

In *Drosophila* the central clock comprises 75 neuron pairs grouped into identifiable clusters that sub-serve different circadian functions (Fig. 1A). The molecular basis of the circadian clock is remarkably conserved from *Drosophila* to mammals (4). This intracellular molecular clock drives clock neurons to express circadian rhythms in electrical excitability including variation in membrane potential and spike firing. Clock neurons are depolarized and fire more during the day than at night and circadian changes in expression of clock-controlled genes encoding membrane proteins such as ion channels and transporters likely contribute to these rhythms (5-8). Such cyclical variations in activity play a critical role in synchronizing different clock neurons and conveying circadian signals to other parts of the nervous system and body (9, 10). Furthermore, they provide positive feedback to the molecular clock, without which it can rapidly dampen (7, 11, 12).

Light resets the circadian clock every morning in order to synchronize it to the environment via Timeless (Tim) degradation after activation of the blue-light photoreceptor Cryptochrome (Cry), Quasimodo (Qsm) and potentially also visual photoreceptors (13-18). Qsm acts either independently or downstream of Cry and is also able to affect clock protein stability in Qsm-negative neurons by an unknown non-cell autonomous mechanism (16). Recently Cry has been shown to regulate clock neuron excitability via the redox sensor of the Hyperkinetic (Hk) voltage-gated potassium ($K_V$)-ß subunit (19, 20) and here we ask if Qsm affects the clock neurons in a similar way.

Membrane potential is important for control of circadian behavior and manipulation of Shaw and the 'Narrow Abdomen' (NA) channels, which are both expressed and function within clock neurons, influences neuronal electrical activity, the circadian clock and clock controlled behavior in both flies (21-23) and mice (24-26). Firing rate is a key component in mammalian circadian rhythmicity and can be regulated by regional and circadian expression of the sodium potassium chloride co-transporter NKCC, which switches the effects of GABA from inhibitory to excitatory across the day (27, 28).

Here we show that down-regulation or over-expression of the three membrane protein encoding genes *qsm*, *Shaw*, and *NKCC* leads to rhythmicity in LL and that they genetically interact. All three genes are expressed in the well-characterized pigment dispersing factor (Pdf) and Cry positive large ventral lateral neurons (l-LNv) (22, 29), which are important for arousal and light input to the clock (19, 20, 30-34). We use whole-cell recordings of l-LNvs to characterize their physiological properties

and acute light effects across the day and find that Qsm helps to set the circadian state of clock neurons and modifies their response to light possibly by acting via Shaw and NKCC.

# RESULTS

## *Rhythmic LL behavior and interaction of Qsm with Shaw and NKCC*

Light is the most important Zeitgeber that resets the circadian clock. Consequently, wild-type flies displayed arrhythmic behavior in constant dim white light (LL, 10 µW/cm$^2$) while *cry$^{02}$* loss-of-function mutants were rhythmic (Fig. 1B and C and Table S1; cf. (15, 35, 36)). Previously we have shown that Qsm triggers Tim degradation within clock cells and *qsm$^{RNAi}$* (>70% reduction of *qsm* mRNA (16)) in all clock neurons using *tim-gal4* resulted in robust long-period (~27 h) locomotor rhythms in LL (cf. (16)). Light sensitivity of wild-type flies is also influenced by the naturally occurring *s-tim/ls-tim* polymorphism (37, 38) and we therefore performed all our experiments with flies carrying the same *ls-tim* genetic background. We confirmed that *qsm$^{RNAi}$* induces robust and long-period rhythms in LL using two independent *qsm-RNAi* lines. Interestingly, *qsm$^{OX}$* led to shorter, ~13 h rhythms, most likely reflecting the persistence of morning and evening activity peaks in LL.

Since NKCC physically interacts with Qsm in yeast (39) and *Shaw* expression in clock neurons partially overlaps with that of *qsm* (16, 22), we tested whether *NKCC* and *Shaw* would also affect LL behavior. Changing levels of both NKCC and Shaw resulted in flies that were rhythmic in dim LL. Like *qsm$^{RNAi}$*, *NKCC$^{OX}$* flies exhibited long periods (~29 h), while *NKCC$^{RNAi}$* (~70% reduction of mRNA, Fig. S1) and *Shaw$^{RNAi}$* (~90% reduction of Shaw (31)), resulted in ~13 h periods, reflecting potential bimodal behavior similar to *qsm$^{OX}$* (Fig. 1B and C and Table S1). *Shaw$^{OX}$* induced truly and remarkably robust ultradian rhythms with a ~4 h period, indicating compromised circadian clock function (cf. (22)) and uncovering an underlying ultradian rhythm presumably generated by a membrane-based oscillator. These

manipulations did not affect behavior in constant darkness (DD; Table S2) with the exception of $Shaw^{OX}$, which led to arrhythmicity as previously reported (22).

To determine the influence of the *s/ls-tim* polymorphism on the behavioral phenotypes observed here, we also tested $qsm^{OX}$, $qsm^{RNAi}$, $Shaw^{RNAi}$ and $NKCC^{OX}$ in a *s-tim* background and compared their LL-rhythmicity with the same manipulations in an *ls-tim* background. In all cases, *s-tim* caused a dramatic (~50%) reduction of the percentage of rhythmic flies compared to *ls-tim* flies, which was often correlated with a period lengthening (Fig. S2 and Table S1). This behavior fits well with the previous observation that flies carrying the *s-tim* allele are more light sensitive compared to their *ls-tim* counterparts and the dramatic behavioral difference caused by the two alleles underscores their biological importance (37, 38).

Because the *tim-gal4* driver is also expressed in the photoreceptor cells of the compound eyes and therefore can influence potential endogenous expression of *Shaw*, *NKCC*, and *qsm* in these cells, we also tested flies with impaired visual system function under the identical LL conditions. Similar to wild type, flies lacking (i) all external photoreceptors ($gl^{60j}$; (15)), (ii) Phospholipase C-ß required for the visual phototransduction cascade ($norpA^{P41}$; (40)), or (iii) histamine, the principal neurotransmitter of the visual system ($Hdc^{JK910}$; (41)) became virtually arrhythmic during LL (Table S1). These results strongly suggest that the *tim-gal4* mediated LL-rhythmicity is induced by alteration of *Shaw*, *NKCC*, and *qsm* expression within the clock neuronal network.

To directly test for genetic interactions, we determined the effects of altering Shaw and NKCC levels on the LL-rhythmic phenotype induced by reducing Qsm. We used $qsm^{105}$, an intragenic $gal4$ insertion line that reduces $qsm$ expression and induced ~37% LL-rhythmicity in heterozygous ($qsm^{105}$ > +) flies (Fig. 1B and C and Table S1; cf. (16)). Using the $qsm^{105}$ driver, both $Shaw^{OX}$ and $NKCC^{RNAi}$ induced wild-type scores of LL-rhythmicity (i.e. 2%), while the opposite manipulation led to increased rhythmicity scores (54% and 81% respectively). The apparent genetic interaction between $qsm$, $Shaw$, and $NKCC$ suggests that they function in the same pathway. We propose that Qsm influences membrane properties via ion channels (Shaw) and transporter proteins (NKCC) that in turn might influence molecular responses of circadian clock neurons to light.

### *Qsm affects daily changes of physiological properties*

In order to determine how Qsm, Shaw and NKCC might contribute to clock neuronal activity, we examined their neurophysiological role by performing whole-cell recordings from l-LNv with altered $qsm$, $Shaw$ or $NKCC$ expression using $Pdf$-$gal4$ and $uas$-$RFP$ (Fig. S3A and B). We focused on the l-LNv because they represent the electrophysiologically best characterized clock neuronal cell type and because they express $qsm$, $Shaw$, and $NKCC$ (8, 22, 29). Additionally, while genetic studies have shown that LL rhythmicity is mainly driven by LNd and DN1 neurons (16, 42-44), l-LNvs play an important role in the light-input and arousal pathways and are more easily accessible (6, 19, 20, 30-34).

While physiological parameters showed variability between recordings of different animals, in the day, wild-type l-LNvs showed a more depolarized resting membrane

potential (RMP) and a higher spontaneous firing rate (SFR) compared to at night (Fig. 2A and B and Table S3; cf. (5, 33)). $qsm^{OX}$ led to a more hyperpolarized RMP and reduced SFR in day recordings that were very similar to control and $qsm^{OX}$ night recordings. In contrast, $qsm^{RNAi}$ depolarized the neurons and increased SFR at night similar to wild-type and $qsm^{RNAi}$ day levels. We tested two $qsm^{RNAi}$ lines targeting different regions of the $qsm$ transcript (16) and we did not observe a difference (pooled data shown). Interestingly, the RMP and SFR differences seen in wild-type between day and night were abolished in both $qsm^{OX}$ and $qsm^{RNAi}$ l-LNvs. Resembling the changes seen for $qsm$, $Shaw^{OX}$ led to more hyperpolarized, less active neurons and $Shaw^{RNAi}$ to more depolarized and more firing neurons, eliminating the wild-type day/night differences. NKCC manipulations had the opposite effect with $NKCC^{OX}$ depolarizing and activating the neurons, in particular at night, and with $NKCC^{RNAi}$ hyperpolarizing and inactivating the neurons.

### Qsm affects daily changes of K⁺ currents

To elucidate potential mechanisms of how Qsm could affect physiological properties and since manipulating $Shaw$ levels had shown similar effects (Fig. 2A), we tested whether this might be due to alteration of voltage-dependent somatic currents affecting the RMP. We examined the current–voltage relationships of l-LNvs and measured the ensuing currents, that are an aggregate of all outward currents including non-inactivating currents at the end of the voltage pulse, like Shaw (Fig. 2C; cf. (45, 46)). We analyzed the sustained current densities of wild-type l-LNvs at day and night and could not detect any difference around the RMP in our voltage-clamp experiments, most likely because of masking by the leak-current subtraction. At positive holding potentials (+100 mV), however, there was a difference with

current densities at night being >30 pA/pF larger than in the day suggesting more open potassium channels (Fig. 2C and D and Table S4). $Shaw^{OX}$ resulted in a similarly high current density as wild-type l-LNvs recorded at night while $Shaw^{RNAi}$ values were matching wild-type day recordings indicating an involvement of Shaw in the observed day/night difference. Mimicking $Shaw$, $qsm^{OX}$ had similarly high current densities as wild-type l-LNvs at night and $qsm^{RNAi}$ currents matched wild-type day levels. This supports an interaction of Qsm with voltage gated potassium channels like Shaw in setting membrane properties.

### Qsm affects daily changes of the GABA reversal potential

Manipulating NKCC levels had an effect on RMP and SFR (Fig. 2A and B) and while NKCC activity is electrically neutral, NKCC pumps chloride ions in the cell, increasing the intracellular chloride concentration. We therefore tested whether the GABA reversal potential ($E_{GABA}$) of l-LNvs also changes across the day by injecting GABA (10 ms, 25 mM, 10 PSI) in the ipsilateral medulla. In all cases spiking activity was affected (Fig. 2E-G). We measured the induced currents in voltage-clamp and calculated $E_{GABA}$ from the resulting IV curve (Fig. 2H and Table S5). At day this reversal potential was more positive (>10 mV) making GABA input less effective. GABA injections at day or night time increased the reversal potential in $NKCC^{OX}$ brains and had the opposite effect in $NKCC^{RNAi}$ brains where $E_{GABA}$ was more negative (Fig. 2F and H), thereby mimicking the wild-type day/night change. Remarkably, changing Qsm levels also had an effect which was again in the opposite direction to NKCC, with $qsm^{OX}$ decreasing and $qsm^{RNAi}$ increasing $E_{GABA}$ (Fig. 2G and H). Strikingly, in some cases $E_{GABA}$ would be more positive than the RMP and GABA consequently acted as an excitatory neurotransmitter inducing firing

(see Fig. 2G, red arrow in top trace). These results suggest a direct interaction of Qsm with NKCC and their participation in the daily change of GABA efficiency.

### *Qsm interacts with Shaw and NKCC*

To further characterize the interaction of Qsm with Shaw and NKCC, we tested if altering levels of *shaw* and *NKCC* could rescue some of the *qsm*-induced physiological changes. We used *tim-gal4 > qsm$^{RNAi}$* to reduce Qsm expression in all clock neurons and found a similar effect to using *Pdf-gal4.* Again we did not observe a difference between day and night but the resulting firing rate was much higher (Fig. 3 and Table S3) suggesting additional network effects (recall *qsm* expression in PDF[-] clock, and non-clock neurons (16)). We then co-expressed *qsm$^{RNAi}$* with *Shaw$^{OX}$* and *NKCC$^{RNAi}$* and could rescue the elevated firing rate to wild-type levels (Fig. 3) mimicking the behavioral rescue experiments (Fig.1 and Table S1) and further supporting that Qsm interacts cell-autonomously with Shaw and NKCC. Interestingly, *Shaw$^{OX}$* seems to be more effective than *NKCC$^{RNAi}$* and the rescue is less pronounced at night.

In summary, over-expression and RNAi knock-down of *qsm* and *Shaw* resulted in opposing phenotypes compared to *NKCC* manipulations. Interestingly, *qsm$^{OX}$* or *Shaw$^{OX}$* and *NKCC$^{RNAi}$* promote the less active night state while *qsm$^{RNAi}$*, *Shaw$^{RNAi}$* and *NKCC$^{OX}$* push the neurons into the more depolarized day state, eliminating the acute differences between day and night in all cases. Furthermore, Qsm interacts with Shaw and NKCC to affect potassium currents and the GABA reversal potential.

### *Acute light effects on l-LNvs and its modulation by Qsm*

Altering levels of Qsm, Shaw and NKCC using the *tim-gal4* driver led to LL rhythmicity suggesting an impairment of the light input to the circadian clock (Fig. 1). Because the visual system mutants became arrhythmic under the identical LL conditions (Table S1) alteration of Qsm, Shaw and NKCC within clock neurons must be responsible for the LL rhythmicity observed. Although l-LNv neurons are unlikely to be mediators of rhythmic behavior in LL (16, 42-44) they are arousal neurons activated by blue light via Cry (19) and the $K_V$-ß subunit redox sensor Hk (20), that could assemble with Shaw ($K_V$-α subunit). We propose that Qsm influences membrane properties via ion channels (Shaw) and transporter proteins (NKCC) that in turn might influence light arousal in l-LNv and molecular light-resetting of the clock in other clock neurons. Therefore, we tested the response of l-LNvs to light in isolated brains, i.e. in the absence of all canonical photoreceptors including the compound eye, the HB-eyelet and the ocelli leaving only deep brain photosensitive pathways (e.g. Cry) intact.

At night all tested wild-type neurons responded to blue light with a slight depolarization and an increase in firing rate either directly at lights-on (Fig. 4A, right panel, top trace) or after a brief 'startle' response (bottom trace), often outlasting the stimulus by tens of seconds before eventually returning to baseline firing rate. On average spiking would increase by 1-2 Hz to levels near the resting firing rate at day (Fig. 5A and Table S3). In daytime the response was different, more complex and variable. Some neurons (n=2) briefly increased (Fig. 4A, left panel, top trace) or decreased firing (n=5, bottom trace), but on average there was not much change in firing frequency, clearly different to the response at night (Fig. 5A and Table S3) and

suggests a daily variation in responsiveness and light sensitivity of the clock. We occasionally (n=8) observed a 'startle' response with a brief period of either increased firing or stopping of firing immediately after the lights-on transition both at day and night. Consistent with published data (19) the effect was only seen with blue (470 nm) and not with green (555 nm, Fig. S3C) or red light (625 nm, not shown). Corresponding with the Cry absorption spectrum (<540 nm; (47)), $cry^{02}$ l-LNvs did not respond to blue light (Fig. S3D). However, $cry^{02}$ l-LNvs had similar day/night RMP and SFR changes compared to control l-LNv indicating that Cry is not required for the circadian clock regulation of neuronal electrical activity (Fig. 2A and B and Table S3).

$qsm^{OX}$ always led to neurons with diminished response to the light pulse in terms of firing frequency (Fig. 4B). Therefore, $qsm^{OX}$ l-LNvs behaved rather like $cry^{02}$ neurons, the control neurons exposed to green light, or the daytime wild-type neurons. $qsm^{RNAi}$ in contrast always led to an increase in spike rate in response to blue light similar to that seen in wild-type night recordings, but already starting from a higher baseline rate (Fig. 2B and 4C). The average change in firing was very similar to the wild-type night response and was independent of time of day (Fig. 5A and Table S3). Light pulses in the presence of up- or down-regulation of *Shaw* resulted in similar changes as the equivalent alterations of *qsm* expression and *NKCC* manipulations resulted in opposite changes (Fig. 4D-G).

In summary, wild-type flies show a differential day/night response to blue light pulses; at day either no response or a slight reduction of firing and at night a dramatic increase in the firing rate reaching or exceeding day levels. Qsm, Shaw,

and NKCC manipulations showed no such day/night difference, with the response of these flies rather being determined by the respective level of gene expression (up- or down-regulation). $qsm^{RNAi}$, $Shaw^{RNAi}$ and $NKCC^{OX}$ l-LNvs respond to acute blue light with an increase in firing like wild-type neurons at night, while the reverse manipulation abolished light responses, presumably as a consequence of silencing the neurons (Fig. 5A).

## DISCUSSION

Light is the dominant circadian Zeitgeber that resets the molecular clock. In this study we determine how light affects membrane excitability via the membrane proteins Qsm, Shaw and NKCC. Previously we have shown that Qsm contributes to circadian clock light-input with down-regulation in all clock neurons (*tim-gal4*) resulting in robust rhythmic behavior in constant light (LL; (16)). Here we show that over-expression ($qsm^{OX}$) also results in robust LL-rhythmicity, yet with predominantly ~13 h periods, most likely reflecting the persistence of morning and evening activity peaks (Fig. 1B and C and Table S1). Manipulating expression levels of both the potassium channel Shaw and the ion co-transporter NKCC also resulted in LL-rhythmicity, whereas several visual system mutants behaved like wild-type and became arrhythmic. Together, these experiments show that normal LL behavior is impaired in clock neurons mis-expressing *qsm*, *Shaw* and *NKCC* and that the membrane proteins encoded by these genes control rhythmic behavior in LL. Furthermore, rescue of wild-type behavior and neurophysiological properties by reciprocal change of Qsm and Shaw as well as simultaneous reduction of Qsm and NKCC suggests a genetic and perhaps direct interaction of Qsm with Shaw and NKCC. The appearance of robust ~13 h periods in $qsm^{OX}$, $Shaw^{RNAi}$ and $NKCC^{RNAi}$ flies further suggests that these manipulations somehow led to a more robust morning oscillator, which is normally weakened in constant dark conditions.

Clock neurons are more depolarized and fire more during the day than at night and circadian changes in expression of clock controlled genes such as ion channels and transporters are likely to play a part (7, 8). Contributing to this rhythm is a sodium leak current mediated by the putative channel 'Narrow Abdomen' (NA) that has

recently been shown to depolarize *Drosophila* clock neurons during the day and functions in a similar way in the mammalian clock (23). Here we show that Shaw, NKCC, and Qsm also contribute to daily electrical activity rhythms: over-expression and RNAi knock-down of *qsm* and *Shaw* compared to *NKCC* resulted in opposing phenotypes. Interestingly, $qsm^{OX}$ or $Shaw^{OX}$ and $NKCC^{RNAi}$ promote the less active night state while $qsm^{RNAi}$, $Shaw^{RNAi}$ and $NKCC^{OX}$ push the neurons into the more depolarized day state, eliminating the acute differences between day and night in all cases (Fig. 2A and B and Table S3). We have previously shown that Shaw regulates circadian behavior possibly via changing rhythmic PDF release (22) and consistent with our current findings, Shaw regulates membrane potential and firing in *Drosophila* motoneurons (31, 48). NKCC activity is electrically neutral but increases the intracellular $Cl^-$ concentration so that in response to GABA the $GABA_A$ receptor opens, but as a consequence $Cl^-$ presumably leaves the cell down its electrochemical gradient, thereby depolarizing the membrane potential so that GABA becomes effectively an excitatory neurotransmitter (6, 12, 27, 31, 49). Our data show for the first time that in *Drosophila* a similar mechanism occurs in l-LNv consistent with potential NKCC enrichment at dawn (29). The mechanism setting the neuronal state to either day or night via Qsm, Shaw and NKCC is likely to be predominantly cell autonomous as all components have been shown to act or be expressed in the l-LNv (8, 22, 29). Although in an earlier study (16) using *qsm-gal4* lines we did not detect *qsm* expression in the l-LNv, these lines may not faithfully report expression in all *qsm* cells. Now, the finding that *qsm* RNA is enriched in the l-LNv (29) combined with strong effects of two *qsm-RNAi* lines on l-LNv electrical properties presented in this study, indicates that *qsm* is endogenously expressed in these neurons.

Our physiological studies are limited to the PDF-expressing l-LNv neurons. These neurons are unlikely candidates for driving behavioral rhythms in LL (42-44), and we showed previously that *qsm* knockdown in PDF neurons (s-LNv and l-LNv) does not result in robust LL rhythmicity (16). The effects of light on the electrical properties of l-LNv reported here, therefore do not necessarily explain the LL-rhythmicity observed after manipulating *qsm, Shaw*, and *NKCC* in all clock neurons (Fig. 1 and Table S1). However, the electrophysiological results using *tim-gal4* show that Qsm, Shaw, and NKCC could fulfill similar functions in other clock neurons including those crucial for LL-rhythmicity (e.g. LNd and DN1). Additionally, or alternatively, the manipulated l-LNv could generate signals interfering with normal network function, resulting in the observed rhythmic LL behavior.

Although *qsm* is a clock controlled gene (16, 29, 50), the acute blue light effects we observed are too fast to be mediated by transcriptional changes. Therefore, we favor a more direct membrane localized mechanism in which rapid light-dependent post-translational changes of Qsm alter the activity of Shaw and NKCC. Since (i) Cry is required for light-dependent Tim degradation in l-LNv (15), (ii) changing Qsm levels has no effect on Cry levels, and (iii) $qsm^{OX}$ triggers Tim degradation in the absence of Cry (16), the most likely explanation for the results obtained here is that in addition to activating Hk, Cry acts upstream of Qsm, which in turn regulates the activity of Shaw and NKCC.

We assume that Qsm is somehow activated by light since a light pulse at night rapidly increases protein levels (16). Qsm is an extracellular zona-pellucida membrane-anchored protein, and we hypothesize that after light-exposure the

extracellular ZP-domain is cleaved at a conserved furin protease cleavage site; a form of post-translational processing typical for ZP-domain proteins (51). While it seems likely that light structurally modifies Qsm, it is also possible that Qsm signals to Shaw and NKCC in both, membrane-bound and cleaved forms. For example, at night membrane-bound Qsm could block NKCC while light-induced cleavage could release this block and the freed extracellular part could then inactivate Shaw (Fig. 5B). This mechanism is reminiscent to the GPI-anchored extracellular protein SLEEPLESS increasing SHAKER ($K_V$1 channel) activity for regulating *Drosophila* sleep (52).

It is also an open question how Qsm-induced changes of clock neuron activity influence the molecular clock. Recent work shows that in addition to the canonical degradation via Cry and JETLAG (53), Tim is also degraded via a CUL-3 and neuronal activity dependent pathway in DD, that has been implicated to mediate phase delays of the circadian clock (54, 55). In contrast to this activity-dependent CUL-3 pathway, the light responses in the current study depend on Cry. We therefore favor a model, in which the combined function of Qsm, Shaw and NKCC feed into the canonical JETLAG-dependent Tim degradation pathway.

In conclusion, we demonstrate that Qsm affects both daily and acute light responses of l-LNvs forming a novel light-input pathway to the *Drosophila* circadian clock. Qsm possibly signals downstream of Cry and acts on Shaw and NKCC to change clock neuronal activity in response to light. It is likely that Qsm also receives light-input from other (Cry-independent) photoreceptors, because of largely non-overlapping Cry and Qsm expression within the clock neuronal network (16).

# MATERIALS AND METHODS

## *Animals*

Flies were raised with a 12 h:12 h light dark (LD) cycle with lights on at ZT 0 (Zeitgeber time) on standard *Drosophila* medium (0.7% agar, 1.0% soya flour, 8.0% polenta/maize, 1.8% yeast, 8.0% malt extract, 4.0% molasses, 0.8% propionic acid, 2.3% nipagen) at 25°C and collected between ~3-5 days post eclosion. The following flies used in this study were previously described or obtained from the Bloomington, Vienna or NIG fly stock centers: $gl^{60J}$ (15), $norpA^{P41}$ (40), $Hdc^{JK910}$ (41) and $cry^{02}$ (56). For wild-type control recordings either *Pdf-RFP* (8) or *Pdf-gal4* (57) crossed to *uas-mCD8-RFP* (BL27392) or *uas-mCherry* (BL52268) were used. For the behavioral controls wild-type (*y w*) and *tim-gal4:16* (58) flies were used. Experimental genotypes including *uas-qsm#5* ($qsm^{OX}$, (16)), *uas-qsmRNAi* (VDRC15394) and *uas-qsmRNAi(2)* ($qsm^{RNAi}$, (16)), *uas-shawWT12B* ($Shaw^{OX}$, (22)), *uas-shawRNAi* ($Shaw^{RNAi}$, (22)), *uas-CG31547RNAi* ($NKCC^{RNAi}$, NIG2509R-2) were crossed to *tim-gal4:16* for behavioral tests and either *Pdf-gal4 ; uas-mCD8-RFP* or *Pdf-gal4 ; uas-mCherry* flies for electrophysiological recordings. $qsm^{105}$, a *gal4*-line inserted in the *qsm* 1$^{st}$ intron that reduces *qsm* expression (16), was used to test for behavioral interactions of *qsm* with *Shaw* and *NKCC*. For respective tests of physiological interaction *Pdf-RFP ; tim-gal4:16 uas-qsm$^{RNAi(2)}$* flies were crossed to either *uas-mCD8-GFP* (BL30001) or *uas-shawWT12B* and *uas-CG31547RNAi*. Note that in both sets of electrophysiology experiments (i.e., those involving *Pdf-gal4* and *tim-gal4*, the ratio of *gal4* to *uas* constructs was always 1:2. To test for Cry dependent effects *Pdf-RFP* was crossed into the $cry^{02}$ background (56). Light sensitivity of wild-type flies is influenced by the naturally occurring *s-tim/ls-tim* polymorphism (37, 38). *ls-tim* flies are less light-sensitive compared to *s-tim* flies and in conjunction with

1  *jetlag* mutants *ls-tim* elicits LL-rhythmicity (37, 38). Therefore all fly stocks used in

2  this study were genotyped for *timeless* and *jetlag* polymorphisms as described (37,

3  38), and if necessary were crossed into the *ls-tim* background to allow comparison

4  between genotypes (all stocks were *jetlag+*). To reveal the effect of the *s/ls-tim*

5  polymorphism, selected genotypes were also analyzed in an *s-tim* background

6  (Table S1).

7

8  ***Generation of transgenic flies***

9  A flag-HA tagged *uas* construct of CG31547-PB (UFO03679) was obtained from

10  DGRC (59) (http://www.fruitfly.org/EST/proteomics.shtml). *uas-CG31547-flgHA* was

11  integrated into the *ZH-attP-86Fb* landing site using the ΦC31 integrase system to

12  generate *NKCC$^{OX}$* (60). The eye-expressed 3xP3-RFP cassette present in the

13  landing site was eliminated by Cre-mediated excision as described (60).

14

15  ***RNA isolation and RT-qPCR***

16  To test the efficiency of *NKCC$^{RNAi}$*, 5-10 day old flies were frozen at ZT 2 and 20

17  heads of each genotype were collected over dry ice. The total RNA was extracted

18  using an RNeasy kit (QIAGEN) according to the manufacturers' instructions and

19  finally eluted in RNase-free water and stored at -80°C. cDNA synthesis was

20  performed with Reverse Transcription Reagents Kit (Applied Biosystems) in 20 µl

21  reactions using 1 µg of total RNA. To verify mRNA expression level of *NKCC*,

22  dilutions of cDNA were used for PCR with *rp49* primers, followed by DNA

23  electrophoresis on 2% agarose gels to visualize the PCR products. Taqman probes

24  for *NKCC* (CatNo. 4351372, ThermoFisher) were applied to determine the amount of

25  mRNA. Real-time assays were performed using an ABI GeneAMP PCR system

9700 using the standard program, and $C_T$ (threshold cycle) values were applied to determine the amount of RNA in each genotype. The relative concentrations were calculated using the comparative $C_T$ method, and *RPL$_{32}$* was used as control.

### *Behavior*

Analysis of locomotor activity of 4-5 day old male flies was performed using the *Drosophila* Activity Monitor System (DAM2, Trikinetics Inc., Waltham, MA, USA) with individual flies in recording tubes containing food (2% agar, 4% sucrose). The DAM monitors, as well as an DEnM environmental monitor (Trikinetics Inc.), were located inside a light- and temperature-controlled incubator (Percival Scientific Inc., Perry, IA, USA) where the fly's activity was monitored for 4 days in 12 h:12 h LD followed by 7 days under constant light (LL, 10 $\mu$W/cm$^2$) conditions at 25°C. Plotting of behavioral activity, period calculations and the determination of rhythmic statistics (R.S.) were performed using either a signal-processing tool-box (61) implemented in Matlab (MathWorks, Natick, MA, USA) or the ImageJ (http://rsb.info.nih.gov/ij/) plug-in ActogramJ (62). LL-rhythmicity was determined on the basis of an R.S. value >1.5 and classified according to the period length of individual flies either being bimodal and circadian (~12 h or ~24 h respectively) or ultradian (< 10 h). Significance of rhythmicity for ultradian periods was determined using periodogram analysis in ActogramJ (p-level set to 0.001), each period was subsequently verified by visual inspection of the actograms. For simplicity the value of the overall rhythmicity includes flies of all categories but the average period was calculated based on the prevalent period for each genotype.

### *Electrophysiological recordings*

Patch-clamp recordings were performed as described previously (63). Flies were decapitated and brains dissected either at ZT 1-3 (day condition) or ZT 13-15 (night condition). For recordings at night, dissections were performed on a M205C stereomicroscope (Leica, Wetzlar, Germany) set to minimal white light and experiments were conducted under red light illumination. Whole fly brains were acutely dissected in extracellular saline solution containing (in mM): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 $NaH_2PO_4$, 20.7 $NaHCO_3$, pH 7.2. Some brains were briefly transferred in saline containing 20 U/ml papain and 1 mM L-cysteine in order to aid removal of the ganglion sheath. After removal of the photoreceptors, lamina, air sacks and trachea, a small incision was made over the position of the l-LNv neurons in order to give easier access for the recording electrodes. The brain was then placed ventral side up in the recording chamber, secured using a custom-made mammalian brain slice harp and during recordings continuously perfused with aerated (95% $O_2$, 5% $CO_2$) saline solution. Neurons were visualized using a x63 lens and a 555 nm LED light source on an upright Zeiss microscope (Examiner.Z1, Carl Zeiss Microscopy GmbH, Jena, Germany). l-LNv neurons were identified on the basis of their fluorescence, size and position (Fig. 1B, C). Generally, a single recording was performed from one l-LNv per brain at room temperature (20-22°C). If a recording from a second neuron was performed in the same brain, it was on the opposite side of the brain and at least 20 min after the first. Whole-cell current and voltage clamp recordings were performed using glass electrodes with 8-18 MΩ resistance filled with intracellular solution (in mM: 102 K-gluconate, 17 NaCl, 0.94 EGTA, 8.5 HEPES, 0.085 $CaCl_2$, 1.7 $MgCl_2$ or 4 Mg·ATP and 0.5 Na·GTP, pH 7.2) and an Axon MultiClamp 700B amplifier, digitized with an

Axon DigiData 1440A (sampling rate: 20 kHz; filter: Bessel 10 kHz) and recorded using pClamp 10 (Molecular Devices, Sunnyvale, CA, USA). Capacitance, series resistance and leak currents were corrected for and voltage clamp traces were offline low-pass filtered (8-pole Bessel, cut-off 100 Hz). All chemicals were purchased from Sigma (Poole, UK).

### *Light and GABA application*

In order to test the effect of light on the activity of the l-LNvs a blue light pulse (470 nm) was applied using the fluorescence kit and appropriate filter of the microscope (Colibri, Zeiss) set at 50% intensity (7.3 mW/cm$^2$). As controls other wavelengths (green 555 nm and red 625 nm) were also tested. The experimental design consisted of 1 min recording before light application, the 30 s light exposure and further 2.5 min recording afterwards. To test for changes in the GABA reversal potential, GABA (25 mM) was injected (10 ms pulse) in the ipsilateral medulla via a glass pipette (1-3 MΩ) and a Picospritzer III (10 PSI; Parker Hannifin, NH, USA).

### *Data analysis/Statistics*

The liquid junction potential of the recordings was calculated as 13 mV and was subtracted from all the membrane voltages. Resting membrane potential (RMP) was measured after stabilizing for 2-3 min after break into whole cell configuration. The spontaneous firing rate (SFR) and spike frequency (F) were measured using 10 s bins throughout the experiment. To quantify the effect of blue light on the firing rate, the average frequency of the 30 s baseline before light application was subtracted from the average of the 30 s light on ($F_{on}$-$F_{off}$). A cell was included in the analysis if the access resistance was less than 50 MΩ and only tonically firing neurons were

analyzed (a total of three bursting neurons were rejected). GABA reversal potentials ($E_{GABA}$) were calculated from the IV-curves resulting from the currents elicited by pressure injected GABA at different holding potentials in voltage clamp mode. Sustained $K^+$ currents were measured from the average of the last 100 ms of a 1 s voltage pulse obtained from neurons held at a potential of -80 mV and stimulated to potentials up to +100 mV in 10 mV intervals. The current density was calculated by normalizing the measured currents with the cell capacitance. Since over-expression and RNAi knock-down of *qsm*, *Shaw* and *NKCC* eliminated the day/night differences of physiological parameters, recordings to measure $E_{GABA}$ and the $K^+$ currents of l-LNvs with altered Qsm, Shaw or NKCC levels were performed at both day and night. Again, we did not observe a difference between day and night recordings and therefore the data were pooled. All values are given as mean and standard deviation (SD). The Kolmogorov-Smirnov test was used to test for normality, the t-test to calculate significant differences between wild-type control flies at day and at night for RMP, SFR and $F_{on}$-$F_{off}$ (indicated by [#]), a 1-way ANOVA followed by Dunnett's multiple comparison test for differences between wild-type and test flies within the day or night condition for RMP, SFR and $F_{on}$-$F_{off}$ (indicated by *), or followed by Tukey's multiple comparison test for differences in $E_{GABA}$ and $K_V$ current densities (indicated by *). The statistical tests were performed using Prism (GraphPad Software Inc., La Jolla, CA, USA) and the figures arranged in Illustrator (Adobe Systems Inc., San Jose, CA, USA).

# ACKNOWLEGMENTS

# FIGURE LEGENDS

**Figure 1.** *Drosophila* circadian clock and LL behavior.

(**A**) Cartoon of a frontal view of the *Drosophila* brain showing the ~75 pairs of clock neurons forming several groups with distinct function: 6 dorsal lateral neurons (LNd); 4 large and 4 small PDF-expressing ventral lateral neurons (LNv) and a single PDF-negative neuron (5th s-LNv); 3 lateral posterior neurons (LPN); dorsal neurons (16 $DN_1$, 2 $DN_2$, 40 $DN_3$) and the potential light input pathways (yellow arrows) via classical photoreceptors, ocelli, the HB-eyelet and $Cry^+$ neurons. (**B**) Double-plotted actograms of exemplary individual flies of the prevalent rhythm recorded for the first 3 days in LD (grey — lights off; white — lights on) and then in constant dim light for the indicated genotypes. (**C**) Bar graph specifying the percentage of flies showing circadian or bimodal (black bars), ultradian (light grey bars) or arrhythmic (open bars) behavior for each genotype as indicated by small letters. Numbers in bars are n. See also Tables S1 and S2 and Fig. S1 and S2.

**Figure 2.** Daily changes of physiological properties of l-LNvs.

(**A**) Resting membrane potential (RMP) changes of wild-type neurons (black bars; means and SD, n indicated), l-LNvs with altered levels (*Pdf-gal4*, solid bars are over-expression, open bars RNAi knock-down) of *qsm* (blue bars), *Shaw* (green bars) and *NKCC* (red bars) and $cry^{02}$ mutants (purple bars) in the day (left side) and at night (right side). (**B**) Spontaneous firing rate (SFR) of the same genotypes as in A. Dotted red line represents wild-type day values and dashed black line night values. (**C**) Sustained current densities (measured as indicated by green bar in example shown in insert) for wild-type l-LNvs at day (open black circles; mean and SD) and night (closed black circles), $Shaw^{OX}$ (closed green circles), $Shaw^{RNAi}$ (open green circles),

*qsm$^{OX}$* (closed blue circles) and *qsm$^{RNAi}$* (open blue circles) l-LNvs. (**D**) Quantification

and statistical analysis of the sustained current density recorded at +100 mV holding

potential. (**E-G**) Representative examples of the GABA response of a wild-type l-LNv

(**E**) at day (left panels) and night (right panels), *NKCC$^{OX}$* and *NKCC$^{RNAi}$* (**F**) as well as

*qsm$^{OX}$* and *qsm$^{RNAi}$* (**G**). Top traces are current clamp responses to GABA (10 ms,

25 mM, 10 PSI) applied via pressure injection in the medulla at arrowhead. Bottom

traces show the same neuron and GABA pulse in voltage clamp held at the indicated

potentials. Note the red arrow in (G) pointing to occasional excitatory GABA effects

for *qsm$^{RNAi}$*. (**H**) Quantification of E$_{GABA}$ for the indicated genotypes. * $p<0.05$, **

$p<0.01$, *** $p<0.001$; 1-way ANOVA with Dunnett's (A, B) or Tukey's (D, H) *post hoc*

test (*) or t-test ($^{\#}$). See also Tables S3-5.


**Figure 3.** Physiological interaction of *qsm* with *Shaw* and *NKCC*.

(**A**) Representative examples of l-LNvs co-expressing *qsm$^{RNAi}$* with *GFP* (*tim-gal4*,

control, top traces), *Shaw$^{OX}$* (middle traces) and *NKCC$^{RNAi}$* (bottom traces) at day

(left) and night (right side). MP, membrane potential. (**B**) Quantification (means and

SD, n indicated) and statistical analysis of resting membrane potential (RMP) and

spontaneous firing rate (SFR) showed a rescue of the *qsm$^{RNAi}$* phenotype by both

*Shaw$^{OX}$* and *NKCC$^{RNAi}$*. For reference the dotted red line represents wild-type day

values and the dashed black line night values (from Fig. 2A and B). * $p<0.05$, **

$p<0.01$, *** $p<0.001$; 1-way ANOVA with Tukey's *post hoc* test. See also Table S3.


**Figure 4.** Acute blue light response of l-LNvs at day and night.

(**A**) Response of wild-type neurons to blue light at day (left) and night (right side). (**B-**

**G**) l-LNvs with altered *qsm*, *Shaw* or *NKCC* levels (*Pdf-gal4*) showed no differential

response depending on time of day (left and right panels). Note the 'startle' response in C and E and the different scale in bottom graphs of F. In each panel the top traces show an example of a current-clamp recording of a l-LNv for 1 min before, 30 s during and the 2.5 min after exposure to light (indicated by blue bar). The bottom graphs show a quantification of the light response from multiple recordings. N.B. In the genotypes not responding to acute light ($qsm^{OX}$, $Shaw^{OX}$, $NKCC^{RNAi}$), 18 neurons were not or only very occasionally spiking, while the other neurons that did spike regularly did not change their firing rate. However, some neurons (n=8) showed a 'startle' response with briefly increased or decreased spiking (e.g. C and E, left panels). The examples shown are from occasionally spiking neurons. Mean, solid line; SD, pale grey background; n, indicated; MP, membrane potential; F, firing frequency. See also Fig. S3.

**Figure 5.** Blue light effects on different genotype l-LNvs and circadian model. (**A**) Quantification (means and SD, n indicated) of the response of the indicated genotypes (*Pdf-gal4*) to light as measured by the activity in the 30 s light on ($F_{on}$) minus the background activity in the 30 s before the light pulse ($F_{off}$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$; 1-way ANOVA with Dunnett's *post hoc* test (*) or t-test ([#]). (**B**) Cartoon of the proposed model of l-LNv cellular changes underlying the membrane potential and the firing differences between day and night. Qsm receives light input from Cry and affects Shaw and NKCC activity. Additionally, Cry activates the $K_V$-ß subunit redox sensor (Hk) and both Qsm and Cry degrade Tim at day. In the day, Qsm is cleaved from the membrane, activity of Shaw is low, the sodium leak channel NA is active and NKCC activity is high resulting in a more positive RMP and higher spiking rate. In the night Qsm is membrane bound and Shaw levels are high while

1    there is less NA and NKCC resulting in more negative RMP and less spiking. See

2    also Table S3.

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