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

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We have characterized a 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 an Na^+, K^+, Cl^- cotransporter (NKCC) and the Shaw K^+ channel ($dK_V3.1$). Because of light-dependent degradation of the clock protein Timeless (Tim), constant illumination (LL) leads to a breakdown of molecular and behavioral rhythms. Both overexpression (^{OX}) and knockdown (^{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} led 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 increased firing at night and no net response during the day, whereas altering Qsm, NKKC, or Shaw levels abolished these day/night differences. Finally, coexpression 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.

circadian rhythms | light input | membrane excitability | GABA reversal potential | potassium currents

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 well-being of an individual to anticipate these changes, and this anticipation 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 that 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 intake, and social interactions (3).

In *Drosophila* the central clock comprises 75 neuron pairs grouped into identifiable clusters that subserve different circadian functions (Fig. 1*A*). 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 the 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, which can dampen rapidly without such feedback (7, 11, 12).

Light resets the circadian clock every morning to synchronize the clock to the environment via Timeless (Tim) degradation after activation of the blue-light photoreceptor Cryptochrome (Cry), Quasimodo (Qsm), and potentially also visual photoreceptors (13–17). Qsm acts either independently or downstream of Cry and also is 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 voltage-gated potassium (K_V)-β subunit (Hk) (18, 19), 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, both of which are expressed and function within clock neurons influence neuronal electrical activity, the circadian clock, and clock-controlled behavior in both flies (20–22) and mice (23–25). The firing rate is a key component in mammalian circadian rhythmicity and can be regulated by regional and circadian expression of the sodium potassium chloride cotransporter NKCC, which switches the effects of GABA from inhibitory to excitatory across the day (26, 27).

Here we show that down-regulation or overexpression of the three membrane proteins encoded by the genes *qsm*, *Shaw*, and

Significance

Most organisms developed a circadian clock 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. 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 light-input pathway to the circadian clock. The membrane-anchored extracellular protein Quasimodo affects both the daily changes in physiological properties and light responses of brain clock neurons, possibly acting upstream of the potassium channel Shaw and the Na^+, K^+, Cl^- ion transporter NKCC.
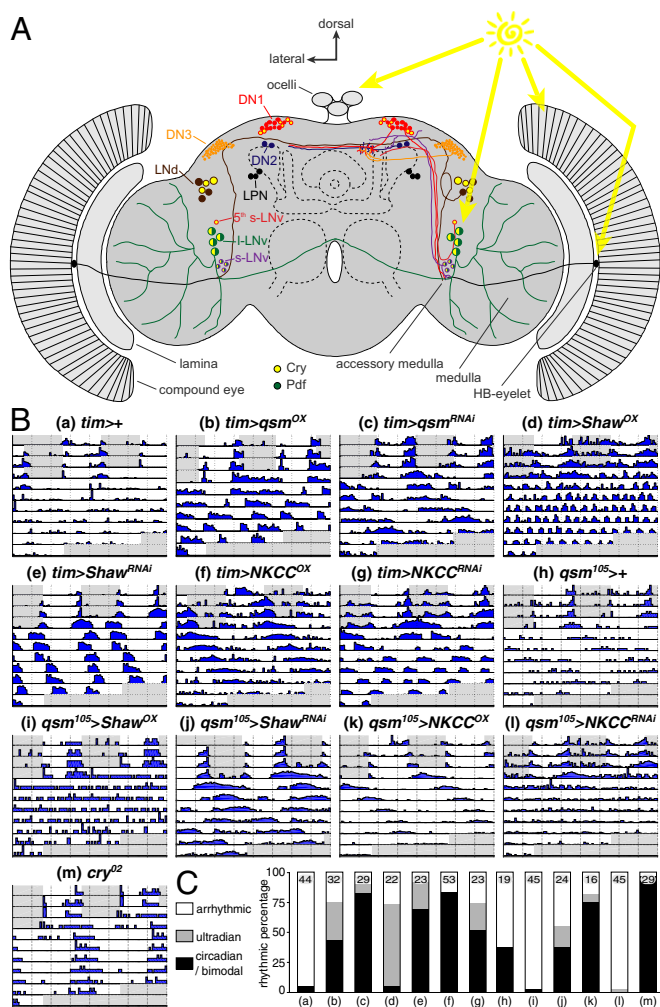
Fig. 1. The *Drosophila* circadian clock and LL behavior. (*A*) Cartoon of the *Drosophila* brain showing the ~75 pairs of clock neurons forming several groups [six dorsal lateral neurons (LNd); four large and four small Pdf-expressing ventral lateral neurons (LNv) and a single Pdf-negative neuron (fifth s-LNv); three lateral posterior neurons (LPN); and 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 indicated genotypes recorded for the first 3 d in a 12-h:12-h light/dark cycle (LD) (gray, lights off; white, lights on) and then in LL. (*C*) Bar graph specifying the percentage of flies showing circadian or bimodal (black bars), ultradian (light gray bars), or arrhythmic (open bars) behavior for each genotype as indicated by small letters referencing the subpanels in *B*. Numbers in bars state the number of flies of each genotype.

NKCC leads to rhythmicity in constant illumination (LL) and that these genes interact. All three genes are expressed in the well-characterized pigment-dispersing factor (Pdf)- and Cry-positive large ventral lateral neurons (l-LNv) (21, 28), which are important for arousal and light input to the clock (18, 19, 29–32). We used whole-cell recordings of l-LNvs to characterize their physiological properties and acute light effects across the day and found that Qsm helps 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 the Interaction of Qsm with Shaw and NKCC.
Light resets the circadian clock, and consequently, wild-type flies displayed arrhythmic behavior in constant dim white light (LL, 10 µW/cm^2), whereas *cry^{02}* loss-of-function mutants were rhythmic (Fig. 1 *B* and *C* and Table S1) (15, 33). Previously

we have shown that Qsm triggers Tim degradation within clock cells, and knockdown of *qsm* [*qsm^{RNAi}*, i.e., >70% reduction of *qsm* mRNA (16)] in all clock neurons using *tim-gal4* resulted in robust long-period (~27-h) locomotor rhythms in LL (16). We confirmed that *qsm^{RNAi}* induces robust and long-period rhythms in LL using two independent *qsm-RNAi* lines. Interestingly, *qsm* overexpression (*qsm^{OX}*) led to shorter, ~13-h rhythms, most likely reflecting the persistence of morning and evening activity peaks in LL.

Because NKCC interacts physically with Qsm in yeast (34) and because *Shaw* expression in clock neurons partially overlaps with the expression of *qsm* (16, 21), 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}* flies, flies overexpressing NKCC (*NKCC^{OX}* flies) exhibited long periods (~29 h), whereas NKCC knockdown [*NKCC^{RNAi}*; an ~70% reduction of mRNA (Fig. S1)] and Shaw knockdown [*Shaw^{RNAi}*; an ~90% reduction of Shaw (29)] resulted in ~13-h periods, reflecting potential bimodal behavior similar to that of *qsm^{OX}*. Shaw overexpression (*Shaw^{OX}*) induced remarkably robust ultradian rhythms with an ~4-h period, indicating compromised circadian clock function (21) 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 (21).

The light sensitivity of wild-type flies is also influenced by the naturally occurring *s-tim/ls-tim* polymorphism (35, 36), and we therefore performed experiments with both alleles. In all tested cases (*qsm^{OX}*, *qsm^{RNAi}*, *Shaw^{RNAi}*, and *NKCC^{OX}*), *s-tim* caused a dramatic (~50%) reduction in the percentage of rhythmic flies compared with *ls-tim* flies and often was correlated with period lengthening (Fig. S2 and Table S1). This result fits well with the previous observation that flies carrying the *s-tim* allele are more light sensitive than their *ls-tim* counterparts, and the dramatic behavioral difference caused by the two alleles underscores their biological importance (35, 36).

Because the *tim-gal4* driver is also expressed in the photoreceptor cells of the compound eyes, we also tested flies with impaired visual system function. Similar to wild-type flies, flies lacking (*i*) all external photoreceptors (*gl^{60j}*) (15), or (*ii*) phospholipase C-β required for the visual phototransduction cascade (*norpA^{P41}*) (37), or (*iii*) histamine, the principal neurotransmitter of the visual system (*Hdc^{JK910}*) (38), became virtually arrhythmic during LL (Table S1). These results strongly suggest that the *tim-gal4*–mediated LL rhythmicity is induced by the alteration of *qsm*, *Shaw*, and *NKCC* expression within the clock neuronal network.

To test directly 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. 1 *B* and *C* and Table S1) (16). With the *qsm^{105}* driver, both *Shaw^{OX}* and *NKCC^{RNAi}* induced wild-type scores of LL rhythmicity (i.e., 2%), whereas the opposite manipulation led to increased rhythmicity scores (54% and 81%, respectively). The apparent genetic interaction among *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 the molecular responses of circadian clock neurons to light.

Qsm Affects Daily Changes in Physiological Properties.
To determine how Qsm, Shaw, and NKCC might contribute to clock neuronal activity, we examined their neurophysiological roles by performing whole-cell recordings from l-LNvs with altered *qsm*, *Shaw*, or *NKCC* expression using *Pdf-gal4* and *uas-RFP* (Fig. S3 *A* 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, 21, 28).

Although physiological parameters showed variability among recordings of different animals, wild-type l-LNvs showed a more depolarized resting membrane potential (RMP) and a higher spontaneous firing rate (SFR) in the day than at night (Fig. 2 A and B and Table S3) (5, 31). In daytime recordings, qsm^{OX} led to a more hyperpolarized RMP and reduced SFR that were very similar to control and qsm^{OX} nighttime recordings. In contrast, qsm^{RNAi} depolarized the neurons and increased SFR at night to levels similar to those in wild-type and qsm^{RNAi} l-LNvs. We tested two qsm^{RNAi} lines targeting different regions of the qsm transcript (16) and did not observe a difference between the two lines (pooled data are shown). Interestingly, the differences in daytime and nighttime RMP and SFR in wild-type l-LNvs 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 $Shaw^{RNAi}$ led to more depolarized and more actively firing neurons. NKCC manipulations had the opposite effect, with $NKCC^{OX}$ depolarizing and activating the neurons, in particular at nighttime, and with $NKCC^{RNAi}$ hyperpolarizing and inactivating the neurons.

Qsm Affects Daily Changes in K⁺ Currents and the GABA Reversal Potential, and Qsm Interacts with Shaw and NKCC.

To elucidate potential mechanisms by which Qsm could affect physiological properties, we tested whether the effect might be caused by alterations in voltage-dependent somatic currents affecting the RMP. We examined the current–voltage relationships of l-LNvs and measured the non-inactivating currents that include Shaw (Fig. 2 C and D and Table S4) (39, 40). We analyzed the sustained current densities of wild-type l-LNvs at daytime and nighttime and could not detect any difference around the RMP, most likely because of masking by the leak-current subtraction. There was a difference, however, at positive holding potentials (+100 mV), with current densities at night being >30 pA/pF larger than in the day, suggesting more potassium channels were open at night. $Shaw^{OX}$ resulted in a high current density similar to that recorded in wild-type l-LNvs at night, whereas $Shaw^{RNAi}$ values matched wild-type daytime recordings, indicating that Shaw is involved in the observed day/night difference. Mimicking Shaw, qsm^{OX} had high current densities similar to those in wild-type l-LNvs at night, and qsm^{RNAi} currents matched wild-type day levels. This observation suggests that Qsm interacts with voltage-gated potassium channels such as Shaw in setting membrane properties.

Manipulating NKCC levels had an effect on RMP and SFR, and although NKCC activity is electrically neutral, NKCC pumps chloride ions into 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) into the ipsilateral medulla. In all cases spiking activity was affected. We measured the induced currents and calculated E_{GABA} from the resulting I–V curve (Fig. 2 E–H and Table S5). During the day the E_{GABA} was more positive (>10 mV), making GABA input less effective. GABA injections in the daytime or nighttime increased the E_{GABA} in $NKCC^{OX}$ brains and had the opposite effect in $NKCC^{RNAi}$ brains in which the E_{GABA} was more negative, thereby mimicking the wild-type day/night change. Changing Qsm levels also had an effect that again was in the opposite direction of NKCC, with qsm^{OX} decreasing and qsm^{RNAi} increasing the E_{GABA}. Strikingly, in some cases the E_{GABA} was more positive than the RMP and GABA consequently acted as an excitatory neurotransmitter that induced firing (Fig. 2G, red arrow in the top trace). These results suggest that Qsm interacts with NKCC and that they participate in the daily change in GABA efficiency.

To characterize the interaction of Qsm with Shaw and NKCC further, we tested whether 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 an effect similar to that seen with 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. We then coexpressed qsm^{RNAi} with $Shaw^{OX}$ and $NKCC^{RNAi}$ and could rescue the
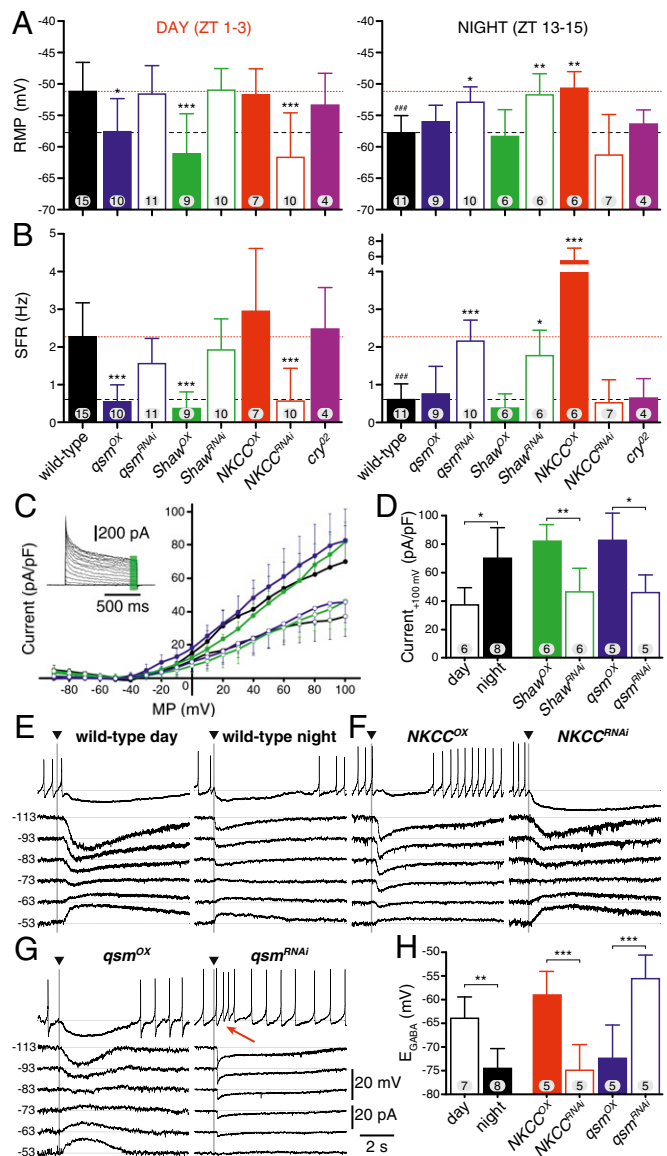

Fig. 2. Daily changes in the physiological properties of l-LNvs. (A) Changes in the RMP of wild-type neurons (black bars) and l-LNvs with altered levels (Pdf-gal4) of qsm (blue bars), Shaw (green bars), and NKCC (red bars), and cry[02] mutants (purple bars) in the day (Left) and at night (Right). Solid colored bars represent overexpression and open bars indicate RNAi knockdown of the respective genes. Data are presented as means; whiskers indicate the SD; the numbers in bars state the numbers of neurons for each genotype. The dotted red line represents wild-type daytime values, and the dashed black line indicates wild-type nighttime values. (B) SFR of the genotypes in A. (C) Current densities (measured at time point indicated by the green bar in the example shown in the inset) for wild-type l-LNvs in the daytime (open black circles) and nighttime (closed black circles), Shaw[OX] l-LNvs (closed green circles), Shaw[RNAi] l-LNvs (open green circles), qsm[OX] l-LNvs (closed blue circles), and qsm[RNAi] l-LNvs (open blue circles). Data are presented as means; whiskers indicate the SD. (D) Quantification of the current density at +100 mV. (E–G) Representative examples of the GABA response of a wild-type l-LNv (E), NKCC[OX] and NKCC[RNAi] (F), and qsm[OX] and qsm[RNAi] (G) during the daytime (Left) and nighttime (Right). The top trace in each panel is the current-clamp response to GABA (10 ms, 25 mM, 10 psi) at the arrowhead. Bottom traces show the same neuron and the 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; one-way ANOVA with Dunnett's (A and B) test or Tukey's (D and H) post hoc test (*) or t test (#).
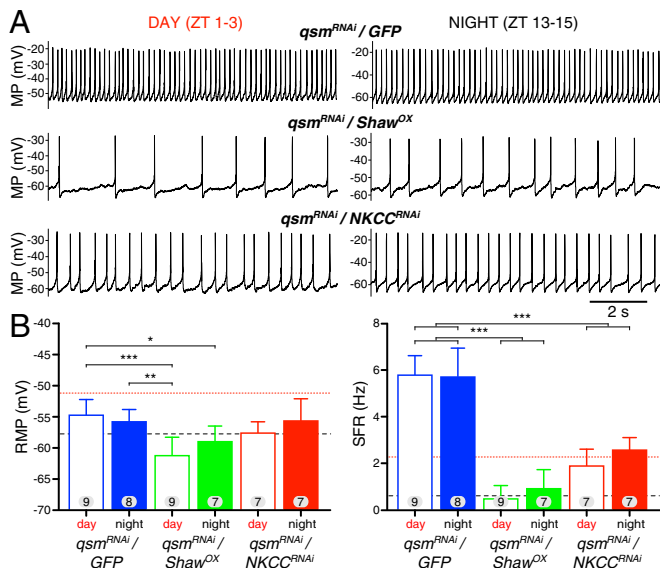
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Fig. 3. Physiological interaction of *qsm* with *Shaw* and *NKCC*. (*A*) Representative traces of l-LNvs coexpressing *qsm^{RNAi}* with *GFP* (*tim-gal4*, control) (*Top*), *Shaw^{OX}* (*Middle*), and *NKCC^{RNAi}* (*Bottom*) in the daytime (*Left*) and nighttime (*Right*). MP, membrane potential. (*B*) Quantification and statistical analysis of RMP (*Left*) and SFR (*Right*) showed a rescue of the *qsm^{RNAi}* phenotype by both *Shaw^{OX}* and *NKCC^{RNAi}*. For reference, the dotted red line indicates wild-type daytime values, and the dashed black line indicates nighttime values (from Fig. 2 *A* and *B*). Data are presented as means; the whiskers indicate SD. The numbers in the bars show the number of recorded neurons for each genotype. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA with Tukey's post hoc test.

elevated firing rate to wild-type levels, mimicking the behavioral rescue experiments and further supporting a cell-autonomous interaction of Qsm with Shaw and NKCC. Interestingly, *Shaw^{OX}* seems to be more effective than *NKCC^{RNAi}*, and the rescue was less pronounced at night.

In summary, overexpression and RNAi knockdown of *qsm* and *Shaw* resulted in opposing phenotypes compared with *NKCC* manipulations. Interestingly, *qsm^{OX}* or *Shaw^{OX}* and *NKCC^{RNAi}* promote the less active nighttime state, whereas *qsm^{RNAi}*, *Shaw^{RNAi}* and *NKCC^{OX}* push the neurons into the more depolarized daytime state, eliminating the acute differences between daytime and nighttime in all cases. Furthermore, Qsm interacts with Shaw and NKCC to affect potassium currents and the E_{GABA}.

Effects of Acute Light on l-LNvs and Its Modulation by Qsm. Altering the levels of Qsm, Shaw, and NKCC led to LL rhythmicity, suggesting an impairment of the light input to the circadian clock. Because the visual system mutants became arrhythmic, the alteration of Qsm, Shaw, and NKCC within clock neurons must be responsible for the observed LL rhythmicity. Although l-LNv neurons are unlikely to be mediators of rhythmic behavior in LL (16, 41–43), they are arousal neurons that are activated by blue light via Cry (18). 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. 4*A*) or after a brief startle response (Fig. 4*A*, *Bottom*), often outlasting the stimulus by tens of seconds before eventually returning to the baseline firing rate. On average, the spiking in response to blue light at night increased by 1–2 Hz to levels near the resting firing rate in the daytime (Fig. 5*A* and Table S3). In the daytime the response was different, more complex, and variable. Some neurons briefly increased ($n = 2$) or decreased firing ($n = 5$), but on average there was not much

change in firing frequency, a response clearly different from that observed at night and suggesting daily variation in the responsiveness and light sensitivity of the clock. We occasionally ($n = 8$) observed a startle response with a brief period of either increased firing or no firing immediately after the lights-on transition both at day and night. Consistent with published data (18), the effect was seen only with blue (470 nm) and not with green (555 nm) (Fig. S3*C*) or red (625 nm) light. *cry^{02}* l-LNvs did not respond to blue light (Fig. S3*D*) but had day/night changes in RMP and SFR similar to those in control l-LNv, indicating that Cry is not required for the regulation of neuronal electrical activity by the circadian clock.

qsm^{OX} always led to neurons with diminished firing frequency in response to the light pulse (Fig. 4*B*). 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 the spike rate in response to blue light similar to that seen in wild-type night recordings but starting from a higher baseline rate (Fig. 4*C*). The average change in firing was very similar to the wild-type nighttime response and was independent of the time of day (Fig. 5*A* and Table S3). Light pulses in the presence of up- or down-regulation of *Shaw* resulted in changes similar to those seen with the equivalent alterations of *qsm* expression, and *NKCC* manipulations resulted in opposite changes (Fig. S3 *E–H*). In summary, wild-type flies show a differential day/night response to blue-light pulses, but no such difference was seen in flies with Qsm, Shaw, and NKCC manipulations, the response of these flies being determined instead by the respective up- or down-regulation of gene expression.

Discussion

Light is the dominant circadian zeitgeber that resets the molecular clock. In this study we determined how light affects membrane
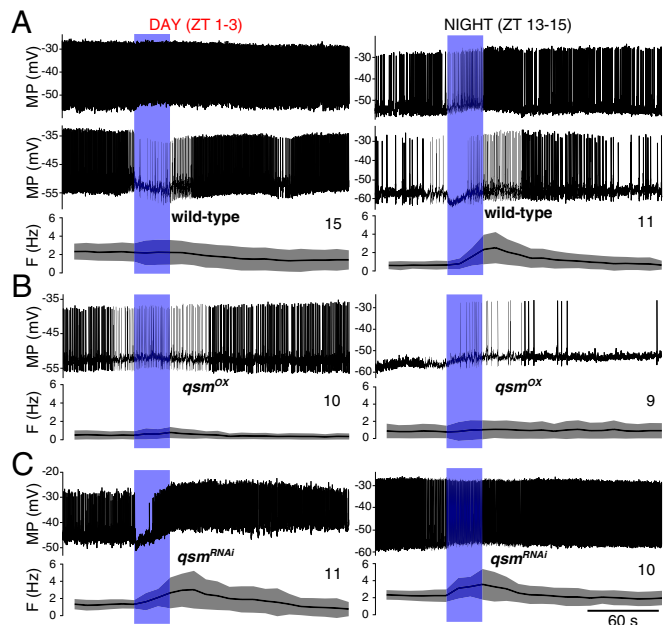

Fig. 4. Acute blue-light response of l-LNvs in daytime and nighttime. (*A*) Response of wild-type neurons to blue light in the daytime (*Left*) and nighttime (*Right*). ZT, zeitgeber time. (*B* and *C*) l-LNvs with altered *qsm* levels (*Pdf-gal4*) showed no differential response depending on the time of day but did show a differential response depending on the level of expression. (*Upper*) Examples of traces of a current-clamp recording of a l-LNv for 1 min before, 30 s during, and for 2.5 min after exposure to light (indicated by blue bar). (*Lower*) Quantification of the light response from multiple recordings. The solid line indicates the mean, and gray shading indicates the SD. The number of recorded neurons for each genotype is indicated. F, firing frequency; MP, membrane potential.
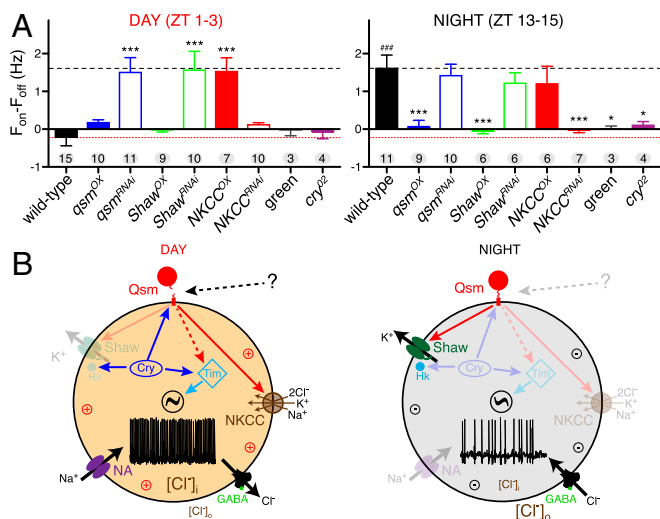
Fig. 5. The effects of blue light on different genotype l-LNvs and the circadian model. (*A*) Quantification of the response of the indicated genotypes (*Pdf-gal4*) to blue light as measured by the activity in the 30-s light on (F_{on}) period minus the background activity in the 30 s before the light pulse (F_{off}). Data are shown as means; whiskers indicate SD. The number of recorded neurons for each genotype is indicated. *$P < 0.05$, ***$P < 0.001$; one-way ANOVA with Dunnett's post hoc test (*) or *t* test (#). (*B*) Model of l-LNv cellular changes underlying the membrane potential and the firing differences between daytime and nighttime. Qsm receives light input from Cry and affects Shaw and NKCC activity. Additionally, Cry activates Hk and Qsm, and light-activated Cry degrades Tim. Because Qsm is also expressed in Cry-negative clock neurons (16), it is likely that non–cell-autonomous mechanisms also activate this pathway (this possibility is indicated by the question mark). How, and to which extend, Qsm, Shaw and NKCC influence TIM stability is unclear (dashed line). In the daytime, 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 nighttime Qsm is membrane bound, and Shaw levels are high, but there is less NA and NKCC, resulting in more negative RMP and less spiking.

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 LL (16). Here we show that overexpression (*qsm^OX*) also results in robust LL rhythmicity, but with predominantly ~13-h periods, suggesting a more robust morning oscillator that is normally weakened in DD conditions (Fig. 1 and Table S1). Manipulating the expression levels of both the potassium channel Shaw and the ion cotransporter NKCC also resulted in LL rhythmicity, whereas several visual system mutants behaved like wild-type flies and became arrhythmic. These experiments show that the membrane proteins encoded by *qsm*, *Shaw*, and *NKCC* control rhythmic behavior in LL. Furthermore, the rescue of wild-type behavior and neurophysiological properties by reciprocal changes of Qsm and Shaw or by the simultaneous reduction of Qsm and NKCC suggests that Qsm interacts genetically and perhaps directly with Shaw and NKCC.

Clock neurons are more depolarized and fire more during the daytime, and circadian changes in the 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 NA that recently has been shown to depolarize *Drosophila* clock neurons (22). Here we show that Shaw, NKCC, and Qsm also contribute to daily electrical activity rhythms: overexpression and RNAi knock-down of *qsm* and *Shaw* compared with *NKCC* resulted in opposing phenotypes. Interestingly, *qsm^OX* or *Shaw^OX* and *NKCC^RNAi* promote the less active nighttime state, whereas *qsm^RNAi*, *Shaw^RNAi*, and *NKCC^OX* push the neurons into the more depolarized daytime state, eliminating the acute day/night differences in all cases (Fig. 2 and

Table S3). We have shown previously that Shaw regulates circadian behavior (21) and that, in agreement with our current findings, Shaw regulates membrane potential and firing in *Drosophila* motoneurons (29, 44). NKCC activity is electrically neutral but increases the intracellular Cl^- concentration so that the $GABA_A$ receptor opens in response to GABA but, as a consequence, Cl^- presumably exits the cell down its electrochemical gradient, thereby depolarizing the membrane potential so that GABA effectively becomes an excitatory neurotransmitter (6, 12, 26, 29, 45). Our data show that in *Drosophila* a similar mechanism occurs, which is consistent with potential NKCC enrichment in l-LNv at dawn (28). The mechanism setting the neuronal state to either daytime or nighttime via Qsm, Shaw, and NKCC is likely to be predominantly cell-autonomous, because all components have been shown to act or to be expressed in the l-LNv (8, 21, 28). Although in an earlier study (16) using *qsm-gal4* lines we did not detect *qsm* expression in the l-LNv, these lines may not report expression faithfully in all *qsm* cells. Now, the finding that *qsm* RNA is enriched in the l-LNv (28), combined with the 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 (41–43), and we showed previously that *qsm* knockdown in Pdf neurons (s-LNv and l-LNv) does not result in robust LL rhythmicity (16). Therefore the effects of light on the electrical properties of l-LNv reported here do not necessarily explain the LL rhythmicity observed after manipulating *qsm*, *Shaw*, and *NKCC* in all clock neurons. 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 DN_1). 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, 28, 46), 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 posttranslational changes of Qsm alter the activity of Shaw and NKCC. Because (*i*) Cry is required for light-dependent Tim degradation in l-LNv (15), (*ii*) changing the Qsm level 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 reported 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 activated by light because a light pulse at night rapidly increases protein levels (16). Qsm is an extracellular zona-pellucida (ZP) 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 posttranslational processing typical for ZP-domain proteins (47). 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, whereas light-induced cleavage could release this block, and the freed extracellular part could inactivate Shaw (Fig. 5*B*). This mechanism is reminiscent to the mechanism by which the GPI-anchored extracellular protein Sleepless increases Shaker (K_V1 channel) activity for regulating *Drosophila* sleep (48).

How Qsm-induced changes in clock neuron activity influence the molecular clock remains an open question. Recent work shows that, in addition to the canonical degradation via Cry and Jetlag (49), Tim is also degraded via a Cul-3– and neuronal activity-dependent pathway in DD that has been implicated in mediating phase delays in the circadian clock (50, 51). 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 functions of Qsm, Shaw, and NKCC contribute to the canonical Cry- and Jetlag-dependent Tim-degradation pathway.

In conclusion, we demonstrate that Qsm affects both daily and acute light responses of l-LNvs, and therefore (Qsm) presumably contributes to light-input 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.

Materials and Methods

Flies were obtained from fly stock centers or were generated using the ΦC31 integrase system (52) and were raised under standard conditions. The efficiency of RNAi knockdown was confirmed using quantitative RT-PCR (RT-qPCR) and a standard RNeasy kit (Qiagen). Analysis of fly activity was performed using the *Drosophila* Activity Monitor (DAM) System and either a MatLab tool-box (53) or ActogramJ (54). Electrophysiological recordings were performed as previously described (55). Prism (GraphPad) was used for statistical tests. Detailed procedures can be found in *SI Materials and Methods*.

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