Rhythmic expression of period \((\text{per})\) and timeless \((\text{tim})\) genes in central circadian pacemaker neurons and prothoracic gland cells, part of the peripheral circadian oscillators in flies, may synergistically control eclosion rhythms, but their oscillatory profiles remain unclear. Here we show differences and interactions between peripheral and central oscillators using \textit{per}-luciferase and cytosolic \(\text{Ca}^{2+}\) reporter (yellow cameleon) imaging in organotypic prothoracic gland cultures with or without the associated central nervous system. Isolated prothoracic gland cells exhibit light-insensitive synchronous \textit{per}-transcriptional rhythms. In prothoracic gland cells associated with the central nervous system, however, \textit{per} transcription is markedly amplified following 12-h light exposure, resulting in the manifestation of day–night rhythms in nuclear PER immunostaining levels and spontaneous \(\text{Ca}^{2+}\) spiking. Unlike PER expression, nuclear TIM expression is associated with day–night cycles that are independent of the central nervous system. These results demonstrate that photoreception and synaptic signal transduction in/from the central nervous system coordinate molecular ‘gears’ in endocrine oscillators to generate physiological rhythms.
N
early all forms of life depend on the circadian clock system, for which transcriptional rhythms of clock genes are a common intracellular mechanism, to enable temporal optimization of physiological activities and behaviours. For holometabolous insects such as Drosophila melanogaster, the timing of eclosion (emergence of the adult insect from the pupal case) is critical for survival, so pupal circadian clock genes likely regulate cellular processes underlying eclosion. Indeed, the dramatic effects of Drosophila period (per) mutations on the circadian rhythms of eclosion identified per as an important clock gene in flies. Endocrine cells in the ring gland (RG) include both the prothoracic gland (PG) and corpus allatum (CA) cells, which release ecdysteroids and juvenile hormones, respectively. PG and CA cells comprise the largest cell clusters that express the clock genes per and timeless (tim) in the pupae of Drosophila. PG cells are considered to be circadian pacemaker cells gating eclosion timing, because when tim is over-expressed in these cells, arrhythmic eclosion occurs under conditions of constant darkness (DD). A reduction in the rhythmic release of ecdysteroids from PG cells, which depends on intracellular inositol 1,4,5-trisphosphate and Ca\(^{2+}\) signalling, has been proposed as the mechanism of initiating the endocrine cascade required for eclosion. Interrelations between clock gene oscillations and cellular physiological activities, however, have not been clearly characterized.

Whether PG cell oscillations are solely sufficient to drive eclosion rhythms is also an unsettled question, because arrhythmic eclosion under DD has been observed in mutant flies with ablated central circadian pacemaker neurons or lateral neurons (LNs); or flies lacking pigment-dispersing factor (PDF), a neuropeptide released from LNs, that efferents terminate on a pair of neurons containing prothoracicotropic hormone that project to PG cells, suggesting that PG cell oscillations are under the influence of LNs. On the other hand, circadian rhythms in nuclear translocation of per protein (PER) are resistant to the Na\(^{+}\) channel blocker tetrodotoxin (TTX) in PG cells in RG cultures associated with the central nervous system (CNS–RG complexes) maintained under a 12-h:12-h light–dark (LD) cycle. Thus, the importance of neural networks on the physiological activity of PG cells and clock-gene transcriptional oscillations in PG cells is currently obscure.

It is widely accepted that the circadian clock system in animals consists of central pacemaker neurons and various peripheral oscillator cells. For example, in mammals, circadian rhythms in PER-immunoreactivity (ir) and/or per promoter-driven luciferase (per-luc) levels were observed not only in central pacemakers (that is, hypothalamic suprachiasmatic nucleus neurons) but also in fibroblasts, the liver, lungs, and muscles. Also, in flies, circadian rhythms in PER-ir and/or per-luc levels have been reported not only in LNs, but also in Malpighian tubules. PG cells, antennae, legs, and wings. The discovery of molecular oscillations in various types of cells introduces de novo concepts of ‘peripheral clock’ or ‘peripheral oscillators’, and the fundamental differences between peripheral and central oscillators need to be elucidated. In mammalian fibroblasts, the amplitude of the per-luc oscillation in each cell is maintained, but synchrony is lost during the process of cell culture. Thus, it has been suggested that inter-cellular communication may be the principal difference in the mechanisms underlying central and peripheral oscillators.

Recently, Sellix et al. succeeded in imaging single-cell bioluminescence in dorsal LNs and large ventral LNs in Drosophila whole-brain cultures and demonstrated sustenance of tim promoter-driven luciferase rhythms in these cells. In Drosophila peripheral cells, however, oscillatory profiles have not been visualized at the single-cell level. Therefore, in the present study, stable RG cultures were established, with or without the associated CNS, and circadian dynamics of per transcription, nuclear PER/TIM-ir and cytosolic free-Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{i}\)) in single PG cells were visualized. The results demonstrate that per-transcriptional rhythms in PG cells were phase-coupled in isolated RG cultures, and that these rhythms were amplified by CNS-dependent photoreception. Because the photic input also amplified PER-ir and Ca\(^{2+}\) spiking rhythms, the dominant output from PG cells seems to depend on neural input. On the other hand, nuclear TIM-ir rhythms in PG cells were driven by local photoreception within the RG, and thus we demonstrate dissociable clock components as a profile of this endocrine oscillator.

Results

Per-transcriptional rhythms in PG cells. Organotypic cultures of CNS–RG complexes or cultures of isolated LNs were established (Fig. 1a,b, Supplementary Fig. S1). To examine oscillatory gene expression profiles, a deep-cooled electron multiplier (EM) charge-coupled-device (CCD) camera was used for bioluminescence video imaging in individual PG cells from transgenic flies expressing per-luc. Long-lasting circadian oscillations in per-luc expression were observed in PG cells in both CNS–RG complexes and isolated LNs (Fig. 1c,f, Supplementary Movies 1 and 2). PG cell oscillations in the CNS–RG complex were initiated in the proximal portion of the RG, which receives projections from the CNS, and the waves were propagated to the distal end (Supplementary Fig. S2, Supplementary Movie 1). In contrast, PG cell oscillations in the isolated RG did not exhibit directional wave propagation. Oscillations in each individual PG cell, however, were synchronized (Supplementary Movie 2). These results provide direct evidence for the presence of self-sustaining circadian oscillators in Drosophila endocrine cells, and demonstrate the effects of an attached CNS on the rhythms.

Moreover, when the CNS–RG complex was transferred from LD to DD, we observed a high amplitude per transcription signal for one 12-h cycle, then an immediate reduction in amplitude for the remaining time under DD (Fig. 1c). This LD-to-DD transition response was blocked with the removal of synaptic input from the CNS, either pharmacologically with TTX (0.3 μM) and the L-type Ca\(^{2+}\) channel inhibitor nimodipine (2 μM; Fig. 1d) or by physical isolation of the RG from the CNS (Fig. 1f). In addition, the LD-to-DD transition response was blocked in the CNS–RG complexes cultured from cry\(^{6}\) mutants, which contain a loss-of-function mutation for the circadian photoreceptor cryptochrome (CRY) (Fig. 1e). The LD-to-DD response was reproducible in the CNS–RG complex, when the cultures were re-exposed to 12 h of light (Fig. 1f, Supplementary Fig. S3). This transition response seems to depend on prior photic input to the CNS–RG complex. The subsequent reduced-amplitude expression in the CNS–RG complex maintained for several days under DD was similar to that observed in the isolated RG. Aside from the beginning of the recordings in the CNS–RG complex, the phase of each PG cell oscillation was highly synchronized. These results demonstrate that intrinsic PG cell oscillations in per transcription are of much smaller amplitude than those in PG cells receiving photic signals via the CNS.

To further characterize the oscillatory response of per-luc to light, the CNS–RG complex was exposed to a brief (30-min) light pulse (Fig. 2). The light pulse did not amplify the per-luc oscillations, but produced phase delays or advances, depending on the time of the peak of the tissue-averaged per-luc rhythm, produced bi-directional phase-shifts and desynchronized each individual PG cell rhythm, resulting in a significant reduction in the average oscillation amplitude (Fig. 2b). A significant reduction in the averaged amplitude following a light pulse was observed not only in central pacemakers (that is, hypothalamic suprachiasmatic nucleus neurons) but also in PG cells receiving photic signals via the CNS.
pulse exposure at a critical circadian time corresponds to loss of eclosion rhythms under similar circumstances. This phenomenon termed 'singularity' has been modelled using Per2-luciferase rhythms in mammalian fibroblasts overexpressing melanopsin as an artificial photoreceptor. To our knowledge, however, the present results are the first demonstration of the cellular singularity-like behaviours in multicellular organs. The photic information underlying the phase-shifts is presumably processed in the CNS, because isolation of RG or treatment with TTX and nimodipine for the CNS–RG complex blocked the light-induced phase-shifts (Fig. 2a). Also, CRY may have a pivotal role in the photoreception in the CNS, because transcriptional rhythms in PG cells, we also analysed nuclear PER-\[\text{transcript signal intensity changes in single PG cells in vitro}\]. To examine post-transcriptional rhythms in PG cells, we also analysed nuclear PER-\[\text{transcript signal intensity changes in single PG cells in vitro}\] and \[\text{bioluminescence measurements were conducted in culture medium containing 1M luciferin, without any medium exchange during the entire recording period. White and black bars on the bottom indicate light and dark periods, respectively. Images in (c) and (f) indicate example bioluminescence images collected at the circadian peak and trough. The corresponding GFP images at the end of the experiments are shown in (b).}

**Figure 1 | Visualization of per-transcriptional rhythms in single PG cells in vitro.** (a) GFP expressed in a CNS–RG complex culture from a per-luc/tim-gal4; UAS-GFP/S65T/+ fly after 12 h in vitro. GFP fluorescence was concentrated between brain hemispheres (Br; white dashed lines) in the RG (yellow dashed line), which contains the PG and CA (yellow dotted line). Arrows indicate GFP-positive LNs and dorsal neurons (DNs). Scale bar, 100 μm. (b) GFP-positive cells in a CNS–RG complex culture (left) and an isolated RG (right) after 10 days in vitro. Transmitted light (TL) images show connective tissue spread laterally from the isolated RG, but containing no GFP-positive LNs or DNs. Scale bar, 100 μm. (c) Per transcript signal intensity changes in single PG cells in the CNS–RG complex. Different line types (for example, dashed with two dots) indicate data from a single cell. Oscillations were of high amplitude at the beginning of recordings, which rapidly reduced under DD. Data from four different cultures were superimposed. One organotypic culture re-exposed to 12 h of light (120 lux) exhibited a similar transition at the beginning of recording. (d) A similar experiment, but CNS–RG complexes were cultured in culture medium containing TTX (0.3μM) and nimodipine (Nim; 2μM). The light-to-dark transition response was blocked. (e) A similar experiment, but CNS–RG complexes were cultured from \[\text{cry}^b\] mutants. The light-to-dark transition response was blocked. (f) Per transcript signal intensity changes in single PG cells in isolated RG cultures of wild-type flies. Steady low-amplitude circadian oscillations were observed with no light-to-dark transition. Smaller absolute per transcript signal in isolated RGS compared with CNS–RG complexes. All the above bioluminescence measurements were conducted in culture medium containing 1M luciferin, without any medium exchange during the entire recording period. White and black bars on the bottom indicate light and dark periods, respectively. Images in (c) and (f) indicate example bioluminescence images collected at the circadian peak and trough. The corresponding GFP images at the end of the experiments are shown in (b).
PER-ir in PG cells lasting 5–6 days *in vitro* (Fig. 3a), consistent with previous observations of PER-ir in PG cells *in vitro* and *in vivo*. Circadian rhythms in nuclear PER-ir, however, were below the detection limit in CNS–RG complex cultures maintained under DD (Fig. 3a). Circadian rhythms in nuclear PER-ir were evident in cultured LNs under DD (Fig. 3b); thus, it appears that in DD, the autonomous outputs from central pacemaker neurons were insufficient to manifest nuclear PER-ir rhythms in PG cells. Results from the present study further demonstrate that circadian rhythms in nuclear PER-ir were below the detection limit in PG cells physically isolated from the CNS, regardless of the lighting schedule (Fig. 3a).

These data indicate that photoreception in the CNS, signal transduction to the RG, and amplification in the gene transcriptional rhythms (Fig. 1c) were required for the manifestation of nuclear PER-ir rhythms in PG cells.

Robust rhythms in TIM-ir, but not in PER-ir, levels were reported for PG cells in pupae maintained for 2 days in the dark. Therefore, we further quantified nuclear TIM-ir in our cultures. Interestingly, unlike PER-ir, nuclear TIM-ir rhythms in PG cells were sustained in CNS–RG complex cultures maintained under DD (Fig. 4a). In addition, nuclear TIM-ir rhythms in PG cells were sustained in isolated RG cultures maintained under DD. Most intriguingly, nuclear TIM-ir rhythms in PG cells entrained to LD cycles in isolated RG cultures with a peak at the end of the dark period, and CRY enrolled the LD entrainment within the RG as the peak drifted to the middle of the light period in isolated RG cultures from *cry* mutant flies (Figs 4a and 5a). Because per–luc rhythms neither respond to light (Figs 1 and 2) nor entrain to LD cycles in the isolated RG (Fig. 5b), this represents differential light-input pathways for PG cell molecular gears: *per* transcription and translation are CNS-dependent, whereas those of *tim* may not be.

**Physiological photoresponses in PG cells.** Using *tim-gal4/UAS-cameleon* (YC2.1-82) flies, we also monitored [Ca$^{2+}$]$_c$ in cultured PG cells. PG cells in CNS–RG complex cultures exhibited spontaneous Ca$^{2+}$ spikes with periods on the order of minutes (Fig. 6). The Ca$^{2+}$ spikes were resistant to the depletion of internal Ca$^{2+}$ stores with exposure to thapsigargin (1 μM; Fig. 6a; Supplementary Fig. S6a), but were highly sensitive to the concentration of extracellular Ca$^{2+}$ (Fig. 6a). Baseline [Ca$^{2+}$]$_c$ levels and Ca$^{2+}$ spike frequencies increased when the extracellular Ca$^{2+}$ concentration was switched back from a Ca$^{2+}$-free buffer (Fig. 6a), but the store-operated Ca$^{2+}$ channel blocker SKF96365 (50 μM) had no apparent effect on this rebound response (Supplementary Fig. S6b). TTX (0.3 μM) transiently reduced the Ca$^{2+}$ spiking frequency, but the continued presence of TTX (0.3 μM) in the culture medium had little effect on the Ca$^{2+}$ spiking frequency (Fig. 6b). When the L-type Ca$^{2+}$ channel activator BayK8644 (100 μM) was applied during exposure to TTX, the Ca$^{2+}$ spiking frequency returned to baseline levels (Fig. 6b). Consistent with this, nimodipine (2 μM) reduced the Ca$^{2+}$ spiking frequency in PG cells cultured in TTX-supplemented medium (Fig. 6c). These results suggest that membrane depolarization and Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels evoked Ca$^{2+}$ spikes in PG cells.

**Figure 2** | Light-pulse-induced circadian phase-shifts of single PG cells in the CNS–RG complex. (a) Each trace denotes a representative *per–luc* rhythm in a single PG cell that received a 30-min light-pulse (yellow bar) immediately after (left) or before (right) the peak of *per–luc* rhythms. Triangles on the top denote the peak of *per–luc* rhythms estimated by the waveforms before light-pulse exposure and extended to the end of recordings as a reference. First row: in PG cells in CNS–RG complex cultures, a light-pulse immediately after the peak produced phase-advance and a light-pulse immediately before the peak produced phase-delay in successive *per–luc* rhythms. Second row: an experiment similar to the above, but CNS–RG complex cultures were maintained in medium containing TTX (0.3 μM) and nimodipine (Nim; 2 μM). Light-induced phase-advance and -delay were both blocked by TTX and Nim. Third row: an experiment similar to the above, but CNS–RG complex cultures were cultured from *cry* mutant flies. Light-induced phase-shifts were significantly reduced in the *cry* mutant. Bottom row: in PG cells in isolated RG cultures from wild-type flies, light-pulse-induced phase-shifts were significantly reduced. (b) Synchronous *per–luc* rhythms in individual PG cells (grey-scale traces) located in a CNS–RG complex were desynchronized by a 30-min light-pulse given at the approximate middle of the peak of *per–luc* rhythms. The average amplitude of *per–luc* rhythms (red trace) was significantly reduced after the light-pulse. (c) Circadian time dependency of light-induced phase-shifts of *per–luc* rhythms in PG cells. Circadian time 18 was defined by the peak of *per–luc* rhythms during the pre-exposure period. The second circadian peak after the light pulse was used to estimate phase-shifts. Under control conditions (black circles), light-pulses produced a phase-response curve (hand-fitted curve as dotted line) in which delay shifts switched to advance shifts at circadian time 20. The light-induced phase-responses were reduced by TTX/Nim treatment (red triangles), in PG cells cultured from *cry* mutant flies (green squares) and in PG cells from isolated RG cultures (blue diamonds).
Frequency of Ca\(^{2+}\) spikes in PG cells in isolated RG cultures did not depend on the intensity of blue-violet (435–445 nm) light (Fig. 6d). Therefore, effects of photic signal inputs on Ca\(^{2+}\) spikes in PG cells were further analysed by exposing the CNS to light (Fig. 6e). Exposure of the CNS to 20 min of blue (455–505 nm) light significantly reduced (−12.8 ± 5.0%) the Ca\(^{2+}\) spiking frequency (mean frequency = 0.014 ± 0.001 Hz before blue light exposure and 0.012 ± 0.001 Hz during blue light exposure; number of cells = 110 in 5 organs; P < 0.05, a paired t-test; Fig. 6f), whereas exposure to red (595–645 nm) light had small effects (mean frequency = 0.013 ± 0.001 Hz before red light exposure and 0.012 ± 0.001 Hz during red light exposure; number of cells = 44 in 3 organs, NS; Fig. 6f). The effects of blue light exposure were abolished in CNS–RG complex cultures in TTX supplemented medium (mean frequency = 0.014 ± 0.001 Hz before blue light exposure and 0.015 ± 0.001 Hz during blue light exposure, number of cells = 119 in 5 organs, NS; Fig. 6g). Also, PG cells in the CNS–RG complex cultured from cry\(^{+}\) background flies overall exhibited higher Ca\(^{2+}\) spike frequency (mean frequency = 0.017 ± 0.001 Hz during the entire session; number of cells = 55 in 3 organs; P < 0.01 in comparison with the control cry\(^{+}\) cells, a paired t-test) and blue light exposure only partially reduced spike frequencies (−8.2 ± 10.5%, NS; Fig. 6h). These results indicate that CRY-dependent photoreception in the CNS inhibited PG cell activity via efferent neuronal networks.

Circadian [Ca\(^{2+}\)]\(_c\) dynamics in PG cells. Finally, 4-point-samples were collected within 24 h to examine the effects of day–night lighting schedules on [Ca\(^{2+}\)]\(_c\) in PG cells (Fig. 7). PG cells in CNS–RG complex cultures exhibited robust daily rhythms in Ca\(^{2+}\) spiking frequency (number of cells = 130 in 6 organs): fast fourier transformation (FFT) power average in the middle of the night (zeitgeber time; ZT18) was 26% larger than that in the middle of the day (ZT6; Fig. 7a,b). The Ca\(^{2+}\) spiking frequency was reduced and the day–night variations were diminished when PG cells in CNS–RG complexes were cultured with TTX and nimodipine (number of cells = 140 in 6 organs; FFT power average at ZT18 was 58% that in control cultures). These results indicate that Na\(^{+}\) and Ca\(^{2+}\) channel-dependent CNS activities contribute significantly to the generation of Ca\(^{2+}\) spiking rhythms in PG cells.

In general, a sedative state of [Ca\(^{2+}\)]\(_c\) follows a Ca\(^{2+}\) spike in endocrine cells. Eventually, baseline [Ca\(^{2+}\)]\(_c\) estimated in PG cells in CNS–RG complex cultures exhibited reversed day–night variations, with the highest [Ca\(^{2+}\)]\(_c\) occurring at ZT6 (P < 0.01 compared with ZT0, number of cells = 130 in 6 organs, one way ANOVA followed by a Duncan’s multiple range test; Fig. 7a,c). In addition, the endogenous clock also drives rhythmic baseline [Ca\(^{2+}\)]\(_c\) in PG
cells, because the day–night variations remained visible, with a peak shift from ZT6 to ZT18 in PG cells in the isolated RG (P < 0.01 compared with ZT0, number of cells = 144 in 5 organs, one way ANOVA followed by a Duncan’s multiple range test; Fig. 7c). The intrinsic \([Ca^{2+}]_c\) rhythms in PG cells, however, coincided with little \(Ca^{2+}\) spikes and the amplitudes of circadian variations in baseline \([Ca^{2+}]_c\) in PG cells of the isolated RG were smaller than those reported previously for plants\(^{29}\) and suprachiasmatic nucleus neurons\(^{30}\).

**Discussion**

The present study demonstrates that per-transcriptional rhythms in individual PG cells were autonomous and synchronous, but highly dependent on photoreception in the CNS and efferents from the CNS. Consistently, PER-ir rhythms in PG cells were evident only when they were cultured in the CNS–RG complex under LD cycles. On the other hand, TIM-ir rhythms in PG cells were detected regardless of the CNS. To our knowledge, this is the first report visualizing peripheral cell oscillations with or without the influence of the CNS in an organ culture model and differential PER/TIM oscillations in particular culturing conditions. Furthermore, the present study revealed the spontaneous \(Ca^{2+}\) spiking activities in PG cells, which were reduced by light exposure to the brain, not to the RG. Ablation of the CNS significantly reduced \(Ca^{2+}\) spiking frequency in PG cells, and thus PG cells may receive excitatory inputs from the CNS. Rapid reduction of \(Ca^{2+}\) spiking frequency in PG cells by light exposure to the brain, however, demonstrated that photic signals might be transmitted to PG cells via inhibitory effenter(s). On the basis of these findings, we propose cellular consequences underlying endocrine oscillators and *Drosophila* eclosion rhythms.

The present study demonstrated that the amplitude and photo-entrainability of PG cell per-luc oscillations are highly dependent on the CNS. Interestingly, the result is different from what Liu et al. reported for mammalian fibroblasts,\(^{20}\) because each PG cell per-luc rhythm was synchronized regardless of the synaptic inputs, whereas the amplitude of per-luc rhythms largely depended on the preceding exposure of the brain to light. Predominant post-translational rhythms (that is, PER-ir levels and \(Ca^{2+}\) spiking rhythms) in PG cells were also CNS-dependent, suggesting that intrinsic small amplitude per-transcriptional rhythms in PG cells were insufficient to produce robust cellular physiological rhythms within these cells.
Significant CNS dependence for photic influence on PG cell rhythms may be inconsistent with the standard concepts of peripheral clocks in flies, because PER-ir and per-luc rhythms in Malpighian tubules synchronize to LD cycles via CRY-dependent mechanisms within the tissues, independent of the CNS. Notably, it has been shown that LD entrainment of nuclear PER-ir rhythms is sustained in PG cells in CNS–RG complexes cultured with medium containing 0.3 μM TTX, suggesting CNS-independent PG cell oscillations. The present results, however, demonstrate that circadian rhythms in nuclear PER-ir, if any, were below the detection limits of our curve-fitting analyses in PG cells isolated from the CNS regardless of the lighting schedule. Also, treatment with TTX and nimodipine or physical disconnection of the CNS blocked light-induced amplification of per-luc rhythms in PG cells in CNS–RG complex cultures, suggesting CNS-dependent photoreception and its influence on the circadian oscillations in PG cells. Two explanations may answer the discrepancy between the previous study and ours. First, the previous study did not include less than 60% immunofluorescent signals to estimate PER-ir rhythms and thus, the analytical threshold may underestimate the effect of TTX on PG cell rhythms. Second, TTX may have incompletely blocked CNS influences in the previous study. Indeed, we observed additional effects of Ca^{2+} channel blockers on the Ca^{2+} spiking activity of PG cells under continuous TTX treatment, although the TTX treatment acutely abolished blue light-induced reduction in Ca^{2+} spiking frequencies in PG cells in the present study.

It has recently been shown that a subclass of LNIs, large ventral LNs in adult flies, exhibit light-dependent action potential firing patterns, with photosensitivity largely dependent on CRY. Therefore, it is likely that photoreception in these central pacemaker neurons substantially strengthen PG cell rhythms. Indeed, light-to-dark rebound amplification of per-luc oscillations in PG cells was blocked in the presence of TTX/nimodipine, in isolated RG or in cry^{b} flies, suggesting that such light-dependent neuronal activity and synaptic signal transduction to PG cells may amplify per-transcriptional oscillations in PG cells.

In addition to the significant CNS dependence of per-transcriptional/translational rhythms in PG cells, robust oscillations in nuclear TIM-ir were detected both under LD and DD conditions, regardless of the presence or absence of the CNS. Furthermore, the presence of sustained TIM-ir rhythms even in isolated cry^{b} RG cultures suggests that intrinsic clock mechanisms may generate TIM-ir rhythms. Conversely, immediate synchrony of TIM-ir rhythms to reversed LD cycles in isolated cultures strongly suggests dissociable oscillatory profiles between per and tim in PG cells. This observation was not surprising, because TIM has more direct interaction with and is degraded by CRY following photoreception. A prior report also demonstrated dissociation between PER/TIM-ir rhythms in PG cells under DD. It is important to note, however, that prior results indicating ablation of TIM-ir rhythms under DD in PDF-null mutant (pdf^{Df}) flies are inconsistent with the present results, which indicate CNS-independent TIM-ir rhythms. This discrepancy may simply be due to the number of samples used for these analyses. More importantly, the present results demonstrate that intrinsic TIM oscillations alone could not drive apparent per-transcriptional/translational rhythms and Ca^{2+} spiking rhythms in PG cells. Together with prior results showing arrhythmic eclosion under DD by tim over-expression in PG cells, we suggest that coordination of dissociable clock gears may be needed for physiological rhythm generation in this endocrine oscillator.

The present study revealed the presence of spontaneous Ca^{2+} spikes in PG cells, with peak frequency in the middle of the night and lowest at the onset of light. Also, the present study demonstrates that brief exposure of the CNS to blue light inhibited Ca^{2+} spiking activity in PG cells, and that TTX or cry^{b} mutation blocked this effect. This suggests that physiological outputs from PG cell oscillators are under the strong influence of CRY-dependent photoreception in the brain and synaptic inputs to PG cells. Because release of ecdysteroids from PG cells depends on intracellular Ca^{2+} signalling, the lights-on signal would reduce rhythmic ecdysteroid release from PG cells and trigger the endocrine cascade underlying eclosion. This hypothesis is consistent with Drosophila eclosion rhythms, which are known to peak at dawn. Under DD conditions or without CNS influences, PG cell oscillations as per-luc, nuclear PER-ir, and Ca^{2+} spiking frequencies were rapidly degraded. These results are consistent with the rapid degradation of circadian ecdysteroid release rhythms under DD reported in Rhodnius prolixus. It is possible that the rapid reduction in PG cell activity under DD opens a temporal gate for eclosion, but the damping PG cell oscillators may not be strong enough to drive robust eclosion rhythms.

**Figure 5 | Selective entrainment of nuclear TIM expression rhythms to LD cycles in PG cells in isolated RGs.** (a) Nuclear TIM-ir rhythms in PG cells in isolated RG cultures maintained under standard LD cycles (black circles), reversed LD cycles (red circles), and DD following the reversed LD cycles (blue circles). Peak TIM-ir rhythms are marked on the top by triangles in corresponding colours. TIM-ir rhythms in PG cells were synchronized to the LD cycles in the isolated RG and had free-run under DD with an initial circadian phase coupled to the precedent LD cycles. (b) Representative per-luc rhythms in PG cells, which was cultured under standard LD cycle conditions (black traces) or under reversed LD cycle conditions (red traces) before recordings under DD conditions. Peak of per-luc rhythms estimated in PG cells pre-cultured under standard LD cycle conditions (black triangles) and under reversed LD cycle conditions (red triangles) were marked on the top. Per-luc rhythms in PG cells synchronized to the LD cycles in the CNS–RG complex but not in the isolated RG.
under DD. Thus, additional clock mechanisms may be required to explain circadian eclosion rhythms under DD.

It has been shown that LN5 project to neurosecretory cells that release eclosion hormone \(^3\) and the other pacemaker neurons, the dorsal neurons, project to cells that release crustacean cardioactive peptide \(^3\), both of which regulate eclosion downstream from ecdysteroid in the eclosion pathway. This, together with the present data showing that pupal LN oscillations in PER/TIM-ir levels were robust under DD, suggests that the timing of eclosion is supported via this neuronal pacemaker pathway under DD. Consistent with this, arrhythmic eclosion under DD has been previously observed in LN5-ablated or \(pde^{01}\) flies \(^5\). The passive nature of PG cell rhythms in per transcription, nuclear PER/TIM expression, and physiological output levels raises the question of the physiological implications of the intrinsic rhythms in PG cells. Overexpression of tim in PG cells results in arrhythmic eclosion patterns \(^2\), suggesting that PG cell oscillations do have a role regardless of the LN5. Under in vivo conditions, PG cells may receive more diverse signals (for example, temperature cues) to coordinate various zeitgebers, which we did not address in the present study. Thus, the present results do not rule out a possible role of the intrinsic PG cell oscillations in the formation of eclosion rhythms at the system level.

In conclusion, we examined the influence of CNS inputs on PG cellular circadian rhythms by monitoring circadian rhythms in per transcription, nuclear PER/TIM expressions, and [Ca\(^{2+}\)]\(_{c}\) oscillations in PG cells in the CNS–RG complex or isolated RG cultures. The present results demonstrate that circadian rhythms in per transcription in PG cells were self-sustaining and primarily phase-coupled, even in isolated RG cultures. On the other hand, the amplitude of per transcription was largely dependent on prior photic input to PG cells via the CNS. Because photic input via the CNS also amplifies the circadian Ca\(^{2+}\) spiking rhythms, the amplitude of oscillations in both per transcriptions and physiological processes in PG cells seems to depend on neural input. Intracellular CA\(^{2+}\) is a potent regulator of diverse physiological processes, including ecdysteroid release, thus the observed oscillatory profiles of PG cells likely contribute to rhythmic ecdysteroid release and eclosion under natural light conditions. Following ablation of photic and synaptic inputs, however, per-transcriptional rhythms and CA\(^{2+}\) spiking rhythms were both significantly reduced in PG cells. This reduction may result in a reduction of ecdysteroid release, which then may allow the process of eclosion to proceed; however, the reduced rhythms were not likely to be strong enough to drive rhythmic eclosion independently. Under these circumstances, other circadian

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**Figure 6** Calcium dynamics in PG cells. The CNS–RG complexes (a–c and f–h) and isolated RG (d) were cultured from tim-gal4/UAS-cameleon (YC2.7–82) flies. Representative data from two different cells were plotted for each experiment. (a) The frequency of spontaneous Ca\(^{2+}\) spikes depended on the extracellular Ca\(^{2+}\) concentrations. In contrast, exposure to the Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG; 1 min, 1 \(\mu\)M) had no effect on the frequency of Ca\(^{2+}\) spikes. (b) Effects of TTX (0.3 \(\mu\)M) and the L-type Ca\(^{2+}\) channel activator BayK8644 (100 \(\mu\)M) on Ca\(^{2+}\) spikes in PG cells. TTX reduced the frequency but did not abolish Ca\(^{2+}\) spikes. Exposure to BayK8644 increased the Ca\(^{2+}\) spiking frequency again, even in the presence of TTX. (c) PG cells in CNS–RG complexes cultured in medium containing TTX exhibited Ca\(^{2+}\) spikes similar to control culture conditions. Nimodipine (2 \(\mu\)M) reduced the Ca\(^{2+}\) spiking frequency in PG cells cultured with TTX. (d) The effect of excitation light on [Ca\(^{2+}\)\(_{c}\)] was tested in PG cells in isolated RG cultures using an LED light source. The low-fluorescent signals were amplified using an EM–CCD gain. The relatively noisy traces at 3 \(\times\) 10\(^5\) photons cm\(^{-2}\) s\(^{-1}\) (invisible to eye) were caused by the electrical amplification of the signals. Neither the frequency of Ca\(^{2+}\) spikes nor baseline [Ca\(^{2+}\)]\(_{c}\) levels were affected by excitation light in PG cells in isolated RG cultures. (e) To examine the effects of photic stimulation on Ca\(^{2+}\) spikes (f–h), a micro-light was located near the brain hemisphere. An excitation light for Ca\(^{2+}\)-imaging (435–445 nm) was tightly focussed on the RG. (f) Exposure of the brain to blue (455–505 nm; blue trace) but not red (595–645 nm; red trace) light temporarily blocked Ca\(^{2+}\) spikes in PG cells in CNS–RG complex cultures. The effects of blue light exposure (in two representative cells as solid and dashed lines) were abolished in PG cells cultured with TTX (g) or in PG cells cultured from cry\(^{b}\) background flies (h). All the above results were consistently observed in 44–119 cells in 3–5 organs.
pacemakers (possibly neural) may support rhythmic eclosion. Such a synergistic multiple pacemaker system may guarantee the precise timing of eclosion, which is critical for the survival of holometabolous insects.

Methods

Fly stocks. The tim-gal4,Pdf-gal4 and UAS-GFPFP65ST (each on the third chromosome) lines were kindly provided by Dr Daisuke Yamamoto (Tohoku University, Japan). The per-luc (second chromosome) and cryG (third chromosome) lines were kindly provided by Dr Jeffery C. Hall (Brandeis University, MA, USA). The tim-gal4 and UAS-YC2.1-82 (each on the second chromosome) lines were supplied from the Bloomington stock center (Indiana University, IN, USA). All lines were raised on corn meal-molasses-yeast media at 25 °C under 12:12-h LD cycle conditions.

RG cultures. Prepupae 1–3 h after pupariation were selected and dissected during the first 2 h after lights-on. The CNS–RG or isolated RG were dissected in a buffered salt solution containing (mM) 0.5 CaCl2, 21.5 KCl, 3.3 KH2PO4, 15 MgSO4, 36 NaCl, 4.8 NaHCO3, 4.9 NaH2PO4, 11.1 d-glucose, and 5.3 sucrose, supplemented with 100 units per ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin-B. Isolated tissues were placed on a 0.40-μm cell culture insert (BD Falcon, Franklin Lakes, NJ, USA) and cultured at 25 ± 1 °C in Schneider’s Drosophila medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 500 ng/ml insulin, 100 units per ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin-B. Cultures were incubated in a temperature- and humidity-controllable chamber in which a 13-W fluorescent bulb (120–130 lx) was used to produce 12-h:12-h LD cycles. The culture medium was exchanged every 48–60 h.

Anti-PER and TIM immunocytochemistry. A total of 419 cultured tissues were fixed at different time points using 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature. After fixation, these tissues were blocked in PBS with 10% (v/v) normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and 0.2% (v/v) TritonX-100 (Sigma-Aldrich, St Louis, MO, USA) overnight at 4 °C. These samples were then incubated in 1:1,000 affinity-purified rabbit polyclonal antibody to Drosophila period clock protein (anti-Drosophila PER; Chemicon, Temecula, CA, USA) or in 1:5,000 affinity-purified guinea pig polyclonal antibody to Drosophila TIM39 (kindly donated by Dr Jaga Giebultowicz, Oregon State University, OR, USA) for 3–4 days at 4 °C. Following five 10-min rinses in PBS, the samples were incubated with 1:200 Cy3-conjugated donkey anti-rabbit IgG or with 1:200 Cy5-conjugated anti-guinea pig IgG (Jackson ImmunoResearch) for 48 h at 4 °C. Following five 10-min rinses in PBS, the samples were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole. Immunofluorescence images were viewed using a confocal imaging system equipped with an inverted microscope, UPLSAPO 20×NA0.75 objective, and argon/helium neon lasers (Fluoview 1000, Olympus, Tokyo, Japan). Scanning parameters were unified across specimens. Optical intensity was analysed using Adobe Photoshop CS software (Adobe System, San Jose, CA, USA). PG cells and CA cells were differentiated according to the size of their nuclei: PG cell nuclei were larger than 10 μm and CA cell nuclei were smaller than 7–8 μm. On the basis of this, nuclear PER/TIM levels were calculated for each cell type.

The LNs were identified using green fluorescent protein (GFP) fluorescence in Pdf-gal4/UAS-GFPFP65ST flies. The boundaries of the nuclei of LNs were defined in the GFP image and superimposed on the PER/TIM image, and then the average intensity of nuclear regions was calculated.

Bioluminescence imaging. The CNS–RG complex or isolated RG from per-luc/tim-gal4; UAS-GFPFP65ST/+ prepupae were cultured on 0.40 μm cell culture inserts (Millicell-CM, Millipore, Billerica, MA, USA) under LD cycle conditions for 36–48 h before recording. Normal Schneider’s medium was switched to a medium supplemented with 1 mM beetle luciferin (Promega, Madison, WI, USA)
for 1–2 h before recording. Cultures were then incubated in a temperature-controlled (26.5 ± 0.5 °C) custom-built chamber attached to the inverted microscope stage (TE-2000s, Nikon, Tokyo, Japan) by a three-dimensional piezo motor-driven manipulator (Luigs & Neumann, Ratingen, Germany). The bioluminescence images were viewed using a ×20 objective lens (PlanAPOCHromat 20×/NA.75, Nikon) and collected with a 40× magnification objective on a cooled EM-CCD camera (Cascade II 512B, Photometrics, Tucson, AZ, USA; controlled at −5 °C and 5090×). The images were acquired using a hardware-specific software (Image-Pro-Plus, Media Cybernetics, Bethesda, MD, USA). During the subjective daytime, z-plane focus was adjusted using the motor-driven manipulator under CCD monitoring of the GFP fluorescence image. For this adjustment, bioluminescence imaging was paused and the sample was exposed to a dim blue light (480 ± 10 nm) for 2–10 s using an LED bulb. The intensity of fluorescence was optically inviable but sufficient for detection with the EM-CCD. Individual PG cells in the GFP image were used to estimate the bioluminescence signals located intracellulary.

**Calcium imaging**. The CNS–RGI complex or isolated RGI from tim-gol-d rift UAS-YC2 J.82 prepucce were cultured on 0.4μm cell culture inserts (Millicell-CM) under LD cycle conditions for 48 h before recording. For short-term Ca2+ imaging, cultures were gently removed from a filter cup and transferred into the circulating microscope chamber by which buffered-saline solutions similar to software (Image Pro-Plus, Media Cybernetics, Bethesda, MD, USA). During the environmental warming, 3-plane focus was adjusted using the motor-driven manipulator under CCD monitoring of the GFP fluorescence image. For this adjustment, bioluminescence imaging was paused and the sample was exposed to a dim blue light (480 ± 10 nm) for 2–10 s using an LED bulb. The intensity of fluorescence was optically inviable but sufficient for detection with the EM-CCD. Individual PG cells in the GFP image were used to estimate the bioluminescence signals located intracellulary.

**Statistical analysis**. Means were calculated with standard errors. Nuclear PER and TIM levels from several representative samples were analysed using a regression imaging software (Hamamatsu Photonics) installed on a computer. The spike frequencies of Ca2+ oscillations were calculated with standard errors. Nuclear PER and TIM levels from several representative samples were analysed using a regression imaging software (Hamamatsu Photonics) installed on a computer.

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Author contributions
E.M. performed the experiments, co-analysed the data, and co-wrote the manuscript. A.M. supplied experimental materials and co-designed the study. M.I. co-designed and supervised the study, co-analysed the data, and co-wrote the manuscript.

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