Circadian Regulation of Glutathione Levels and Biosynthesis in Drosophila melanogaster

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Abstract

Circadian clocks generate daily rhythms in neuronal, physiological, and metabolic functions. Previous studies in mammals reported daily fluctuations in levels of the major endogenous antioxidant, glutathione (GSH), but the molecular mechanisms that govern such fluctuations remained unknown. To address this question, we used the model species Drosophila, which has a rich arsenal of genetic tools. Previously, we showed that loss of the circadian clock increased oxidative damage and caused neurodegenerative changes in the brain, while enhanced GSH production in neuronal tissue conferred beneficial effects on fly survivorship under normal and stress conditions. In the current study we report that the GSH concentrations in fly heads fluctuate in a circadian clock-dependent manner. We further demonstrate a rhythm in activity of glutamate cysteine ligase (GCL), the rate-limiting enzyme in glutathione biosynthesis. Significant rhythms were also observed for mRNA levels of genes encoding the catalytic (Gclc) and modulatory (Gclm) subunits comprising the GCL holoenzyme. Furthermore, we found that the expression of a glutathione S-transferase, GstD1, which utilizes GSH in cellular detoxification, significantly fluctuated during the circadian day. To directly address the role of the clock in regulating GSH-related rhythms, the expression levels of the GCL subunits and GstD1, as well as GCL activity and GSH production were evaluated in flies with a null mutation in the clock genes cycle and period. The rhythms observed in control flies were not evident in the clock mutants, thus linking glutathione production and utilization to the circadian system. Together, these data suggest that the circadian system modulates pathways involved in production and utilization of glutathione.

Introduction

Circadian clocks generate a multitude of circadian rhythms in behavioral, neuronal, physiological, and endocrine functions [1,2]. While these rhythms have endogenous periodicity of circa 24 h, in nature they are entrained by light and temperature cycles associated with solar days. Circadian clocks consist of transcriptional and translational feedback loops working in a cell autonomous manner that are largely conserved between Drosophila and humans [3,4]. At the core of the Drosophila circadian clock there are four clock genes: Clock (Clk), cycle (cyc), timeless (tim), and period (per) [5]. They interact in a negative feedback loop, such that loss of function in any of these genes results in disruption of the clock mechanism [6]. The expression levels of per and tim are regulated by transcriptional activators encoded by Clk and cyc. This leads to periodic increase in the levels of PER and TIM proteins. The latter accumulate in cell nuclei, and repress CLK/CYC activators, leading to suppression of per and tim transcription. In addition to per and tim, CLK/ CYC heterodimers activate genes that participate in additional clock feedback loops and a substantial number of clock output genes [7,8]. Clock-controlled output genes modulate a myriad of metabolic and cellular functions, such as the regulation of energy balance, DNA-damage repair and xenobiotic detoxification in both mammals [9–11] and Drosophila [12–14].

There is emerging evidence that circadian clocks regulate processes that protect an organism from oxidative stress. Previously, we reported that levels of reactive oxygen species (ROS) and protein carbonyls fluctuate in a daily rhythm in heads of young wild type flies, whereas they were non-rhythmic and significantly higher in clock deficient per01 mutants [15]. These mutants also accumulated higher levels of protein carbonyls and peroxidated lipids during aging [16,17], suggesting that antioxidant defenses were compromised by the loss of clock function. In mice, deficiency of the clock protein BMAL1 (homolog of fly CYC protein) leads to increased ROS levels in several tissues [18]. However, it is not understood which pathways involved in protecting cells from oxidative stress may be modulated by the circadian system.

To combat oxidative stress and minimize the accumulation of oxidative damage, organisms developed a complex network of antioxidant defenses, capable of ROS removal. Glutathione (GSH)
is a central player in this network, able to protect cells from oxidative stress, regulate activity of detoxification enzymes and mediate redox-sensitive signaling [19,20]. Previous studies reported daily changes in GSH levels in different mammalian organs [21] but the role of circadian mechanism in these fluctuations has not been addressed. Genome-wide analyses of circadian transcriptome in fly heads by microarray [8,22–24], or RNA-seq [25] suggests that the expression of some genes comprising glutathione-synthesizing and conjugating systems may occur in a circadian manner. Here we utilized the genetic tools available in Drosophila to determine whether there is a causal relationship between circadian clocks and GSH-related pathways. We uncovered daily oscillations in glutathione levels in fly heads and investigated the molecular mechanisms underlying this rhythm. We report that the circadian clock is involved in regulating de novo glutathione biosynthesis.

Methods

Fly rearing and strains

Drosophila melanogaster were raised on yeast (35 g/L), cornmeal and molasses diet at 25±1°C, at low density to attain uniform size, under a 12 h light/12 h dark (LD) regimen (where Zeitgeber time (ZT) 0 is time of lights on and ZT 12 is time of lights off). Flies were separated 1–2 days after emergence, and five day old males were used for all experiments. For constant darkness (DD) experiments, flies were collected on the second day of DD where the time when lights would have turned on is designated by CT 0 and off is CT 12. Clock mutants cyc01 [26] and per01 [27] were backcrossed to the Canton S (CS) control line allowing free recombination for at least 8 generations to isogenize the genetic background. All work was completed on heads that were isolated following freezing using a dry ice and sieve method.

Quantitative real-time PCR

Male heads were homogenized in TriReagent following manufacturer’s protocol (Sigma-Aldrich Co., St. Louis, MO) using a Kontes handheld motor. Samples were purified using the RNeasy mini kit (Qiagen, Valencia, CA) with on-column DNAse digestion (Qiagen) or by DNAse I (Takara, Shiga, Japan) followed by sodium acetate precipitation. Synthesis of cDNA was achieved with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer’s protocol (Sigma-Aldrich Co., St. Louis, MO) or by rDNAse I (Takara, Otsu, Shiga, Japan) followed by sodium acetate precipitation. Synthesis of cDNA was achieved with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer’s protocol (Sigma-Aldrich Co., St. Louis, MO) or by rDNAse I (Takara, Otsu, Shiga, Japan) followed by sodium acetate precipitation.

Immunoblot analysis

Samples were collected at 4 h intervals from 15–20 whole heads obtained from CS flies and processed as described [29]. Briefly, proteins were extracted from heads, and ~5 μg of protein was resolved by PAGE for each sample. Immunoblots were performed with antibodies generated against recombinant GCLc and GCLm proteins [29] and anti-actin antibodies (MP Biomedical, Santa Anna, CA) to control for loading. The intensity of signals was analyzed by densitometric scanning, using the digital imaging analysis system with AlphaEase Stand Alone Software (Alpha Innotech Corp., San Leandro, CA). Signals were standardized against the signals obtained for actin or against the densitometry of Coomassie staining.

Glutamate cysteine ligase (GCL) enzyme activity and glutathione levels

GCL enzyme activity was measured as described [30] with some modifications (see Supplementary Methods S1). Briefly, ~50 fly heads were homogenized in 0.3 ml extraction buffer (320 mM sucrose, 1mM PMSF, 1 mM 6-aminohexanoic acid, 10 mM Tris, pH 7.4) and centrifuged at 14000 g for 5 min at 4°C. Low-molecular weight components were removed from the supernatant by ultrafiltration at 14000 g for 10 min at 4°C using Amicon Ultra Centrifugal Filter with a 10 kDa cut-off (EMD Millpore Corp., Billerica, MA). The protein preparations were washed with 0.3 ml buffer (200 mM sucrose, 1 mM PMSF, 1 mM 6-aminohexanoic acid, 10 mM Tris, pH 7.4) by additional ultrafiltration. Protein concentrations were determined using the DC Bio-Rad protein assay (Bio-Rad). GCL activity reaction was performed immediately after protein preparation, as suggested by [31]. The reaction was initiated by mixing 20–35 μg protein with a reaction mixture containing 10 mM ATP, 5 mM L-cysteine, 50 mM L-glutamate, 500 μM acivicin, 20 mM MgCl2, 100 mM Tris-HCl pH 8.2 in a total volume of 150 μl followed by incubation for 15 min at 25°C. Each reaction was duplicated. Time, protein and substrate concentration linearity were determined in pilot experiments. The specificity of the assay was tested using the GCL inhibitor L-buthionine-S, R-sulfoximine. The reaction was terminated by adding an equal volume of freshly prepared 10% (w/v) metaphosphoric acid (MPA) containing 10 μM L-methionine as internal standard for HPLC analysis. Precipitated proteins were removed by centrifugation and the supernatant was filtered through 0.22 μm PTFE membrane syringe filter HPLC/GC/MS detector (Phenomenex, Inc., Torrance, CA). Filtrates were immediately analyzed by HPLC or stored at −80°C for no longer than 24 h before analysis.

GSH content in the head homogenates was quantified by HPLC as described [30] with some modifications (see Supplementary Methods S1). Briefly, 50 heads were homogenized in 200 μl of freshly prepared ice-cold 5% MPA. After 30 min incubation on ice and centrifugation at 16000 g for 20 min at 4°C, the amount of protein in the precipitate was determined using the DC protein assay (Bio-Rad Laboratories) according to manufacturer’s recommendations (Bulletin 1770 US/EG Rev A, Bio-Rad). The supernatant was divided into two aliquots. The first aliquot was neutralized with 1N NaOH, treated with 2 mM N-ethylmaleimide for 30 sec on ice, and terminated with an equal volume of 10% MPA. The second aliquot was adjusted to the final volume of the first aliquot with 5% MPA. Samples were then filtered through 0.22 μm PTFE membrane syringe filter and immediately analyzed by HPLC or stored at −80°C.

Samples from the GCL assay and the free aminothiol (cysteinylglycine, cysteic acid) extractions were analyzed by HPLC equipped with the Agilent 1100 quaternary pump, degasser, thermostated autosampler, and column compartment (Agilent Technologies, Germany). The separation was performed under isocratic elution using a reverse-phase C18 Gemini-NX (3 μm, 4.6x150 mm) column (Phenomenex, Torrance, CA) with the flow rate of 0.75 ml/min. The mobile phase contains 2% (v/v) acetonitrile, 25 mM monobasic potassium phosphate, pH 7.2, adjusted with orthophosphoric acid. Following separation, aminothiols were detected in channel 3 immediately analyzed by the 5600 CoulArray electrochemical detector equipped with a four-channel analytical cell (ESA, Inc., Chelmsford, MA). For measuring γ-GC, we used increasing potentials (+100, +200, +750, +850 mV in channels 1–4, respectively). γ-GC and L-methionine were detected in channel 5 at +750 mV. Calibration standards containing 1, 2, 3 and 4 μM γ-GC (Sigma-Aldrich) were prepared in 5% (w/v) MPA contain-
ing 5 μM L-methionine (Sigma-Aldrich) as an internal standard and injected at regular intervals. Peak areas normalized to an internal standard were used for determining concentrations of γ-GC.

Potentials of +400, +600, +750, and +875 mV were used for GSH detection. GSH was detected in channel 3 at +750 mV. Each sample was injected twice. GSH concentrations were calculated as differences between peak areas corresponding to untreated and N-ethylmaleimide-treated aliquots of the sample. Calibration standards containing 0.1, 0.3, 1, 3, 10 and 30 μM GSH (Sigma-Aldrich) were prepared in 5% (w/v) MPA and injected at regular intervals.

Results

Circadian clock regulates GSH levels in fly heads

We measured GSH levels in heads of wild type Canton S (CS) control flies collected at 4 h intervals around the clock and found significant oscillations with 1.5-fold amplitude such that the highest GSH concentrations were detected in the early morning at ZT 0 followed by a decline to a trough in midday at ZT 8 (Fig. 1A). To test whether the GSH rhythm is controlled by the clock mechanism, we measured GSH in heads of arrhythmic clock mutants with loss of cyc (cyc01) or per (per01) function. In contrast to control CS flies, no significant difference between peak and trough times was found in per01 or cyc01 mutants (Fig. 1B). Furthermore, the trough in levels of GSH observed in the control was absent in the arrhythmic mutants.

Expression of genes involved in glutathione synthesis is modulated by the circadian clock

Given the rhythmic fluctuations in GSH levels, we investigated the daily profiles of the genes involved in GSH biosynthesis. Genes encoding the catalytic (Gclc) and modulatory (Gclm) subunits of the rate-limiting GCL holoenzyme were examined. We also examined the gene encoding second enzymatic step, glutathione synthase (GS). Analysis of the mRNA revealed daily oscillations in the expression of Gclc and Gclm in LD (Fig. 2A and 2B), while no significant diurnal fluctuations were found in the GS mRNA levels (Fig. 2C). The levels of both Gclc and Gclm mRNA oscillated in a rhythmic fashion with a significant, about two-fold amplitude between the peak and trough time points. Interestingly while a sharp peak of Gclc mRNA was detected at late night (ZT 20), the peak of Gclm expression was much broader (ZT 8–16) and phase advanced relative to the Gclc peak (Fig. 2A–B). To determine whether the expression of Gclc and Gclm was regulated by the circadian clock, mRNA levels were examined in per01 and cyc01 mutants at times when wild type flies showed trough and peak expression levels for each gene. In cyc01, Gclc mRNA levels were significantly lower at the time point when control flies showed peak expression (Fig. 2D). In contrast, in the per01 flies, Gclc mRNA levels were significantly higher as compared to the trough time in control flies (Fig. 2G). With regard to Gclm, mRNA levels were intermediate in both clock deficient genotypes, that is, significantly higher in per01 and cyc01 than control flies the trough time point, but significantly lower at ZT 12, the peak time point (Fig. 2E and 2H). GS mRNA levels were not altered in cyc01 or per01 flies (Fig. 2F and 2I).

The observed expression levels of Gclc and Gclm in per01 flies (lack of a trough in the morning) and in cyc01 flies (lack of a peak in the evening) suggest that transcription of both genes is positively regulated by the CYC/CLK protein complex and negatively regulated by the PER protein. Transcriptional activation of Gclc and Gclm by CLK/CRY would be consistent with the recent genome-wide ChIP-chip study showing that CLK/CRY complexes are bound in the vicinity of Gclc and Gclm promoters in a time-dependent manner [7]. However, in both cases, CLK binding occurred near another transcription start site on the opposite DNA strand. Thus, these alternate genes, CG1575 and CG17625, could have been the CLK/CRY targets instead of, or in addition to, Gclc and Gclm. To explore this issue, we conducted qRT-PCR studies. We determined that CG17625 is not expressed in adult heads, consistent with fly atlas data [32] and that CG1575, which is adjacent to Gclc, did not display rhythms consistent with CLK targets (data not shown). As the Gclc gene encodes two isoforms, RA and RB, that share the same coding regions but have distinct 5′ UTR regions [33,34], we determined the daily profile of both transcripts, using subunit-specific primers. Data revealed that both isoforms have rhythmic expression with a significant peak at ZT 20 (Fig. 3). Previous studies showed that deletion of the 5′ UTR associated with the RA transcript results in lethality [34], suggesting that the Gclc-RA isoform is essential for survival.

A key feature of the circadian clock is that rhythmic variations in the mRNA levels of clock genes such as tim are maintained under constant darkness (DD) [5]. Our qRT-PCR analysis of head samples isolated from flies kept in DD revealed that tim maintained a 4-fold mRNA amplitude between CT 4 and CT 12 (Fig. 4A) on the second day in DD. In addition, a significant circadian rhythm

Figure 1. Circadian regulation of GSH levels in Drosophila heads. (A) Daily changes in GSH levels in wild type CS males. Data represents average values ± SEM obtained from 4 independent bio-replicates (total N = 8). Data were analyzed by a 1-way ANOVA and Bonferroni’s post-tests where an asterisk marks significantly lower values relative to ZT 0 (p < 0.05). White horizontal bar marks the time when light is on; black bar denotes darkness. (B) GSH levels were altered in per01 and cyc01 mutants such that no statistical difference was detected between time points where control CS flies showed a peak (ZT 0) and a trough (ZT 8). Bars represent average values ± SEM obtained from 3–4 independent bio-replicates (± SEM). Data in (B) were analyzed by a 2-way ANOVA and Bonferroni’s post-tests. Different subscript letters indicate significant difference between treatment groups. ZT = Zeitgeber Time. doi:10.1371/journal.pone.0050454.g001
in $Gclm$ mRNA levels was evident in DD (Fig. 4B). On the other hand, the $Gclc$ mRNA rhythm was not sustained in DD (Fig. 4C).

GCL enzyme activity displays a daily rhythm which is abolished in clock mutants

In contrast to $Gclm$ mRNA levels, Western blot analysis showed less pronounced but significant changes in GCLm protein levels with a trough at lights on (ZT 0) and a peak in the evening (ZT 12–20) (Fig. 5A). GCLc protein did not show significant changes during the 24 h period (Fig. 5B). It is known that the catalytic activity of the GCL holoenzyme is regulated at different levels and depends on many factors, including interactions between GCLc and GCLm, as well as their concentrations and molar proportions [35–37]. Although only minor variations in the protein levels of individual GCL subunits were observed, the ratios of GCLc to GCLm changed by about 40% around the clock (Fig. 5C), and this could affect GCL catalytic activity. Importantly, we found significant changes in GCL activity in wild type flies throughout the circadian day. The peak in GCL activity was in the morning at ZT 0–4, and the lowest activity was between ZT 8–16 (Fig. 6A).

Next, we examined whether GCL activity was altered in the $per^{01}$ or $cyc^{01}$ mutants. Neither mutant showed a significant difference in GCL activity at the time points when control (CS) flies showed a peak (ZT 0) and trough (ZT 8, Fig. 6B). Thus, we determined that the circadian clock regulates glutathione synthesis by affecting activity of the GCL holoenzyme.

Circadian regulation of GstD1 expression

One of the major defense functions of glutathione is the elimination of xenobiotics, as well as metabolic by-products, by conjugating these compounds to glutathione in reactions mediated by the family of enzymes designated as the glutathione transferases (GSTs). Given the decline in GSH levels in midday (Fig. 1), we examined mRNA levels of glutathione S-transferase D1 ($GstD1$),
a known antioxidant and detoxification response gene in *Drosophila* [38,39]. We found a statistically significant circadian rhythm (p<0.01) in *GstD1* expression levels in heads of wild type CS flies with the peak expression at ZT 8 and the trough 12 hours later at ZT 20 (Fig. 7A). This differential expression was abolished in heads of both *per*01 and *cyc*01 mutants such that similar low levels of *GstD1* mRNA were detected at both ZT 8 and ZT 20 (Fig. 7B). Taken together, these data demonstrate that the circadian clock affects the expression of *GstD1*, as previously suggested by microarray studies [40]. Given that *GstD1* expression in *Drosophila* is induced via *Keap1*/Nrf2 signaling [39], we also examined the transcriptional profiles of *cncC*, (the *Drosophila* homologue of mammalian *Nrf2* gene), and *Keap1* genes. We found no circadian rhythms in *cncC* or *keap1* mRNAs, nor was there any effect of *per* or *cyc* mutations on their mRNA expression levels (Figure S1).

**Discussion**

This study advanced our understanding of the effects of circadian clocks on cellular homeostasis. We found that the

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**Figure 3. Circadian expression of GCLc isoforms.** Daily oscillations in (A) total Gclc mRNA levels were also significant when (B) Gclc-RA and (C) Gclc-RB isoforms were measured separately using isoform-specific primers. The two isoforms share the same coding regions, but have distinct 5' UTR regions. All graphs are average values obtained from 3 independent bio-repeats each normalized to the time point with the lowest expression. An asterisk indicates significant difference from the trough based on a 1-way ANOVA and Bonferroni’s post-tests (p<0.05).

**Figure 4. Circadian rhythm in Gclm expression persists in constant darkness.** (A) *tim* and (B) Gclm mRNA expression show a circadian rhythm in heads of CS flies on the second day of constant darkness. An asterisk indicates a significant difference in the expression level between the trough of each gene and the peak (p<0.05). (C) No significant rhythm was detected in Gcl mRNA levels in wild type flies. Data represents average values obtained from 3 independent bio-replicates (± SEM) and normalized to ZT 0. Significance was calculated by a 1-way ANOVA and Bonferroni’s multiple comparison post-tests. CT=Circadian Time. Shaded horizontal bars indicate subjective day.
the circadian clock and that many metabolites linked to redox show diurnal oscillations in mammals [21,41] the molecular connections we established here between the circadian clock and GSH biosynthesis may be conserved across different phyla.

We show that GSH undergoes circadian fluctuations in *Drosophila* heads, reaching its highest levels in the morning. While diurnal GSH variations were previously reported in different mammalian organs, such as the liver [42], the underlying molecular mechanism was not elucidated. A critical finding of our study is that the generation of the GSH rhythm in *Drosophila* heads involves transcriptional regulation of genes that encode subunits comprising GCL, the first and rate limiting enzyme in glutathione production. Daily rhythms for both *Gclm* and *Gclc* mRNA were discerned in LD with peak expression in the early and late night, respectively. However, *Gclc* mRNA did not show significant fluctuations in DD, suggesting that the rhythm may have dampened or is modulated by LD. On the other hand, the expression of both genes was significantly altered in mutants with defects in the positive or negative arm of the clock loop. Namely, expression of *Gclc* and *Gclm* was lower at the expected peak in *cyc* null flies, which have a disrupted CLK/CYC complex, and higher at the expected trough in *per* null mutants lacking periodic repression of CLK/CYC activity. Thus, our functional genetic data suggest that *Gclc* and *Gclm* may be activated by the CLK/CYC complex. This

Figure 5. Profiles of GCL proteins and their ratio over the circadian day in the heads of wild type CS males. (A) GCLm and (B) GCLc protein levels based on average densitometry of signals obtained on Western blots with anti-GCLc or anti-GCLm antibodies normalized to signals obtained with anti-actin antibodies. Each replicate was normalized to the time point with the lowest expression. (C) Ratio of GCLc to GCLm protein over the circadian day in wild type CS males. (A–C) Data represent average values ± SEM obtained from 8 immunoblots performed with 4 independent bio-replicates. Statistical significance was determined by a 1-way ANOVA and Dunnett’s post-test as denoted by asterisks (p<0.05). doi:10.1371/journal.pone.0050454.g005

Figure 6. Circadian regulation of GCL enzymatic activity. (A) Daily profile of GCL activity in heads of CS flies as measured by the formation of the GCL product, γ-GC. Data represents average values ± SEM obtained from 4 independent bio-replicates (total N = 16). An asterisk indicates a significant difference between the peak and trough time points calculated by 1-way ANOVA and Bonferroni post-tests. (B) GCL activity was altered in *per* and *cyc* mutants such that no statistical difference was detected between time points where control CS flies showed peak at (ZT 0) and trough (ZT 8). Bars show average values ± SEM obtained from 4–5 independent bio-replicates (total N = 16). Data in (B) are analyzed by 2-way ANOVA and Bonferroni’s post-tests. Different subscript letters indicate significant differences between treatment groups (p<0.05). doi:10.1371/journal.pone.0050454.g006
Circadian Control of Glutathione Homeostasis

Conclusion

The rhythm in GSH biosynthesis could be important for many aspects of clock-controlled cellular homeostasis since this prevalent endogenous compound acts as a major antioxidant, regulates activity of detoxification enzymes, and mediates redox-sensitive signaling. GSH functions in the central nervous system also include maintenance of neurotransmitters, and membrane protection [46,47]. Our previous study suggested that ROS and oxidative damage levels fluctuate in heads of wild type flies [15] raising a possibility that GSH rhythms may be linked to these phenotypes. However, the mechanism remains to be elucidated as GSH does not directly react with peroxides. The removal of hydrogen peroxide and other peroxides occurs in high-turnover reactions catalyzed by glutathione peroxidases and peroxiredoxins [48–50]. Interestingly, some of these enzymes display circadian oxidation-reduction cycles in model organisms across phyla, including Drosophila [51].

An important function of GSH is in phase II detoxification, in which GSH is conjugated with xenobiotics and metabolic by-products in reactions catalyzed by glutathione S-transferases [52,53]. We report here that mRNA levels of GstD1, a known antioxidant and detoxification response gene in Drosophila [38,39,54] is expressed rhythmically in heads of wild type flies. This is consistent with previous microarray-based analyses which suggested that GstD1 and several other GSTs are expressed rhythmically in the adult Drosophila head [40,55,56]. Interestingly, GstD1 expression peaks in mid-day, when GSH levels become significantly reduced (compare Fig. 1 and 7). Other GSTs also peak at this time [40], suggesting a scenario where GSH is depleted due to conjugation and then replenished later in the circadian cycle. It has been hypothesized that the clock may coordinate redox responses as part of a strategy to increase the potential for neutralization of toxins during the morning when flies are active [40]. In agreement with this view, we showed that the circadian clock regulates susceptibility to pesticides as well as expression of specific genes that control xenobiotic metabolism [12,13].

Although a significant rhythm in the GSH levels and GCL activity was detected in flies with an intact clock, the rhythm was not apparent in clock mutants. Instead, both of these parameters remained relatively elevated around the clock, more similar to the peak rather than the trough levels of the control (Fig. 1B & 6B). It is conceivable that this enhanced constitutive GSH production may result in an imbalanced redox state, which in turn could compromise redox signaling leading to physiological deficits. Consistent with this inference is the observation of adverse effects.
on fly survivorship when GCLc was over-expressed ubiquitously, resulting in high levels of GSH production [29,34]. S. Radyuk, unpublished observations). In other studies, we showed that accumulation of carbonylated proteins and peroxidized lipids is accelerated in per01 flies relative to age-matched controls [16], and that per01 mutants are more susceptible to neurodegeneration [17]. Taken together, these data suggest that daily fluctuations in GSH may promote the health of the nervous system more efficiently than if GSH is maintained at constitutively elevated levels. Another important point is that while per01 exhibits constant high GSH levels, the expression of the GSH-conjugating enzyme GadJ is significantly reduced in this mutant. This suggests that dysregulation between GSH supply and utilization may occur in clock-deficient flies.

One important question that remains to be addressed is whether rhythms in GSH-biosynthesis are controlled cell-autonomously or systemically. The circadian system in fly heads consists of several clusters of central pacemaker neurons forming a circuit responsible for circadian rhythms of locomotor activity [57]. In addition, retinal photoreceptors, sensory neurons, glia, and other cells contain a molecular clock mechanism, which can function independently of the central pacemaker [6,58]. Transcriptional rhythms that are detected in whole heads may be generated in peripheral oscillators. Nevertheless, at least some central pacemaker neurons appear to be among the cells showing transcriptional Gclc and Gclm rhythms, based on microarray analysis of isolated pacemaker cells [59]. While the range of cells displaying rhythmic GSH biosynthesis remains to be determined, it is likely to be broad. A recent genome-wide study suggests that circadian expression of Gclc may occur in isolated fly brains [25], and our data suggest that Gclc and Gclm expression is also rhythmic in fly bodies (Dani Long and Eileen Chow, unpublished).

What is the biological advantage of adding a circadian level of regulation to GSH biosynthesis? While excessive ROS levels are detrimental to cell function, some levels of ROS are necessary in the organism, as these molecules are responsible for essential processes including cell signaling cascades and immune response. Thus, GSH acts not only as an antioxidant, but also plays a critical role in a plethora of redox-sensitive cellular functions (reviewed in [49]). While over-expression of GCLc in Drosophila neuronal tissue, and thus increased GSH levels, correlated with protection against oxidative stress and extension of lifespan [34,60], recent findings suggests that GSH may rather function via affecting specific metabolic and defense pathways [61]. An array of connections has been recently established between circadian clocks and metabolism in mammals [10,41,62] and in flies [63]. Our present study adds an important novel link to this array by demonstrating circadian control of glutathione, a compound that is critically involved in maintaining human health.

Supporting Information

Figure S1 No significant circadian rhythm was detected in (A) cncC and (B) Keap1 mRNA levels over the circadian day in the heads of wild type CS males. A 1-way ANOVA and Dunnett’s post-test showed p>0.05. No significant difference was observed in (C) cncC or (D) Keap1 mRNA levels at ZT 8 or ZT 20 between wild type (CS), per01 and cyc01 flies. Data were analyzed by a 2-way ANOVA and Dunnett’s post-tests and p>0.05. (A–D) Data represent average values (± SEM) obtained from 3 independent bio-replicates and normalized to ZT 0 (A–B) or ZT 8 (C–D).

Supplementary Methods S1 Validation of the GSH and γ-GC detection methods and improvement of GSH detection in fly heads.

Table S1 Summary of the forward and reverse sequences of PCR primers used for quantitative RT-PCR analysis in alphabetical order.

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Author Contributions

Conceived and designed the experiments: LMB SNR JMG. Performed the experiments: LMB VIK ESC JKR MW SNR JMG. Analyzed the data: LMB ESC VIK JKR SNR. Wrote the paper: LMB ESG WCO SNR JMG.

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