Loss of circadian clock accelerates aging in neurodegeneration-prone mutants

Natraj Krishnan[a,1,#] , Kuntol Rakshit[a,b,1], Eileen S. Chow[a], Jill S. Wentzell[c], Doris Kretzschmar[c] and Jadwiga M. Giebultowicz[a,b,*]

[a]Department of Zoology, Oregon State University, Corvallis, OR, USA

[b]Center for Healthy Aging Research, Oregon State University, Corvallis, OR, USA

[c]CROET- Oregon Health and Science University, Portland, OR 97239 USA

[1]Both authors contributed equally to this work.

[#] Current address

Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Starkville, MS 39762 USA

[*] To whom correspondence should be addressed:
Jadwiga M. Giebultowicz
Oregon State University
Department of Zoology
3029 Cordley Hall
Corvallis, OR 97331 USA

Phone: (541) 737-5530
Fax: (541) 737-0501
E-mail: giebultj@science.oregonstate.edu

Highlights:

- Disruption of the circadian clock shortens lifespan in neurodegeneration-prone mutants of *Drosophila melanogaster*.
- Arrhythmia accelerates neuronal degeneration and impairs motor functions.
- The circadian clock gene *period* appears to function in pathways maintaining neuronal homeostasis.

Abstract

Circadian clocks generate rhythms in molecular, cellular, physiological, and behavioral processes. Recent studies suggest that disruption of the clock mechanism accelerates organismal senescence and age-related pathologies in mammals. Impaired circadian rhythms are observed in many neurological diseases; however, it is not clear whether loss of rhythms is the cause or result of neurodegeneration, or both. To address this important question, we examined the effects of circadian disruption in *Drosophila melanogaster* mutants that display clock-unrelated neurodegenerative phenotypes. We combined a null mutation in the clock gene *period* (per^{01}) that abolishes circadian rhythms, with a hypomorphic mutation in the carbonyl reductase gene *sniffer* (sni^{1}), which displays oxidative stress induced neurodegeneration. We report that disruption of circadian rhythms in sni^{1} mutants significantly reduces their lifespan compared to single mutants. Shortened lifespan in double mutants was coupled with accelerated neuronal degeneration evidenced by vacuolization in the adult brain. In addition, per^{01} sni^{1} flies showed drastically impaired vertical mobility and increased accumulation of carbonylated proteins compared to age-matched single mutant flies. Loss of *per* function does not affect *sni* mRNA expression, suggesting that these genes act via independent pathways producing additive effects. Finally, we show that per^{01} mutation accelerates the onset of brain pathologies when combined with neurodegeneration-prone mutation in another gene, *swiss cheese* (sws^{1}), which does not operate through the oxidative stress pathway. Taken together, our data suggest that the *period* gene may be causally involved in neuroprotective pathways in aging *Drosophila.*

Key words: biological clock; circadian rhythms; neuronal health; protein carbonyls; RING assay

Introduction

Circadian clocks are endogenous timekeeping mechanisms that generate rhythms with circa-24 h periodicity. At the molecular level, circadian clocks consist of cell autonomous networks of core clock genes and proteins engaged in transcriptional-translational feedback loops, which are largely conserved between *Drosophila* and mammals (Yu and Hardin, 2006). Rhythmic activities of clock genes generate daily fluctuations in the expression level of many target genes that underlie cellular, physiological and behavioral rhythms (Allada and Chung, 2010; Schibler, 2007). Disruption of circadian rhythms by environmental manipulations or mutations in specific clock genes lead to various age-related pathologies and may reduce lifespan in mice (Antoch et al., 2008; Davidson et al., 2006; Kondratov et al., 2006; Lee, 2006).

Functional links between circadian rhythms and aging are supported by observations that an impaired circadian system may predispose organisms to neurodegenerative diseases (Gibson et al., 2009). However, the evidence linking disruption of circadian rhythms to premature neurodegeneration is of correlative nature and the mechanisms involved are not yet understood. Studies in the model organism, *Drosophila melanogaster,* showed that a null mutation in the clock gene *period* (per^{01}) is associated with increased susceptibility to oxidative challenge (Krishnan et al., 2008). Furthermore, exposure of aging per^{01} flies to mild oxidative stress increased their mortality risk, accelerated functional senescence, and increased signs of neurodegeneration compared to the age-matched controls (Krishnan et al., 2009). Together, these data suggest that the clock gene *period* may protect the health of the nervous system in aging animals.

Neurodegeneration is a detrimental aging phenotype affecting homeostasis, motor performance, and cognitive functions. Several mutants uncovered in *Drosophila* show these

phenotypes (Kretzschmar, 2005); one of them affects the gene *sniffer (sni)* that encodes for a carbonyl reductase in fruitflies. Carbonyl reductases catalyze the detoxification of lipid peroxides generated by reactive oxygen species (ROS) and help to prevent protein carbonylation (Maser, 2006). Loss of *sni* function leads to a progressive neurodegenerative phenotype with the formation of spongiform lesions in the brain neuropil, and apoptotic cell death of glia and neurons (Botella et al., 2004). Similar to *sni*, mutation in the *swiss cheese (sws)* gene produces age-dependent lesions in the neuropil that are accompanied by apoptotic neuronal death (Kretzschmar et al., 1997). However, the *sws* gene encodes a phospholipase that interacts with Protein Kinase A (PKA) and it has not been connected with oxidative stress (Muhlig-Versen et al., 2005).

Neurodegeneration is often associated with accumulated oxidative damage in the nervous system (Sayre et al., 2001). We previously reported that arrhythmic per^{01} flies show significantly increased levels of lipid peroxidation and protein carbonylation during aging (Krishnan et al., 2009). We therefore hypothesized that the circadian system may contribute to cellular homeostasis by curtailing oxidative damage in the nervous system. To test this hypothesis, we examined aging phenotypes in flies carrying mutations in the clock gene *per* and carbonyl reductase encoded by *sni*. We report that such double mutants show significantly shortened lifespan, accelerated neurodegeneration, and a decline in climbing ability. Interestingly, these effects were not restricted to the *sni* gene alone, because arrhythmia due to loss of *per* function also accelerated neurodegeneration in the *sws* mutant. Together, our data suggest that the core clock gene *period*, functions in neuroprotective pathways that may delay the progression of brain pathologies during aging.

Materials and Methods

Fly rearing and creation of double mutants

D. melanogaster were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25°C in 12-hour light: dark (LD,12:12) cycles (with an average light intensity of ~2000 lx). All experiments were performed between 4 and 8 h after lights-on (or equivalent time in constant light (LL)) in male flies of different ages, as specified in results. To determine lifespan, 3-4 cohorts of 100 mated males of a given genotype were housed in 8 oz round bottom polypropylene bottles (Genesee Scientific) inverted over 60 mm Falcon Primaria Tissue culture dishes (Becton Dickinson Labware) containing 15 ml of diet. Diet was replaced on alternate days without anesthesia after tapping flies to the bottom of the bottle, and mortality was recorded at this time. The per^{01} mutants were previously backcrossed to the Canton S (CS) for 8 generations and sni^{1} mutants were backcrossed to *yellow white* (*y w*). The per^{01} sni^{1} double mutants were created by recombination using per^{01} *w* crossed to *y w* sni^{1} and selecting flies that were per^{01} *w* sni^{1} (sni^{1} was detected by the orange eye color). *y* is localized at 1A5, *per* at 3B1, *w* at 3B6 and *sni* at 7D22. Similarly, the per^{01} sws^{1} double mutants were created by recombination with per^{01} *w* and *y w* sws^{1} *Appl*-GAL4 (as a visible marker proximal of *sws*, which is localized at 7D1, detectable by the orange eye color) and selecting flies that were per^{01} *w* sws^{1} *Appl*-GAL4. The correct genotype was confirmed by external markers, mutant phenotype, and PCR. To determine circadian rhythmicity for each genotype, locomotor activity patterns were monitored in 2-3 independent experiments using the Trikinetics monitor (Waltham, MA). Flies were entrained to LD for 3 days and then recorded for 7 days in constant darkness. Fast Fourier Transform (FFT) analysis was conducted using the ClockLab software (Actimetrics, Coulbourn Instruments). Flies

with FFT values >0.04, which showed a single well-defined peak in the periodogram, were classified as rhythmic and included in the calculation of free-running period using the ClockLab software. The *y w* flies served as control for sni^1 and sws^1 single mutants and double mutants carrying per^{01} allele.

Neuronal degeneration

Paraffin-embedded sections of heads were processed as previously described (Bettencourt da Cruz et al., 2005; Tschape et al., 2002). Briefly, heads were cut in 7 μm serial sections, the paraffin was removed in SafeClear (Fisher Scientific), sections were embedded in Permount, and analyzed with a Zeiss Axioscope 2 microscope using the auto-fluorescence caused by the eye pigment (no staining was used). Experimental and control flies were put next to each other in the same paraffin block, cut, and processed together. Microscopic pictures were taken at the same level of the brain, the vacuoles (identified by being unstained and exceeding 50 pixels in size) were counted and vacuolized area was calculated using our established methods (Bettencourt da Cruz et al., 2005; Tschape et al., 2002). For *sws,* the pictures were taken at the level of the great commissure (z=-1; http://web.neurobio.arizona.edu/Flybrain/html/atlas/silver/horiz/index.html) and the holes in the deutocerebral neuropil were measured as described (Bettencourt Da Cruz et al., 2008). For *sni,* the pictures were taken from sections that contained the ventral deutocerebral neuropil (z=-6; http://web.neurobio.arizona.edu/Flybrain/html/atlas/silver/horiz/index.html), and the vacuoles in all four optic neuropils (lamina, medulla, lobula, and lobula plate) were counted and measured. In both cases, each side of the brain was scored independently (the number of brain hemispheres analyzed for each genotype is indicated in the figures). For a double blind analyses, pictures were taken and numbered, vacuoles were counted, and the area of vacuoles

was measured in pixels in Photoshop and subsequently converted into μm^2 (Bettencourt da Cruz et al., 2005). Statistical analysis was done using one-way ANOVA.

Rapid iterative negative geotaxis (RING) and oxidative damage assays

Vertical mobility was tested using the RING assay as described (Gargano et al., 2005). Briefly, 2 groups of 25 flies of each genotype were transferred into empty vials without anesthesia, and the vials were loaded into the RING apparatus. The apparatus was rapped three times in rapid succession to initiate a negative geotaxis response. The flies' movements in tubes were videotaped and digital images captured 4 s after initiating the behavior. The climbed distance was calculated for each fly and expressed as average height climbed in the 4 s interval. The performance of flies in a single vial was calculated as the average of 5 consecutive trials (interspersed with a 30 s rest). To assess oxidative damage, protein carbonyls were measured in male head homogenates of the various genotypes at 370 nm after reaction with 2,4-dinitrophenylhydrazine (DNPH) using a BioTek Synergy 2 plate reader, as described previously (Krishnan et al., 2008). Results were expressed as $nmol.mg^{-1}$ protein using an extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Gene expression by qRT-PCR

The expression of *sni* gene was measured in per^{01} mutants and CS control flies collected at 4 h intervals around the clock in LD. Total RNA was extracted from fly heads using TriReagent (Sigma). The samples were purified using the RNeasy mini kit (Qiagen) with on-column DNAse digestion (Qiagen), and cDNA was synthesized with iScript (Bio-Rad). Real-time PCR (qRT-PCR) was performed on the StepOnePlus (Applied Biosystems) under default thermal cycling

conditions with a dissociation curve step. Every reaction contained iTaq SYBR Green Supermix with ROX (Bio-Rad), 0.6 ng cDNA, 80 nM primers. Primer sequences are available upon request. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA levels were calculated with respect to the trough levels set as 1 for control flies.

Statistical analyses

Lifespan and survival curves were plotted using Kaplan Meier survival curves and statistical significance of curves assessed using the Log-Rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests (GraphPad Prism v5.0; GraphPad Software Inc. San Diego CA). For statistical analysis of biochemical and gene expression results, one-way ANOVA with post-hoc tests were conducted (GraphPad Instat v3.0).

Results

Loss of circadian rhythms shortens the lifespan of flies mutant for carbonyl reductase

To determine the effects of *per* on longevity of *sni*-deficient flies, we created per^{01} sni^{1} double mutants. Since both genes are localized on the X chromosome (*per* at 3B and *sni* at 7D), we achieved this by recombination using per^{01} *w* and *y w* $sni^{1}$and selecting flies that lost the *y* marker but were orange due to the P-element in sni^{1}. The double mutants were confirmed by phenotype and by PCR using primers within the P-element and *sni*. Several double mutant lines were generated and two lines (referred to as per^{01} sni^{1} line 1 and 2) were selected for further analysis. As expected, both double mutant lines exhibited loss of circadian rhythms due to per^{01}

mutation, while single mutants were mostly rhythmic in DD indicating that they had a functional circadian clock (Table 1).

The lifespan of per^{01} sni^{1} flies was compared to the sni^{1} and per^{01} single mutants, as well as *y w* controls. There was no difference in mean lifespan between both the single mutants (per^{01} and sni^{1}) and the *y w* control (Fig. 1A, Table 2). In contrast, per^{01} sni^{1} double mutants showed very significant ($p<0.001$) reduction of their mean lifespan compared to single mutants. While both double mutant lines were short-lived, we observed significant difference in their lifespan with 32% reduction in recombinant line 1, and 50 % reduction in line 2 (Fig. 1A, Table 2).

We next tested whether disruption of circadian rhythms by non-genetic interventions affect longevity in sni^{1} single mutants. Adult sni^{1} flies were reared in constant light (LL) which interferes with the circadian clock mechanism and causes behavioral arrhythmia (Price et al., 1995). The lifespan of sni^{1} flies maintained in LL was significantly shortened ($p<0.0001$) compared to sni^{1} flies reared in LD 12:12, while *y w* flies showed similar lifespan in both LD and LL (Fig. 1B, Table 2).

Double per^{01} sni^{1} mutants show increased neurodegeneration and reduced climbing ability

Due to its neuroprotective role, loss of carbonyl reductase in sni^{1} mutants results in progressive degeneration with small vacuoles appearing in the first 7-9 days of adult life, and becoming larger and more numerous with progressing age (Botella et al., 2004). We demonstrate that neurodegeneration is dramatically increased in per^{01} sni^{1} double mutants (Fig. 2). Vacuoles were rarely detected in 9 day-old per^{01} fly brains (Fig. 2A), while we consistently observed a few small vacuoles in sni^{1} mutants (Fig. 2B). The vacuolization of the brain was markedly exacerbated in the age-matched per^{01} sni^{1} double mutant flies (Fig. 2C). Both, the area taken up

by vacuoles and average vacuole number increased very significantly in each of the two per^{01} sni^{1} lines examined at 9 days of age (Fig. 2D-E). More pronounced vacuolization was also maintained in 19 day-old per^{01} sni^{1} compared to sni^{1} alone (Fig. 2F). In the next experiment, we asked whether disruption of circadian rhythms by LL, which shortens lifespan of sni^{1} mutants, affects the levels of neurodegeneration. Brains of 10 day-old sni^{1} males maintained in LD or LL were sectioned and examined for vacuole formation. Disrupting the circadian clock by constant light significantly increased brain vacuolization in sni^{1} (Fig. 3), although the effects were less severe than in per^{01} sni^{1} double mutants.

To test whether increased neurodegeneration is associated with altered motor abilities, we conducted the RING assay on 10 day-old single and double mutants along with their controls. The climbing ability of per^{01} flies did not differ significantly from their CS control, while sni^{1} mutants showed modest but significant ($p<0.05$) impairment of climbing ability compared to their *y w* control. Importantly, the average climbing distance was dramatically reduced ($p<0.001$) in both per^{01} sni^{1} double mutant lines compared to the single sni^{1} mutants (Fig. 4).

Protein carbonyl levels are elevated in per^{01} sni^{1} double mutants

Since mutation in the *sni* gene severely attenuates carbonyl reductase expression (Botella et al., 2004), we tested the levels of oxidatively damaged proteins in heads of per^{01} and sni^{1} single mutants as well as per^{01} sni^{1} double mutants by measuring protein carbonyls. Levels of protein carbonyls were significantly increased ($p<0.01$) in heads of both per^{01} and sni^{1} single mutants compared to CS and *y w* controls. Importantly, a significant increase in the protein carbonyl accumulation ($p<0.01$) was detected in both recombinant lines of the per^{01} sni^{1} double mutants compared to the controls and single mutants (Fig. 5).

Expression of sni is not affected in per^{01} mutants

The gene *per* encodes transcriptional co-regulators that may affect the expression of downstream target genes (Claridge-Chang et al., 2001). Because per^{01} alone increases protein carbonylation (Krishnan et al., 2008; Fig. 5), we tested whether *sni* expression might be clock-controlled and therefore altered by the loss of *per* function. We measured the levels of *sni* mRNA around the clock in CS and per^{01} flies by qRT-PCR. The expression of *sni* did not show a daily rhythm in CS flies, and was not significantly reduced in per^{01} mutants compared to controls (Fig. 6). These data suggest that *sni* is not a downstream target of *per*, rather both mutants appear to act through independent pathways causing additive effects.

Neurodegeneration in sws mutant is substantially increased in a per^{01} background

To investigate whether arrhythmicity may affect another neurodegeneration-prone mutant, we compared the neurodegenerative changes caused by a mutation in the *swiss cheese* (*sws*) gene in the wild-type and per^{01} background. The per^{01} sws^{1} double mutants were created by recombination between per^{01} *w* and *y w* sws^{1} *Appl*-GAL4 (see Methods). Locomotor activity assays indicated that 97% of sws^{1} single mutants showed rhythmicity with a free-running period similar as in control *y w* flies, but rhythmicity was mostly lost in per^{01} sws^{1} (Table 1). As reported previously (Kretzschmar et al., 1997), 14 day-old sws^{1} mutant displayed characteristic symptoms of neurodegeneration evidenced by vacuoles in the dorsal neuropil (Fig. 7A, B), which does not occur in *y w* control flies at this age (Fig. 7D). Importantly, the age-matched per^{01} sws^{1} double mutants showed marked increase in the size and number of vacuoles (Fig. 7C

arrows) compared to the *sws*^{1} single mutants. The three-fold increase in vacuolization was highly significant (Fig. 7E, F).

Discussion

Our study demonstrates that disruption of circadian rhythms accelerates aging in two independent mutants that display neurodegenerative phenotypes. We show that lifespan of *sni*^{1} flies is reduced by 32-50% in a *per*^{01} background, which abolishes molecular and behavioral rhythms. Significant lifespan shortening was also observed in *sni*^{1} flies reared in LL, which is known to disrupt circadian systems (Price et al., 1995). Lifespan reduction resulting from the disruption of the clock by either genetic or environmental manipulations strongly suggests that this phenotype is caused by the loss of rhythmicity. However, we cannot exclude that clock-unrelated pleiotropic effects of *per* may be involved in accelerated aging, since PER protein is unstable in LL (Price et al., 1995). Interestingly, studies in mammals have also shown that interfering with the circadian clock mechanism by the knock-out of specific clock genes may lead to shortened lifespan (Davidson et al., 2006; Yu and Weaver, 2011). Premature aging was observed in mice with mutant core clock genes *Bmal1* or *Clock*, which together form the positive feedback loop of the circadian clock (Antoch et al., 2008; Kondratov et al., 2006). Genetic ablation of *per* gene homologs in mice resulted in some aging phenotypes and a significant increase in cancer incidence after gamma-radiation challenge (Lee, 2006). Our previous study showed that exposure of *per*^{01} flies to external oxidative challenge, significantly increased their mortality risk (Krishnan et al., 2009). Here we show that lifespan is compromised even further when loss of *per* function is combined with an internal oxidative stress caused by carbonyl

reductase deficiency. Taken together, these data suggest that intact circadian clocks promote longevity under various homeostatic challenges.

We show that that *per*-null related arrhythmia is associated with premature loss of neuronal integrity. A significant increase in the number and size of vacuoles was observed in the brains of per^{01} sni^{1} double mutants in LD, or the sni^{1} mutant in LL compared to sni^{1} males kept in LD. The increased deterioration of the nervous system might be the cause of the shortened lifespan in these flies because it has been shown that several neurodegenerative mutants are short-lived (Kretzschmar et al., 1997; Tschape et al., 2002). Consistent with the neuronal damage, per^{01} sni^{1} flies showed precipitous loss of climbing ability at the age of 10 days, whereas sni^{1} flies with a functional clock showed only modest (albeit significant) climbing impairment at this age. These data provide experimental evidence suggesting that the disruption of circadian rhythms, which is also observed in human neurodegenerative diseases, may be a causative factor contributing to these pathologies. Indeed, we showed previously that the loss of the clock by itself can lead to neurodegenerative symptoms in per^{01} mutants later in life (Krishnan et al., 2009). Interestingly, there is also evidence for a reverse relationship such that progressive neurodegeneration may contribute to the loss of clock function in both flies and mice (Morton et al., 2005; Rezaval et al., 2008).

Carbonyl reductase encoded by *sni,* acts as a neuroprotective enzyme against oxidative stress (Botella et al., 2004); therefore, we tested the levels of oxidatively damaged proteins in heads of single and per^{01} sni^{1} double mutants. Consistent with our previous report (Krishnan et al., 2009), there was a significant increase in the protein carbonyl levels in per^{01} mutants, and these levels were even higher in sni^{1} mutants (Fig. 4). While protein carbonyl levels were further elevated in per^{01} sni^{1} flies, this was not as dramatic as the increase in brain damage observed in

these double mutants at the same age (Fig. 2). It is possible that other oxidatively damaged species might accumulate to higher levels in per^{01} sni^{1} flies since deficiency in carbonyl reductase activity may also contribute to increased lipid peroxidation (Martin et al., 2011; Sgraja et al., 2004).

To address the nature of per^{01} and sni^{1} interactions, we tested daily profiles of *sni* expression in heads of wild-type CS flies and clock-deficient per^{01} mutants. The levels of *sni* mRNA did not change significantly across circadian time points in control flies and neither were they different in per^{01} mutants. These data suggest that *per* does not regulate *sni* expression, consistent with the exacerbated neurodegeneration observed in double mutants. The effects of *per* mutation appear indirect and additive suggesting more general protective functions of this clock gene. This is further supported by the fact that loss of *per* function resulted in accelerated neuronal damage in *sws* mutant, which increases neurodegeneration via different mechanisms. This gene encodes a phospholipase, thereby interfering with the phospholipid homeostasis (Muhlig-Versen et al., 2005). These data show that detrimental effects of *per*-null allele are not specific to the sni^{1} mutant with increased oxidative stress, but also extend to the sws^{1} mutant, which does not appear to act via the oxidative stress pathway. However, the increase in vacuolization was greater in sni^{1} than in sws^{1} mutant (Fig. 2, 7), suggesting that *per* mutation may affect *sni* via multiple pathways that may or may not be related to oxidative damage.

While our results suggest that a functional circadian system improves the performance of neurodegeneration-prone mutants, the mechanisms involved remain to be investigated. We hypothesize that the circadian clocks slow down the accumulation of neuronal damage in aging organisms by synchronizing the activities of enzymes involved in cellular homeostasis. Indeed, microarray studies of daily gene expression profiles suggested synchronous circadian

fluctuations in the expression of some protective enzymes such as glutathione-S-transferases, in fly heads (Wijnen and Young, 2006). Our previous data demonstrated daily fluctuations in the levels of mitochondrial ROS and carbonylated proteins in flies with a functional clock, indicative of a daily rhythm in the removal of oxidative damage (Krishnan et al., 2008). In the absence of the circadian clock, enzymes working in a specific pathway may become dysregulated, leading to the impaired removal of oxidative damage. Consistent with this idea, we reported increased levels of oxidatively damaged lipids and proteins in per^{01} mutants during aging (Krishnan et al., 2009). It was also shown that oxidative stress may impair the clock function in *Drosophila* (Zheng et al., 2007).

Circadian clocks are involved in the regulation of response to genotoxic stress and xenobiotics in both mice and fly (Antoch et al., 2008; Beaver et al., 2010; Gachon and Firsov, 2011). Therefore, increased neurodegeneration in arrhythmic sni^{1} and sws^{1} mutants may involve additional pathways beyond ROS homeostasis. In mice, the loss of an essential clock component encoded by *Bmal1* causes a variety of premature aging phenotypes (Kondratov, 2007). Interestingly, treatment with antioxidants reversed some aging symptoms, but had no effect on other age-related pathologies such as sarcopenia (Kondratov et al., 2009). This suggests that both ROS-dependent and independent mechanisms may contribute to neuroprotection in organisms with functional clocks.

In summary, we show that disrupting clocks in flies has a profound impact on neurodegeneration-prone mutants. Circadian rhythm disturbances such as sleep disorders, which are commonly observed during aging, are exacerbated in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington disease (Sterniczuk et al., 2010; Wu and Swaab, 2007). Our functional study, which involved the manipulation of a clock gene to assess its

neuroprotective role, substantiates the possibility that arrhythmia is not a mere correlation, but may actually contribute to the onset of neurodegenerative disorders. Conversely, intact circadian clocks appear to promote the health of the nervous system during aging.

Acknowledgements

We thank the anonymous reviewers for insightful comments. This research was supported in part by NIH R21AG038989 and R21 NS075500 grants to JMG and R01 NS047663 to DK. KR is supported by NSF IGERT in Aging Sciences Fellowship at OSU (DGE 0965820).

References

Allada, R., Chung, B. Y., 2010. Circadian organization of behavior and physiology in *Drosophila.* Annual Review of Physiology. 72**,** 605-624.

Antoch, M. P., et al., 2008. Disruption of the circadian clock due to the Clock mutation has discrete effects on aging and carcinogenesis. Cell Cycle. 7**,** 1197-204.

Beaver, L. M., et al., 2010. Circadian clock regulates response to pesticides in *Drosophila* via conserved Pdp1 pathway. Toxicol Sci. 115**,** 513-20.

Bettencourt da Cruz, A., et al., 2005. Disruption of the MAP1B-related protein FUTSCH leads to changes in the neuronal cytoskeleton, axonal transport defects, and progressive neurodegeneration in *Drosophila.* Mol Biol Cell. 16**,** 2433-42.

Bettencourt Da Cruz, A., et al., 2008. Swiss Cheese, a protein involved in progressive neurodegeneration, acts as a noncanonical regulatory subunit for PKA-C3. J Neurosci. 28**,** 10885-92.

Botella, J. A., et al., 2004. The *Drosophila* carbonyl reductase sniffer prevents oxidative stress-induced neurodegeneration. Curr Biol. 14**,** 782-6.

Claridge-Chang, A., et al., 2001. Circadian regulation of gene expression systems in the *Drosophila* head. Neuron. 32**,** 657-71.

Davidson, A. J., et al., 2006. Chronic jet-lag increases mortality in aged mice. Curr Biol. 16**,** R914-6.

Gachon, F., Firsov, D., 2011. The role of circadian timing system on drug metabolism and detoxification. Expert Opin. Drug Metab. Toxicol. 7**,** 147-158.

Gargano, J. W., et al., 2005. Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila.* Exp Gerontol. 40**,** 386-95.

Gibson, E. M., et al., 2009. Aging in the circadian system: considerations for health, disease prevention and longevity. Exp Gerontol. 44**,** 51-6.

Kondratov, R. V., 2007. A role of the circadian system and circadian proteins in aging. Ageing Res Rev. 6**,** 12-27.

Kondratov, R. V., et al., 2006. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. Genes Dev. 20**,** 1868-73.

Kondratov, R. V., et al., 2009. Antioxidant N-acetyl-L-cysteine ameliorates symptoms of premature aging associated with the deficiency of the circadian protein BMAL1. Aging (Albany NY). 1**,** 979-87.

Kretzschmar, D., 2005. Neurodegenerative mutants in *Drosophila*: a means to identify genes and mechanisms involved in human diseases? Invert Neurosci. 5**,** 97-109.

Kretzschmar, D., et al., 1997. The swiss cheese mutant causes glial hyperwrapping and brain degeneration in *Drosophila.* J Neurosci. 17**,** 7425-32.

Krishnan, N., et al., 2008. Circadian regulation of response to oxidative stress in *Drosophila melanogaster*. Biochem Biophys Res Commun. 374**,** 299-303.

Krishnan, N., et al., 2009. The circadian clock gene *period* extends healthspan in aging *Drosophila melanogaster*. Aging (Albany, NY). 1**,** 937-948.

Lee, C. C., 2006. Tumor suppression by the mammalian *Period* genes. Cancer Causes Control. 17**,** 525-30.

Martin, H. J., et al., 2011. The *Drosophila* carbonyl reductase sniffer is an efficient 4-oxonon-2-enal (4ONE) reductase. Chem Biol Interact. 191**,** 48-54.

Maser, E., 2006. Neuroprotective role for carbonyl reductase? Biochem Biophys Res Commun. 340**,** 1019-22.

Morton, A. J., et al., 2005. Disintegration of the sleep-wake cycle and circadian timing in Huntington's disease. J Neurosci. 25**,** 157-63.

Muhlig-Versen, M., et al., 2005. Loss of Swiss cheese/neuropathy target esterase activity causes disruption of phosphatidylcholine homeostasis and neuronal and glial death in adult *Drosophila*. J Neurosci. 25**,** 2865-73.

Price, J. L., et al., 1995. Suppresion of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. EMBO J. 14**,** 4044-4049.

Rezaval, C., et al., 2008. A functional misexpression screen uncovers a role for enabled in progressive neurodegeneration. PLoS One. 3**,** e3332.

Sayre, L. M., et al., 2001. Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr Med Chem. 8**,** 721-38.

Schibler, U., 2007. The daily timing of gene expresion and physiology in mammals. Dialogues Clin. Neurosci. 9**,** 257-272.

Sgraja, T., et al., 2004. Structural insights into the neuroprotective-acting carbonyl reductase Sniffer of Drosophila melanogaster. J Mol Biol. 342**,** 1613-24.

Sterniczuk, R., et al., 2010. Characterization of the 3xTg-AD mouse model of Alzheimer's disease: Part 1. Circadian changes. Brain Res. 1348**,** 139 -148.

Tschape, J. A., et al., 2002. The neurodegeneration mutant lochrig interferes with cholesterol homeostasis and Appl processing. Embo J. 21**,** 6367-76.

Wijnen, H., Young, M. W., 2006. Interplay of circadian clocks and metabolic rhythms. Annu Rev Genet. 40**,** 409-48.

Wu, Y.-H., Swaab, D. F., 2007. Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease. Sleep Med. 8**,** 623-636.

Yu, E. A., Weaver, D. R., 2011. Disrupting the circadian clock: gene-specific effects on aging, cancer, and other phenotypes. Aging (Albany NY). 3**,** 479-93.

Yu, W., Hardin, P. E., 2006. Circadian oscillators of *Drosophila* and mammals. J Cell Sci. 119**,** 4793-5.

Zheng, X., et al., 2007. FOXO and insulin signaling regulate sensitivity of the circadian clock to oxidative stress. Proc Natl Acad Sci U S A. 104**,** 15899-904.

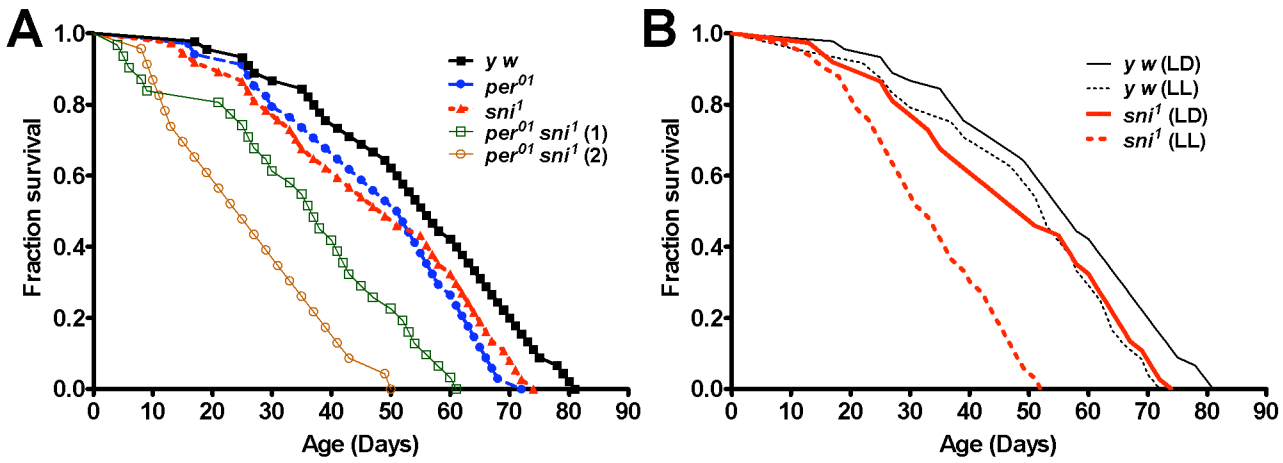

Figure 1. Loss of circadian rhythms dramatically shortens the lifespan of *sni*^{1} mutants.

A) Survival curves for *y w*, *per*^{01}, *sni*^{1}, and *per*^{01} *sni*^{1} double mutant lines **B**) Lifespan of *sni*^{1} and *y w* in 12h light: dark (LD) cycles and constant light (LL), which disrupts the circadian clock function.

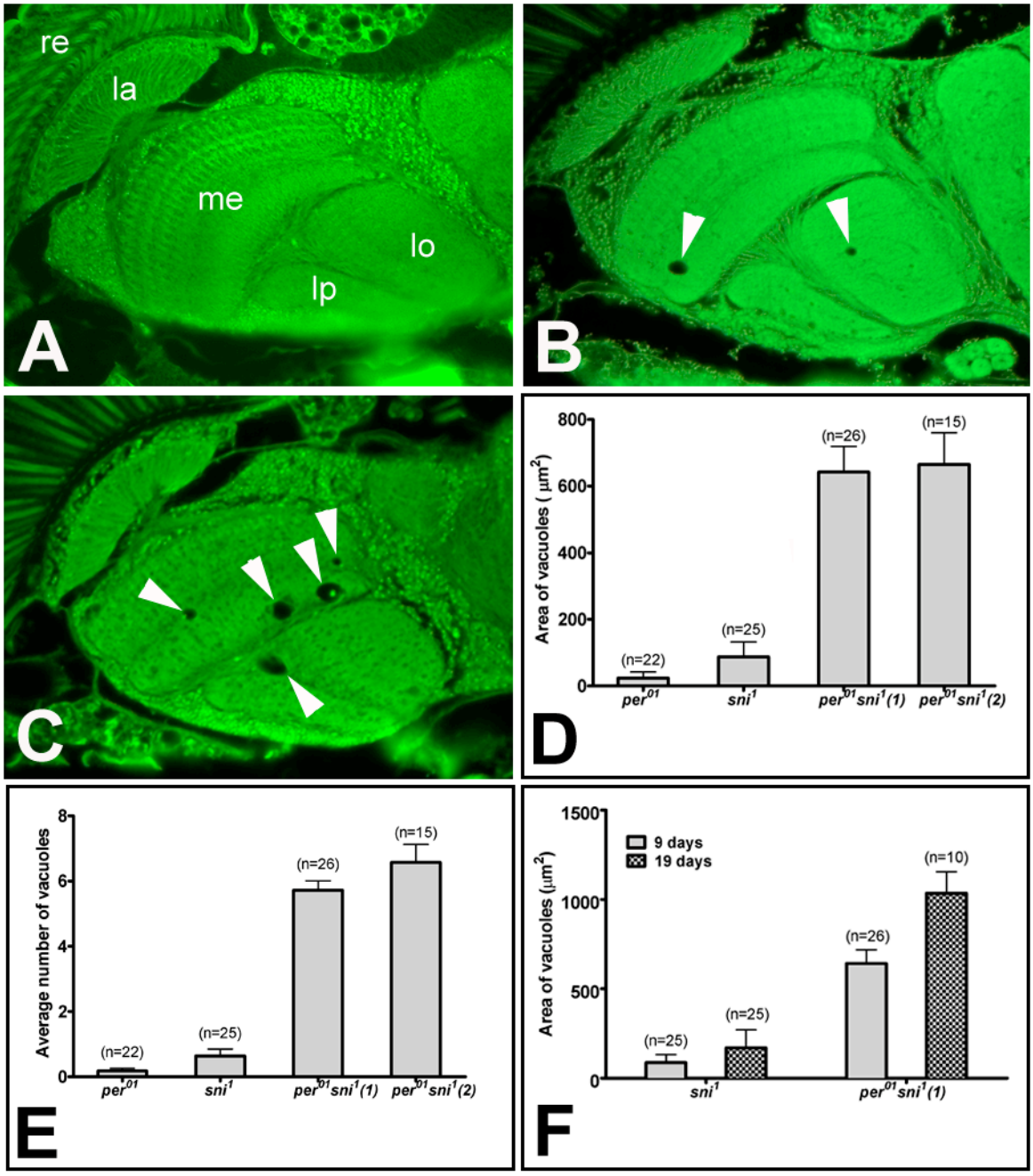

Figure 2. Interfering with the clock increases neurodegeneration in *sni*^{1} mutants.

A-C) Paraffin head sections from 9 day-old males (scale bar=25µm, re=retina, la=lamina, me=medulla, lo=lobula, lp=lobula plate). **A)** No vacuoles are detectable in the brain of a *per*^{01} fly. **B)** A *sni*^{1} fly brain shows a few vacuoles (arrows). **C)** Brains of *per*^{01} *sni*^{1} double mutant show increase in the size and number of vacuoles. **D)** Bar graph showing the mean ± SEM area

of all vacuoles /brain hemisphere. There is a significant difference between the *sni*^{1} and *per*^{01} *sni*^{1} line 1 ($p=2.9x10^{-6}$), and *sni*^{1} and *per*^{01} *sni*^{1} line 2 ($p=1.6x10^{-5}$). **E)** The mean number of vacuoles /brain hemisphere is increased in *per*^{01} *sni*^{1} compared to *sni*^{1} alone [*sni*^{1} to *per*^{01} *sni*^{1} (1): $p=8.42x10^{-12}$; *sni*^{1} to *per*^{01} *sni*^{1} (2): $p=6.5x10^{-8}$]. **F)** Comparison of the vacuolization between 9 and 19 day-old flies shows that the phenotype is progressive with age for both *sni*^{1} ($p=0.036$) and *per*^{01} *sni*^{1} line 1 ($p=0.03$). **D-F)** The number of brain hemispheres (n) examined to calculate the average values is indicated on the top of each bar.

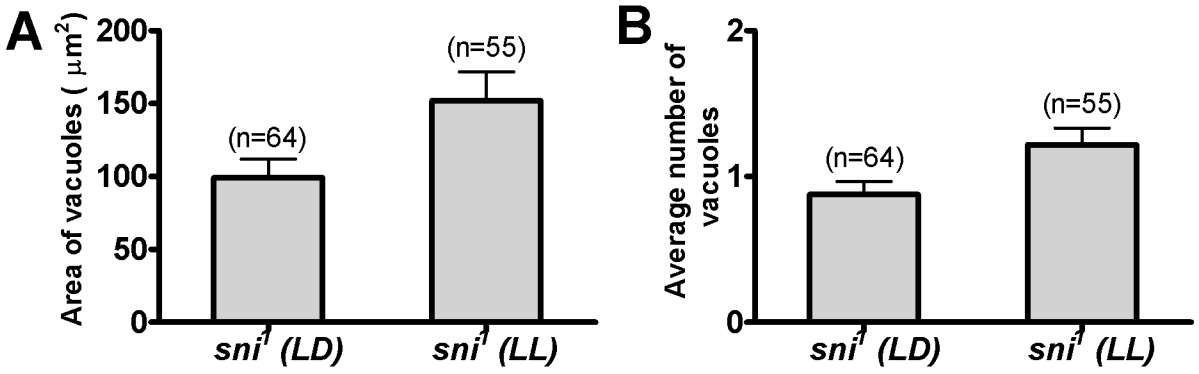

Figure 3. Disrupting the circadian clock by constant light increases vacuolization in *sni*^{1} mutant. A) 9 day-old *sni*^{1} flies maintained in constant light (LL) show significant increase in the mean area of vacuoles /brain hemisphere compared to 9 day-old *sni*^{1} flies in 12h light: dark (LD, 12:12) cycles ($p=0.024$). **B)** The mean number of vacuoles is also significantly higher in *sni*^{1} mutant kept in LL ($p=0.018$). **A-B)** The number of brain hemispheres (n) examined to calculate the average values is indicated on the top of each bar.

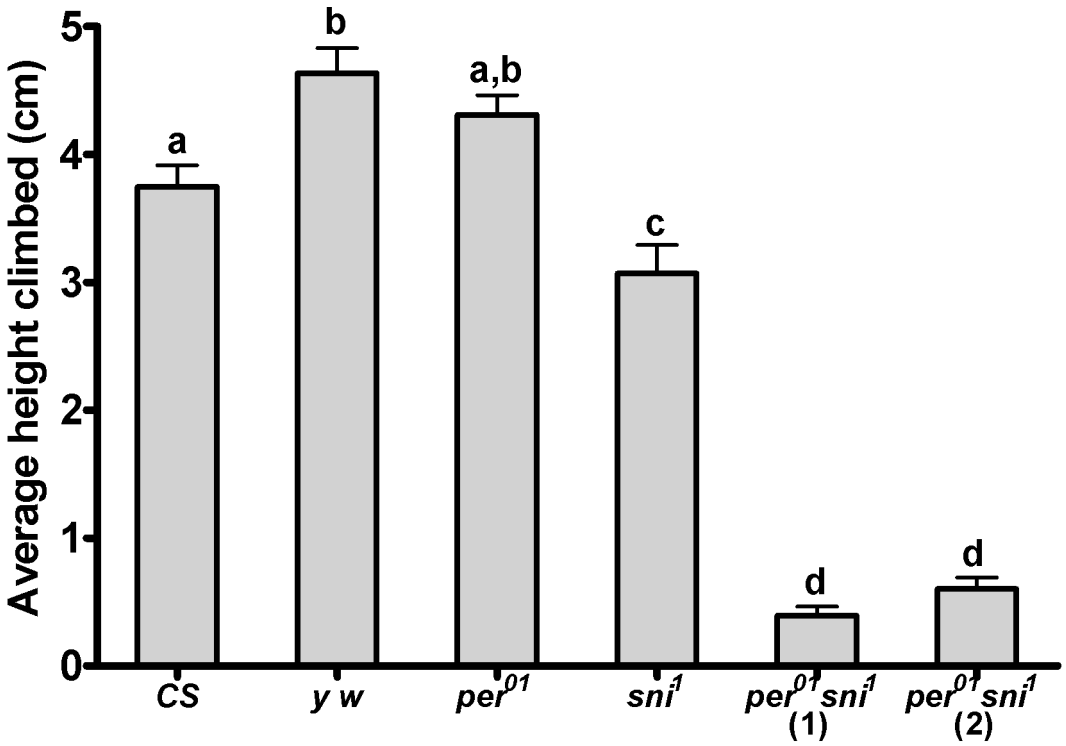

Figure 4. ***per*^{01} *sni*^{1} **double mutants show accelerated mobility impairment.** Vertical mobility was measured by the RING assay in 10 day-old males of the indicated genotypes. Bars represent mean height climbed (± SEM), based on testing 2 vials per genotype, each containing 25 flies. Bars with different superscripts are significantly different at $p<0.01$.

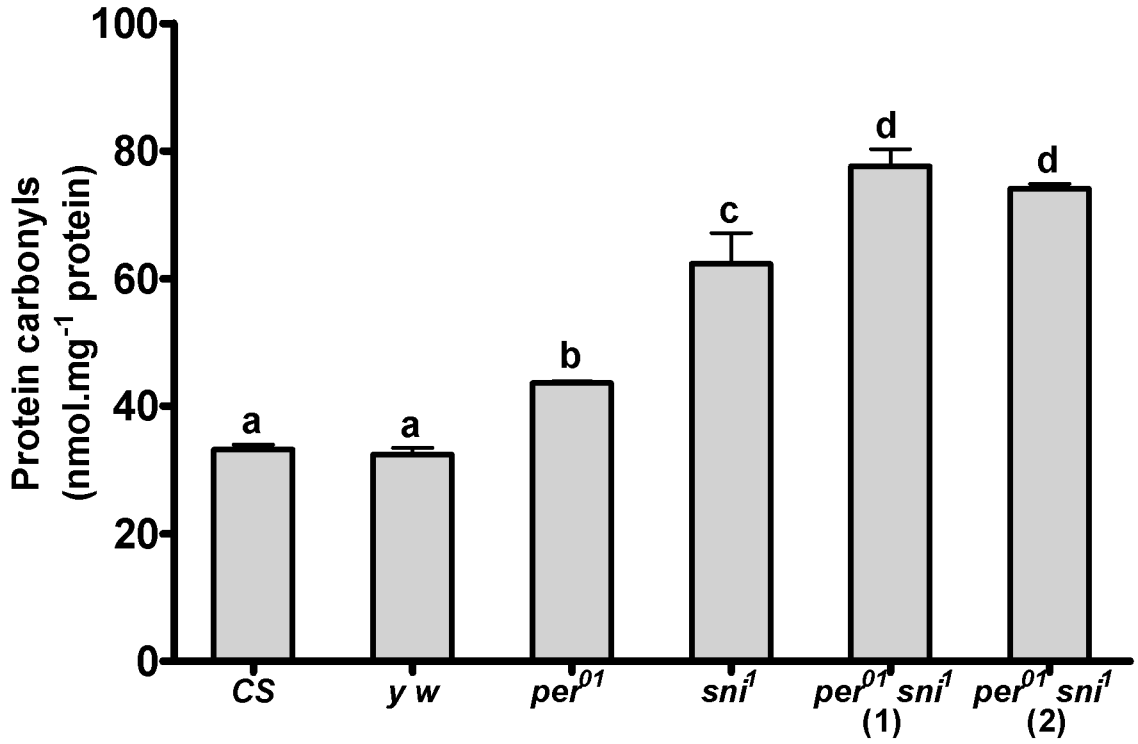

Figure 5. Oxidative damage in the form of protein carbonyls accumulates to higher levels in *per*^{01} *sni*^{1} flies. Protein carbonyl levels were measured in heads of 10 day-old males of the indicated genotypes. Both *per*^{01} and *sni*^{1} single mutants had higher protein carbonyls than their respective CS and *y w* controls. Protein carbonyls were further elevated in *per*^{01} *sni*^{1} double mutants, compared to *per*^{01} or *sni*^{1} single mutants. Bars represent mean carbonyl levels (± SEM), based on testing 3 independent sets of flies each containing 75 flies in 3 technical repeats of 25 flies each. Bars with different superscripts are significantly different at $p<0.01$.

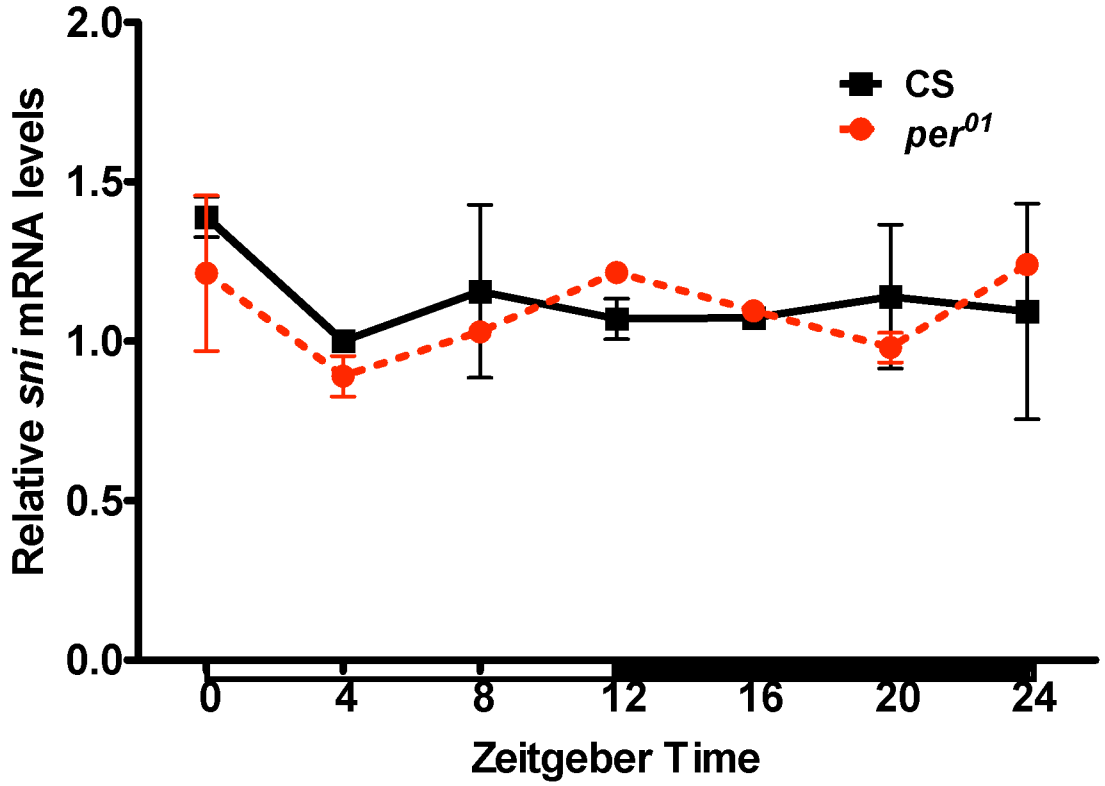

Figure 6. Relative *sni* mRNA levels are not significantly different between CS and per^{01} mutants. Expression profile of *sni* was analyzed by qRT-PCR in heads of flies collected at 4 h intervals in LD, 12:12 cycles. White and black horizontal bars indicate periods of light and darkness, respectively.

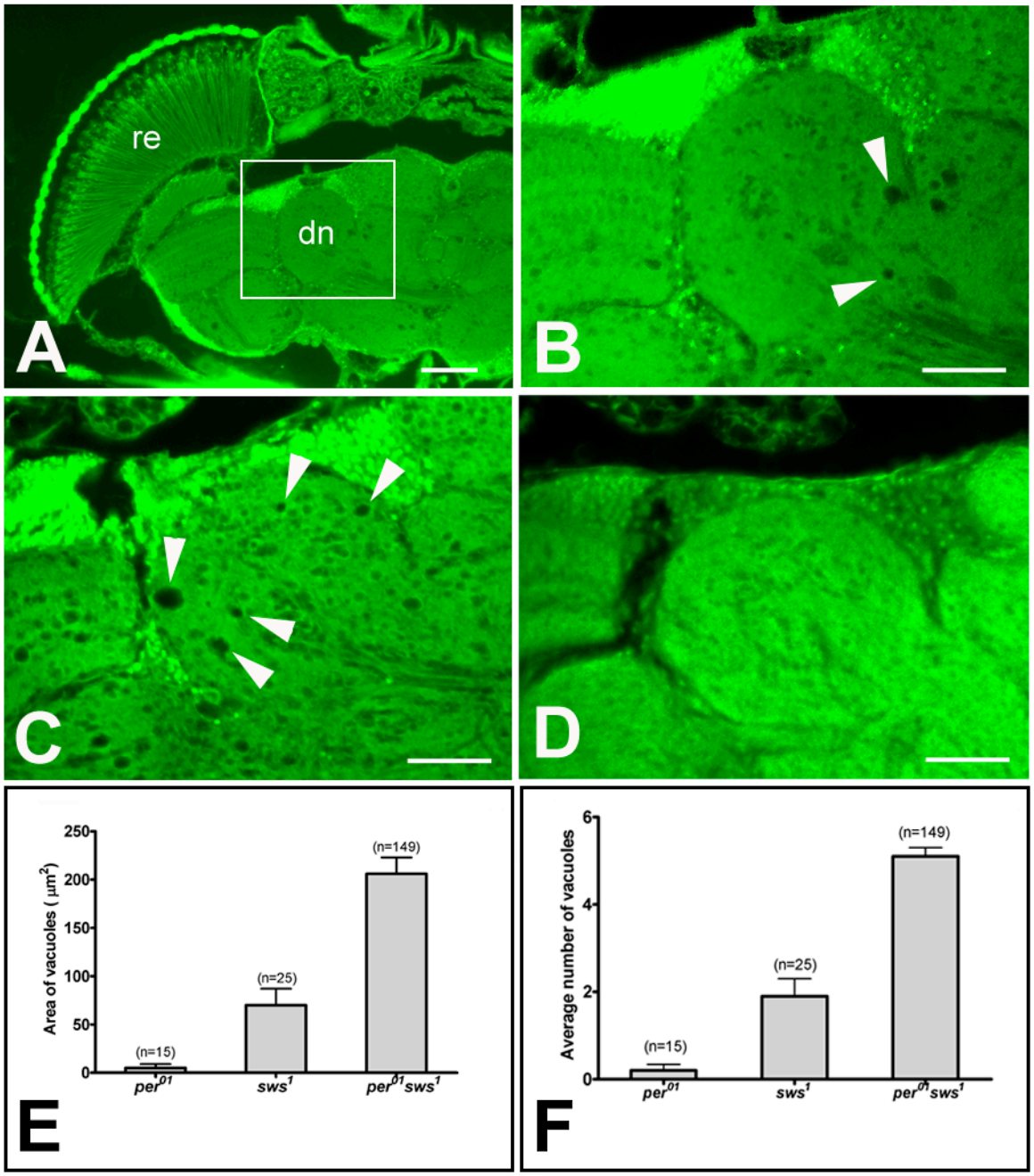

Figure 7. Loss of *per* function increases neurodegeneration in *sws* mutants. A) Paraffin head sections from a 14 day-old old *sws*^{1} fly show widespread degeneration (arrows) characteristic for this mutant (scale bar=50µm, re=retina, ol=optic lobes, dn=deutocerebral neuropil). **B)** Magnification from A (box), showing the deutocerebral neuropil that was used for measurements. **C)** Age-matched *per*^{01} *sws*^{1} double mutants show increase in the size and number

of vacuoles compared to *sws*^{1} single mutants. **D)** Age-matched *y w* control does not show vacuoles in this area. **B-D)** Scale bar=25µm**. E)** Bar graph showing significant difference in the mean area of all vacuoles in the deutocerebral neuropil between *sws*^{1} and *per*^{01} *sws*^{1} ($p=1.9\times10^{-8}$). **F)** The mean number of vacuoles /brain hemisphere is also significantly higher in the double mutant compared to *sws*^{1} alone (p=0.0033). **E-F)** The number of brain hemispheres (n) examined to calculate average values is indicated on the top of each bar.

Table 1: Table showing the percentage of rhythmic flies and mean period length in flies of indicated genotypes. Locomotor activity was monitored in 2-3 independent experiments and the total number of flies (n) analyzed for each genotype is indicated.

Genotype	n	% Rhythmic	Period
y w	30	93	23.87
***sni*^{1}**	30	70	23.77
***per*^{01}**	30	0	-
***sws*^{1}**	31	97	23.70
***per*^{01} *sni*^{1} (1)**	14	0	-
***per*^{01} *sni*^{1} (2)**	21	0	-
***per*^{01} *sws*^{1}**	23	13	24.11

Table 2. Disruption of circadian rhythms shortens lifespan in *sni*^{1} mutants. Median and mean ± SEM lifespan (days) is shown for indicated genotypes with n = sample size. Statistical comparison was conducted using one-way ANOVA with Tukey-Kramer multiple comparison's test. Values with different superscripts are significantly different at $p<0.001$.

Genotype	Regime	n	Median	Mean ± SEM
y w	LD	300	55	55.2 ± 0.7^{a}
y w	LL	300	52	53.8 ± 0.3^{a}
***per*^{01}**	LD	295	51.5	55.7 ± 0.4^{a}
***sni*^{1}**	LD	197	49	55.1 ± 0.9^{a}
***sni*^{1}**	LL	303	37	37.1 ± 0.4^{b}
***per*^{01} *sni*^{1} (1)**	LD	194	37	35.7 ± 1.0^{b}
***per*^{01} *sni*^{1} (2)**	LD	268	25	27.1 ± 0.5^{c}