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Loss of circadian clock accelerates aging in neurodegeneration-prone mutants

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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*.

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 leads 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

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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