affective responses in behaving animals[12–15]. Thus, one consequence of pharmacological intervention in dopamine system dynamics is a disruption in this finely tuned associative process. Such a condition may underlie improper associative events, such as those thought to contribute to inappropriate affect in schizophrenia[23–25] or heightened distractibility in attention-deficit hyperactivity disorders[26,27]. □

Methods

Materials

Haloperidol was a gift from McNeil Laboratories, and was dissolved in dilute lactic acid, then further diluted with 0.9% saline to a concentration of 0.5 mg ml^{-1}.

Animal preparation

All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (250–350 g weight) were anaesthetized with an initial injection of 400 mg kg^{-1} of 8% chloral hydrate, administered intraperitoneally, and placed into a stereotaxic device that was modified to allow contained odour delivery to the nose at a flow rate of 1.5 l min^{-1}, with delivery of odour within 0.5 s. Supplemental anaesthesia (8% chloral hydrate) was delivered by a lateral tail vein catheter as necessary to maintain suppression of hindlimb withdrawal reflex. Temperature was monitored and maintained at ∼37 °C. Coordinates for recordings were determined using a stereotaxic atlas, as follows: LAT −5.0 lateral, −3.3 caudal from bregma.

Intracellular recordings

Recordings were performed as described previously[22]. Briefly, electrodes were constructed using borosilicate glass tubing and filled with 2% biocytin in 3 M potassium acetate (Sigma). Impedances were measured in situ and ranged from 45–75 MΩ. Hyperpolarizing d.c. pulses were used to determine input resistance, and only the linear portion of the plot was included for this analysis. Mean resting membrane potential and standard deviation were determined from 30-s sampling periods, and action potentials were eliminated from this analysis. The area under the odour-evoked PSP was analysed by measuring the first 5 s of the area under the odour-evoked PSP as the baseline, and subtracting out the area under spontaneously occurring baseline PSPs in the 5 s immediately preceding odour presentation. These baseline-corrected responses to odours were averaged for data analysis. Recording electrode placements were identified as previously described[22]. Neurons were not included in this study if the resting membrane potential was less polarized than −65 mV, if their action potentials did not overshoot 0 mV, if the measured input resistance was below 20 MΩ, or if they were found to lie outside the LAT.

Pavlovian conditioning

A pavlovian conditioning procedure was performed by pairing of an odour with a foot-shock. A foot-shock was delivered by two 28-g needles inserted in the lateral side of the foot contralateral to the neuronal recordings. Each odour (anise or almond) was presented at least two times, for 10 s, with a 60-s delay between presentations. One odour was chosen to be paired with the foot-shock. Paired odour selection was counterbalanced. This first odour was paired with the foot-shock (4 s, 2–5 mA, 20 Hz, 0.2-ms duration pulses) such that the foot-shock was presented 5 s after the odour began. The foot-shock intensity chosen was dependent on the level of depolarization achieved in the neuron by the foot-shock. Typically, an intensity of 4–5 mA was chosen, evoking a response that was subthreshold to spike generation. This pairing was performed 5–8 times at 60-s intervals. After these pairings, each odour was presented at least twice, 10 s each, at 60 s intervals. In some experiments, after the first odour was paired with the foot-shock, 0.9% saline (0.4 ml) or haloperidol (0.4 ml of 0.5 mg ml^{-1}) was administered, and the rat was subjected to exactly the same procedure, substituting the non-paired odour for the previously paired odour.

To examine habituation, the odours were presented to a separate group of rats in a fashion identical to the conditioning protocol, but without foot-shocks. Only neurons that displayed an initial response to odour presentation were used to examine habituation.

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Competing interests statement

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Stress response genes protect against lethal effects of sleep deprivation in *Drosophila*

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Sleep is controlled by two processes: a homeostatic drive that increases during waking and dissipates during sleep, and a circadian pacemaker that controls its timing[1]. Although these two systems can operate independently[2,3], recent studies indicate a more intimate relationship[4,5]. To study the interaction between homeostatic and circadian processes in *Drosophila*, we examined

homeostasis in the canonical loss-of-function clock mutants *period* (*per^{01}*), *timeless* (*tim^{01}*), *clock* (*Clk^{jrk}*) and *cycle* (*cyc^{01}*)[6–9]. *cyc^{01}* mutants showed a disproportionately large sleep rebound and died after 10 hours of sleep deprivation, although they were more resistant than other clock mutants to various stressors. Unlike other clock mutants, *cyc^{01}* flies showed a reduced expression of heat-shock genes after sleep loss. However, activating heat-shock genes before sleep deprivation rescued *cyc^{01}* flies from its lethal effects. Consistent with the protective effect of heat-shock genes, was the observation that flies carrying a mutation for the heat-shock protein *Hsp83* (*Hsp83^{08445}*)[10] showed exaggerated homeostatic response and died after sleep deprivation. These data represent the first step in identifying the molecular mechanisms that constitute the sleep homeostat.

A sleep-like state has been described in *Drosophila melanogaster* on the basis of its similarities to mammalian sleep[11,12]. This state is characterized by increased arousal thresholds and is regulated homeostatically[11,12]. Like mammalian sleep, it is abundant in young flies, decreases in older animals and is modulated by stimulants and hypnotics[11]. Perhaps the most important similarity between mammals and flies is homeostatic regulation: when flies are kept awake, they show a large compensatory increase in sleep the next day[11,12].

In mammals, the circadian pacemaker alternately promotes and maintains both wakefulness and sleep[13,14]. Although the circadian pacemaker and the sleep homeostat can interact, little is known about the underlying mechanisms. To evaluate this relationship, homeostasis was evaluated in clock mutants maintained in constant darkness (DD) and deprived of sleep for 3, 6, 9 and 12 h (Fig. 1). Under these conditions, sleep is evenly distributed across the day (Fig. 1a). Upon release from sleep deprivation, wild-type *Canton-S* (*Cs*) flies recover ~30–40% of the sleep that they lost within 12 h (ref. 11). *per^{01}* and *Clk^{jrk}* showed a more prominent sleep rebound, reclaiming ~100% of lost sleep within 12 h (Fig. 1a–e). *tim^{01}* flies did not show a homeostatic response after 3–6 h of sleep deprivation[12] but displayed a sleep rebound similar to that of *per^{01}* and *Clk^{jrk}* flies after 7, 9 and 12 h of sleep deprivation ($P > 0.05$; 7-h data not shown).

Surprisingly, *cyc^{01}* mutants showed an exaggerated response to 3 h of sleep deprivation, reclaiming ~3 min of sleep over baseline for each minute of sleep lost (Fig. 1b). Further increasing sleep debt produced a change in the regulation of sleep not seen in other clock mutants (Fig. 1c–e): *cyc^{01}* flies showed large increases in sleep that persisted for as long as the flies were recorded (up to 16 days). These
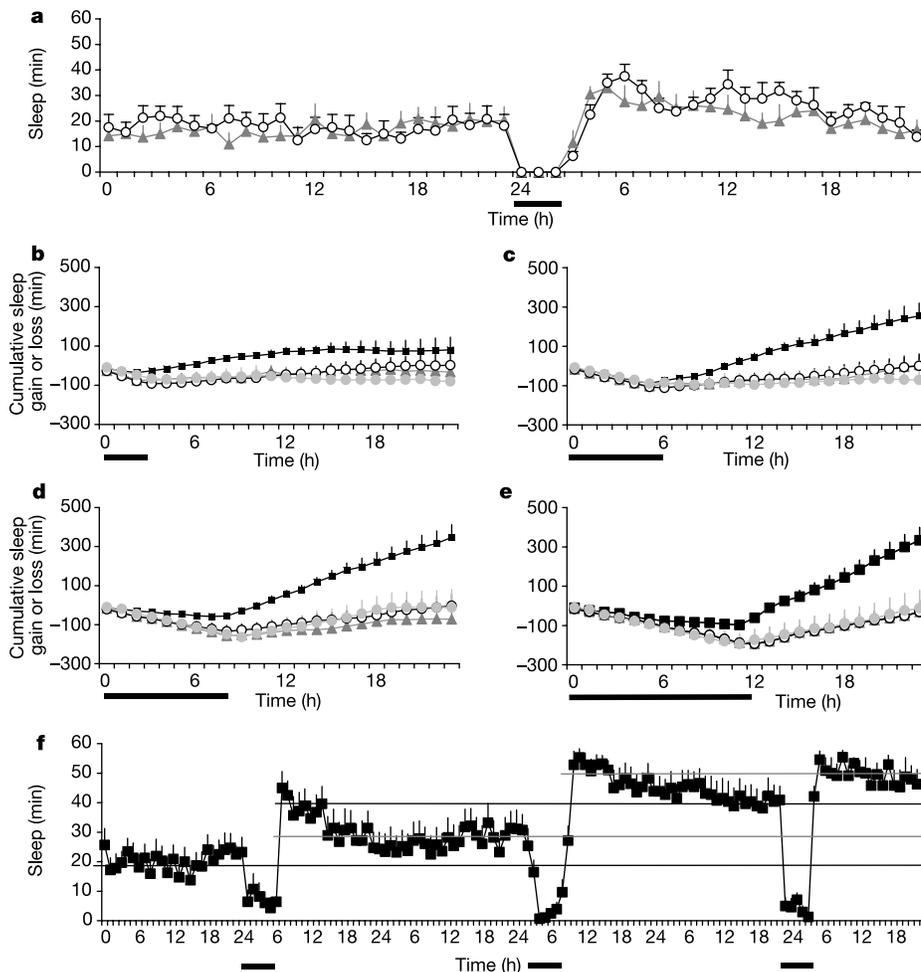
Figure 1 Sleep homeostasis is altered in circadian mutants and is markedly increased in *cyc^{01}* flies. **a**, Illustration of sleep during a typical experiment. Undisturbed *per^{01}* and *Clk^{jrk}* flies sleep for ~20 min each hour under DD. When flies are kept awake for 3 h they show an initial increase in sleep followed by a return to baseline. **b**, Cumulative sleep lost or gained during sleep deprivation and subsequent recovery. A negative slope indicates sleep lost, a positive slope indicates sleep gained; when the slope is zero, recovery is complete. **c–e**, *cyc^{01}* flies show a disproportionate increase in sleep that depends on the length of the deprivation. **f**, *cyc^{01}* flies continually increase baseline levels of sleep after repeated sleep deprivation. Black squares, *cyc^{01}*; grey triangles, *Clk^{jrk}*; open circles, *per^{01}*; grey circles, *tim^{01}*; horizontal lines reflect new setpoint; black bars indicate periods of sleep deprivation.

Table 1 Mortality in clock mutants during 12 h of sleep deprivation

Genotype	No. of trials	n	Mortality (%)	Range (%)
cyc^{01}	16	251	33 ± 4	12–60
Clk^{Jrk}	9	138	0	n.a.
per^{01}	7	106	0	n.a.
tim^{01}	5	87	0	n.a.
Cs	20	540	0	n.a.

periods of quiescence were associated with increased arousal thresholds, indicating that the deprivation had produced an increase in the amount of sleep and did not result in an injured fly. If sleep deprivation produced an increased need for sleep in cyc^{01} flies, they should show higher amounts of sleep when sleep deprived for a second time. Indeed, deprivations of an additional 6 h resulted in further increases in sleep (Fig. 1f).

The extreme sensitivity of cyc^{01} flies was revealed when sleep deprivation extended past 10 h: the flies began to die. This effect was not observed in wild-type flies, in mutant lines representing 45 other genetic loci, or in other clock mutants, indicating that the mutations do not in themselves increase vulnerability to sleep deprivation (Table 1, and Supplementary Information). Note that the most sensitive of the clock mutants (cyc^{01}) is the only one that does not cycle.

To determine whether death in the cyc^{01} flies was due to the stimuli used for sleep deprivation, flies were deprived of sleep for 30 min each hour for 24 h, ensuring that the flies received the same number of stimuli that accrued during 12 h of sleep deprivation but without producing 12 h of continuous wakefulness. No deaths were observed after this protocol, indicating that the deprivation stimulus was not responsible for the deaths (Fig. 2a). Further supporting this conclusion, *stress-sensitive B* ($sesB^1$) flies, which are extremely sensitive to mechanical shock[15], survived 12 h of sleep deprivation and showed activity patterns during the deprivation that were similar to those of wild-type flies ($P = 0.36$, data not shown). We also deprived cyc^{01} flies of sleep by gentle handling as described previously[11]. The proportion of flies that succumbed to sleep deprivation and the size of the homeostatic response in surviving flies were indistinguishable from the automated deprivation method (two trials, $n = 32$, $P = 0.67$). Similar results were obtained

with a rotating deprivation apparatus described previously[8].

To determine whether death in cyc^{01} flies is due specifically to sleep deprivation or to hypersensitivity to any environmental challenge, per^{01}, tim^{01}, Clk^{Jrk}, cyc^{01} and Cs flies were subjected to several stressors including heat stress, oxidative stress, starvation, desiccation and physical stress. cyc^{01} flies were as sensitive, but no more so than other genotypes to desiccation and vortex-mixing (Fig. 2e, f; $P > 0.05$) and survived longer than per^{01}, tim^{01} and Clk^{Jrk} flies when challenged with heat, oxidative stress and starvation (Fig. 2b–d; $P < 0.01$). Cs flies, which have an intact clock, were more resistant to starvation and desiccation than tim^{01}, Clk^{Jrk} and cyc^{01} flies. These data indicate that cyc^{01} mutants are vulnerable to prolonged wakefulness in itself and are not merely hypersensitive to non-related stressors.

To confirm that this phenotype maps to the cyc locus, we crossed cyc^{01} homozygotes with flies carrying the appropriate deficiency $Df(3L)kto2/TM6B$, Tb^1. The resulting cyc^{01}/Df transheterozygote flies showed an exaggerated homeostatic response and deaths after 12 h of sleep deprivation (data not shown). Furthermore, cyc^{01} heterozygotes with and without a functioning clock ($cyc^{01}/+$ and $per^0w;cyc^{01}/+$) also showed exaggerated homeostasis (data not shown). We evaluated cyc^{01} st and $yw;cyc^{01}$ flies to determine whether the background would influence the phenotype; it did not do so (data not shown). Nor was the phenotype changed in aged flies (25 days old; data not shown). Similarly to females, male cyc^{01} flies have been shown to have aberrations in sleep homeostasis (J. Hendricks, personal communication). We found that cyc^{01} males were less sensitive to stress than other clock mutant males, recovered 100% of lost sleep (compared with 300% in cyc^{01} females) and died after 12 h of sleep deprivation, indicating that homeostasis is dissociable from lethality (data not shown). Interestingly, homeostatic regulation of sleep is also sex-dependent in humans[16].

Prolonged sleep deprivation (2–4 weeks) is invariably fatal in normal rats[17]. Is the rapid demise after a few hours of sleep deprivation the result of an anomalous reaction in cyc^{01} mutants, or is it an increased susceptibility to the lethal consequences of sleep loss? Individual Cs flies were kept awake for 70 h by tapping on their tubes when they stopped moving, as described above, to ensure that lethality was not due to excessive handling; 2 of 12 Cs flies died after ~60 h of continuous wakefulness, whereas 2 more died by ~67–
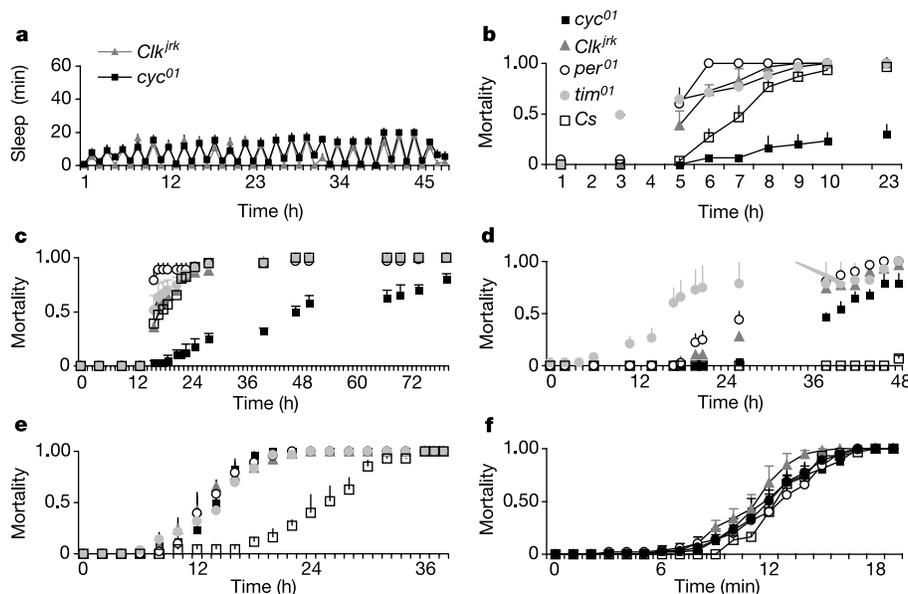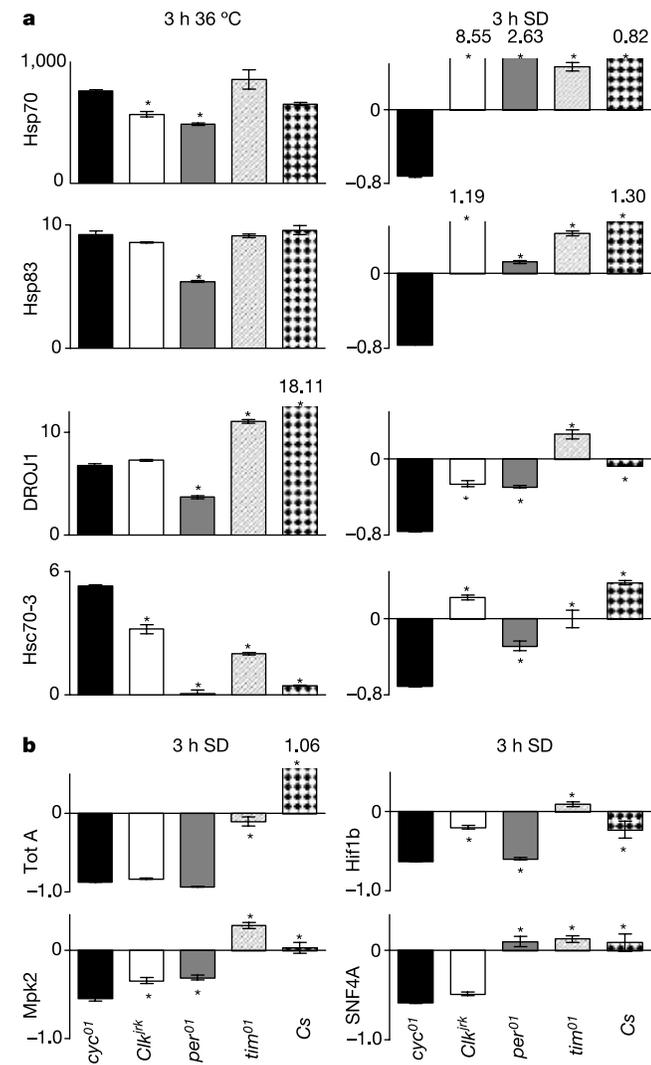

Figure 2 cyc^{01} flies are resistant to stress. **a**, The amount of sleep in cyc^{01} and Clk^{Jrk} flies deprived of sleep for 30 min each hour. **b–f**, Mortality in cyc^{01}, Clk^{Jrk}, per^{01} and tim^{01} flies in response to heat stress (36 °C) (**b**), oxidative stress (20 μM paraquat) (**c**), starvation (**d**), desiccation (**e**) and physical stress (vortex-mixing) (**f**).

70 h. The behaviour of the flies during the last hours of the sleep deprivation protocol resembled that seen in cyc^{01} flies, indicating that the deaths were due to sleep loss and not to the deprivation stimulus itself. To test this, we kept an additional group of Cs flies ($n = 10$) awake by using a different deprivation method and found again that flies began to die between 60 and 70 h. These data indicate that sleep does indeed serve a vital biological role in the fly and that specific mutations that increase susceptibility to death might help to clarify such a role.

Given that cyc^{01} flies are equally well or better equipped than other clock mutants to tolerate chronic heat and other stressors, why do they die in response to sleep deprivation? There is much evidence that stress response genes can protect an organism during challenging conditions[18]. We therefore examined the ability of heat and sleep deprivation to activate stress response genes, by using real-time quantitative polymerase chain reaction (QPCR). All clock mutants responded to 3 h of heat with an induction of genes such as $hsp70$, $Hsp83$, $droj1$ and $hsc70-3$ coding for chaperone proteins (Fig. 3a)[19-21]. After 3 h of sleep deprivation, the levels of these genes were

near baseline in Clk^{jrk} flies (with the exception of $hsp70$) and were unchanged or mildly increased in per^{01}, tim^{01} and Cs flies (see Supplementary Information for gene expression profiles after longer deprivation protocols). Interestingly, levels of chaperone proteins are also elevated after sleep deprivation in rodents[22]. However, sleep deprivation produced a decrease in the expression of these genes in cyc^{01} flies. Genes activated by qualitatively different stressors, including metabolic stress ($SNF4a$, $Hif1$), chemical stress ($mpk2$) and humoral stress ($turandot$), were reduced in all lines[23-26], indicating that sleep deprivation is not inherently stressful (Fig. 3b).

To evaluate the relationship between heat-shock genes and sleep deprivation in cyc^{01} flies, we induced heat-shock genes before sleep deprivation. When 12 h of sleep deprivation was preceded by 3 h of heat exposure at 36 °C, the mortality rate was reduced compared to unheated cyc^{01} flies (Fig. 4a; note that one would have predicted increased mortality because preheating results in a further 3 h of wakefulness). Moreover, heat exposure reduced homeostatic drive in cyc^{01} and Clk^{jrk} flies (Fig. 4b; Clk^{jrk} data not shown). When cyc^{01} flies were pre-exposed to 37 °C, homeostasis was reduced further (Fig. 4b).

We also show the importance of heat-shock genes in sleep deprivation by examining flies mutant for $Hsp83$ ($Hsp83^{08445}$)[10]. After 12 h of sleep deprivation, $Hsp83^{08445}$ mutants exhibited a mortality rate similar to that of cyc^{01} and showed a homeostatic response corresponding to fivefold that of wild-type flies (three trials; $n = 48$; Fig. 4c, d). The sensitivity to sleep deprivation in $Hsp83^{08445}$ mutants is present even in heterozygous flies, which have only a modest reduction in gene product. Heterozygous $Hsp83^{08445}$ flies displayed a sleep rebound that was not statistically different from either homozygous $Hsp83^{08445}$ or heterozygous $Hsp83^{e6A}$ flies ($P > 0.10$; data not shown). However, both $Hsp83$ heterozygotes exhibited a sleep rebound that was significantly different from that of $Hsp60^{RA75}$ heterozygotes[27], indicating that a limited set of chaperone proteins are involved in homeostasis ($P < 0.05$; Fig. 4d). Finally, whereas preheating cyc^{01} flies prevented the lethal effects of sleep deprivation, this did not occur in $Hsp83^{08445}$ flies (Fig. 4c). It should be noted that we have evaluated sleep homeostasis in mutant lines representing 45 other genetic loci; cyc^{01} and
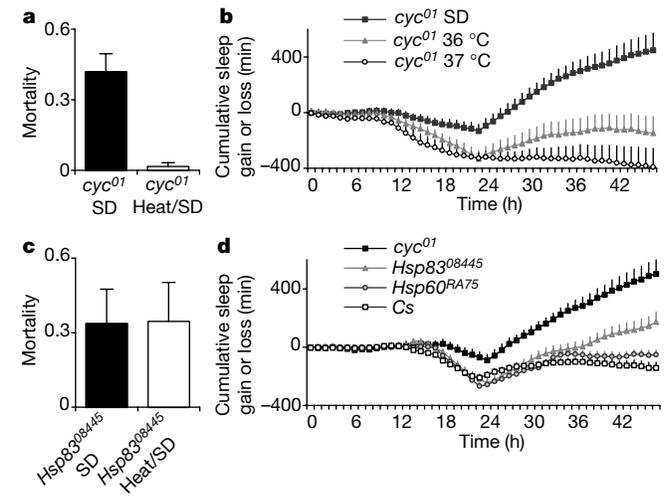

Figure 3 Expression of heat-shock genes is reduced in cyc^{01} flies after 3 h of sleep deprivation. **a**, Change in gene expression after either 3 h of heat exposure (left panels) or 3 h of sleep deprivation (SD) (right panels) shown as percentage deviation from control values. **b**, Expression of stress response genes after 3 h (SD). Values for data extending beyond the axis are shown above respective bars. Asterisks indicate values that are significantly different from cyc^{01} ($P < 0.05$).

Figure 4 Heat protects cyc^{01} flies from the lethal effects of sleep deprivation. **a**, Mortality after 12 h sleep deprivation in cyc^{01} flies and cyc^{01} flies pretreated for 3 h at 36 °C. **b**, Cumulative sleep lost or gained during sleep deprivation and subsequent recovery in untreated cyc^{01} flies and preheated flies. **c**, Mortality after 12 h SD in $Hsp83^{08445}$ flies and $Hsp83^{08445}$ flies pretreated for 3 h at 36 °C. **d**, Cumulative sleep lost or gained during sleep deprivation and subsequent recovery in untreated cyc^{01} and $Hsp83^{08445}$.

$Hsp83^{08445}$ flies are the only mutants that show both an exaggerated homeostatic response and death after sleep deprivation. Although it is unlikely that these are the only two genes involved in the sleep homeostat, it is worth noting that their mammalian homologues have been shown to interact physically[28]. Nevertheless, it is possible that the increased homeostatic response and lethality that we observed in cyc^{01} mutants is due to factors other than $Hsp83$.

Although it is believed that sleep is an essential biological process, its function remains a mystery[29]. So far, death after chronic total sleep deprivation in the rat provides the best evidence in support of a vital role for sleep[30]. Our data reinforce these findings and indicate that the vital role of sleep extends beyond mammals; the data also indicate a connection between vulnerability to sleep loss and increased homeostatic drive. Most importantly, the observation that the induction of certain chaperone proteins protects against the lethal effects of sleep loss provides a first hint about the functional targets of sleep and its molecular mechanisms. □

Methods

Source and maintenance of flies

Flies were cultured at 25 °C, 50–60% humidity, 12 h:12 h light:dark cycle, on yeast, dark corn syrup and agar food as described[8]. per^{01}, $yw;tim^{01}$, $Clk^{jrk}sc$, $cyc^{01};ry$, $yw;cyc^{0}$ and $cyc^{01}st$ flies were obtained from J.C. Hall (Brandeis University), $per^{0}w;cyc^{01}$ flies from P. Hardin (University of Houston), and $Df(3L)kto2/TM6B$, Tb^{1}, $Hsp83^{08445}$, $Hsp83^{e6a}$ (recessive lethal) and $HSP60^{RA75}$ (recessive lethal) flies from the Bloomington *Drosophila* Stock Center. Sleep-activity patterns were monitored with the *Drosophila* Activity Monitoring System (Trikinetics) as described previously[8].

Sleep deprivation

To ensure that flies were awake during the sleep deprivation procedure, we developed a system that coupled the Trikinetics activity monitors with the deprivation apparatus. The Sleep Nullifying Apparatus (SNAP) tilted asymmetrically from −60° to +60° such that the sleeping flies were displaced during the downward movement 10 times per minute. Flies were also deprived of sleep by gentle handling; when an individual fly began sleeping the experimenter would gently tap on the tube. Finally, flies were deprived by rotation as described previously[8].

Procedure

Female flies 3 d old were individually placed into 65-mm glass tubes in the Trikinetics activity monitoring system under DD. Sleep deprivation was conducted after one full day of baseline, either by the SNAP method or by gentle handling. Flies remained in the Trikinetics monitors during baseline, sleep deprivation and recovery. Cumulative difference plots were calculated for each individual fly first by subtracting the minutes of sleep during deprivation and recovery from the corresponding baseline value and summing the difference score with the preceding hour. A negative slope indicates that sleep is being lost; a positive slope indicates sleep gained and a slope of zero indicates that recovery is complete. Sleep rebound was calculated as a ratio of the amount of sleep recovered divided by that lost, that is [(maximum value when the slope was zero − minimum value)/minimum value]. Statistical significance was assessed for sleep rebound by using a one-way analysis of variance (ANOVA) for genotype. The total numbers of replications and the number of flies of each genotype that were sleep deprived with each method are as follows. For 3 h, gentle handling 5 replications, 80 flies; SNAP 8 replications, 128 flies. For 6 h, gentle handling 4 replications, 64 flies; SNAP 6 replications, 96 flies. For 9 h, gentle handling 1 replication, 16 flies; SNAP 4 replications, 64 flies. For 12 h, gentle handling 2 replications, 32 flies; SNAP 14 replications, 224 flies; and for the rotator 1 replication, 26 flies.

Stress tests

All stress tests were conducted on 3-day-old female flies. During each test, three vials containing 10 flies each were evaluated for each genotype. During each reading, the number of dead flies in a vial was expressed as percentage of the total number of flies. The mean ± s.e.m. of the three vials was calculated for each genotype. A representative example from one of four independent replications for each stress test is shown in Fig. 2. Thus a total of 120 flies were evaluated for each genotype for each stress test. Heat tests were performed at 36 °C in flies maintained on 1% agar, 5% sucrose. Oxidative stress was evaluated in flies maintained at 25 °C in vials with 20 mM paraquat dissolved in 1% agar, 5% sucrose. Flies were starved by placing them in vials with a Kimwipe saturated with water. Desiccation was produced in vials without food or water. Physical stress was evaluated by vortex-mixing flies at high speed for 6 min, after which the flies were allowed 1 min to recover and the number of incapacitated flies was counted. Flies were then vortex-mixed for 2 min and given 1 min to recover, during which time incapacitated flies were again counted. This protocol was repeated until all flies were dead.

QPCR

Total RNA was isolated from fly heads with Trizol (Invitrogen, Carlsbad, California) by following the manufacturer's protocol. Reverse transcription reactions were performed in parallel on DNAse-I-digested total RNA as described previously[22]. Reverse transcription

products were stored at −80 °C until use. PCR reactions to measure levels of artificial transcript were done to confirm uniformity of reverse transcription within sample groups and between samples. Comparable reverse transcription reactions within a sample group were pooled. All reverse transcriptions were performed in quadruplicate. A minimum of three QPCR replications were performed for the sleep deprivation experiments and two for the heat experiments. Values were expressed as a percentage of untreated animals and were evaluated by using a one-way ANOVA for genotype. Dunnett post hoc tests were used to identify values that differed significantly from those of the cyc^{01} flies.

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Competing interests statement

The authors declare that they have no competing financial interests.