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BASIC SCIENCE

Age-Related Reduction of Recovery Sleep and Arousal Threshold in *Drosophila*

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Study Objectives: Physiological studies show that aging affects both sleep quality and quantity in humans, and sleep complaints increase with age. Along with knowledge about the negative effects of poor sleep on health, understanding the enigmatic relationship between sleep and aging is important. Because human sleep is similar to *Drosophila* (fruit fly) sleep in many ways, we addressed the effects of aging on sleep in this model organism.

Methods: Baseline sleep was recorded in five different *Drosophila* genotypes raised at either 21°C or 25°C. The amount of sleep recovered was then investigated after a nighttime of sleep deprivation (12 h) and after chronic sleep deprivation (3 h every night for multiple nights). Finally, the effects of aging on arousal, namely, sensitivity to neuronal and mechanical stimuli, were studied.

Results: We show that fly sleep is affected by age in a manner similar to that of humans and other mammals. Not only do older flies of several genotypes have more fragmented sleep and reduced total sleep time compared to young flies, but older flies also fail to recover as much sleep after sleep deprivation. This suggests either lower sleep homeostasis and/or a failure to properly recover sleep. Older flies also show a decreased arousal threshold, i.e., an increased response to neuronal and mechanical wake-promoting stimuli. The reduced threshold may either reflect or cause the reduced recovery sleep of older flies compared to young flies after sleep deprivation.

Conclusions: Further studies are certainly needed, but we suggest that the lower homeostatic sleep drive of older flies causes their decreased arousal threshold.

Keywords: aging, *Drosophila*, sleep deprivation, arousal threshold, arousal

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Significance

Sleep is important for health and wellness. Human sleep changes with age, for example sleep quantity and quality, but there is little understanding of the cause. In this paper we monitored sleep in several strains of the fruit fly *Drosophila melanogaster*, which is a model organism for many aspects of human biology. Compared to young flies, older flies have more fragmented sleep (more awakening), reduced total sleep, a lower arousal threshold (easier to wake up), and fail to recover as much sleep after sleep deprivation. The effects of aging in flies are almost identical to those in humans, making *Drosophila* attractive for future genetic and mechanistic studies on aging and sleep.

INTRODUCTION

Aging is accompanied by alterations in many biological processes, including sleep. Physiological studies have documented profound changes in several aspects of sleep in healthy older individuals compared to young individuals. Similar changes have been reported in animals, and several of them also occur progressively with age.[1–7] Key changes include an increase in sleep fragmentation, e.g., more waking events during sleep, a reduction in total sleep time, and an even larger reduction in slow wave sleep time and electroencephalographic slow-wave sleep activity. There is also a reduction in the response to sleep loss, i.e., recovery sleep or sleep rebound. This ability to make up for lost sleep, typically the night after missing substantial sleep or during the weekend to compensate for lost sleep during the workweek or school week, illustrates the homeostatic aspect of sleep regulation.[3,8–10]

Epidemiological studies are also relevant and have reported an increased frequency of sleep complaints among elderly individuals including insomnia.[11–13] This triggers a higher rate of hypnotic prescriptions in this population, despite the fact that the medications are known to have limited efficacy and increased side effects due to chronic use.[14] Understanding the mechanisms by which the regulation of sleep is affected by age is therefore of major importance, to improve the quality of life in aging societies and to trigger the discovery of innovative therapies.

Drosophila melanogaster (fruit fly) is a popular animal model for the study of aging because of its short lifespan as well as its genetic and molecular similarities with other organisms.[15,16] Many physiological processes are also studied in *Drosophila* because of their conservation with mammals. For example, nearly 75% of the genes that cause disease in humans are reported to have a fly ortholog.[17]

Drosophila has also become a valuable model for the study of sleep. In addition to its facile genetics, flies share with mammals many features of sleep. Flies sustain long periods of rest associated with an increased arousal threshold (a sleep-like state). They also show alternating deep and light sleep stages as well as changes in brain activity during sleep (i.e., a decrease of brain local field potential during long sleep episodes[18]). They respond to stimulants such as caffeine in a manner similar to that of mammals,[19,20] and they also share with mammals several genes regulating sleep and wakefulness.[21–23] Moreover and as previously mentioned, an important parameter is the recovery aspect of sleep, which is believed to reflect homeostatic regulation and occurs similarly in flies and mammals. For example, sleep-deprived flies as well as mammals can recover lost sleep. This occurs in both systems by increasing the intensity of the subsequent sleep episode, and the magnitude of the increase has been correlated with the prior duration of wakefulness. More intense sleep is characterized by a longer sleep episode duration as well as an increased arousal threshold. In the case of mammals, it is also characterized by an increase in electroencephalographic slow-wave activity (delta power) during deep or stage N3 sleep.[18,24–31]

Because the molecular and genetic features that underlie the relationship between sleep and aging are unknown, it might be

worthwhile to further explore this relationship in *Drosophila*. In this study, we show that aging causes increased sleep fragmentation and lower sleep rebound in two different wild-type fly strains, similar to what has been reported in healthy humans. We also show that older flies have a decreased arousal threshold, including an increased response to mild stimulation of two different wake-promoting systems. The results indicate that a decreased arousal threshold is a fundamental aspect of sleep regulation in older animals.

METHODS

Fly Stocks and Rearing Conditions

Canton-S (*Canton S*) and white[1118] (w^{1118}) are standard *Drosophila* laboratory strains.

TH-GAL4 and *Pdf-GAL80* were described in the literature.[32,33] *UAS-dTrpA1* (*UAS-dTrpA1*; second chromosome) was a gift from Dr. Paul Garrity and *DvPdf-GAL4* was provided by Dr. J. H. Park. *TH-GAL4* and *UAS-dTrpA1* were backcrossed with w^{1118} at least 10 times before being used. A stable *DvPdf-GAL4*; *Pdf-GAL80* line was made and called *Ecell-GAL4* (i.e. *Evening Cell-GAL4* flies). TH-GAL4 flies carry the *TH-GAL4* transgene and express the *GAL4* protein in dopaminergic (*TH*) cells. When crossed with flies carrying the *UAS–dTrpA1* transgene, their progeny express *dTrpA1* in *TH* cells. This line was always assayed in parallel with its two control lines (flies carrying either one copy of the *TH-GAL4* transgene or one copy of the *UAS-dTrpA1* transgene). *GAL80* is a protein that neutralizes the *GAL4* protein. When both *DvPdf-GAL4* and *Pdf-GAL80* are expressed together in one fly, the *GAL4* protein is active in the *DvPdf* cells not expressing the *Pdf* peptide. These cells are called the *Evening* cells. Thus, when *DvPdf-GAL4*; *Pdf-GAL80* flies were crossed with *UAS-dTrpA1* flies, their progeny expressed the *dTrpA1* channel only in *Evening* cells. These flies were used along with control flies carrying either one copy of the *DvPdf-GAL4*; *Pdf-GAL80* transgene or one copy of the *UAS-dTrpA1* transgene (also see a review on the GAL4/UAS, GAL80 system[34]).

Flies were reared on standard yeast/cornmeal-based *Drosophila* medium in a 12-h light-dark (LD) incubator. They were collected within 48 h of eclosion, allowed to mate for 2 to 3 days, and either directly used for behavioral assays or placed in vials in single-sex groups of 20 to 25. Flies were fed a standard 10% (wt/vol) sucrose–yeast diet (SY10%) *ad libitum* throughout adult life. Flies were reared and housed at 25°C for non–temperature-sensitive experiments. For all temperature-sensitive *UAS-dTrpA1* manipulations, flies were raised at 21°C.

Sleep Recording

Adult locomotor activity was assayed using the Trikinetics *Drosophila* Activity Monitoring System (Waltham, MA). Flies were loaded individually into 5 × 65 mm glass tubes using CO_2 anesthesia (< 2 min) containing 5% agarose with 2% sucrose, and the tubes placed in the activity monitors. Sleep is defined as a minimum of a consecutive 5 min of inactivity.[19,20] Sleep analysis was performed using MATLAB software (MathWorks, Natick, MA).

Mechanical Sleep Deprivation Assay

This assay was performed in an incubator at 25°C with a 12-h LD cycle. Flies had 2 to 3 days of habituation followed by a baseline day. The subsequent day, flies were mechanically sleep deprived during the 12-h dark period followed by a recovery day. Sleep deprivation was replicated by attaching monitors to a customized vortexer (Analog Multi-Tube Vortexer, speed range: 1,200–2,400 rpm, VWR, Radnor, PA; customization by Trikinetics (Waltham, MA)). The vortexer was placed on an antivibration cushion on the bottom of the incubator, while control flies were placed on an antivibration platform on the top shelf. The vortexer was controlled by Trikinetics software (Waltham, MA) programmed to stimulate for 1.2-sec pulses at random intervals lasting 5–15 sec. Usually, 100% of the young and middle-aged flies survived 12-h sleep deprivation, whereas survival of older flies approached 75%. Due to differential sensitivity to the manipulation, a rotation speed of 4.5 was used for *Canton S* and 5.5 for w^{1118}. Compared to baseline, 75% to 100% sleep loss was observed in *Canton S* and 50% to 100% sleep loss in w^{1118}. Several experiments were performed and pooled together (four to five per genotype/age). In each experiment of this assay, all the different ages of the same genotype were tested simultaneously. However, all ages were not always recorded together in the genetic sleep deprivation assay described later in this section. Thus, the strategy of pooling experiments of a same assay was chosen to avoid having any environmental factor influencing one specific age/assay, and then introducing an unintended external bias into the analysis. In this way, environmental factors have been hopefully neutralized. In addition, experiments were pooled together to maximize statistical power.

Sleep loss was calculated in two steps. First, the amount of sleep of each individual fly during the12-h sleep deprivation period was subtracted from the amount of sleep during the 12-h baseline night of this same fly. The identical procedure was followed for control flies. Mean sleep loss for control flies from the same age was calculated. Finally, mean sleep loss of control flies was subtracted from each sleep-deprived fly. Thus, normalization was to the baseline of each individual and then further normalized to control flies. Sleep gain was calculated in a similar manner (step one: sleep gain during the recovery period compared to baseline; step two: subtraction of the mean of the control flies). Sleep recovered was calculated by taking the ratio sleep gain/sleep loss and expressing it as a percentage.

Genetic Sleep Deprivation Assay

Sleep deprivation was induced by stimulating wake-promoting cells (*TH* and *E cells*) with the temperature-sensitive *dTrpA1* channel. This assay was performed in an incubator at 21°C (off-temperature) with a 12-h LD cycle. The design was similar to the mechanical sleep deprivation assay, except that sleep deprivation was induced by increasing the temperature from 21°C to 28°C–29°C (on-temperature) during the 12-h dark period.

Several experiments were performed. Each time, the experimental line (*TH-GAL4/+ > UAS-dTrpA1/+* (*TH*) or *Ecell-GAL4/+ > UAS-dTrpA1/+*(*E cell*)) and its controls (*UAS-dTrpA1/+* (*UAS*) and *TH-GAL4/+* (*GAL4*) or *Ecell-GAL4/+*) from either one, two, or three ages were recorded together.

Each genotype and age was replicated once and both replicates were pooled together. Sleep analysis was performed as for the mechanical sleep deprivation assay. In this case, however, all three lines were compared first to their own references (individual baseline, recovery period) and then to either the *UAS* control line or to *GAL4* control line.

Chronic Sleep Deprivation Assay

Young and middle-aged *TH flies* and their controls (*UAS* and *GAL4*) were raised at 21°C and loaded into individual tubes to record their sleep in a 12h/12h LD conditions as previously described. After 3 days of habituation, a baseline day was recorded (day 1) followed by 6 days (day 2 to 7) when *TH* flies were sleep deprived during the first 3 h of the dark period. Sleep deprivation was induced by an increase of the temperature from 21°C to 28°C (wake-promoting *TH* cells stimulation by activating the temperature-sensitive channel *dTrpA1*). At the end of the 3-h sleep deprivation period, the temperature was reduced to 21°C. Sleep gain was calculated by comparing the amount of total sleep during the 21-h recovery period compared to the baseline 21 h. Then, mean sleep gain from either *UAS* or *GAL4* control lines was subtracted from the experimental line (*TH*).

Arousal Assay

Middle-aged and young *CS* flies were placed in individual glass tubes on the vortexer, as previously described, in the mechanical sleep deprivation assay. After 2 habituation days and 1 baseline day, flies were shaken for 0.6 sec once every hour during 48 h, and their activity recorded to identify whether or not this stimulus was able to wake them up within the 2 min following the pulse (arousal threshold assessment). Activity analysis was performed 5 min before each pulse. Inactive flies during that time were considered asleep. Those flies were then analyzed during the 2 min following the pulse to assess the arousal effect of the mechanical stimulus. The number of aroused flies was summed from time points where at least 20% of the flies were asleep during the 5 min before the pulse. The percentage of flies asleep during the 5 min prior to the pulse was also reported.

For the subtle mechanical stimulation experiment, individual flies were loaded into 96-well plates and placed close to a small push-pull solenoid. The tap number of the solenoid was directly driven by an Arduino UNO board (Smart Projects, Italy). We used one tap every 10 min from ZT12 to ZT24 as a modest stimulus during the night. Fly behavior was recorded by a Logistic C910 web camera (Logitech. Newark, CA) without an infrared filter. We used time-lapse software to capture snapshots at 10-sec intervals. Fly sleep was calculated by Pysolo software (www.pysolo.net[35]) and transformed into a MATLAB readable file. The activity and sleep analyses were performed with a signal-processing toolbox implemented in MATLAB (MathWorks, Natick, MA) as described previously.

Excitability Assay

This assay is identical to the genetic deprivation assay, except that, during the 12-h dark period stimulation, the temperature was slightly increased to 23°C for *TH* line and their controls, and to 25°C for *Ecell* line and theirs controls. Again, each genotype and age was replicated once and data from both replicates were pooled together. Sleep loss was calculated by subtracting the baseline dark period data from the 12-h stimulation data for the experimental line and its two control lines (experimental Δ and control Δ; hourly values). Then, mean sleep loss obtained for each control (i.e., control Δ for *UAS* or for *GAL4*) was subtracted from the sleep loss of the experimental line (i.e., experimental Δ). This was done in a manner similar to the genetic sleep deprivation assay and chronic sleep deprivation assays previously described. Finally, cumulative differences were calculated.

At the end of each experiment, a temperature check was performed by increasing the temperature to 28°C–30°C for 16 to 24 h to verify that the flies were well sleep deprived as expected at any age. (See previous discussion on genetic sleep deprivation assay.)

Statistical Analysis

Statistical analysis was performed with SigmaStat (v11, Systat Software, Inc., San Jose, CA). Comparisons between age, conditions, and/or genotypes were done by either two-way analysis of variance or one-way analysis of variance followed by *post hoc* analysis (Student *t* test for two groups and Tukey test for three groups or more). For non-parametric data, Kruskal-Wallis analyses followed by Dunn test (multiple comparisons) were performed. Significance was set at $P < 0.05$ for all statistical tests.

RESULTS

Sleep Duration and Sleep Consolidation Decrease with Age

To investigate the effect of age on sleep variables, two different genotypes were first assayed during 24-h baseline conditions at 25°C. w^{1118} and *Canton S* flies were compared at young, middle, and old age. Small groups of flies were reared and aged together to allow social interactions. These interactions are known to affect behavior, including sleep behavior.[36] A longitudinal study may yield more accurate results because baseline data and long-term effects can be determined for each individual fly. However, this type of experiment is cumbersome for *Drosophila*. Moreover, it has been shown that longitudinal sleep studies mirror data obtained in cross-sectional studies.[6] We therefore chose to do a cross-sectional rather than a longitudinal study.

Both strains showed a significant decrease of sleep duration (total sleep) and an increase of sleep fragmentation as a function of age (Figure 1A–1D, 1F–1I). During the dark period, the dramatic sleep fragmentation phenotype was illustrated by a striking increase in mean sleep episode number and a decrease in mean sleep episode duration (Figure 1C, 1D, 1H, 1I). Mean sleep episode duration was also decreased during the light period. However, the number of sleep episodes varied among genotypes and ages during the light period, i.e., decreased with age in w^{1118} but increased or was not clearly monotonically decreasing in *Canton S*. Another genotype-dependent trait was sleep latency. It was clearly increasing with age in w^{1118} flies in both light and dark periods, while significantly decreasing

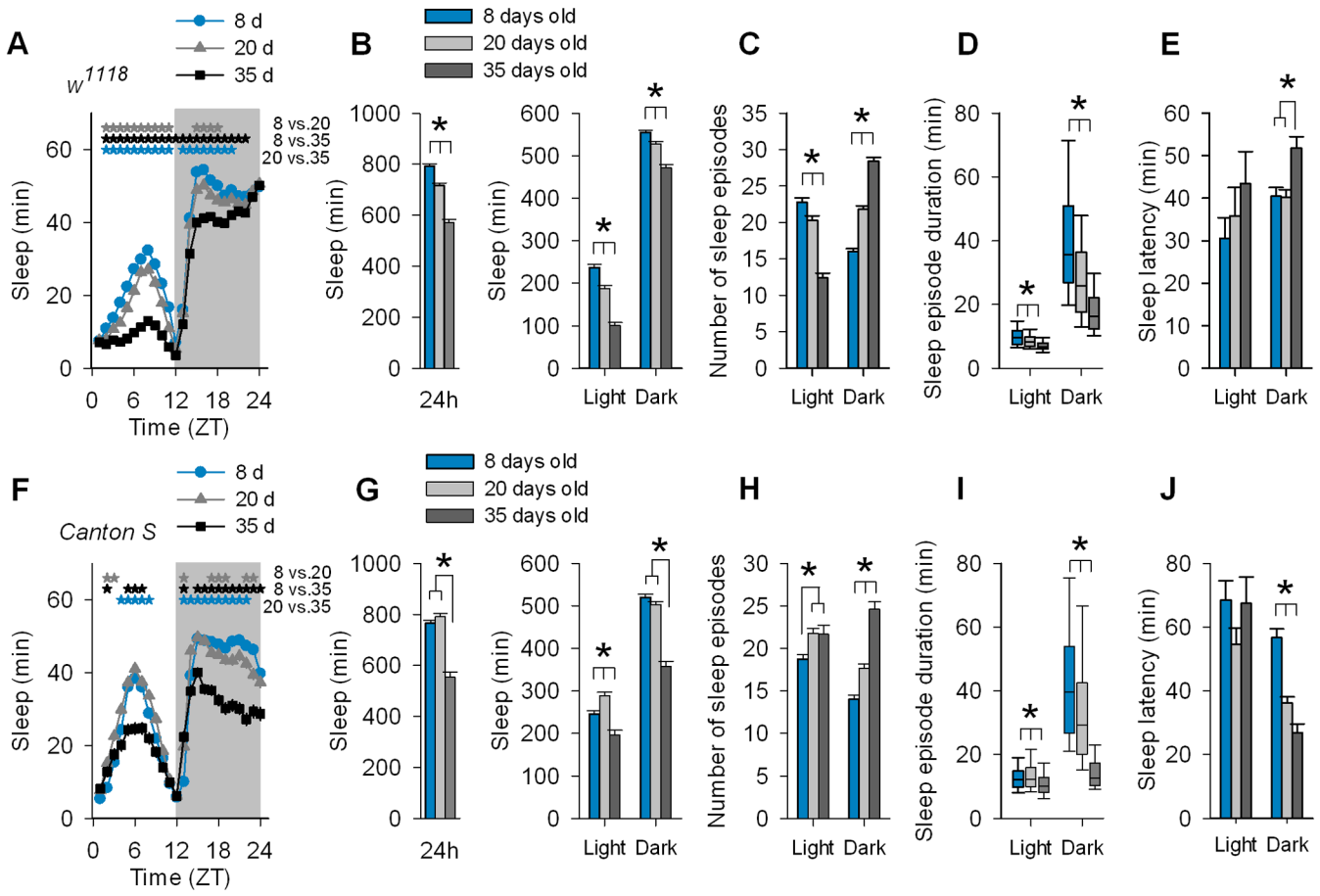

Figure 1—Graphical representation of how aging decreases sleep and increases sleep fragmentation under baseline conditions. **A**,**F**. Time courses of sleep (hourly values; mean ± standard error of the mean) during 24-h baseline in w^{1118} and *Canton S* flies at three different ages. (Two-way analysis of variance [ANOVA] factors Age [P < 0.0001], Time [P < 0.0001] and their interaction [P < 0.0001]; significant difference between ages: Tukey test [P < 0.05; stars]; gray areas illustrate 12-h dark periods). **B**,**G**. Total sleep duration decreased with aging in w^{1118} and *Canton S* flies in both light and dark periods. **C**,**D**,**H**,**I**. With aging during the 12-h dark period (major sleep period), the mean sleep episode duration shortened and number of sleep episodes increased, i.e., aging correlated with more fragmented sleep in both w^{1118} and *Canton S* flies. During the light period, the duration of mean sleep episodes still decreased as flies aged. However, the total number of sleep episodes varied with age and genotype. (One-way ANOVA factor Age [P < 0.003]; Tukey test [P < 0.05; star with connected lines]; for the mean sleep episode duration: Kruskal-Wallis one-way ANOVA on ranks, factor Age [P < 0.0001]; Dunn test [P < 0.05; star with connected lines]). **E**,**J**. Sleep latency was increased during the dark period in w^{1118} but decreased in *Canton S* flies (for sleep latency-light period: one-way ANOVA factor Age [P ≥ 0.22]; for sleep latency-dark period: one-way ANOVA factor Age [P = 0.0001]; Tukey test [P < 0.05; star with connected lines]). n = 166–245/age (w^{1118}), n = 145–186/age (*Canton S*).

during the dark period in *Canton S* flies (Figure 1E, 1J). In summary, total sleep decreased and nighttime sleep fragmentation increased as a function of age.

Importantly, similar if not identical sleep phenotypes were observed in different strains raised at 21°C. This temperature was used to prevent activation of the dTrpA1 gene during development and adulthood. dTrpA1 is a temperature-sensitive cation channel, which is genetically expressed in a chosen subset of neurons and fires (activates) when the flies are placed in a warm environment (28°C–30°C) but is silent when flies are maintained at the lower temperature (21°C). This genetic tool allows remote activation of neurons without developmental effects. The strains used in this study were *TH-GAL4/+;UAS-dTrpA1/+* (*TH*) and their controls, *UAS-dTrpA1/+* (*UAS*) and *TH-GAL4/+* (*GAL4*), in a w^{1118} background (Figure S1A–S1I in the supplemental material). At 21°C, all of these strains behaved in a manner similar to w^{1118} wild-type flies, i.e., there was no evidence of dTrpA1 activation. The *TH-GAL4* cells express tyrosine hydroxylase (TH), and these dopaminergic neurons have strong wake-promoting activity when dTrpA1 is expressed in these cells and activated.[37,38]

When these strains are raised at 21°C, they age more slowly than wild-type flies at 25°C, i.e., their lifespan was increased by about twofold as seen on their survivorship curves (Figure S2 in the supplemental material). Age-related sleep deterioration also appears later, reflecting physiological age rather than chronological age (Figure S1 in the supplemental material) as previously described.[6] We therefore assayed sleep in 8, 40, and 70-day-old at 21°C flies (*TH* and their *GAL4* and *UAS* controls; Figure S1A–S1I in the supplemental material). The results were quite similar to those from 8, 20, and 35- day-old flies maintained at 25°C (w^{1118} and *Canton S* flies; Figure 1).

Recovery Sleep after Sleep Deprivation Decreases with Age

To assess the effect of age on sleep homeostasis (recovery sleep), flies were sleep deprived during the 12-h dark period; sleep was then analyzed during the following 24 h. Importantly, two different sleep deprivation techniques were used and compared: standard mechanical sleep deprivation (shaking) and genetic sleep deprivation. The latter technique involved activating the temperature-sensitive dTrpA1 channel in wake-promoting dopaminergic cells as described previously.[37,38] The temperature was increased from 21°C to 28°C at lights off (Zeitgeber time [ZT]12) and then maintained at 28°C for the 12 h of sleep deprivation. This occurred during what would be normal nighttime sleep. The temperature was then returned to 21°C at lights on (ZT0), which defines the end of the sleep deprivation period and the beginning of the 24-h recovery period. Average sleep loss during the 12 h of deprivation was more than 80% compared to the baseline dark period. This was true with both deprivation methods (Figures 2 and 3).

In the *TH* and *Canton S* flies, sleep loss (in minutes) caused by sleep deprivation was lower in older flies compared to younger flies. This might be because older flies sleep less than younger flies during the baseline dark period (ceiling effect; Figure 1A, 1B, 1F, 1G, 2N, 3G, 3K and Figure S1A–S1E in the supplemental material). Older flies therefore cannot lose as much total sleep as younger flies. Indeed, when sleep loss is expressed as a percentage of sleep duration during the baseline dark period, older flies lose as much or even more sleep than younger flies with both methods (Figure S3 in the supplemental material).

Sleep gain during the following 24-h recovery period was decreased in older w^{1118} and *Canton S* flies compared to both control flies and baseline conditions (Figure 2A–2E, 2H–2L). Importantly, sleep recovered or sleep rebound, which is the sleep gain expressed as a percentage of sleep loss, was also decreased with age (Figure 2F, 2G, 2M, 2N). Identical recovery sleep phenotypes were observed in *TH* flies compared to their controls (*GAL4* or *UAS*; Figure 3A–3F, 3H–3J), indicating that a decrease in sleep rebound with age is probably general. Interestingly, the same conclusion applies to genotype-dependent differences in recovery sleep, i.e., *Canton S* flies recovered overall less sleep than w^{1118} (Figure 2F, 2M).

To investigate further sleep homeostasis and aging, we decided to mimic chronic, modest sleep loss in humans. Therefore, we deprived flies of 3 h of nighttime sleep every day for 6 consecutive days. Young and middle-aged *TH* flies as well as their *UAS* and *GAL4* controls were shifted from 21°C to 28°C at the beginning of every dark period and then shifted back to 21°C after 3 h (Figure 4A–4C). The flies were then maintained at 21°C for 21 h, to allow for possible sleep recovery, after which they were shifted again to 28°C for 3 h.

The amount of sleep lost each day was somewhat different as a function of age during the 6 days of 3 h of deprivation in the *TH* strain compared to *GAL4* control flies (Figure 4D). Indeed, sleep loss progressively increased in young flies, whereas it decreased in middle-aged flies (e.g., day 2 and day 6 were significantly different from each other in both age groups [Tukey test: $P < 0.001$]). This difference was not seen when *TH* sleep loss was normalized to its *UAS* control due to a slightly different behavior of this strain compared to the *GAL4* strain during the assay (Figure 4A, 4B, and 4G). Clear conclusions on sleep loss, therefore, could not be drawn.

Consistent with the aforementioned 12-h sleep deprivation results, young *TH* flies recovered progressively more sleep during their subsequent 21-h recovery periods, whereas the middle-aged flies did not recover any lost sleep; on the contrary, they tended to lose even more sleep (Figure 4E, 4F, 4H, and 4I). The increased recovery sleep in young flies was particularly obvious during the light period, i.e., the active period (Figure 4A). This suggests that nighttime and daytime sleep are at least somewhat interchangeable, arguing against completely separate regulation of these two sleep events in flies.[39] We also speculate that the increased reliance on daytime sleep recovery reflects the lack of sufficient recovery sleep capacity in the remaining 9 h of the night. This could be because sleep is already nearly maximal during that time and/or because the first 3 h of the night is especially important for sleep.

Arousal Threshold and Neuronal Sensitivity to Stimuli Increase with Age

To assess arousal threshold as a function of age during sleep, young and middle-aged *Canton S* flies were exposed to a mild mechanical stimulus every hour for 48 h. Only time points when more than 20% of the flies were asleep before the stimulus were included in the analysis.

As expected from results under baseline conditions, the number of flies asleep before the stimulus was higher in young flies compared to middle-aged flies (Figure 5A and Figure 1A, 1B, 1F, 1G). Nonetheless, a higher percentage of middle-aged flies were awakened by the stimulus compared to young flies (Figure 5B). This indicates that the arousal threshold decreases with age, suggesting in turn that this change in arousal threshold may be related to the decreased sleep rebound with age.

We then considered that the decrease in arousal threshold might be paralleled by age-related changes in the response to modest activation of arousal (wake-promoting) neurons. To address this possibility, the genetic sleep deprivation protocol was modified so that mild dTrpA1 stimulation could be compared between young, middle-aged, and older flies. The wake-promoting strains and their controls were exposed to a small increase of temperature, from 21°C to 23°C or to 25°C rather than the usual protocol of increasing the temperature to 28°C–30°C to cause full-blown dTrpA1 activation and severe sleep deprivation as described previously. The lower temperatures are below those normally used to activate the dTrpA1 channel. Two different groups of arousal neurons were assayed: the *TH* cells previously described and the evening (*E*) cell subset of the circadian system (*Ecell-GAL4/+;UAS-dTrpA1/+* and their controls: *Ecell-GAL4/+* (*GAL4*) and *UAS-dTrpA1/+* (*UAS*), see also Methods). Suppressing neuronal activity of these *E* cells strongly decreased locomotor activity.[40] Because *E* cell activity had not been assayed for its effect on sleep, we stimulated *E* cells with dTrpA1 at 28°C–30°C. Sleep was inhibited, similar to the 28°C to 30°C dTrpA1 stimulation of dopaminergic neurons. Comparable sleep rebound also followed the activation of these two sets of wake-promoting neurons (Figure S4 in the supplemental material and Figure 3).

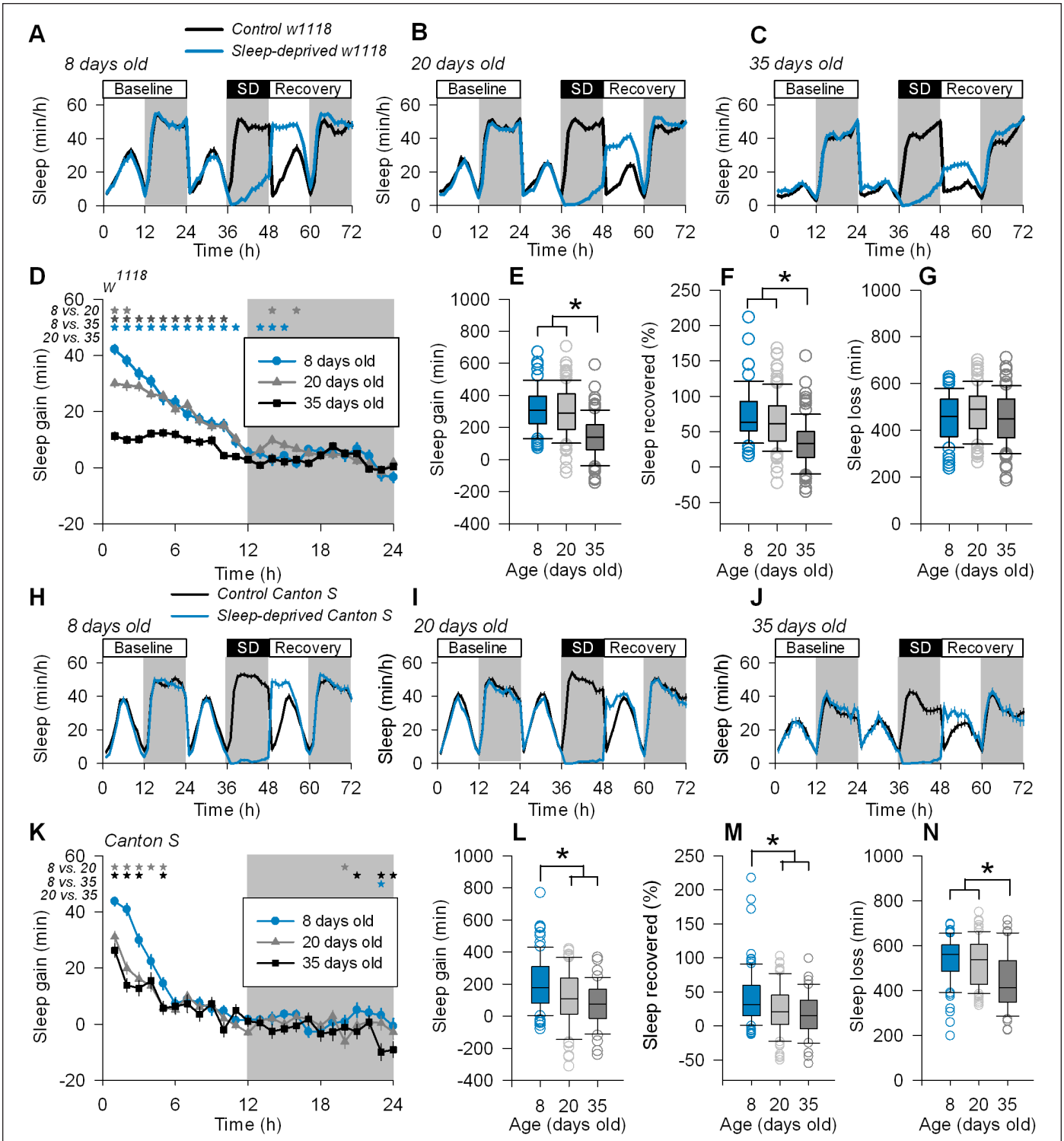

Figure 2—Graphical representation of how recovery sleep decreases with age after 12 h of mechanical sleep deprivation. **A**–**C**, **H**–**J**. Sleep time course (hourly values; mean ± standard error of the mean) of w^{1118} and *Canton S* flies at three different ages (8, 20, and 35 days old). The 24-h baseline day was followed by a 12-h sleep deprivation (SD) during the subsequent dark period (gray area) and then a 24-h recovery period. **D**,**K**. Hourly time course of sleep gain during the 24-h recovery period after 12 h of mechanical sleep deprivation in *Canton S* and w^{1118} flies was significantly decreased with age. (Two-way analysis of variance [ANOVA], factors Age [P < 0.0001], Hour [P < 0.0001] and interaction [P < 0.0001]; Tukey test [P < 0.05; stars]). Sleep gain represents the amount of sleep gained during the recovery period compared to both baseline values and normalized by control flies (see Methods). **E**,**F**,**L**,**M**. Sleep gain and sleep recovered during the 24-h recovery period were significantly decreased with age (Kruskal-Wallis one-way ANOVA on ranks, factor Age [P < 0.001]; Dunn test [P < 0.01]; star with connected lines). Sleep recovered displays sleep gain as a percentage of sleep loss (sleep gain/sleep loss*100). **G**,**N**. Sleep loss (min) was not significantly different in w^{1118} but decreased in older *Canton S* compared to middle-aged and young flies (Kruskal-Wallis one-way ANOVA on ranks, factor Age in w^{1118} [P = 0.07] and in *Canton S* [P < 0.001]; Dunn test [P < 0.05; star with connected lines]). n = 69–108 (w^{1118}); n = 44–82 (*Canton S*).

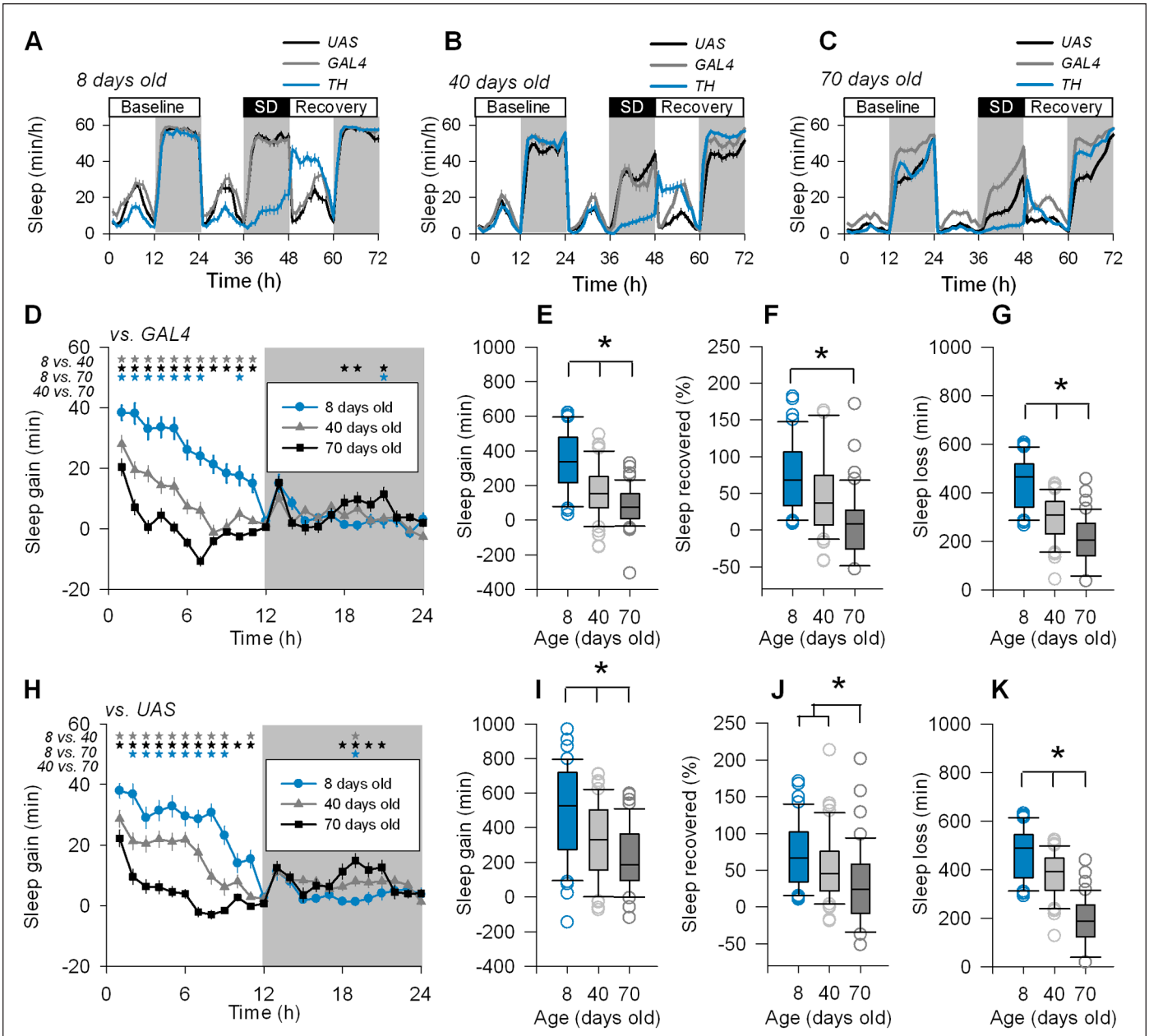

Figure 3—Graphical representation of how recovery sleep decreases with age after 12 h of genetic sleep deprivation. **A–C.** Sleep time course (hourly values; mean ± standard error of the mean) of *TH* flies and control flies (*GAL4* and *UAS*) at three different ages (8, 40, and 70 days old). The 24-h baseline day was followed by 12 h of sleep deprivation (SD) during the subsequent dark period (gray area) and a 24-h recovery period. **D**,**H**. Hourly time course of sleep gain during the 24-h recovery period after 12 h sleep deprivation (28°C; *TH* cell stimulation) in *TH* flies was significantly decreased with age compared to control flies (either *GAL4* **(D)** or *UAS* **(H)**. For analysis details, see Methods. For both **D** and **H**: Two-way analysis of variance [ANOVA], factor Age [P < 0.0001], Hour [P < 0.0001] and interaction [P < 0.0001]; Tukey test [P < 0.05; stars], n = 41–53. **E**,**F**,**I**,**J**. Sleep gain and sleep recovered during 24-h recovery period were significantly decreased with age (Kruskal-Wallis one-way ANOVA on ranks, factor Age [P < 0.0001]; Dunn test: [P < 0.05; stars]). Sleep recovered displays sleep gain as a percentage of sleep loss (sleep gain/sleep loss *100). **G**,**K**. Sleep loss (min) was decreased with age (Kruskal-Wallis one-way ANOVA on ranks; factor Age [P < 0.0001]; Dunn test: [P < 0.001; stars]); n = 41–52.

Importantly, activation of dTrpA1 at these subthreshold temperatures in both *TH* and *E* cells sleep-deprived older flies, whereas young flies were either not affected or barely affected. The sleep deprivation effect is best seen by the cumulative difference between the wake-promoting lines (*TH* and *Ecell*) and their respective *GAL4* and *UAS* control flies (Figure 5C–5F; for more experimental details, see Methods). It should be noted that when the temperature was increased to a high temperature (28°C to 30°C; strong stimulation) 12 to 24 h after the end of the assay, total sleep of both young and older flies was strongly suppressed as in Figure 3A–3C, 3G, 3K, and Figure S4A and S4B in the supplemental material (see also and Methods).

To support this conclusion, flies were subjected to a mild but persistent mechanical stimulation throughout the 12 h of night. The young flies were insensitive to the stimulation (1 tap/10 min) as they had comparable sleep levels to the baseline

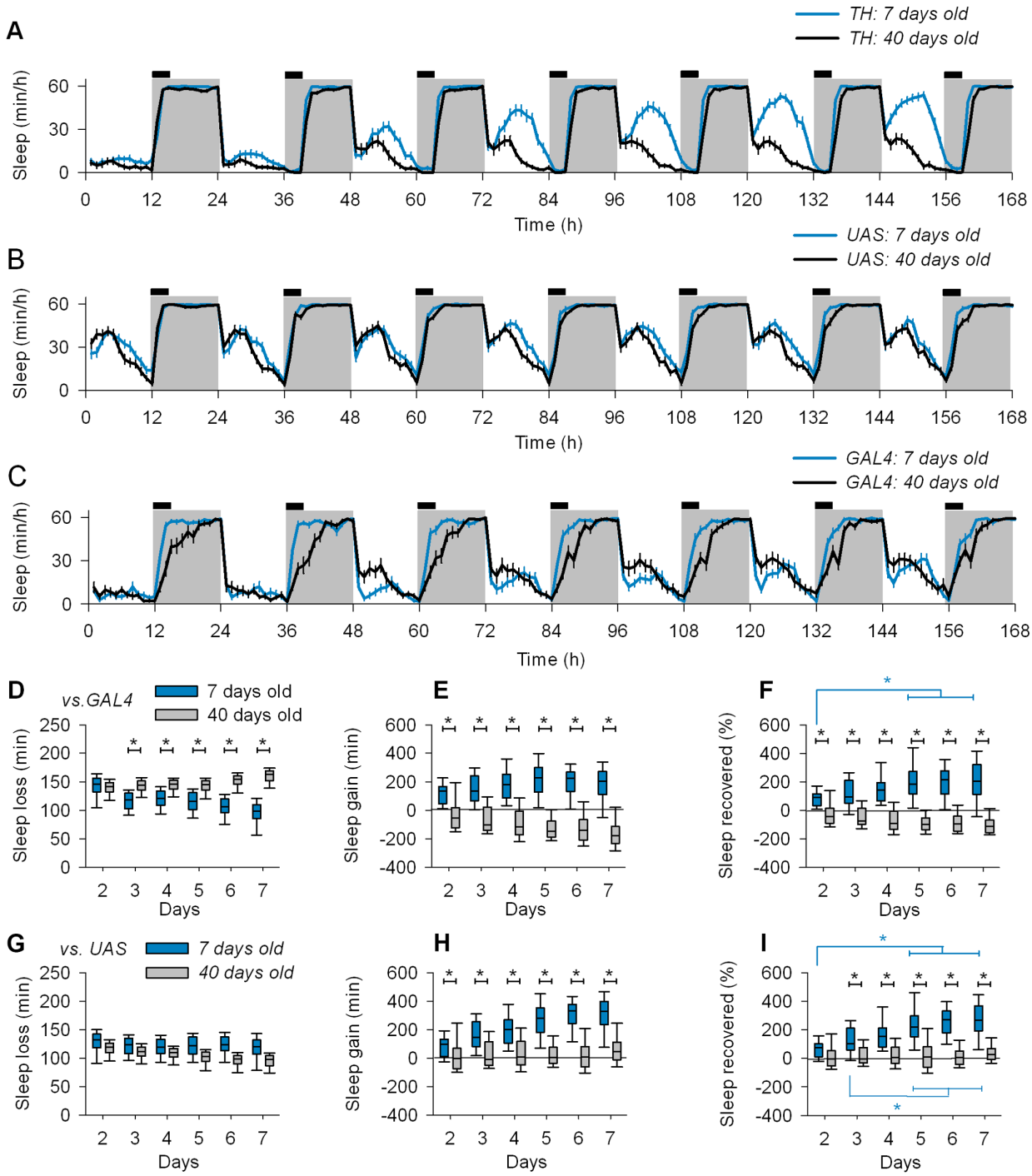

Figure 4—Graphical representation showing how young flies recover progressively more sleep after a daily 3-h sleep deprivation period compared to middle-aged flies (chronic sleep deprivation). After a baseline day (day 1), young (7 day old) and middle-aged (40 day old) *TH* flies were sleep-deprived during the first 3 h (black rectangles; 28°C) of the dark period (gray area) during 6 consecutive days. Between each sleep deprivation period, flies had 21 h to recover lost sleep (21°C). *UAS* and *GAL4* flies were used as controls. **A**,**B**,**C**. As illustrated by the sleep time course (hourly values; mean ± standard error of the mean) of young (blue line) and middle-aged (black line) *TH*, *UAS*, and *GAL4* flies, young flies recovered progressively more sleep after the daily 3 h of sleep deprivation (*TH*, **A**) than middle-aged flies. **D**,**G**. The amount of sleep loss during the 3 h of sleep deprivation varied among age and days. (vs. *GAL4*: two-way analysis of variance [ANOVA], factors Age [$P < 0.0001$], Day [$P = 0.001$], and interactions [$P < 0.0001$]; vs. *UAS*: two-way ANOVA, factors Age [$P < 0.0001$], Day [$P = 0.001$], and interactions [$P < 0.0001$]; Tukey test for factor Age [$P < 0.0001$; black stars]). **E**,**F**,**H**,**I**. During the 21-h recovery periods following each 3-h sleep deprivation, young *TH* flies gained/recovered more sleep than middle-aged *TH* flies (Sleep gain/recovered vs. *GAL4* flies [**E**,**F**]: two-way ANOVA, factors Age [$P < 0.0001$], Day [$P \geq 0.4$] and interactions [$P \leq 0.0003$]; Sleep gain/recovered vs. *UAS* flies [**H**,**I**]: two-way ANOVA, factors Age [$P < 0.0001$], Day [$P < 0.0001$] and interactions [$P \leq 0.0001$]; Tukey test for Age [$P \leq 0.03$; black stars]). In addition, only young flies showed a progressive increase of sleep recovered with each additional 3-h sleep deprivations (**H**,**I**: Tukey test [$P < 0.05$; star with connected blue lines]). Sleep recovered displays sleep gain as a percentage of sleep loss (sleep gain/sleep loss *100; see also Methods for analysis details; n = 16–31 flies).

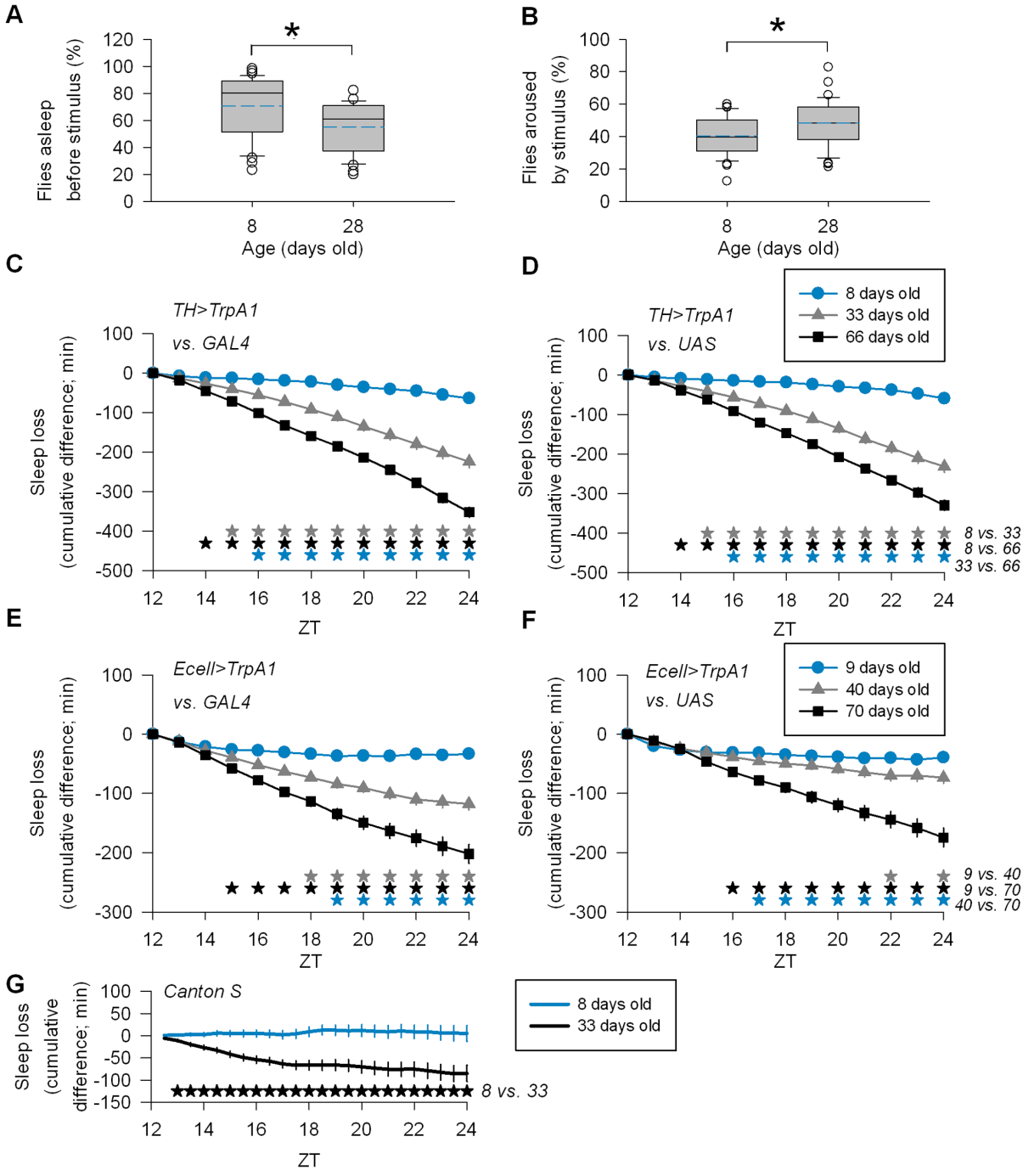

Figure 5—Graphical representation showing how older flies are more affected by wake-promoting stimuli than younger flies. Mild mechanical stimuli were applied to young and middle-aged *Canton S* flies. **A**,**B**. The percentage of flies asleep before each stimulus was higher in young flies compared to middle-aged flies, and the percentage of flies awakened by each stimulus was lower in young flies compared to middle-aged flies (one-way analysis of variance [ANOVA], factor Age [P ≤ 0.0145]; Student *t*-test [P < 0.0146; star with connected lines], n = 38 (young), n = 34 (middle-aged); blue dashed line in boxplots = mean, black solid line = median). During the 12-h dark period, a mild increase of temperature was applied to young, middle-aged, and old *TH* flies, *Ecell* flies, and their respective *UAS* and *GAL4* controls. Older flies were more sensitive to this mild wake-promoting stimulus than younger flies. **C**,**D**. Cumulative difference (mean ± standard error of the mean) between the baseline dark period (21°C) and the experimental dark period (23°C) of *TH* flies normalized by either *UAS* **(C)** or *GAL4* **(D)** control flies. There was an age-dependent sensitivity to wake-promoting stimuli (two-way ANOVA, factors Age [P < 0.0001], Hour [P < 0.0001], and interaction [P < 0.0001]; Tukey test [P < 0.05; stars], n = 51–55 [*TH*], n = 57–64 [*GAL4*], n = 53–62 [*UAS*]). **E**,**F**. Similar age-dependent sensitivity to wake-promoting stimuli was observed in *Ecell* flies (two-way ANOVA, factors Age [P < 0.0001], Hour [P < 0.0001] and interaction [P < 0.0001]; Tukey test [P < 0.05; stars]; n = 51–55 (*Ecell*), n = 57–64 (*GAL4*), n = 53–62 (*UAS*)). **G**. Cumulative difference between the baseline dark period and the experimental dark period of young and middle-aged *Canton S* flies during a 12-h subtle mechanical stimulation experiment (1 tap/10 min as described in Methods; mean ± standard error of the mean). Young fly sleep was not disrupted, whereas middle-aged flies showed a significant sleep reduction (two-way ANOVA, factors Age [P < 0.0001], Time [P < 0.0001], and interaction [P < 0.0001]; Tukey test [P < 0.05; black stars]; n = 46 (young), n = 40 (middle-aged)).

day. However, sleep in middle-aged flies was significantly disrupted as they experienced 85 min of sleep loss during the night of mechanical stimulation (Figure 5G). The results taken together indicate that age decreases arousal threshold, i.e., it increases the sensitivity to wake-promoting stimuli, which may be related to the relative failure of older flies to achieve robust sleep rebound.

DISCUSSION

We show here that middle-aged and old flies manifest increased sleep fragmentation and a lower total sleep time compared to young flies. Moreover, aging alters sleep homeostasis, by reducing recovery sleep after sleep deprivation (lower sleep rebound). Finally, there is a decreased arousal threshold and an increased response to neuronal stimulation of several wake-promoting systems in older flies compared to younger flies. This last result suggests increased arousal tone, which might contribute to the lower sleep rebound and the decreased sleep maintenance (increased sleep fragmentation with age). Alternatively, the increased arousal tone might result from the reduced homeostatic sleep drive (sleep need). As most published human data tend to favor the interpretation that homeostatic sleep need declines with age,[10,41] we favor the second possibility.

Similar to previous human and animal studies,[6,19,31,42,43] flies from different genetic backgrounds show increased sleep fragmentation with age. This is particularly striking in the major sleep period (dark period or nighttime), during which the number of sleep episodes increased and the mean sleep episode duration decreased for all five genotypes tested. Total sleep duration was also decreased in older flies. This was often but not always monotonic; i.e., middle-aged flies slept an intermediate amount, indicating that the decrease was not just due to the infirmities of old age. (Exception: the *Canton S* background in which total sleep in old flies but not middle-aged flies was distinguishable from that of young flies.) These results support several previous human and fly studies.[8,9,31,42] We note, however, that the opposite has also been reported in mammals and flies, namely that total sleep duration was either not affected or actually increased with age.[3,6,44,45]

Interestingly, rearing flies at a lower temperature (21°C) not only increased lifespan but also delayed the sleep deterioration, suggesting that these sleep/wake changes reflect a changing physiology rather than a strictly chronological influence.[6] Although the mechanisms that underlie the relationship between age and sleep are still not understood, recent studies in rodents as well as *Drosophila* suggest a role for stress and the unfolded protein response: modulation of the unfolded protein response leads to either an increase or a decrease of unfolded proteins, which then affects age-dependent sleep changes as well as lifespan.[5,7]

Sleep of older flies was more easily disrupted by a mild mechanical stimulus using two different mechanical assays. Moreover, the response to mild stimulation of two neuronal wake-promoting systems (dopaminergic cells and E cells) increased with age. Although both cell types in older flies could be more sensitive to the weak stimulation of the dTrpA1 channel, a more parsimonious interpretation is that the arousal threshold decreases with age in *Drosophila* as in humans, e.g., older adults are more easily aroused from nighttime sleep by auditory stimuli than young adults.[46]

Interestingly, sleep quality deterioration as a function of age has been observed with insomnia patients. These patients also show higher cortical activation during sleep compared to good sleepers,[47,48] and similar activation has been observed in middle-aged individuals compared to young individuals.[8] Higher cortical activity may therefore reflect a higher vulnerability to challenges that can disrupt sleep. Something similar might occur in *Drosophila* and give rise to the lower arousal threshold, which may even underlie the reduced sleep rebound observed in older flies. An inability to disengage from active wake processes, to disinhibit sleep, might also interfere with sleep initiation. This is indeed what has been observed in w^{1118} flies and *TH* flies where, particularly during nighttime, sleep latency was increased with age (Figure 1E and Figure S1I in the supplemental material). However, this trait seems to be genotype dependent; the opposite was true for *Canton S* flies during the dark period (Figure 1J).

Although middle-aged and old flies still respond to sleep deprivation by increasing total sleep time the following day compared to baseline and nonsleep-deprived flies, recovery sleep was lower than what occurred in young flies. This conclusion was obtained with different genotypes, with very different sleep deprivation methods and even under the two different environmental conditions (low and normal temperatures). Although genetic background can influence the amount of recovery sleep (i.e., w^{1118} flies recovered more sleep than *Canton S* flies), and sleep deprivation duration or timing can also affect the time course of recovery sleep (i.e., 3-h versus 12-h sleep deprivation; end of sleep deprivation at ZT15 versus ZT24), the general conclusion still stands: young flies recover more sleep than old flies.

It is notable that a recent study reached a different conclusion,[7] namely, recovery sleep was similar in old versus young flies after 6 h of mechanical sleep deprivation. Moreover, old flies recovered lost sleep at a slower pace, i.e., gradually during the subsequent 12 h of recovery rather than predominantly during the first 4 h as in young flies. In our experiments, however, old flies had a recovery sleep time course quite similar to that of young flies, i.e., predominantly during the first part of the light period. We also analyzed the second day of recovery sleep and found no more sleep recovered for middle-aged and older flies compared to young flies (data not shown). This discrepancy in the time course of recovery sleep could be explained by a genotype difference, i.e., they used $w\ CS^{10}$ flies, whereas we used w^{1118}, *Canton S*, *TH* and control flies; similar discrepancies between genotypes have been reported in rodents.[3] Other possibilities are a technical or experimental difference (i.e., the other study used virgin females, and we used mated females), a sleep deprivation duration difference (i.e., the other study used 6-h sleep deprivation, whereas we used 12- and 3-h sleep deprivation) and a different timing of sleep deprivation start (i.e., they started sleep deprivation in the middle of the dark period, whereas we started our deprivations at the beginning of the dark period). In any case, the difference between young and old flies was without exception in our hands and robust over several genotypes as well as protocols.

It is also consistent with what has been previously reported in human studies: aging decreases sleep recovery after sleep deprivation, particularly quality sleep or deep sleep.[10,49,50]

One interpretation of our results is that the age-related reduction of total sleep duration and of recovery sleep reflects a decline of homeostatic sleep drive (sleep need). The sleep fragmentation and reduced sleep maintenance of older individuals (flies) would also be secondary to this reduced homeostatic sleep drive. In other words, older flies do not need as much sleep as young flies and are then less affected by sleep loss. An alternative interpretation is that sleep is less efficient or less successful in decreasing homeostatic sleep need in older flies (and people), due for example to a decreased arousal threshold. An effect of aging on arousal threshold might then be upstream of the two other aging phenotypes, namely, sleep fragmentation and the decrease of recovery sleep.

Importantly, the two possibilities lead to opposite predictions in term of alertness/sleepiness during waking periods. The first predicts that healthy older individuals have a similar or even reduced sleepiness compare to younger individuals, whereas the second predicts an increase in sleepiness in older individuals. Future *Drosophila* studies are needed to determine whether this is also the case for flies. This could be addressed by asking whether the reduced recovery sleep after sleep deprivation adversely affects alertness and cognitive performance of old flies compared to young flies. New techniques developed to assess sleep stages and sleep intensity in flies[18,51] should also facilitate distinguishing between the two possibilities. A clear answer will enhance our understanding of the effects of aging on fly sleep as well as the human-fly comparison.

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