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amnesiac regulates sleep onset and maintenance in *Drosophila melanogaster*

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ABSTRACT

The adenylate cyclase/cAMP signaling pathway and adult mushroom bodies (MBs) have been shown to play an important role in sleep regulation in *Drosophila*. The *amnesiac* (*amn*) gene, encodes a neuropeptide that is homologous with vertebrate pituitary adenylate cyclase-activating peptide (PACAP), is expressed in dorsal paired medial (DPM) neurons and is required for the middle-term memory (MTM) in flies. However, the role of *amn* on regulation of sleep is as yet unknown. Here we provide evidence that *amn* plays a major role on sleep maintenance and onset in *Drosophila*. Flies with the *amnesiac* allele, loss-of-function *amn*^{X8} mutation, showed a fragmented sleep pattern and short sleep latency. Moreover, homeostatic regulation was disrupted in *amn*^{X8} mutants after sleep deprivation. Sleep maintenance was also influenced by disruption of neurotransmission in DPM neurons with increased sleep bout number and decreased sleep bout length. Furthermore, age-related sleep fragmentation and initiation were inhibited in *amn*^{X8} mutant flies. These data suggest that *amn* is required in initiation and maintenance of sleep.

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Sleep disorder is a general problem for both young and old. How to maintain a good sleep is an emerging problem. Mammalian research on sleep maintenance is difficult to perform because of the complex mechanisms involved and problems associated with carrying out genetic studies. Studies have shown that characteristics of sleep, such as homeostasis and age-associated sleep fragmentation [1,2], are shared between mammals and flies [3–5]. For this reason, *Drosophila* can be used as a model organism for investigating sleep.

Some signaling pathways and neural circuits involved in the regulation of sleep have been identified in *Drosophila* [6–13]. Cyclic-AMP-dependent protein kinase A (PKA) activity has a reverse relationship with sleep duration [13]. The expression of a catalytic subunit of PKA in MBs has been found to decrease sleep amount significantly [8].

Previous studies showed that *amn* encodes a neuropeptide that has limited homology to the mammalian neuropeptide (PACAP) [14,15], which is related to PKA activity tightly. The *amn* gene is specifically expressed in DPM neurons [16]. The axons of DPM neurons innervate the vertical and horizontal lobes of MBs [16], which is an important brain region regulating memory and sleep in *Drosophila* [8,9]. Previous studies on the *amn* gene have focused on its

function in olfactory memory in *Drosophila* [17]. Mutations in the *amn* gene, or blockade of DPM neurons, disrupt MTM [18]. However, the function of *amn* on sleep has not yet been investigated.

In this study, we found that flies with *amn* gene mutation showed fragmented sleep and shortened sleep latency. Their sleep rebound was also impaired after exposure to sleep deprivation. The same fragmented sleep phenotype was induced when neurotransmission from DPM neurons to mushroom bodies was interrupted by cell-specific expression of tetanus toxin. Interestingly, unlike wild-type flies, the *amn*^{X8} mutants did not exhibit age-related sleep fragmentation and shortened sleep latency. To account for those finding, we suggest that sleep onset and maintenance in *Drosophila* is dependent on the *amn* gene.

Materials and methods

Fly strains. All flies used in the experiments were reared on cornmeal, sugar, yeast, and agar medium [19], at 23 ± 1 °C with 55 ± 5% relative humidity on a 12 h:12 h light:dark cycle.

Stocks of wild-type (a *white* line that was extensively isogenized to Canton-S wild-type), *amn*^{X8} (X chromosome, 19A *amn* gene deletion, a mutant that lacks the entire predicted *amn* open reading frame), c316-GAL4 (third chromosome P{GAL4}element) and MZ717-GAL4 (second chromosome, 41 F P{GAL4}) flies were kindly provided by Dr. Scott Waddell (University of Massachusetts Medical School); UAS-TNT-E (tetanus toxin light chain) and its inactive toxin UAS-IMPNT-V were as previously described [20].

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Aged flies were cultured in the same incubator from eclosion to death, and they were transferred to fresh media containing the same food every 2–3 days.

Behavioral analysis. Individual virgin flies, 3–5 days old, were transferred into monitor tubes (5 mm × 65 mm) containing media [19] (airproofed with paraffin) at one end, enabling the continuous recording over days. Locomotor activity was recorded continuously, in 1 min bins, using a *Drosophila* Activity Monitoring System (DAMS; Trikinetics, Waltham, MA). Sleep was defined as periods of 5 min with no recorded activity [21]. Data collections were over a period of 3 days after about 36 h of environmental adaptations in the monitors. All tests were performed in the incubator with temperature at 23 ± 1 °C and $55 \pm 5\%$ relative humidity, lights on at ZT0 (circadian time 06:00) and lights off at ZT12 (circadian time 18:00).

Sleep deprivation (SD) was performed as described by Huber et al. [1]. In brief, DAMS monitors containing the flies were placed vertically into a framed box, which could rotate 180° clockwise or counter clockwise (2–3 revolutions per minute) under the control of a motor. The monitor was dropped 1 cm at the nadir of every rotation, causing flies to fall to the bottom of the tube. The experimental procedure for sleep deprivation included one adaptation day, two baseline days, one sleep deprivation day and two recovery days. Sleep rebound was calculated as the loss of sleep and gain of sleep on an individual basis. Lost of sleep was the amount of sleep during the SD period relative to baseline sleep, while gain of sleep was calculated as the amount of sleep during recovery time relative to baseline sleep.

Data analysis. We used ClockLab software (Actimetrics) and software developed in our own Lab to analyze sleep data. Sleep latency was defined as cumulative time from ZT12 to initiation of the first sleep episode [21]. Measurement of the distribution of the

duration of sleep episodes (5–15 min, 15–50 min, 50–150 min, 150–500 min, >500 min) was conducted as described previously [8]. The fraction of time spent sleeping was calculated as the percentage of sleep episodes relative to total sleep for each respective period.

Statistical analysis was conducted by two-tailed, unpaired Student's *t*-tests or one-way ANOVA followed by the Tukey–Kramer HSD test as the *post hoc* test. Data are presented as mean behavioral responses and standard errors of the mean (SEM) are represented by error bars. Differences between groups were considered significant if the probability of error was less than 0.05 ($p < 0.05$).

Results

amn^{X8} mutants showed a fragmented sleep phenotype and short sleep latency

To explore the potential role of the *amn* gene on sleep, we investigated sleep pattern in *amn*^{X8} mutants, the loss-of-function mutation of the *amn* gene in *Drosophila*. Compared with wild-type flies, we found that *amn*^{X8} mutants maintained normal circadian rhythms under LD conditions (Supplementary Fig. 1) but their sleep patterns changed significantly. In *amn*^{X8} flies, the daily number of sleep bouts increased significantly (Fig. 1A; $p < 0.001$) but without a remarked variation in the total amount of sleep (Supplementary Fig. 2A) within a LD cycle. Correspondingly, average sleep duration within one LD cycle decreased significantly (Fig. 1B; $p < 0.001$) in *amn*^{X8} mutant flies. On further analysis we found that the increase in sleep bout number was more marked during the light phase of LD conditions than during the dark phase (Supple-

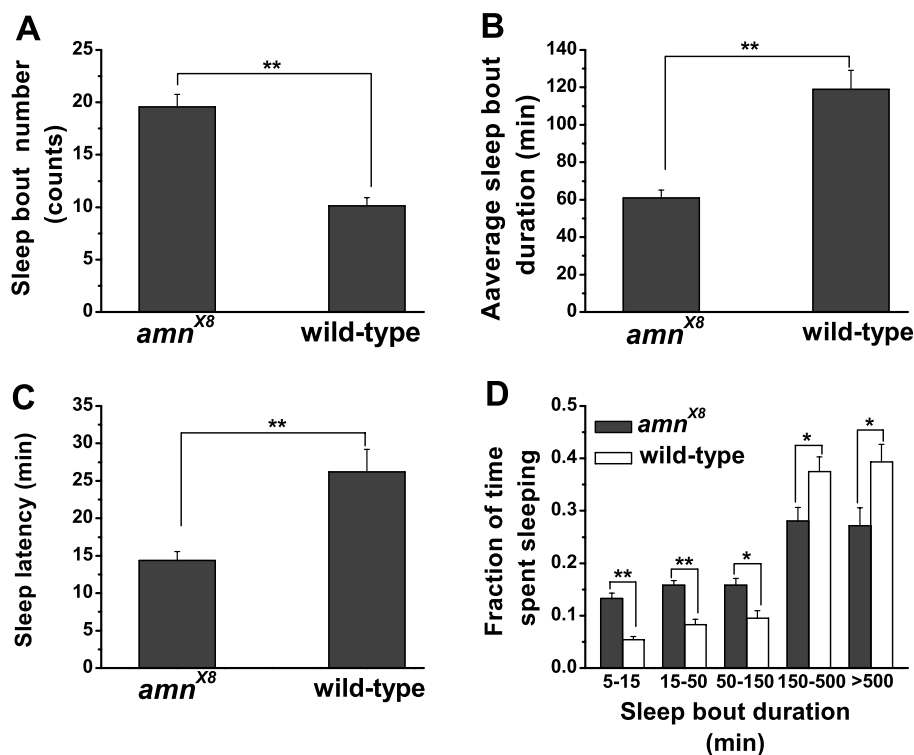


Fig. 1. Loss-of-function *amn*^{X8} mutation caused shortened sleep latency and fragmented sleep in *Drosophila* under a LD cycle. The numbers of replication was the same in the treatment and in the control, $n = 46$. Statistical significance ($p < 0.05$; $p < 0.001$) or not significant (n.s.) is indicated. (A) Sleep bout numbers increased significantly in *amn*^{X8} mutants compared to controls. (B) Average sleep bout duration decreased significantly in *amn*^{X8} mutant flies. (C) Mutant flies showed shortened sleep latency. (D) Mutant flies showed increased percentages of short-duration sleep episodes compared with control flies during one LD cycle. Fraction of time spent sleeping is percentage change in sleep episodes relative to total amount of sleep within a given period (5–15 min $p < 0.001$; 15–50 min $p < 0.001$; 50–150 min $p < 0.05$; 150–500 min $p < 0.05$; > 500 min $p < 0.05$).

mentary Fig. 2C), while average sleep bout duration decreased significantly during both environmental conditions (Supplementary Fig. 2B).

In *Drosophila*, sleep is made up of a series of episodes, with long sleep periods at night and short sleep periods during the daytime [21]. To determine detailed changes in sleep patterns, we examined the sleep structure of the *amn^{X8}* mutants. While wild-type flies exhibited a dispersed sleep architecture, however, *amn^{X8}* mutants had more short-sleep episodes (5–15 min; 15–50 min) and fewer long-sleep episodes (150–500 min; >500 min) (Fig. 1D), especially during the daytime (Supplementary Fig. 2D and E).

These results showed that *amn^{X8}* mutants had a fragmented sleep phenotype. This phenomenon pushed us to explore sleep latency, which is a parameter indicating sleep onset by estimates interval time from light off at zeitgeber time 12 to initiation of the first sleep episodes [21,6], is presumably reflects sleep pressure in flies. Unsurprisingly, compared with the wild-type flies, *amn^{X8}* mutant flies showed shortened sleep latency under a LD cycle (Fig. 1C; $p < 0.001$). This indicates that the sleep onset is affected by *amn^{X8}* mutation. Taken together with these results, we surmised that *amn^{X8}* is required for sleep onset and maintenance in *Drosophila*.

Homeostatic regulation of sleep is abnormal in *amn* mutants

Homeostatic regulation of sleep is similar in flies and mammals, with marked sleep rebound after hours of sleep deprivation [1]. We wondered therefore whether *amn^{X8}* mutant flies, with a fragmented sleep phenotype, would have a normal sleep homeostasis. To answer this question, we performed sleep deprivation experi-

ments in *amn^{X8}* mutant flies. Surprisingly, no obvious sleep rebound was found in *amn^{X8}* mutant flies after 24-h of mechanical sleep deprivation. Neither the sleep bout duration nor the number of sleep bouts were significantly altered in *amn^{X8}* mutant flies in contrast to the wild-type flies (Fig. 2B and C), which were observed to have significant sleep rebound immediately after sleep deprivation (Fig. 2A; $p < 0.001$).

Previous reports have suggested that 12 h of sleep deprivation is enough to produce a marked effect on the following sleep in wild-type flies [1]. Therefore, we used 12-h sleep deprivation treatment during the daytime and night time. We found that amount of sleep and sleep bout duration showed no rebound in *amn^{X8}* mutant flies after 12-h of sleep deprivation (Fig. 2D, Supplementary Fig. 3A; $p = 0.50$, $p = 0.64$). Wild-type flies showed significant sleep rebound after 12-h of sleep deprivation during dark phase (Fig. 2D; $p < 0.05$) while 12-h daytime sleep deprivation treatment did not cause a significant sleep rebound at the following night. For night time sleep may have arrived to a high level even under normal conditions [1].

It appears that neither sleep patterns nor sleep structure was significantly altered by sleep deprivation in *amn^{X8}* mutant flies (Fig. 2E; Supplementary Fig. 3B). Interestingly, the shallow sleep latency has little change before and after sleep deprivation in *amn^{X8}* mutant flies (Fig. 2F; baseline $p < 0.001$; recovery $p < 0.05$). This indicates that sleep onset is less affected by sleep deprivation in *amn^{X8}* mutant flies.

These data imply that the minimal change on sleep latency and fragmented sleep phenotype after sleep deprivation might be caused by the abnormal homeostatic regulation of sleep in *amn^{X8}* mutant flies.

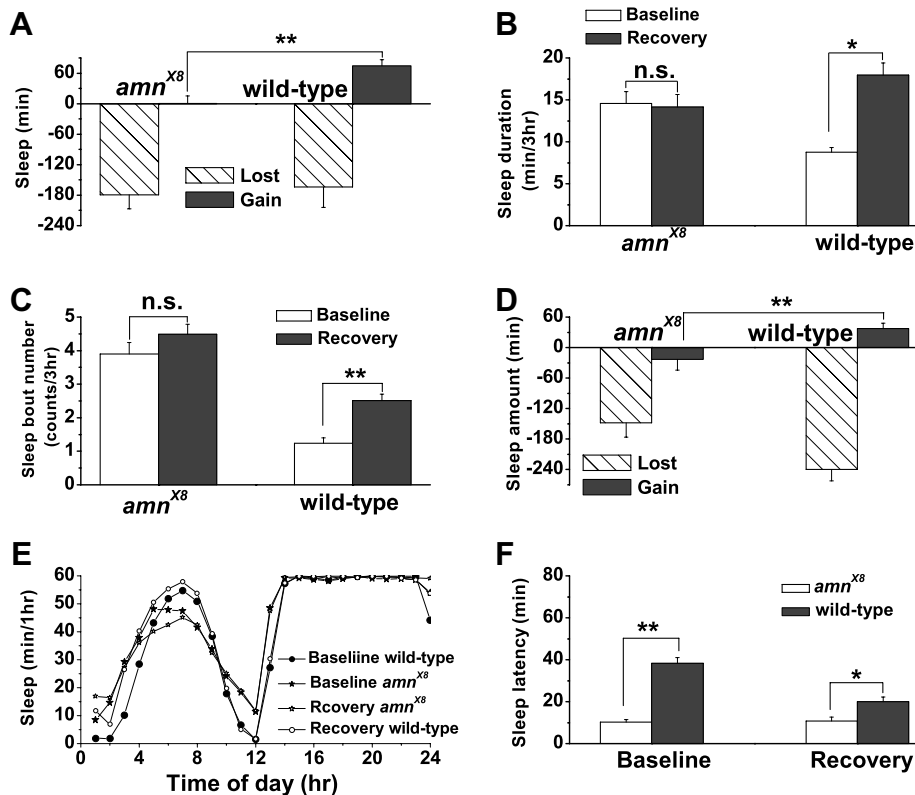


Fig. 2. The *amn^{X8}* mutation affects sleep homeostasis. Statistical significance ($*p < 0.05$; $**p < 0.001$) or not significant (n.s.) is indicated. (A–C) *amn^{X8}* $n = 60$, control $n = 55$. (D–E) *amn^{X8}* $n = 43$, control $n = 45$. (A) Compared with control flies, the mutants showed poor sleep rebound while they lost a lot of sleep during 24-h of SD. (B, C) Compared with mutant flies, control flies showed prolonged average sleep bout duration and increased sleep bout number within 3 h after 24-h SD. (D) Mutant flies showed obvious sleep loss during 12-h SD in dark phase and poor sleep rebound during recovery. (E) Sleep pattern was little affected by 24-h SD in *amn^{X8}* mutant flies. (F) Sleep latency has significant decrease before and after SD.

Disruption of neurotransmission in DPM neurons affects sleep maintenance

The *amn* gene is expressed mainly in DPM neurons in the fly brain. We wondered, therefore, whether DPM neurons were involved in sleep. To investigate this, we used the GAL4/UAS system to target expression of tetanus toxin [20] to disrupt neurotransmission from DPM neurons. Our data showed that sleep bout number was increased and sleep bout duration was shortened by targeted expression of c316-GAL4 driven UAS-TNT in flies (Fig. 3A; $p < 0.001$; B; $p < 0.001$). As expected, distribution of sleep episodes was in accord with that of *amn*^{X8} mutant flies, with more short sleep episodes and less long sleep episodes compared to control flies (Fig. 3C). Thus, disruption of neurotransmission in DPM neurons with the UAS-TNT transgene mimics the fragmented sleep phenotype in *amn*^{X8} mutant flies.

Therefore, we wondered whether the sleep latency is similar to that in *amn*^{X8} mutant flies. However, sleep latency was not so obvious in the c316; uas-tnt flies, while it was shortened remarkably in Mz717;-uas-tnt flies (Supplementary Fig. 4, $p < 0.001$). This may be due to fine distinctions in the location of expression resulting from the different promoters; expression of c316-GAL4 is specific to DPM neurons, while expression of MZ717-GAL4 is more extensively in the fly brain [18].

These results confirm our suggestion that DPM neurons, as a major locus of AMN, may be involved in the regulation of sleep maintenance in *Drosophila*, and its function on sleep onset need to be examined in detail in future.

Sleep latency and fragmentation exhibited limited change with aging

It has been shown that sleep tends to be fragmented in aging flies [4]. Therefore, we wondered whether the sleep phenotype

in *amn*^{X8} mutants would fragment more with aging. Unexpectedly, aged *amn*^{X8} mutant flies showed no significant change in sleep bout number (Fig. 4A), or total amount of sleep (Supplementary Fig. 5). Furthermore, average sleep bout duration (Fig. 4B) and distribution of sleep episodes showed no significant differences with aging in *amn*^{X8} mutants (Fig. 4C, 5–15 min; Supplementary Table. 1).

Concerning to age-related sleep, sleep onset is an important aspect to care. After sleep latency examination, we could not find significant difference in *amn*^{X8} mutant flies with aging (Fig. 4D). We also compared locomotion in *amn*^{X8} mutant and control flies, locomotor activity of the former was similar to the latter (data not shown). Together, these phenomena indicate that the shortened sleep latency and the fragmented sleep caused by the *amn*^{X8} mutation may contribute to age-related sleep initiation and fragmentation.

Discussion

amn has been studied in olfactory memory since it was found by Quinn et al. [22], but its function on sleep has not been reported yet. In this study, we found the important function of *amn* on sleep regulation. The shortened sleep bout duration and sleep latency of *amn*^{X8} mutants suggest that *amn* plays role on sleep maintenance and onset. Normally, wild-type flies tend to sleep less at light phase [21], but *amn*^{X8} mutant flies initiate more short sleep episodes during the daytime. Increased sleep bouts number and shortened sleep latency may be a compensatory mechanism to maintain adequate sleep in the mutants. However, the *amn*^{X8} mutant flies showed no obvious rebound after sleep deprivation. We postulated that the effect caused by the *amn*^{X8} mutation was already too great to be further influ-

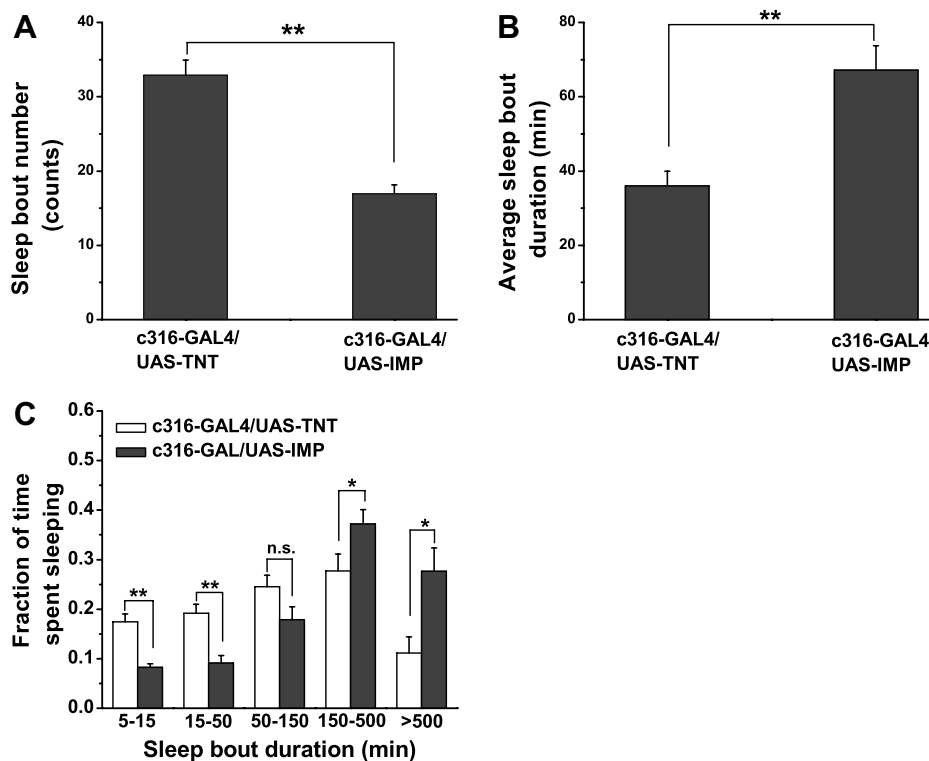


Fig. 3. Disruption of neurotransmission in DPM neurons leads to fragmented sleep. Statistical significance ($*p < 0.05$; $**p < 0.001$) or not significant (n.s.) is indicated. Expression of UAS-TNT-E and its inactive toxin (UAS-IMP-TNT-V) were driven by a c316-GAL4 promoter. Treatment $n = 23$, control $n = 24$. (A) Sleep bout number increased significantly after disruption of neurotransmission of DPM neurons. (B) Average sleep bout duration was shortened significantly in the disrupted flies. (C) Percentage of short-duration sleep episodes (5–15 min $p < 0.001$; 15–50 min $p < 0.001$; 50–150 min $p < 0.05$) was increased and percentage of long-lasting sleep episodes (150–500 $p = 0.150$; > 500 $p < 0.05$) tend to decrease in the disrupted flies.

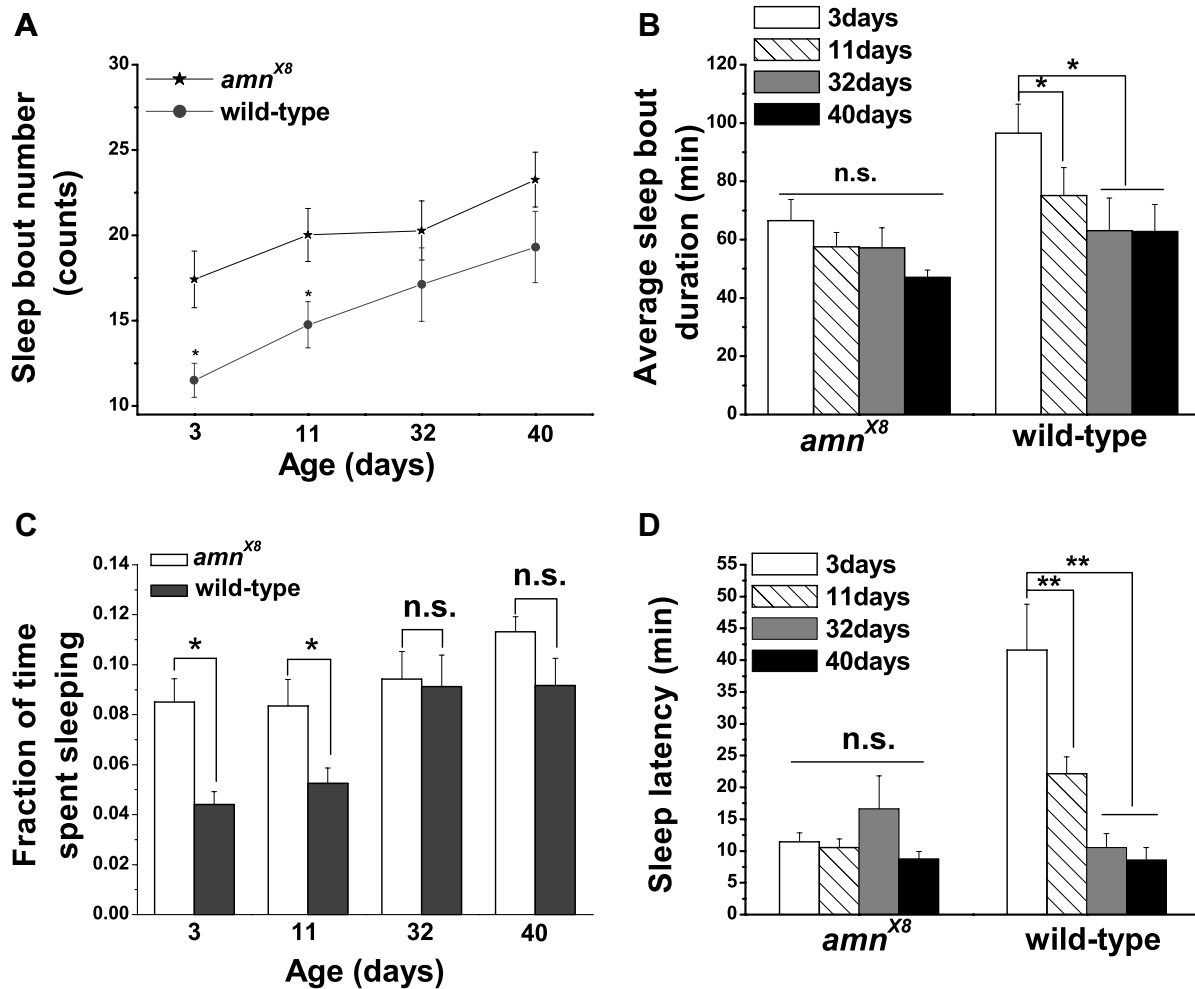


Fig. 4. *amn*^{X8} mutant flies did not show significant change on sleep latency and sleep fragmentation. Statistical significance ($p < 0.05$; $**p < 0.001$) or not significant (n.s.) is indicated. Replication numbers of *amn*^{X8} and control flies used in this experiment were the same, $n = 16$. (A) Sleep bout number did not change significantly with aging in *amn*^{X8} mutant flies. (B) Comparing with young and adult flies, average sleep bout durations showed no significant change in aged *amn*^{X8} mutant flies. (C) Percentage of short-duration of sleep episodes (5–15 min) appeared minor difference with aging in *amn*^{X8} mutants. (D) In *amn*^{X8} mutant flies, sleep latency is similar from young to old.

enced by mechanical sleep deprivation. Taken together, these results indicate that *amn*^{X8} mutation undermined original balance on sleep regulation in flies.

AMN peptide is homologous to neuropeptide (PACAP) in vertebrates [14]. Studies showed that PACAP mediates many physiological functions through L-type Ca^{2+} channels via a cAMP signaling pathway [23]. The *amn*^{X8} mutation affects the cAMP signaling pathway directly in fly brain [24]. It is well known that PKA is a cAMP-dependent protein kinase. As a major factor in cAMP signaling pathway, PKA activity is involved in regulating sleep bout duration in *Drosophila* [13]. Flies showed shortened sleep bout duration with high PKA activity and prolonged sleep bout duration with low PKA activity [13]. Previous studies have proposed that PKA activity is enhanced in *amn* mutant flies [25]. Combined with our results, we propose that enhanced PKA activity may underlie the short sleep bout duration and short sleep latency in *amn*^{X8} mutant flies.

AMN neuropeptide is expressed mainly in DPM neurons that spread throughout the MBs lobes [16]. MBs play a key role in the regulation of memory and sleep in *Drosophila* [9,27]. Previous investigations have showed that MTM can be blocked by the disruption of neurotransmission in DPM neurons [18,26]. Studies showed that the enhanced PKA activity in MBs leads to shortened sleep bout duration in flies [8] and average bout length is reduced with disruption of neurotransmission in MBs [9]. In the present study we found that flies showed fragmented sleep phenotype

after disruption of neurotransmission from DPM neurons similar to that of *amn*^{X8} mutants. This suggests that DPM neurons have a function for sleep maintenance. Taken together with close relationship of MBs and DPM neurons [16] and their role in regulating sleep and memory, our results support the proposal of DPM neurons regulate sleep maintenance operating via the cAMP signaling pathway [26].

Studies showed that the age-dependent memory impairment was absent in *amn* flies [28]. This premature age-related memory impairment in *amn* mutant flies was maybe due to an increase in cAMP and PKA activity in MBs [25]. Our studies showed that the fragmented sleep phenotype and sleep initiation had minimal change with aging in *amn*^{X8} mutant flies. Together with age-related effects on memory, these evidences tempt us to speculate that altered cAMP and PKA activity are involved in age-associated sleep maintenance and onset in *amn*^{X8} mutant flies. Additional experiment will be required to elucidate it.

Collectively, the results of our study provide new insights concerning the *amn* gene, which has been identified as a key point for MTM in *Drosophila*, is also involved in sleep. The regulation of sleep has long been a matter of discussion [29]. However, there is insufficient evidence to clarify the problem. We propose that *amn* may acts as an important factor on sleep onset and maintenance regulating via cAMP signaling pathway, and sleep in aged flies is related to this gene tightly. Our results in sleep regulation of DPM neurons

further enhancing the understanding of the *amn* gene on sleep. Combined with previous reports on memory consolidation [16], we propose that *amn* may links memory consolidation with sleep maintenance. Further research is needed to determine the precise role that the *amn* gene plays in memory consolidation and sleep maintenance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.119.

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