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Pan-neuronal knockdown of the *c-Jun N-terminal Kinase* (*JNK*) results in a reduction in sleep and longevity in *Drosophila*.

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Abstract Sleep is a unique behavioral state that is conserved between species, and sleep regulation is closely associated to metabolism and aging. The fruit fly, *Drosophila melanogaster* has been used to study the molecular mechanism underlying these physiological processes. Here we show that the *c-Jun N-terminal Kinase* (*JNK*) gene, known as basket (*bsk*) in *Drosophila*, functions in neurons to regulate both sleep and longevity in *Drosophila*. Pan-neuronal knockdown of *JNK* mRNA expression by RNA interference resulted in a decrease in both sleep and longevity. A heterozygous knockout of *JNK* showed similar effects, indicating the molecular specificity. The *JNK* knockdown showed a normal arousal threshold and sleep rebound, suggesting that the basic sleep mechanism was not affected. JNK is known to be involved in the insulin pathway, which regulates metabolism and longevity. A *JNK* knockdown in insulin-producing neurons in the pars intercerebralis had slight effects on sleep. However, knocking down *JNK* in the mushroom body had a significant effect on sleep. These data suggest a unique sleep regulating pathway for *JNK*.

Introduction

Sleep is a behavioral state characterized by an extended immobile period with a decreased sensitivity to outside stimuli and is considered to be conserved across a variety of species [1]. The discovery of sleep and sleep mutants in the fruit fly, *Drosophila melanogaster* has accelerated the study of sleep [2,3]. *Drosophila* has been used as a model organism for studying lifespan, and there is a growing evidence to suggest that the relationship between sleep and lifespan is important. Sleep deprivation of the cyc^{01} mutant resulted in a reduced lifespan [4] and short sleep mutants *Shaker*, *sleepless* and *Hyperkinetic* have a short lifespan [5-7]. Interestingly, another short sleep mutant *fumin (fmn)*, which is a mutation of the dopamine transporter gene, showed a similar lifespan to the wild type when under conventional diet conditions [8,9]. However, *fmn* had a reduced lifespan when under high calorie diet conditions. High calorie diet also caused a reduction in sleep, and sleep time was reduced from approximately 30% to 10% of the day in 20 day-old *fmn* flies (Yamazaki et al, accompanying paper). These data suggest a close relationship between metabolic signaling, lifespan and sleep regulation.

During the search for the genes responsible for the difference between control and *fmn* flies on high calorie diet, we found that many genes show differential expression in the head (data not shown). To clarify the functions, we knocked down a selection of genes using RNA interference (RNAi) lines obtained from the Vienna *Drosophila* RNAi Center (VDRC). We then examined the sleep phenotypes and identified novel sleep related genes. Here we describe a pan-neuronal knockdown of the ortholog of mammalian *c-Jun N-terminal Kinase* (*JNK*), named basket (*bsk*) in *Drosophila*, which resulted in a reduction in both sleep and longevity.

Materials and Methods

Fly stocks, food conditions and genetic crosses

All stocks were raised at 25°C in 60% humidity with a 12h light/dark cycle on a conventional cornmeal, yeast, glucose agar medium (see below for the composition). All the experiments using live flies were performed at 25°C. Two independent RNAi lines for *JNK* under the control of the upstream activating sequence (UAS) [Transformant ID: 34138 (II) and 104569 (II)] and the w^{1118} line, which is the genetic background of the RNAi lines, were from VDRC. Gal4 drivers [*elav-Gal4* (X), OK107 (IV), eas^{alaP} (X)] were from the Bloomington *Drosophila* Stock Center, Indiana University, Indiana, USA. dilp2-Gal4 (II) was a kind gift from Dr. Linda Partridge. The *JNK* mutant, bsk^{147e}, has been described previously [10] and was a kind gift from Dr. Julian Ng [11].

Fly food preparation

The conventional food contained: 8% glucose, 2% dry bakery yeast, 8% cornmeal, 3.2% wheat malt, approximately 0.2 % molasses, 1% propionic acid, 1% of 10% *para*-hydroxybenzoate in 95% ethanol and 0.5 % agarose. The high calorie food (10% SY) contained: 10% sucrose, 10% yeast extracts, 8% cornmeal, 1% propionic acid and 0.5% agarose. The low calorie food (1% SY) is same as 10% SY except for both sucrose and yeast extract are 1%.

Measurement of knockdown efficiency

Total RNAs were prepared from 8 to 15 frozen heads of flies, using Trizol (Sigma, St. Louis, MO). 1.8 μg of total RNAs were used for polyA$^+$ RNA purification with an Oligotex-dT, mRNA purification kit (TaKaRa, Tokyo, Japan). Then, entire polyA$^+$ RNA samples were reverse transcribed to synthesize cDNA using an oligo(dT)20 primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). The primers used were 5'-CCATCCAATA TAGTTGTAAA GGCCG-3' and 5'-CGAGGGTGAT GGTGTACCTAA TTGC-3' for *JNK* as described by Yoshioka et al.[12], and 5'-TGGTACGACAACGAGTTTGG-3' and 5'-TTTCAGGCCGTTTCTGAAGT-3' for *GAPDH2*. Densitometric analysis was performed using the ImageJ 1.43 software (NIH, Bethesda, MA, USA). The mRNA levels of *JNK* were first normalized by those of a housekeeping gene, *GAPDH2* in all of the RNAi and control flies. Then, the values of the RNAi flies were divided by those of the control flies and expressed as a percentage.

Sleep analysis

Activity was monitored by the number of times an individual fly crossed an infrared beam when housed in a 65 mm glass tube. The number of beam crossings were scored for every 1 min using the Trikinetics *Drosophila* Activity Monitoring System (DAM2 and DAM5, Waltham, MA, USA). Except for the longevity experiments, 2- to 7-d-old male flies were used for behavioral analysis and the data were collected continuously for three days under either a 12 hr light/dark (LD) cycle or constant dark (DD) conditions. Sleep was defined as 5 min or longer continuous periods without any activity and was calculated using a Microsoft Excel based program as described previously [13].

Stimulation study

To examine the responsiveness to stimuli during sleep, we used an automatic mechanical-stimulator which was developed in our laboratory by coupling the DAM2 (Trikinetics) with a small vortex mixer, the mini MIXER N-20M (Nissin, Tokyo, Japan) and controlled by custom-built software on a LabVIEW (National Instruments, Austin, TX, USA) platform. Flies were individually placed in glass tubes in the mechanical-stimulator and maintained in DD followed by a three day LD cycle. Mechanical stimuli were applied to the flies by vortexing at regular intervals, at circadian time (CT) 14, 16, 18, 20 and 22, and repeated for 1 week. The strength of the stimuli was adjusted empirically by changing the power supply voltage and duration of action of the mixer. Typically, a stimulation by the maximum voltage (3.9 V) or that by a quarter of the maximum voltage was used as a strong or mild stimulus, respectively, and 200 or 600 msec was used for a short or a long stimulus respectively. Whether flies responded or not against the stimulus was determined by the immediate movement within 1 min, which is consistent with the criteria described previously [8].

Sleep deprivation

Sleep deprivation analysis was performed using the automatic mechanical- stimulator described above. Flies were stimulated using a 10 sec strong stimulus once per 5min for 12hr of subjective night and their activities were

recorded throughout the experiments. The efficiency of sleep deprivation and the amount of rebound sleep during the first 6 hr of the following subjective day period were then calculated.

Statistical analysis

Data were analyzed as described in the figure legends.

Results & Discussion

To knockdown the *JNK* (*bsk*) gene, we crossed two independent *UAS-JNK RNAi* lines from the VDRC (104569 and 34138, designated as Line1 and Line2) with *elav-Gal4* and examined the sleep of the progeny. Both Line1 and Line2 showed an increase in the total daily locomotor activity and a decrease in sleep (Fig. 1A-C). Since Line2 yielded a smaller number of progeny, possibly due to the extensive

Figure1

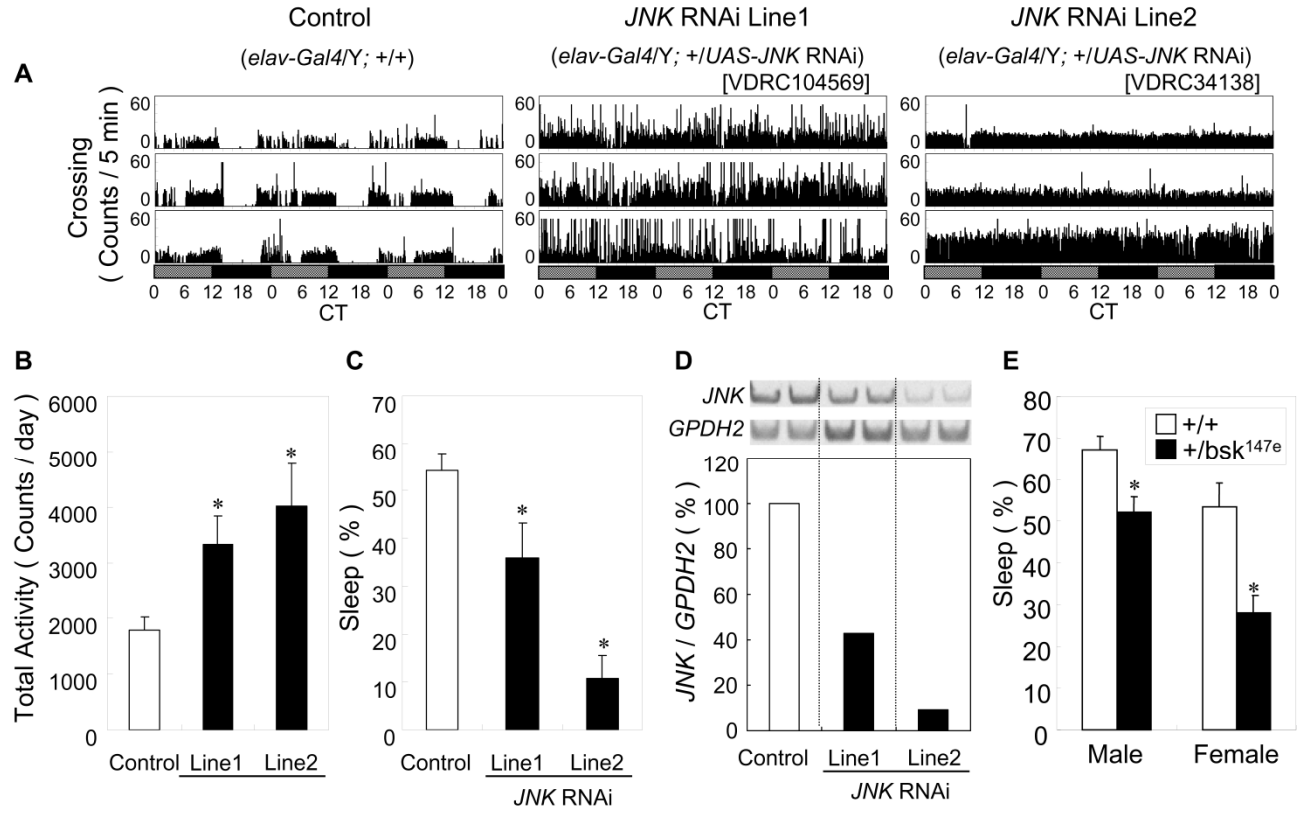

Figure 1. A reduction in sleep for *JNK* knockdown and knockout flies. A, Locomotor activity profiles of three representative flies from the control (*elav-Gal4* /Y;+/+, left panel), *JNK RNAi* Line1 (*elav-Gal4*/Y; *UAS-JNK RNAi*[VDRC 104569]/+, middle) and Line2 (*elav-Gal4*/Y; *UAS-JNK RNAi*[VDRC 34138]/+, right) for three days in constant dark (DD) conditions. The *JNK RNAi* was pan-neuronally expressed using the*elav-Gal4* driver. Gray and black bars under the horizontal axis indicate subjective day and night respectively. CT: circadian time. B, total daily activity and C, total daily sleep of the control and *JNKRNAi*Line1 and Line2 in DD. Data are presented as the mean ± SEM. Asterisks indicate statistically significant differences between the control and RNAi flies: *$p<$ 0.05, Student's t test, n = 16, 16, 3 for each group respectively. D, efficiency of the *JNK* gene knockdown. The expression levels of *JNK* genes in the head of *JNK RNAi* Line1 and Line2 flies are evaluated as the relative values to the control. As described in the Materials and Methods, each mRNA level was quantified by qPCR and first normalized to *GAPDH2.* Then the values were normalized to the average of the independent control samples that are set at 100%. E, total sleep of the male and female bsk^{147e} heterogeneous *JNK* mutant flies in DD conditions. Data are presented as the mean ± SEM. For the statistical significance between the control and RNAi flies: student's t test, *$p<$ 0.05, n = 8 for each group.

expression of the RNAi, further experiments were done using Line1. Total locomotor activity was increased two fold in Line1 and nearly three fold in Line2, and the total sleep decreased by 30% in Line1 and 70% in Line2 (Fig. 1B,C). The efficiency and targeting specificity of each *JNK RNAi* transgene was assessed by quantification of RT-PCR products on conventional gel electrophoresis as described in the Materials and Methods. While control GAPDH mRNA levels were almost similar between samples (data not shown), both Line1 and Line2 significantly down-regulated *JNK* mRNA, and Line2 was more effective than Line1 (Fig. 1D). Thus the knockdown efficiencies of the two lines correlated with their sleep phenotypes, supporting the specificity of *JNK* knockdown on sleep phenotypes.

To further confirm the specificity of the knockdown, since RNAi may involve off-target effects on other genes, we examined the sleep phenotype of the *JNK* gene knockout flies. Because homozygous knockout flies are lethal, we examined the heterozygous mutant of *JNK* and found a reduction in sleep for both male and female flies (Fig. 1E). These results also indicated that a decrease in *JNK* gene expression results in short sleep phenotype.

We further investigated the detailed properties of sleep for neuronal *JNK* knockdown flies. In order to examine their arousal threshold, we applied mechanical stimuli of four different magnitudes (mild short-stimuli, mild long-stimuli, strong short-stimuli and strong long-stimuli) to both the control and *JNK* knockdown flies during the dark period. As shown in Fig. 2A, the four different magnitudes of stimuli induced various levels of arousal in control flies and *JNK* knockdown flies exhibited a similar response compared to the control.

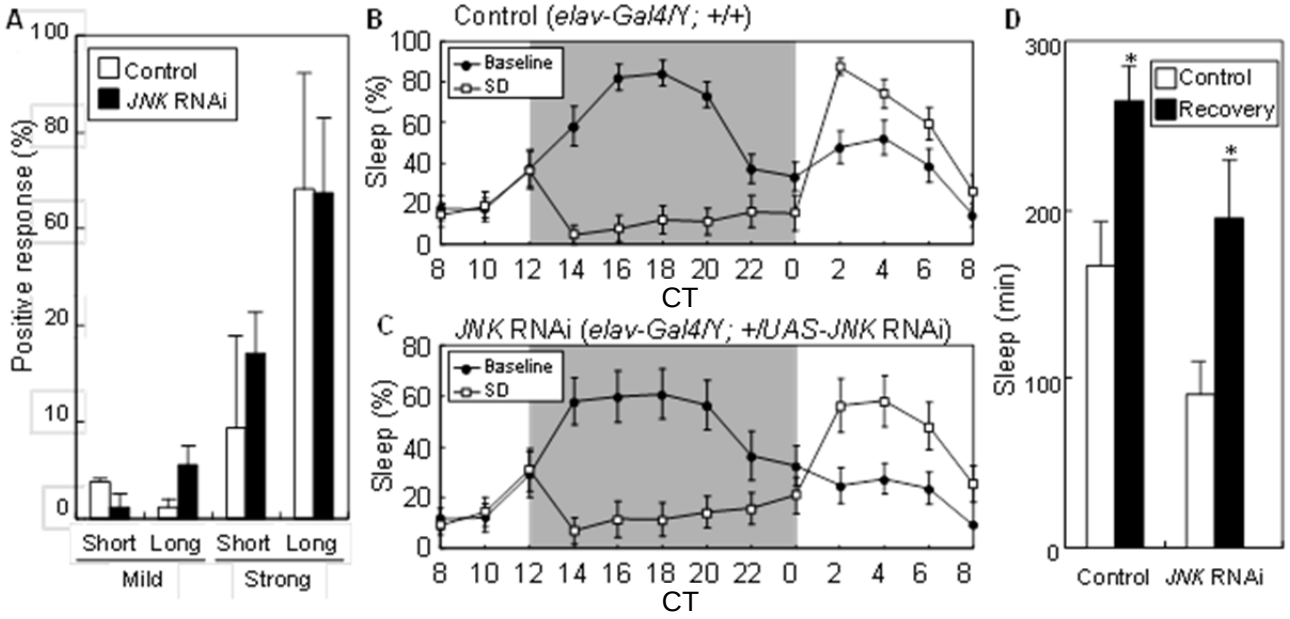

Figure 2. Properties of sleep for *JNK* knockdown flies. A, Normal responsiveness to mechanical stimuli of sleeping *JNK RNAi* flies. Mechanical stimuli were applied to the male flies and the positive response rate (the ratio of the number of flies responding against stimuli to the number of sleeping flies before the stimuli) was calculated. The control (*elav-Gal4*/Y; +/+) and the *JNK RNAi* (*elav-Gal4*/Y; *UAS-JNK RNAi* /+) flies showed an almost equivalent response rate to all stimuli regardless of strengths and lengths (n = 16 for all groups). Data are presented as the mean ± SEM. B, C, D, the homeostatic response to sleep deprivation in *JNK RNAi* flies. Sleep profiles were plotted in 2 hr bins over 2 days under DD conditions for control (*elav-Gal4*/Y; +/+) (**B**) and *JNK RNAi*(*elav-Gal4*/Y; *UAS-JNK RNAi* /+) flies (**C**). The white and gray backgrounds of the horizontal axis indicate subjective day and night respectively. Twelve hours of sleep deprivation (SD) was started at CT 12. The values for control (closed circles) and sleep-deprived flies (open square) are the group average for 2 hr (n =16). (D) The amount of sleep during the first 6 hr of the baseline day (open bars) and recovery day (solid bars) were quantified. Significant sleep rebound was observed in both strains: Student's t test; *p< 0.01. Data are presented as the mean ± SEM.

This is an interesting property of the *JNK* knockdown that is distinct from other short sleep mutants. The dopamine transporter mutant, *fmn*, showed hyperresponsiveness (a lower arousal threshold), while the *calcineurin A-14F* knockdown showed hyporesponsiveness (a higher arousal threshold), although both flies sleep as little as the *JNK* knockdown flies [8,14]. Thus the sleep phenotype of *JNK* knockdown flies is unique in this respect. Next we deprived the flies of sleep and examined sleep rebound. Since the *JNK* knockdown flies showed an intact arousal response to mechanical stimuli, their sleep could be deprived efficiently. After depriving the flies of sleep for 12 hr during the night period, *JNK* knockdown flies showed a clear rebound increase in sleep during the following day time (Fig. 2B,C) and the magnitude of the rebound was comparable to that of the control (Fig. 2D). The intact arousal threshold and the sleep rebound indicated that the basic mechanism of sleep regulation was not affected in the *JNK* knockdown flies.

Since JNK has been reported to be involved in lifespan regulation [15,16], we next examined the effect of neuronal *JNK* knockdown on longevity. As mentioned earlier, the change in the diet condition differentially affected the short sleep mutant, *fmn* (Yamazaki et al., accompanying paper). We determined the longevity of *JNK* knockdown flies using both low calorie (1%SY: 1% sucrose and 1% yeast extract) and high calorie (10% SY: 10% sucrose and 10% yeast extract) conditions.

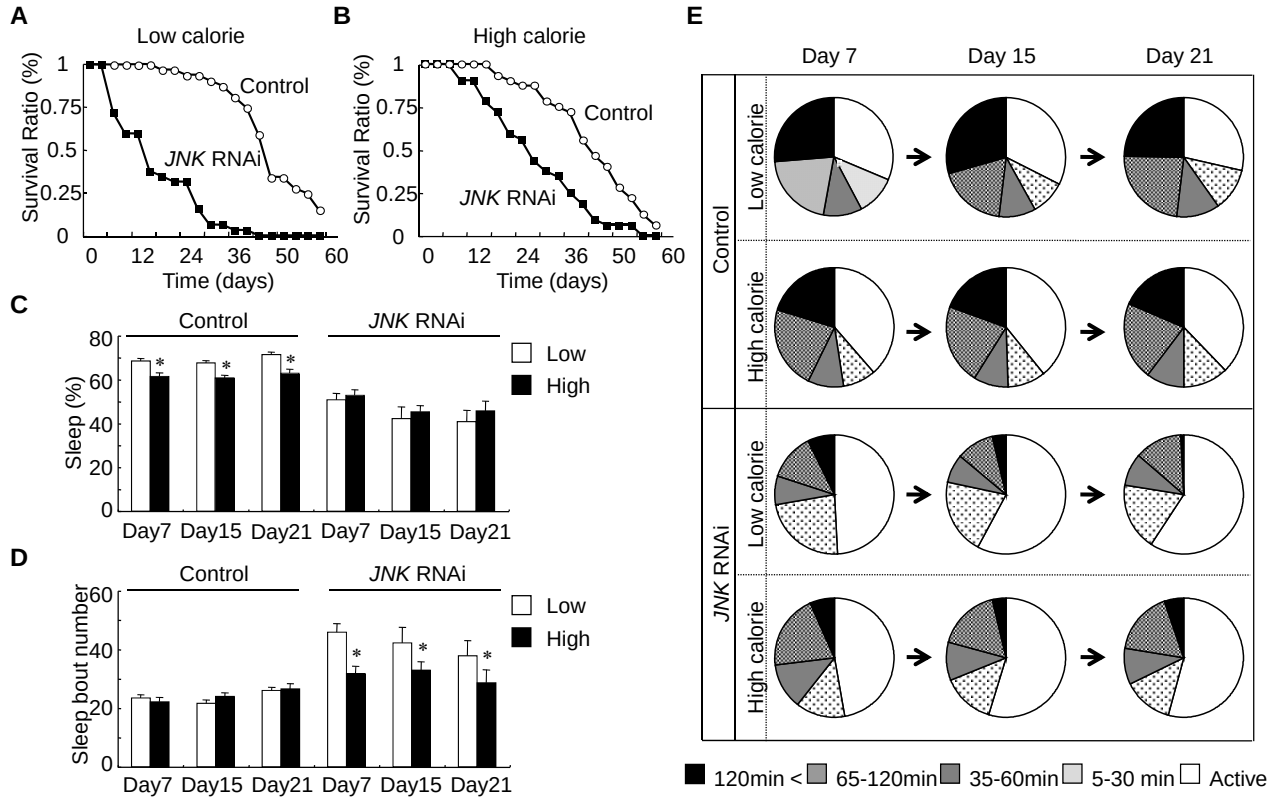

Figure 3. Calorie effect on lifespan and sleep in *JNK RNAi* flies. A, B, Survival curves of control (open circles) and *JNK RNAi* flies (closed square) under both low (1% SY) and high (10% SY) calorie conditions in LD. Compared to the control, *JNK RNAi* flies had a shorter lifespan under both dietary conditions. Control flies had a slightly longer lifespan under the low calorie diet condition than the high calorie diet condition, while *JNK RNAi* flies had longer lifespan under the high calorie diet condition. C, total daily sleep and D, sleep bout number of control and *JNK RNAi* flies under low (white bars) and high (black bars) calorie conditions in LD. Data are presented as the mean ± SEM. The statistical significance between the low calorie diet condition and the high calorie diet condition at each time point: Student's *t* test; $*p < 0.05$, $n = 8$~32 for each group. E, sleep properties. Sleep bouts are classified by their length and their proportion among the total number of sleep and activity bouts are plotted in pie charts.

As shown in Fig. 3A,B, the *JNK* knockdown flies had a shorter lifespan than the control flies in both conditions, but the difference was more evident with 1% SY. In terms of longevity, the low calorie diet was worse for *JNK* knockdown flies.

Caloric conditions strongly affected not only lifespan but also sleep in *fmn* mutants (Yamazaki et al., accompanying paper). Thus we examined the effects of calorie conditions on the sleep of *JNK* knockdown flies. Since *JNK* knockdown flies had a reduced longevity, we also analyzed the effects of diet on sleep in relation to aging. As shown in Fig. 3C, the amount of sleep was reduced in control flies when under high calorie diet conditions. This was consistent with recently published results [17]. In *JNK* knockdown flies however, a high calorie diet had no effect on sleep amount. On the other hand, the sleep bout number was significantly increased in *JNK* knockdown flies when under low calorie diet conditions than when under high calorie diet conditions (Fig. 3D). These results indicated more fragmentation of sleep occurred in the flies that were fed on a low calorie diet. We also analyzed the distribution of sleep bout lengths. Sleep bouts were classified into four groups according to their length and their proportion to the total number of sleep and activity bouts were plotted in a pie chart. The *JNK* knockdown flies had a reduced proportion of longer sleep bouts (120<, plotted as a black portion in Fig. 3E) compared with the control flies. Thus, *JNK* knockdown flies showed a shorter lifespan and more fragmented sleep when under low calorie diet conditions. This is in contrast to *fmn* flies, where high calorie conditions affected both sleep time and lifespan (Yamazaki et al., accompanying paper). These data demonstrate the unique properties of *JNK* knockdown flies.

In order to explore the mechanism of how *JNK* regulates sleep, we used region specific Gal4 drivers to knockdown *JNK*. As shown in Fig. 4, *JNK* knockdown using two different mushroom body (MB) Gal4 drivers, eas^{alaP} (eas-Gal4) and OK107 revealed a reduction in sleep. However, *JNK* knockdown by dilp2-Gal4, an insulin producing pars intercerebralis (PI) neuron specific Gal4 driver, did not induce a significant effect. This was an unexpected result, since the PI reportedly links nutritional state with lifespan [18].

In this study, we demonstrated that the *Drosophila* ortholog of mammalian *JNK*, which was originally isolated as a morphological mutant named *bsk* [19], is a novel sleep regulating gene. We demonstrated that pan-neuronal knockdown of *JNK* resulted in short sleep and reduced longevity. The short sleep phenotype shown by the *JNK* knockdown flies is unique in that it does not affect the arousal threshold.

Recent studies have indicated that the MB is an important area for sleep regulation. Ablating the MB developmentally or inhibiting the MB neurons induces short sleep [20,21]. Thus we used MB-Gal4 drivers, OK107 and eas-Gal4, to knockdown *JNK*, which resulted in a reduction in sleep. Both of these drivers coverthe majority of neurons arborizing in the MB [22].

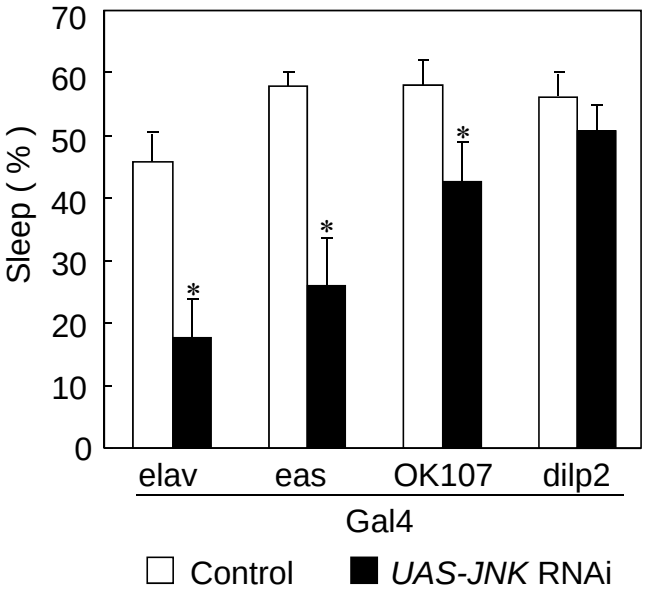

Figure 4. Region specific *JNK* knockdown.Total daily sleep for three days in DD of male flies for each of the genotypes of control flies (*elav-Gal4*/Y; +/+, *eas-Gal4*/Y; +/+, +/Y; +/+; +/+; OK107/+, +/Y; *dilp2-Gal4*/+) and *JNK RNAi* flies (*elav-Gal4*/Y; *UAS-JNK RNAi*/+, *eas-Gal4*/Y; *UAS-JNK RNAi* /+, +/Y; *UAS-JNK RNAi* /+; +/+; OK107/+, +/Y; *dilp2-Gal4*/ *UAS-JNK RNAi*). Control and *JNK RNAi* flies were the progenies generated by crosses between the flies with region specific Gal4 drivers (*elav-Gal4*, *eas-Gal4*, OK107 and *dilp2-Gal4*) and control (*+/+*) flies or *JNK RNAi* (*UAS-JNK RNAi*) flies. A significant reduction in sleep was observed in progenies from *elav-Gal4*, *eas-Gal4* and OK107 but not from *dilp2-Gal4*. Data are presented as the mean ± SEM. Student's *t* test; **p*< 0.01.

On the other hand, sleep was not affected significantly when *JNK* knockdown was induced in the PI by Dilp2-Gal4, where *JNK* signaling inversely regulates the production of insulin like peptide. Although there is a controversy over the relationship between diet-restriction induced longevity extension and insulin like peptide signaling [23], *JNK* has been reported to function in the PI to extend longevity [15,16]. In these studies, *JNK* is supposed to function in the PI. This is in contrast to the function of *JNK* in sleep described in this study.

Taken together, although the mechanism is still unknown, our data points to a novel sleep regulating function for *JNK*, which may be independent of its function in metabolic regulation.

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