Drosophila ATF-2 Regulates Sleep and Locomotor Activity in Pacemaker Neurons[▽]

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Stress-activated protein kinases such as p38 regulate the activity of transcription factor ATF-2. However, the physiological role of ATF-2, especially in the brain, is unknown. Here, we found that *Drosophila melanogaster* ATF-2 (dATF-2) is expressed in large ventral lateral neurons (l-LN_s) and also, to a much lesser extent, in small ventral lateral neurons, the pacemaker neurons. Only l-LN_s were stained with the antibody that specifically recognizes phosphorylated dATF-2, suggesting that dATF-2 is activated specifically in l-LN_s. The knockdown of dATF-2 in pacemaker neurons using RNA interference decreased sleep time, whereas the ectopic expression of dATF-2 increased sleep time. dATF-2 knockdown decreased the length of sleep bouts but not the number of bouts. The ATF-2 level also affected the sleep rebound after sleep deprivation and the arousal threshold. dATF-2 negatively regulated locomotor activity, although it did not affect the circadian locomotor rhythm. The degree of dATF-2 phosphorylation was greater in the morning than at night and was enhanced by forced locomotion via the dp38 pathway. Thus, dATF-2 is activated by the locomotor while it increases sleep, suggesting a role for dATF-2 as a regulator to connect sleep with locomotion.

The transcription factor ATF-2 is a member of the ATF/CREB family and has a bZIP-type DNA-binding domain (18, 34). In mammals, ATF-2 is ubiquitously expressed, with the highest expression level observed in the brain (53). ATF-2 forms either a homodimer or heterodimers with c-Jun, which directly binds to the cyclic AMP (cAMP) response element (CRE; 5'-TGACGTCA-3') (17, 18, 34). ATF-2 bears the *trans*-activation domain in its N-terminal region, which contains the sites phosphorylated by stress-activated protein kinases such as p38. Stress-activated protein kinases phosphorylate ATF-2 at Thr-69 and Thr-71 in the N-terminal transcriptional activation domain and thereby enhance its *trans*-activating capacity (16, 32, 54). Phosphorylated ATF-2 (P-ATF-2) activates the transcription of various genes, including apoptosis-inducing genes, which are involved in the regulation of the proliferation or the differentiation of various types of cells (5, 33, 35). The study using the knockout mice indicated that ATF-2 family transcription factors are required for the development of the placenta and fetal liver (7, 33), and it also acts as a tumor susceptibility gene of mammary tumors (35).

Drosophila melanogaster has one homologue of ATF-2 (dATF-2) that has the bZIP-type DNA-binding domain and the p38 phosphorylation sites in its C- and N-terminal regions, respectively (44). Like mammalian ATF-2, the *trans*-activating capacity of dATF-2 is activated via phosphorylation at Thr-59 and Thr-61 by *Drosophila* p38 (dp38) in response to various stresses, such as osmotic stress. dATF-2 also binds to the CRE

as a homodimer or a heterodimer with *Drosophila* Jun (dJun) and activates the transcription of the target genes (44). Recently, we demonstrated that dATF-2 in the fat body, the fly equivalent of the mammalian liver and adipose tissue, plays a critical role in the regulation of fat metabolism by activating the transcription of the *Drosophila* phosphoenolpyruvate carboxykinase gene (38). However, the physiological role of ATF-2, especially in the brain, remains unknown.

Sleep has been identified in almost every animal species and serves an essential function. In addition, sleep-like states also have been identified in several invertebrates. *Drosophila* recently has emerged as a useful system to study sleep (22, 46). Sleep is tightly regulated in a homeostatic manner. A homeostatic drive increases during waking and disappears during sleep. Furthermore, the timing of sleep is controlled by the circadian system, which ensures that sleep occurs at the appropriate time of day. Although the key mechanisms controlling the circadian timing of sleep are well understood, those determining the amount of sleep remain unclear.

Here, we have demonstrated that the knockdown of dATF-2 in pacemaker neurons decreases sleep time and increases locomotor activity. The degree of dATF-2 phosphorylation was enhanced by forced locomotion via the dp38 pathway. Thus, dATF-2 plays a critical role in the regulation of sleep and locomotion.

MATERIALS AND METHODS

Fly stocks. All flies were reared at room temperature on standard medium containing agar, dry yeast, corn meal, glucose, and propionic acid. The *Drosophila melanogaster* strains used in this study were w¹¹¹⁸ (wild type), UAS-dATF-2IR R-2 (38), UAS-dATF-2^{WT} (38), UAS-GFPnls, UAS-p38DN (2), UAS-bskDN (1), UAS-dMekk1 (26), pdf-GAL4 (40), c253-GAL4 (42), p38a¹ (11), and dMekk1^{Ur36} (26). Since *Drosophila* behavior such as sleep is highly influenced by the genetic background, the genetic backgrounds of the lines being compared

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must be as similar as possible. To do this, the UAS-dATF-2IR R-2 and UAS-dATF-2^{WT} lines were backcrossed to a Canton-S stock with the w¹¹¹⁸ mutation six times. Thus, the control (w¹¹¹⁸), UAS-dATF-2IR R-2, and UAS-dATF-2^{WT} lines have almost the same genetic background. Since the pdf-GAL4 line also had the same genetic background, the control, pdf>dATF-2 IR, and pdf>dATF-2^{WT} lines had the same genetic background.

Generation of phosphorylated dATF-2 (P-dATF-2)-specific antibody. Rabbit polyclonal antibodies were raised against a synthetic peptide corresponding to a region containing p38 phosphorylation sites (from leucine 53 to lysine 67). The sequence of the peptide was L-F-A-A-D-Q-pT-P-pT-P-T-R-L-I-K, in which Thr-59 and Thr-61 were phosphorylated. For immunoblotting, the antiserum was affinity purified using the antigen conjugated to *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow (GE Healthcare Life Sciences). Anti-dATF-2 antibodies were previously described (38).

Western blotting. The pact5C-FLAG-dATF-2 expression plasmid was reported previously (44). S2 cells were maintained in Schneider's medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma) and 10 mg/ml of penicillin-streptomycin (GIBCO). S2 cells were grown to about 50% confluence in 9-cm dishes and then transfected with 10 μ g of pact5C-FLAG-dATF-2 expression plasmid or 10 μ g of the empty pact5C vector by the CaPO₄ method. Forty-eight hours after transfection, cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, 1% NP-40, 0.1% deoxycholate, 0.1% sodium dodecyl sulfate, 400 mM NaCl, 50 mM EDTA, 0.1 mM Na₃VO₄, 50 mM NaF, 10 mM β -glycerophosphate, 10 mM Na₂P₂O₇). In some cases, cells were treated with 0.5 M sorbitol for 15 min before lysate preparation. Lysates proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blotting analysis with anti-dATF-2 or anti-P-dATF-2 antibodies (1:1,000).

Immunocytochemistry. For each line, 15 male flies/vial were entrained in a 12-h LD cycle at 25°C for 3 to 5 days. Flies were anesthetized on ice at each zeitgeber time (ZT2, ZT5, ZT8, ZT11, ZT14, ZT17, ZT20, and ZT23). After a 5-s wash in 70% ethanol on ice, six to eight brains were dissected in phosphate-buffered saline (PBS) within 30 min and stored in ice-cold fixative (4% paraformaldehyde in PBS), followed by fixation in fresh fixative at room temperature for 30 min. After three washes with PBS-TX (0.3% Triton X-100 in PBS) at room temperature, the tissue was soaked in blocking solution (2% normal donkey serum in PBS-TX) for 60 min at 4°C and then incubated with primary antibody for 48 to 72 h at 4°C. The primary antibodies used in this study were rat anti-TIM (1:2,000; a gift of A. Sehgal), rabbit anti-PDF (1:10,000; a gift of F. Rouyer), rabbit anti-dATF-2 (1:100), and rabbit anti-phosphorylated dATF-2 (1:10); all primary antibodies were diluted in blocking solution. Tissue then was incubated in secondary antibody for 24 to 48 h at 4°C. The secondary antibodies used in this study were fluorescein isothiocyanate-conjugated donkey anti-rat antibody (1:1,000; Jackson ImmunoResearch) for TIM staining and Cy5-conjugated donkey anti-rabbit antibody (1:1,000; Chemicon) for PDF, dATF-2, and P-dATF-2 staining.

Confocal images were obtained using an LSM510 (Zeiss) laser-scanning microscope. For quantitative immunostaining experiments, identical laser power and acquisition settings were used. The mean pixel density of images was measured by LSM510 3.2 software at a depth of 8 bits. The signal located specifically at the large ventral lateral neurons (l-LN_vs) was assayed by dividing the mean pixel density measured in the region of interest (1 μ m by 1 μ m) placed over the l-LN_vs by that measured in a region outside the l-LN_vs. Thus, the relative amount of each protein was calculated as $([A]_{\text{l-LN}_v} - [A]_{\text{outside l-LN}_v}) / ([A]_{\text{outside l-LN}_v})$, in which $[A]$ means the staining intensity of dATF-2, P-dATF-2, or TIM. The relative amount of each protein was quantified in 10 to 16 hemispheres of the brain. The values obtained by this method were used to compare various strains (see Fig. 3B and 8), times (see Fig. 9D), and stress conditions (see Fig. 6B and C). For instance, in Fig. 2B, the values of the dATF-2 RNA interference (RNAi) line and the dATF-2 overexpression line are indicated relative to that of the control line.

Behavioral analysis. Individual male flies were entrained in a 12-h LD cycle for 3 days and transferred to glass tubes for monitoring. Their locomotor activity at 25°C was scored in 1-min bins using the *Drosophila* activity monitoring system (DAMS; Trikinetics, MA). Data were collected continuously for 3 days using a 12-h LD cycle and then 2 weeks in constant darkness (DD). The period of locomotor activity rhythm was determined by a chi-square periodogram using ClockLAB (Actimetrics). The total sleep time, the sleep bout duration, and the number of sleep bouts were calculated based on the sleep definitions described previously (9, 27). As reported by previous studies (22, 46), we have defined a rest bout as >5 min of no activity. Quantitative analyses were performed with at least 32 flies, and all behavioral experiments were replicated in each genotype.

Sleep homeostasis and arousal threshold. To disrupt rest behavior, mechanical stimuli were applied with a timer-controlled rotating shaker (CM-1000;

Eyela Co., Ltd., Tokyo, Japan), which has a 6-mm turning diameter. The intensity of stimuli was graded as level 1 (1,000 rpm), level 2 (1,500 rpm), and level 3 (2,000 rpm). The observation of rest rebound after sleep deprivation was performed as follows: after 2 baseline days, the DAM2 activity monitor was shaken at level 2 once every minute (1 s/1 min) for 12 h until the lights were turned on (ZT12 to ZT0). The level 2 stimulation in every minute was sufficient to keep all flies awake during nighttime. The activity/rest behavior was monitored continuously for 3 postdeprivation days. The gain-of-rest behavior (sleep rebound) was calculated individually based on the data for baseline days. To evaluate the stimulus intensity required for waking the resting flies, one-off stimulation was done as follows: after 2 baseline days, the DAM2 monitor was shaken at three different levels once (1 s) at midnight (ZT19), when the rest is most consolidated (22). Before the stimulation, all tested flies exhibited sleep behavior (>5-min rest) during 30 min. After the stimulation, the start time at which the stimulated flies resumed the sleep behavior was analyzed until lights were turned on. The flies showing no sleep behavior until 30 min after the stimulation were defined as fully woken animals.

Treatment with forced locomotion. For testing the effects of forced locomotion, a vial with 15 flies was rotated for 1 h at the speed of $\sim$ 2 cm/s, with tapping every 5 min to keep the flies walking. The flies' brains were dissected subsequently and processed as described above.

Luciferase reporter assays. pact5C-dClk and pact5C-*Renilla* luciferase expression plasmids were constructed in a way that was similar to that for the pact5C-FLAG-dATF-2 expression plasmid. A series of truncated *timeless* (*tim*) reporters (from bp +50 to -2400, -1900, -1600, -1300, -850, or -500, where +1 is the RNA start site) were constructed into the pGL3(R2.2)-Basic vector using a PCR-based method. Site-directed mutagenesis was used to introduce point mutations into possible dATF-2 binding sites (at -1460 or -1820) within the 1.9-kb *tim* promoter, with a complementary primer set of 5'-ATTGTGAGTGAGTGAATTGGTAAAATGAGAGTGT-3' (-1472 to -1439) or 5'-TGATCGCACAACTTAAGTGGAGTGGAACTTTGG-3' (-1796 to -1829), respectively. S2 cells were maintained in Schneider's medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma) and 10 mg/ml of penicillin-streptomycin (GIBCO). S2 cells were grown to about 50% confluence in 12-well tissue culture plates and then transfected with 1 μ g of *tim* reporter construct together with the pact5C-FLAG-dATF-2 expression plasmid (1 μ g), pact5C-dClk (0.05 or 0.1 μ g), and the internal control plasmid pact5C-*Renilla* luciferase (0.15 μ g) by the CaPO₄ method. The total amount of plasmid DNA introduced was adjusted to 3 μ g with the empty pact5C vector. The transfection reagents were washed off and replaced with Schneider's medium 10 h later, and after another 20 h, luciferase activities were measured using the dual-luciferase assay system (Promega). The transfection efficiency was normalized with *Renilla* luciferase activity expressed from the internal control plasmid pact5C-*Renilla*.

Statistical analysis. Data of behavioral analysis, luciferase assay, and fluorescence intensities obtained from immunostaining studies are presented as means $\pm$ standard errors of the means (SEM). Student's *t* test was used to compare all of the results. *P* values of <0.05 were considered significant.

RESULTS

dATF-2 is expressed predominantly in l-LN_vs and, to a much lesser extent, in s-LN_vs. To determine which regions of the brain express dATF-2, fly brains were stained with an anti-dATF-2 antibody. dATF-2 staining was detected in a subset of lateral neurons and colocalized with green fluorescent protein (GFP) carrying a nuclear localization signal (GFPnls) that was expressed by GAL4 driven by the neuropeptide pigment-dispersing factor (*pdf*) gene promoter (Fig. 1A). The *pdf*-GAL4 construct drives gene expression in small and large lateral neurons (s-LN_vs and l-LN_vs) (40). Figure 1A and B show the z-dimension composite of serial 10- μ m images of brain stained with anti-dATF-2 antibody, demonstrating that these data contain the dATF-2 signals of almost the whole brain. The results showed that dATF-2 is expressed in l-LN_vs and also, to a much lesser extent, in s-LN_vs. In addition to l-LN_vs and s-LN_vs, we also found punctate staining that might be neurites extending from LN_vs (Fig. 1B). Although anti-dATF-2 antibody also stained the optic lobe, several cells on

FIG. 1. Expression of dATF-2 in the pacemaker neurons. (A) dATF-2 is expressed predominantly in l-LN_vs and, to a much lesser extent, in s-LN_vs. Fly brains expressing GFPnls (green) using the *pdf*-GAL4 driver were stained with anti-dATF-2 antibody (red). The two images showing GFPnls and dATF-2 are merged in the right panel. Arrows and arrowheads indicate l- and s-LN_vs, respectively. Scale bar, 100 μ m. (B) dATF-2 expression in the brain. The wider region of the brain stained with anti-dATF-2 antibody (red) is shown. The region indicated in panel A is surrounded by a square. Punctate staining, which might be neurites extending from LN_vs, are shown by double arrowheads. The asterisk indicates a nonspecific signal that also was obtained using preimmune serum. Scale bar, 300 μ m. (C) Preparation of the P-dATF-2-specific antibody. S2 cells were transfected with the Flag-dATF-2 expression plasmid and treated with or without sorbitol. Whole-cell lysates were prepared and analyzed by Western blotting using anti-Flag or anti-P-dATF-2 antibody. IB, immunoblot. (D) Phosphorylated dATF-2 is expressed only in l-LN_vs. Flies expressing GFPnls (green) using the *pdf*-GAL4 driver were maintained on a 12-h LD cycle. At ZT5, the brains were stained with anti-P-dATF-2 antibody and analyzed using confocal microscopy. Arrows and arrowheads indicate l-LN_vs and s-LN_vs, respectively. Scale bar, 50 μ m. (E) P-dATF-2 is not expressed in s-LN_vs of larval brain. The larval brains expressing GFPnls (green) using the *pdf*-GAL4 driver were stained with anti-dATF-2 (upper) or anti-P-dATF-2 (lower) antibody and analyzed as described above.

the brain surface, and several large single cells (each one a pair on the ventral and dorsal sides of LN_vs), all of these cells also were stained with the preimmune sera. These results indicate that LN_vs were the only cells stained with anti-dATF-2 anti-

FIG. 2. dATF-2 is not expressed in mushroom bodies. Fly brains that expressed GFPnls (green) in mushroom bodies using the *c253* driver were stained with anti-dATF-2 antibody (red). The same confocal microscopy optical sections are shown. The two images showing GFPnls and dATF-2 are merged in the right panel.

body specifically. Although the s-LN_vs and the dorsal lateral neurons were recently reported to be neuronal substrates for the morning and evening oscillators (15, 51, 52), the function of l-LN_vs remains unknown. The dATF-2 signals did not colocalize with GFP expressed by the *c253*-GAL4 driver, which directs gene expression in adult mushroom bodies (12) (Fig. 2). Thus, dATF-2 is not expressed in mushroom bodies, which play a critical role in sleep regulation (27, 42).

Phosphorylated dATF-2 is expressed only in l-LN_vs. dATF-2 is activated via phosphorylation at Thr-59 and Thr-61 by dp38a and dp38b (44). To detect the activated form of dATF-2 in the brain, we generated an antibody that can recognize dATF-2 that is phosphorylated at these sites. To confirm the specificity of this antibody, FLAG-tagged dATF-2 was overexpressed in S2 cells, and cells were subjected to osmotic stress. This antibody recognized only dATF-2 that was phosphorylated in response to osmotic stress (Fig. 1C). Although the anti-dATF-2 antibody stained l-LN_vs and, also, to a much lesser extent, s-LN_vs (Fig. 1A), the anti-P-dATF-2 antibody stained only l-LN_vs and not s-LN_vs (Fig. 1D). dATF-2 is localized mainly in the nucleus in l-LN_vs (Fig. 1A), while P-dATF-2 is mainly in the cytoplasm (Fig. 1D), although some amounts of P-dATF-2 are in the nucleus. ATF-2 was reported to localize both in the cytoplasm and the nucleus (6). Further, ATF-2 contains the nuclear export sequence in its leucine zipper region (31), suggesting that its subcellular localization is modulated by the dimerization partner. Therefore, the phosphorylation of dATF-2 in l-LN_vs could change the dimerization partner, leading to an enhancement in the localization in the cytoplasm. This may function to suppress the continuous induction of the ATF-2 target genes.

s-LN_vs project to centers that are associated with sleep and activity. l-LN_vs do not, and their physiological function remains unknown. Moreover, the PDF driver expresses in both s-LN_vs and l-LN_vs, and distinguishing between the two types of cells is difficult sometimes. To further confirm that P-dATF-2 is not expressed in s-LN_vs, we have stained larvae in which the only the precursors of the s-LN_vs and a subset of the dorsal neurons, but not l-LN_vs, are present as demonstrated previously (for example, see reference 20). Although dATF-2 signals were detected in larval s-LN_vs, no P-dATF-2 signal was found (Fig. 1E). These results further support the notion that the phosphorylation of dATF-2 occurs only in l-LN_vs.

Enhanced locomotor activity and reduced sleep time of dATF-2 knockdown flies. To examine the role of P-dATF-2 in l-LN_vs, we used dATF-2 knockdown flies and dATF-2-overex-

FIG. 3. Up- and downregulation of dATF-2 in l-LN_s. (A) Analysis of the brains of *dATF-2* knockdown flies, in which *dATF-2* dsRNA was expressed using the *pdf*-GAL4 driver, and of *dATF-2*-overexpressing flies. Genotypes are as follows. *dATF-2* knockdown flies (*pdf*>IR), *pdf*-GAL4/+ *UAS-dATF-2IR*/+; *dATF-2*-overexpressing flies (*pdf*>dATF-2WT), *pdf*-GAL4/+ *UAS-dATF-2*/+; control flies (*pdf*-G/+), *pdf*-GAL4/+. Arrows indicate l-LN_s. Scale bar, 50 μ m. (B) Signal intensities from immunostaining images were quantified, and the average of the total intensity of 10 to 16 independent hemispheres was obtained. The values of the *dATF-2* RNAi line and the *dATF-2* overexpression line are indicated relative to that of the control lines by a bar graph, with SEM. ***, $P < 0.001$.

pressing flies. We recently established transgenic fly strains bearing inverted repeats (IRs) of 500-bp DNA sequences corresponding to the NH2 termini of dATF-2 coding regions under the control of upstream activating sequences (UAS) for the GAL4 transcription factor (UAS-IR-dATF-2) (38). Using this transgenic fly line, we previously demonstrated that the dATF-2 mRNA levels were reduced to ~40% of the wild-type level in the fat body when dATF-2 double-stranded RNA (dsRNA) was expressed using the *c564*-GAL4 driver (38). Further, the dATF-2 mRNA level in dATF-2 transgenic flies was two- to fivefold higher than the wild-type level. To test the effectiveness of dATF-2 transgenic RNAi, we examined the ability of IR-dATF-2 to reduce endogenous dATF-2 expression. When dATF-2 dsRNA was expressed in pacemaker neurons using the *pdf*-GAL4 driver, the dATF-2 signal from anti-dATF-2 antibodies in l-LN_s was reduced by about 48% (Fig. 3A and B). On the other hand, when dATF-2 was ectopically expressed using the *pdf*-GAL4 driver, the dATF-2 signals increased about 84%. Since the *pdf*-GAL4 construct drives gene expression not only in l-LN_s but also in s-LN_s, the level of dATF-2 in s-LN_s also was changed in these transgenic flies. However, P-dATF-2, the activated form of dATF-2, is ex-

pressed only in l-LN_s. Thus, the *pdf*-GAL4 driver can be used to modulate the amount of P-dATF-2 in l-LN_s, although we cannot exclude the possibility that the effect is due to the change in nonphosphorylated dATF-2 in s-LN_s.

The localization of dATF-2 to pacemaker neurons raised the possibility that dATF-2 regulates behavior such as locomotor activity rhythm and sleep. The locomotor activity of control flies showed a diurnal pattern with two peaks at dawn and evening, respectively, under 12-h LD conditions (Fig. 4A). Using the *pdf*-GAL4 driver, both the *dATF-2* knockdown flies and *dATF-2*-overexpressing flies exhibited circadian patterns of locomotor activity that were similar to those of control flies. The free-running period in constant darkness of *dATF-2* knockdown and *dATF-2*-overexpressing flies ($t = 24.7 \pm 0.21$ h and 25.2 ± 0.40 h, respectively) were similar to the period for control flies ($t = 24.5 \pm 0.25$ h). These results indicated that dATF-2 does not affect the circadian locomotor rhythm. However, the locomotor activity of *dATF-2* knockdown flies was 59% higher than that of control flies, whereas *dATF-2*-overexpressing flies exhibited a 34% lower locomotor activity (Fig. 4A, right). These results suggest that dATF-2 suppresses locomotor activity but does not affect its circadian regulation.

Drosophila melanogaster flies exhibit a sleep-like state that is under both circadian and homeostatic regulation (22, 46). We examined whether the dATF-2 level affects the regulation of sleep. In this study, we defined sleep as behavioral immobility lasting 5 min or more, as reported previously (22, 46). Under 12-h LD conditions, the *dATF-2* knockdown flies slept less than control flies (408 ± 32 min versus 563 ± 32 min per day), whereas *dATF-2*-overexpressing flies slept more (692 ± 31 min per day) (Fig. 4B). We obtained similar results under DD conditions (Fig. 5A). When we defined sleep as behavioral immobility lasting 30 min or more, the effect of *dATF-2* knockdown or overexpression was even more dramatic (Fig. 5B and C). This was due to decreases and increases, respectively, in the length of each sleep episode in *dATF-2* knockdown and in *dATF-2*-overexpressing flies, not to decreases and increases in the number of sleep bouts (Fig. 4C). Thus, dATF-2 affects the duration of sleep in flies.

Phosphorylation of dATF-2 in l-LN_s shows a diurnal rhythm and is enhanced by exercise. The anti-P-dATF-2 antibody specifically stained l-LN_s at ZT5 (Fig. 1D). Since dATF-2 in pacemaker neurons appears to be involved in the regulation of sleep and locomotor activity, we examined P-dATF-2 levels in l-LN_s at various times during the 12-h LD cycle. The amount of P-dATF-2 was relatively high at early morning and midmorning (ZT2 and ZT5) time points, while it was low during the night (ZT17 to ~ZT23) (Fig. 6A). In contrast, dATF-2 levels in l-LN_s appeared to be similar at various times (data not shown). Thus, the phosphorylation of dATF-2 in l-LN_s shows a diurnal rhythm. *Drosophila melanogaster* has two locomotor activity peaks, one in the morning and another in the evening. The level of P-ATF-2 increases at ZT2, after the morning peak of locomotor activity, and then reaches a peak at ZT5, when the locomotor activity is at a minimum. Thus, the correlation between locomotor activity and P-ATF2 levels holds for the morning peak of activity but not the evening peak.

Our observations suggested a possible link between dATF-2 phosphorylation and locomotor activity. To investigate this

FIG. 4. *dATF-2* regulates locomotor activity and sleep. (A) Increased locomotor activity of *dATF-2* knockdown flies. The locomotor activity records of male control (pdf-G/+), *dATF-2* knockdown (pdf>IR), and *dATF-2*-overexpressing flies (pdf>dATF-2WT) are shown. Adult flies were entrained to cycles of 12-h LD, and their locomotor activity was measured for 3 days in 12-h LD. The white and black horizontal bars above the panels indicate light and dark periods, respectively. The curves connect mean values $\pm$ SEM (pdf-G/+, $n = 29$; pdf>IR, $n = 25$; pdf>dATF-2WT, $n = 31$). Asterisks indicate the values that are statistically different from those of control flies. *, $P < 0.05$. On the right, the locomotor activity of three types of flies is shown with SEM. ***, $P < 0.001$. (B) Decreased sleep of *dATF-2* knockdown flies. Daily time course (30-min intervals) of the amount of sleep in male control (pdf-G/+), *dATF-2* knockdown (pdf>IR), and *dATF-2*-overexpressing flies (pdf>dATF-2WT). Curves connect mean values $\pm$ SEM (pdf-G/+, $n = 29$; pdf>IR, $n = 25$; pdf>dATF-2WT, $n = 31$). *, $P < 0.05$. (C) Shortened sleep bout duration of *dATF-2* knockdown flies. (Left) Sleep bout number per 24 h in 12-h LD is indicated, with SEM. (Right) Sleeping episodes during a 24-h period were categorized based on their duration, and the total amount of sleep in each category per day is indicated, with SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

further, we examined whether forced locomotion induced the phosphorylation of dATF-2. We provided flies with forced locomotion at ZT8, when flies normally show lower locomotor activity, by forcing them to walk for 1 h. This enhanced the level of P-dATF-2 in l-LN_vs (Fig. 6B). Thus, forced locomotion appears to enhance the phosphorylation of dATF-2 in l-LN_vs. We observed that forced locomotion at night (ZT20) also stimulated the phosphorylation of dATF-2 (Fig. 6B). This strongly suggests that the peak of P-dATF-2 in the morning is not due to photoperiodic reg-

ulation and coincides with the morning peak of locomotor activity. We further gave different lengths of forced locomotion at ZT10, when flies are relatively active and have a low amount of sleep. Forced locomotion for 1 or 2 h enhanced the level of phosphorylation of dATF-2, but forced locomotion for 30 min did not (Fig. 6C). Thus, the stimulation of dATF-2 phosphorylation needs at least 1 h of forced locomotion, and longer locomotion does not enhance the dATF-2 phosphorylation any further. However, since enforced locomotion always causes sleep deprivation and non-

FIG. 5. *dATF-2* levels affect sleep in DD conditions. (A) Daily time course (30-min intervals) of the amount of sleep, defined here as behavioral immobility lasting 5 min or more, in male control (*pdf>G/+*), *dATF-2* knockdown (*pdf>IR*), and *dATF-2*-overexpressing flies (*pdf>dATF-2WT*). Adult flies were entrained to cycles of 12-h LD, and their locomotor activity then was measured in DD for 3 days. Curves connect the mean values $\pm$ SEM. (*pdf>G/+*, $n = 29$; *pdf>IR*, $n = 25$; *pdf>dATF-2WT*, $n = 31$). *, $P < 0.05$. (B and C) Daily time course (30-min intervals) of the amount of sleep, defined here as behavioral immobility lasting 30 min or more. Experiments were performed as described above in 12-h LD (B) or DD (C).

specific stress, we cannot completely exclude the possibility that the enhanced phosphorylation of dATF-2 is caused by sleep deprivation or nonspecific stress.

dATF-2 affects sleep homeostasis and the arousal threshold. dATF-2 is known to be activated via phosphorylation by dp38

in response to various stresses (11, 44). As described above, dATF-2 in *l-LN_vs* is phosphorylated in response to forced locomotion (Fig. 6B), while the level of dATF-2 in *l-LN_vs* affects the sleep amount (Fig. 4B). These results allowed us to speculate that dATF-2 is phosphorylated in response to loco-

FIG. 6. Phosphorylation of dATF-2 in l-LN_s shows a diurnal rhythm and is enhanced by forced locomotion. (A) The diurnal rhythm of dATF-2 phosphorylation in l-LN_s. Flies expressing GFPnls (green) using the *pdf*-GAL4 driver were maintained on a 12-h LD cycle, indicated by the white and black horizontal bars, respectively, below the panel on the right. At various times, the brains were stained with anti-P-dATF-2 or anti-dATF-2 antibodies and analyzed using confocal microscopy. Typical staining patterns at ZT5 and ZT17 are shown on the left. Arrows indicate l-LN_s. Scale bar, 50 μ m. The signals of P-dATF-2 and dATF-2 in l-LN_s at various times were measured (data not shown), and the ratios are indicated by bar graphs on the right. (B) Forced locomotion enhances the phosphorylation of dATF-2. The amount of P-dATF-2 in l-LN_s at ZT8 or ZT20 was measured as described above using flies that had 1 h of forced locomotion or no forced locomotion. Typical staining patterns at ZT8 are shown on the left, and the amount of P-dATF-2 in l-LN_s is indicated as the value relative to that of flies without forced locomotion by the bar graph (with SEM) on the right. Arrows and arrowheads indicate l-LN_s and s-LN_s, respectively. Scale bar, 50 μ m. (C) Effect of different lengths of forced locomotion on the phosphorylation of dATF-2. The amount of P-dATF-2 in l-LN_s at ZT10, when flies are relatively active and have small amounts of sleep, was measured using flies that had different lengths of forced locomotion.

motor activity and induces sleep. If this is the case, dATF-2 may affect sleep homeostasis and the arousal threshold. To examine the effect of the dATF-2 levels on sleep homeostasis, sleep rebound after sleep deprivation was measured using three types of flies, *dATF-2* knockdown, *dATF-2*-overexpressing, and control flies. When sleep is deprived during nighttime, sleep rebound is most clearly observed in the morning period immediately after sleep deprivation (22). Therefore, we measured the sleep rebound during 2 h in the morning immediately after sleep deprivation during nighttime. When sleep was deprived by treatment with mild vibration once per min for 12 h during ZT12 and ZT0, *dATF-2*-overexpressing flies exhibited a significant increase of sleep rebound compared to the sleep rebound of *dATF-2* knockdown and control flies (Fig. 7A and B). There was no significant difference in sleep rebound between *dATF-2* knockdown and control flies. The dATF-2 signal detected by anti-dATF-2 antibodies in l-LN_s was reduced by about 48% in the *dATF-2* knockdown flies used in this study (Fig. 3), but more severe reduction could be required to see the effect on sleep homeostasis.

To investigate the role of dATF-2 in the arousal threshold, three types of flies were examined at various strengths of one

1-s vibrations during the night, when rest is most consolidated (22), and the number of flies that did not resume sleep behavior until 30 min after stimulation was measured. Before the stimulation, all tested flies exhibited sleep behavior (>5 min rest) during 30 min. The number of flies that did not sleep after stimulation (animals awakened by stimulus) increased when the vibration strength was enhanced (Fig. 7C). The *dATF-2* knockdown flies were the most sensitive to this arousal stimulus, while the *dATF-2*-overexpressing flies were the most insensitive (Fig. 7C). These results indicate that the dATF-2 level is correlated with the arousal threshold. Thus, the dATF-2 level in l-LN_s affects both sleep homeostasis and the arousal threshold, suggesting that dATF-2 acts as a sleep regulator.

Phosphorylation of dATF-2 in l-LN_s is controlled by the dp38 pathway. To investigate whether the dp38 pathway regulates dATF-2 phosphorylation in l-LN_s, we examined P-dATF-2 levels in a *dp38a* mutant and in a *dMekk1* mutant. *dMekk1* is a kinase upstream of dp38a. In these mutants, the level of P-dATF-2 in l-LN_s was lower than that in control brains (Fig. 8A). Furthermore, when a dominant-negative form of dp38b was expressed in l-LN_s using the *pdf*-GAL4

FIG. 7. Role of dATF-2 in sleep homeostasis and arousal threshold. (A and B) Increased sleep rebound response after sleep deprivation in flies overexpressing dATF-2. Sleep in male control (pdf-G/+), dATF-2 knockdown (pdf>IR), and dATF-2-overexpressing flies (pdf>dATF-2WT) early in the morning after a 12-h sleep deprivation (ZT12 to ~ZT0) (red) or before sleep deprivation (gray) is shown with SEM (30-min intervals). (A) Curves connect mean values $\pm$ SEM ($n = 32$ for each). (B) The amount of sleep gains for 2 h immediately after sleep deprivation is shown, with SEM. *, $P < 0.05$. (C) Alterations in the arousal threshold depending on dATF-2 expression. Three types of flies ($n = 32$ of each) were treated with different intensities of vibration once (1 s at ZT19) during the night, and the number of flies that did not resume sleep behavior (a rest of more than 5 min) until 30 min after vibration is indicated with SEM. Since all flies tested were exhibiting sleep behavior before vibration, the number of flies that had no sleep after vibration is correlated with the arousal threshold. *, $P < 0.05$; **, $P < 0.01$.

driver, the level of P-dATF-2 was decreased (Fig. 8B). On the other hand, the level of P-dATF-2 was increased when dMeKK1 was overexpressed in l-LN_s using the *pdf*-GAL4 driver. The overexpression of the dominant-negative form of Bsk (Basket), *Drosophila* JNK, did not strikingly affect the phosphorylation status of dATF-2, which is consistent with our previous observation that dATF-2 is not phosphorylated by Bsk (44). From these results, we conclude that dATF-2 phosphorylation in l-LN_s is regulated by the dp38 pathway. It was previously demonstrated that dp38 phosphorylates dATF-2 and that the overexpression of dp38 or dMKK3 enhanced the *trans*-activating capacity of dATF-2 (44), while the p38 inhibitor, SB203580, suppressed the *trans*-activating capacity of dATF-2 (38). Thus, the P-dATF-2 levels appear to correlate with the dATF-2 activity.

FIG. 8. Phosphorylation of dATF-2 is regulated by the dp38 pathway. (A) dp38 pathway mutants show decreased dATF-2 phosphorylation. The amount of P-dATF-2 in l-LN_s at ZT5 was measured as described in the legend to Fig. 3 using flies with the indicated genotype. The amounts of P-dATF-2 are indicated as values relative to that of wild-type flies (with SEM) in the bar graph. ***, $P < 0.001$. (B) Effect of dp38 and MeKK1 on the phosphorylation of dATF-2. The amount of P-dATF-2 in l-LN_s at ZT5 was measured as described in the legend to Fig. 2 using flies expressing dMeKK1 or the dominant-negative (DN) form of dp38b or Basket using the *pdf*-GAL4 driver. The amounts of P-dATF-2 are indicated as the values relative to that of control flies (pdf>Gal4), with SEM, in the bar graph. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

dATF-2 directly regulates *tim* transcription. We previously looked for putative dATF-2 target genes by DNA array analysis using RNA prepared from S2 cells treated with dATF-2 dsRNA (44). In that study, dATF-2 dsRNA strongly downregulated the *tim* gene. Since *tim* encodes a protein that is key for circadian rhythm (19) and sleep homeostasis (22), we examined whether dATF-2 directly regulates the transcription of the *tim* gene. In a reporter assay using the *tim* promoter-containing luciferase reporter, dATF-2 activated transcription from the 2.5-kb *tim* promoter fragment about 12-fold; truncation to 1.9-kb did not affect the degree of activation by dATF-2 (Fig. 9A). Further truncation to 1.6, 1.3, 0.85, and 0.5 kb gradually decreased the dATF-2-dependent activation of the *tim* promoter, suggesting the presence of multiple dATF-2-binding sites in the region between -1900 and -500. Searching the DNA sequence in this region revealed the presence of the CRE half-site at -1460 and -1820. The disruption of either or both of these sites significantly reduced the degree of activation by dATF-2. These results indicate that dATF-2 directly activates the *tim* promoter. ATF-2 forms a heterodimer with Jun (17) and also with C/EBP α (50), and ATF-2 in these complexes recognizes the half-site of CRE. Thus, the half-site of CRE in the *tim* promoter may be responsible for the dATF-2-dependent activation. The transcription factor dClock, which contains the basic helix-loop-helix-Pas domain, activates the *tim* promoter by directly binding to the E box (3, 14). Both dClock and dATF-2 activated the *tim* promoter, and transcription stimulation was additive when they were coexpressed (Fig. 9B).

We also analyzed the levels of TIM in l-LN_s in dATF-2 knockdown flies, dATF-2-overexpressing flies, and control flies. The level of TIM in the early morning (at ZT2) was very low, as reported previously (24, 36, 45, 57), because TIM is degraded by light, but a small amount of TIM was

FIG. 9. Transcriptional activation of the *tim* gene by dATF-2. (A) Analysis of the *tim* promoter. A reporter plasmid in which the mutated *tim* promoter region was linked to the luciferase gene was transfected into S2 cells together with the dATF-2 expression plasmid or a control plasmid. The luciferase activity was measured. The reporter plasmids used are shown schematically on the left. X indicates the mutated sites containing CRE-like sequences, in which the sequence matched with the consensus CRE is indicated by boldface letters. The ratio of luciferase activity in the presence of dATF-2 to the activity without dATF-2 is indicated as the change (*n*-fold) in activation. The averages and standard deviations from three experiments are indicated. (B) dATF-2 activates the *tim* promoter together with dClk. A reporter plasmid in which the 1.9-kb *tim* promoter region was linked to the luciferase gene was transfected into S2 cells together with the dATF-2 expression plasmid (0, 0.5, or 1.0 μ g) and the indicated amount (+, 50 ng; ++, 100 ng) of the dClk expression plasmid or control plasmid. The luciferase activity was measured. The averages and standard deviations from three experiments are indicated. (C and D) Decreased level of TIM in *dATF-2* knockdown flies. Control, *dATF-2* knockdown, and *dATF-2*-overexpressing flies, all of which expressed GFPnls (green) using the *pdf*-GAL4 driver, were maintained on a 12-h LD cycle. At various times, fly brains were stained with anti-TIM antibody and analyzed using confocal microscopy. (C) Typical staining patterns at ZT2 are shown. Arrows indicate l-LN_s. Scale bar, 50 μ m. (D) TIM signals in l-LN_s at various times were measured, and the average of the total intensity of 10 to 16 independent hemispheres is indicated using a bar graph, with SEM. Asterisks indicate values that are statistically different from those for control flies at each time point. **, $P < 0.01$; ***, $P < 0.001$.

detectable (Fig. 9C and D). In *dATF-2* knockdown flies, no TIM immunoreactivity was visible at all at ZT2. Further, the level of TIM in *dATF-2*-overexpressing flies was higher than that in control flies at ZT2. These results suggest that dATF-2 induces TIM expression at ZT2. On the other hand, the levels of TIM in *dATF-2* knockdown flies were similar to those of control flies at ZT8, ZT14, and ZT20, although the signal at ZT8 was very weak, like that at ZT2 (Fig. 9D and 10). The upregulation of TIM in the *dATF-2*-overexpressing flies was observed at ZT14 but not at ZT8 and ZT20. These results are consistent with the data shown in Fig. 6A, in that the level of P-dATF-2 is relatively high in the early morning and midmorning compared to that at other times. Thus, P-dATF-2 upregulates TIM expression at ZT2, and this upregulation is coupled to dATF-2 phosphorylation, which is diurnal. The *tim* mRNA levels are low at ZT2, and the TIM levels at that time do not contribute to rhythm formation. These results also are consistent with our observations that the knockdown or overexpression of dATF-2 did not affect locomotor rhythm.

DISCUSSION

In summary, dATF-2 is expressed in l-LN_s and, to a much lesser extent, in s-LN_s, while P-dATF-2 is detected only in l-LN_s, suggesting that dATF-2 is activated specifically in l-LN_s. The degree of the phosphorylation of dATF-2 in l-LN_s is enhanced by forced locomotion and also increases at ZT2 after the morning peak of locomotor activity and reaches a peak at ZT5, when the locomotor activity is at a minimum. During the preparation of the manuscript, it was reported that the phosphorylation of ATF-2 in the chick pineal gland exhibits a circadian rhythm with a daytime peak (49). The down-regulation of dATF-2 in l-LN_s and s-LN_s enhanced locomotor activity and reduced sleep time. Similar results were obtained using either male or female flies. Furthermore, the dATF-2 level affects sleep homeostasis and the arousal threshold. Thus, dATF-2 appears to be a regulator connecting sleep with locomotion.

Since P-dATF-2, the activated form of dATF-2, is expressed only in l-LN_s, P-dATF-2 in l-LN_s appears to reduce locomotion.

FIG. 10. Levels of TIM in *dATF-2* knockdown flies. Fly brains were stained with anti-TIM antibody as described in the legend to Fig. 8B. Typical staining patterns at ZT8, ZT14, and ZT20 are shown. Arrows indicate l-LN_s. Scale bar, 50 μ m.

tor activity and to increase sleep time in response to certain kinds of stress induced by locomotor activity. Each hemisphere of the *Drosophila* brain contains four l-LN_s and five s-LN_s (28). s-LN_s have been thought to be the most important master neurons of clock neurons, because strong oscillations of *Per* and *Tim* are continuously maintained over several days in s-LN_s in DD (55). We have detected P-dATF-2 only in l-LN_s, although nonphosphorylated dATF-2 also is expressed in s-LN_s at a lower level than that in l-LN_s. We also found that there were almost no P-dATF-2 signals detected in larvae, which do not have l-LN_s (Fig. 1E). However, l-LN_s do not project to centers that are associated with sleep and activity, which is in contrast to s-LN_s, suggesting that l-LN_s are not involved in the regulation of sleep and activity. On the other hand, it was shown that neurite fibers of l-LN_s and s-LN_s are interconnected (28), suggesting that there is some local circuitry from l-LN_s to s-LN_s. In addition, it was recently reported that the receptor Han for PDF, which affects not only the activity rhythm but also the activity level (21), is expressed only in l-LN_s but not in s-LN_s (25), raising the possibility that Han-mediated PDF signaling contributes to the coordinated interaction of both neurons. Taking our results together with these reports, it might be possible that P-dATF-2 in l-LN_s affects the function of s-LN_s via the interaction between both neurons.

The downregulation of dATF-2 in l-LN_s led to a decrease in the levels of TIM at ZT2. It was previously reported that *tim* mutant flies have smaller amounts of sleep and poorly consolidated sleep compared to that of wild-type flies (29, 47). In addition, *tim* mutants exhibit higher locomotor activity compared to that of wild-type flies during the light phase under 12-h LD conditions (10), suggesting that the low level of TIM during the light phase has some role in suppressing locomotor activity. These phenotypes of *tim* mutants are consistent with the results that dATF-2 knockdown flies have shortened sleep bout durations and higher locomotor activity (Fig. 4). Thus, the decreased TIM expression may, at least partly, contribute to the observed phenotype of dATF-2 knockdown flies, although other uncharacterized target genes of dATF-2 also may play important roles. It also was reported that *tim* mutant flies do not exhibit the normal sleep rebound after sleep deprivation,

indicating a defect in the homeostatic regulation of sleep (22, 48). This is consistent with our observations that dATF-2 also is involved in the homeostatic regulation of sleep (Fig. 7). dATF-2 is phosphorylated in response to locomotion or fatigue at ZT2 to ~ZT5 and may gradually accumulate the target gene products, including TIM, which then would promote sleep and suppress activity after reaching the threshold level. Since forced locomotion also should cause sleep deprivation, sleep deprivation may also stimulate the phosphorylation of dATF-2 and accumulate the target gene products such as TIM, which then may induce sleep rebound. Thus, dATF-2 might act as a modulator of sleep and activity in response to fatigue, but not as a master key regulator of sleep and activity. Thus, the peak of P-dATF-2 and sleep may not necessarily be the same.

The phosphorylation of dATF-2 in l-LN_s is mediated by the dp38 pathway, which is activated by various signals, including inflammatory cytokines (8). Inflammatory cytokines such as interleukin-1 and tumor necrosis factor α are induced in peripheral tissues by exercise (37), and they act on the brain (13). Therefore, this kind of signaling cascade may activate ATF-2 in the brain. It is also notable that in mice, transforming growth factor α inhibits locomotor activity and disrupts circadian sleep-wake cycles via epidermal growth factor receptors on neurons in the hypothalamic subparaventricular zone (30). Since ATF-2 also is activated by a signaling cascade via the epidermal growth factor receptor (39), a similar pathway could be involved in activating dATF-2.

It was reported that *Drosophila* transcription factor dCREB2 (the CRE-binding protein), the *Drosophila* homolog of CREB, also is involved in the regulation of sleep homeostasis. dCREB2 mutants have an increased homeostatic sleep rebound, whereas CREB activity increases after rest deprivation (23). In addition, dCREB2 activity cycles with a 24-h rhythm, and dCREB2 mutation shortens the circadian locomotor rhythm (4). Furthermore, the forced expression of cAMP-dependent protein kinase A in the mushroom bodies led to decreased sleep bout duration (42), although no evidence has been shown that this is due to enhanced dCREB2 activity in the mushroom bodies. Although both dATF-2 and dCREB2 bind to the same CRE and affect sleep regulation, there are some obvious differences between these factors. P-dATF-2 is

expressed only in l-LN_s, as shown in the present study, while dCREB2 was previously reported to be expressed in most cell bodies, but not in neuropils, in the brain (56). Thus, it is unknown which region in the brain is critical for the regulation of sleep and locomotor activity by dCREB2. Moreover, dATF-2 is likely to be activated via dp38 in response to various stresses, such as forced locomotion. Since the production of tumor necrosis factor alpha and interleukin-1, which are known to activate the p38–ATF-2 pathway, is enhanced by fatigue (37, 41), these cytokines may be good candidates to activate dATF-2 in l-LN_s in response to locomotion. On the other hand, various neurotransmitters such as 5-hydroxytryptamine, which regulates the cAMP level, could modulate dCREB activity via protein kinase A. Thus, dATF-2 and dCREB2 may regulate sleep and locomotor in response to different stimuli.

It was previously reported that mutant flies of heat shock protein Hsp83 exhibited exaggerated sleep homeostatic responses and died after sleep deprivation (48). In addition, the transcription factor Cycle, which is a basic helix-loop-helix–PAS protein essential for the transcription of *period* and *tim* (43), is responsible for the induction of the genes encoding heat shock protein after sleep deprivation. At present, it is unknown which neurons express these heat shock proteins to regulate sleep homeostasis. However, dATF-2 also could induce heat shock proteins, because dATF-2 induces the expression of various gene products that protect from stress. In fact, we previously observed that a chaperon gene is a target of dATF-2 (44). Further study is required to examine whether dATF-2 is correlated with the sleep homeostat by regulating the expression of heat shock genes in the brain.

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