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Article

A Sleep-Promoting Role for the *Drosophila* Serotonin Receptor 1A

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Summary

Background: Although sleep is an important process essential for life, its regulation is poorly understood. The recently developed *Drosophila* model for sleep provides a powerful system to genetically and pharmacologically identify molecules that regulate sleep. Serotonin is an important neurotransmitter known to affect many behaviors, but its role in sleep remains controversial.
Results: We generated or obtained flies with genetically altered expression of each of three *Drosophila* serotonin receptor subtypes (d5-HT1A, d5-HT1B, and d5-HT2) and assayed them for baseline sleep phenotypes. The data indicated a sleep-regulating role for the d5-HT1A receptor. d5-HT1A mutant flies had short and fragmented sleep, which was rescued by expressing the receptor in adult mushroom bodies, a structure associated with learning and memory in *Drosophila*. Neither the d5-HT2 receptor nor the d5-HT1B receptor, which was previously implicated in circadian regulation, had any effect on baseline sleep, indicating that serotonin affects sleep and circadian rhythms through distinct receptors. Elevating serotonin levels, either pharmacologically or genetically, enhanced sleep in wild-type flies. In addition, serotonin promoted sleep in some short-sleep mutants, suggesting that it can compensate for some sleep deficits.
Conclusions: These data show that serotonin promotes baseline sleep in *Drosophila*. They also link the regulation of sleep behavior by serotonin to a specific receptor in a distinct region of the fly brain.

Introduction

Sleep is an essential part of animal physiology, with humans spending more than one-third of their lives in the sleep state [1, 2]. Sleep is also a complex process influenced by both genetic and environmental components. Despite the clear necessity for sleep and the extensive investigation of this process, the brain structures that drive sleep, the cellular mechanisms involved in sleep regulation, and the function of sleep are still unclear.

Multiple approaches, including activity monitoring, arousal threshold measurements, rebound sleep following sleep deprivation, responsiveness to sleep-altering drugs, and electrophysiological studies, indicate that rest in *Drosophila* shares features with mammalian sleep [3–5]. Studies in flies by both a candidate gene approach and large-scale genetic screens have identified several genes involved in sleep regulation, including the cAMP-dependent protein kinase (PKA), cAMP response element binding protein (CREB), a potassium channel, *Shaker*, and the dopamine transporter *fumin* [6–8]. A study in mammals confirmed a connection between CREB activity and the control of the sleep/wake cycle, suggesting that mechanisms of sleep regulation are conserved from flies to mammals [9].

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter widely distributed in the central and peripheral nervous systems of both mammals and insects [10, 11]. The idea that serotonin is involved in the regulation of sleep-wake cycles was proposed some time ago [12], but its role remains unclear [13, 14]. Reducing serotonin levels through pharmacological treatment or surgical ablation of serotonergic cells causes insomnia, suggesting that serotonin promotes sleep [15]. On the other hand, neuronal activity of serotonergic neurons and the timing of serotonin release suggest that it is associated with the waking state [16]. Knockout mouse models for several serotonin receptor subtypes support an association between serotonin and sleep but have not clarified the mechanistic link. Overall, the results of many different approaches support the idea that serotonin suppresses rapid eye movement (REM) sleep [14, 17–19]; however, the effects on non-REM (NREM) sleep are debatable [13, 20].

In *Drosophila*, serotonergic neurons send projections to most brain regions [11, 21]. Four serotonin receptors have been identified in the *Drosophila* genome, d5-HT1A, d5-HT1B, d5-HT2, and d5-HT7 [22–24]. They are all G protein-coupled receptors and share considerable sequence similarity with their mammalian homologs. Conserved effects of serotonin on the regulation of complex behaviors in flies and mammals were demonstrated in fly models of addiction, aggression, and circadian entrainment [25–27]. We considered the possibility that serotonin might affect sleep/arousal regulation in *Drosophila*. Therefore, we tested the baseline sleep phenotypes of flies with genetically modified expression of three *Drosophila* serotonin receptors. Flies carrying a truncated d5-HT1A receptor had decreased sleep amount and poor consolidation, while flies with reduced levels of the other two receptors, d5-HT1B and d5-HT2, showed no baseline sleep abnormalities. The short and fragmented sleep phenotype of the d5-HT1A receptor mutant was rescued by a transgene of d5-HT1A expressed specifically in adult mushroom bodies. In addition, elevating serotonin levels pharmacologically or genetically promoted baseline sleep in wild-type flies. A serotonin precursor also enhanced sleep in some known sleep mutants but failed to have effects when chemical neurotransmission was blocked in serotonergic cells, indicating that increased extracellular serotonin is required to promote sleep. We propose that the serotonin system is important for baseline sleep control

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in *Drosophila* and involves the function of the d5-HT1A receptor in adult mushroom bodies.

Results

Baseline Sleep Phenotypes in Flies with Modified Levels of *Drosophila* Serotonin Receptors

We collected or generated loss-of-function mutants of the *Drosophila* homologs of the mammalian 5-HT1 and 5-HT2 classes of receptors, which have been implicated in sleep regulation [14, 17, 19, 28, 29]. Baseline sleep phenotypes were studied under alternating light-dark conditions in 7- to 10-day-old female flies bearing mutations in the following genes: d5-HT1A, d5-HT1B (both of which are *Drosophila* homologs of the mammalian 5-HT1A receptor), and d5-HT2. Total daily sleep and sleep bout length were used to describe overall sleep and sleep consolidation [30, 31].

We previously studied the d5-HT1B receptor and demonstrated that it functions in the circadian response to light [27]. To assay the effect of d5-HT1B on sleep, we expressed UAS-d5-HT1B and UAS-d5-HT1BRNAi transgenes in different cell types by means of a variety of drivers including *elav*-Gal4 for panneuronal expression, *tim*-Gal4 for expression in clock cells, *Ddc*-Gal4 for expression in serotonin- and dopamine-producing cells, and d5-HT1B-Gal4 for expression in locations that express endogenous receptor. The effects of the UAS-d5-HT1B and UAS-d5-HT1BRNAi transgenes on increasing or decreasing, respectively, the levels of d5-HT1B protein and on circadian photosensitivity were shown previously [27]. Daily sleep was compared between flies overexpressing d5-HT1B and those that had d5-HT1B expression knocked down through RNAi. Regardless of the Gal4 driver used to express the UAS transgenes or of the level of d5-HT1B receptor in a given region of the brain, no significant differences were found in either the total amount of sleep or in sleep consolidation measured as the average length of sleep bouts (Figure 1A, data not shown). These data suggest that the d5-HT1B receptor is not involved in the regulation of baseline sleep in *Drosophila*.

The mammalian 5-HT2 receptor is implicated in several psychopathological conditions in humans, including schizophrenia and eating disorder [32–34]. Pharmacological studies in mammals suggest that serotonin acts on 5-HT2 receptors in the thalamus to produce an arousing effect [35]. On the other hand, mouse knockouts of 5-HT2A and 5-HT2C have mildly reduced NREM sleep [28, 29]. Analysis of the *Drosophila* homolog of the 5-HT2 receptor is limited [24, 36], in part due to the lack of loss-of-function mutants.

We obtained a fly line from the Exelixis collection carrying a piggybac insertion in the third intron of the d5-HT2 gene [37, 38] (see Figure S1 in the Supplemental Data available with this article online). Due to the presence of dual splice donor sites in this P element, the d5-HT2 transcript is disrupted, producing a loss-of-function allele. Specifically, the expression of three exons downstream of the insertion, which encode essential intracellular and transmembrane domains, is affected (Figure S1). Sleep analysis indicated that neither total sleep nor sleep bout length was significantly different between flies with reduced d5-HT2 levels and controls (Figure 1B), suggesting that this receptor subtype is not involved in the regulation of fly baseline sleep.

The d5-HT1A receptor, like the d5-HT1B receptor, is a homolog of the mammalian 5-HT1A receptor. It shares more than 80% homology with d5-HT1B and is located in close cytogenetic proximity. To generate a loss-of-function mutant of d5-HT1A, we obtained a fly line with a P element insertion 2.5 kb downstream of its coding region [39]. We then carried out crosses to excise this element and screened ~500 independent P excision lines. One P excision event generated an imprecise excision deleting more than 5 kb of genomic sequence including the 3′ coding region of d5-HT1A. In a baseline sleep assay, flies carrying deletions in d5-HT1A showed significantly reduced sleep and sleep bout length as compared to control flies from a precise excision line that does not affect the d5-HT1A receptor (Figure 1C).

The analysis of flies with modified expression levels of three *Drosophila* serotonin receptor subtypes suggested that d5-HT1A is a specific receptor subtype that regulates baseline sleep in flies. We carried out detailed molecular and behavioral studies focused on the d5-HT1A mutant flies in the following experiments.

Characterization of the Lesion in d5-HT1A Mutants

To confirm that we had generated a loss-of-function mutant of d5-HT1A, we characterized the deletion in the P excision line by genomic PCR and RT-PCR (Figures 2A and 2B). Although 5-HT1A transcripts were still expressed in these flies, the last two exons, which encode the sixth and seventh transmembrane domains, the C-terminal end, and a part of the third intracellular loop, were deleted (Figure 2B). In addition, the transcript produced by the neighboring gene CG15117, which encodes a putative metabolic enzyme, was also affected (Figure 2A, see Discussion).

Since flies carrying the deletion were capable of producing a truncated form of the receptor, we cloned and tested the full-length and truncated receptors in an S2 cell culture system. The truncated receptor was expressed diffusely in the cytoplasm, in contrast to the full-length receptor that localized largely to the cell surface (Figure 2C). In response to serotonin, the full-length receptor on the cell surface was internalized and formed clusters while the truncated receptor showed no changes in its cytoplasmic distribution (Figure 2C). Thus, consistent with previous functional studies of C-terminal truncated G protein-coupled receptors [40, 41], the lesion in the d5-HT1A gene leads to altered subcellular localization and loss of the response to serotonin. We surmise that flies carrying the truncated receptor are loss-of-function mutants of d5-HT1A.

Phenotypic Analysis of d5-HT1A Mutant Flies

The d5-HT1A mutant did not have visible defects in body and brain development or in locomotion. In circadian behavioral assays, d5-HT1A mutant flies showed normal free-running rhythms, although the strength of the rhythm was reduced (Figure 3A). We infer that the reduced rhythm strength was due to increased nighttime activity resulting from the decrease in sleep. Unlike flies with reduced levels of the d5-HT1B receptor [27], the d5-HT1A mutants did not show increased circadian photosensitivity as measured by light-induced phase

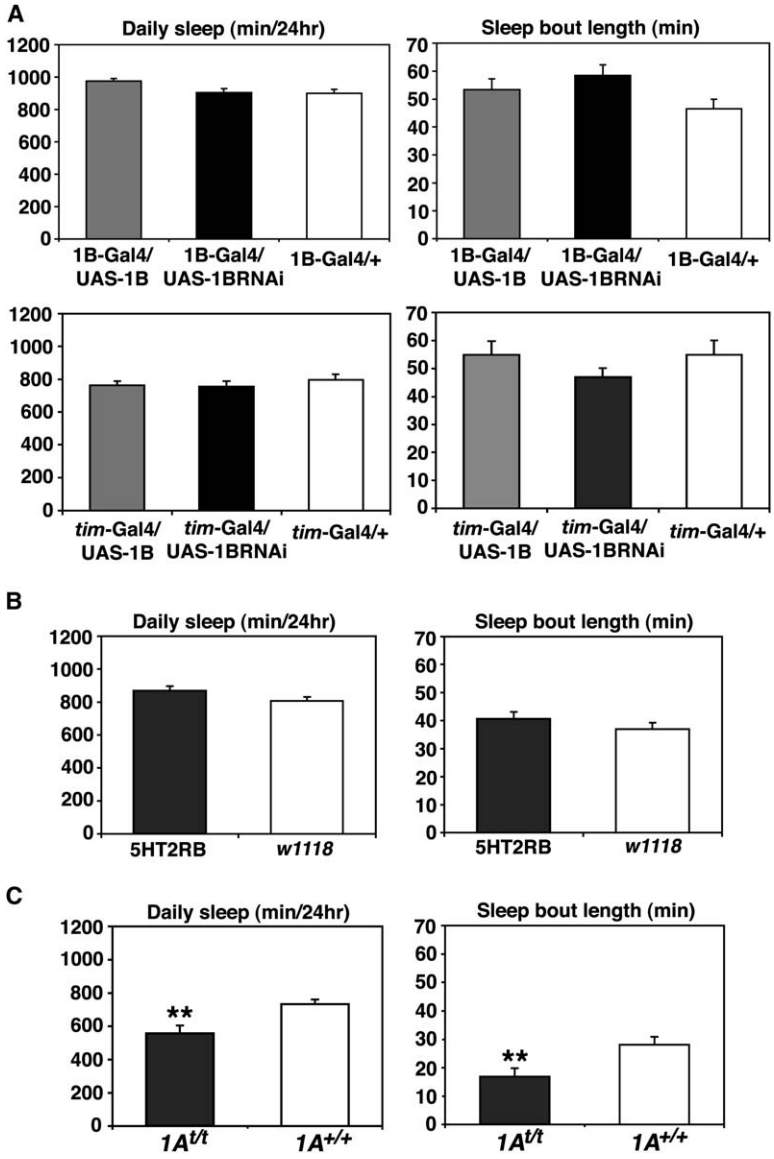

Figure 1. Baseline Sleep Phenotypes in Loss-of-Function Mutants of Three *Drosophila* Serotonin Receptors

(A) Female flies with modified levels of the d5-HT1B receptor have similar baseline sleep levels and sleep bout lengths as control flies. Average daily sleep and average sleep bout length of flies expressing UAS-d5-HT1B or UAS-d5-HT1BRNAi under the control of d5-HT1B-Gal4 (1B-Gal4) or *tim*-Gal4 (TG) are shown.

(B) Daily sleep and bout length in flies with reduced levels of the d5-HT2 receptor (5-HT2RB) are similar to those of background control flies (w^{1118}).

(C) Flies with a lesion in the d5-HT1A receptor show a reduction in both daily sleep and sleep consolidation. d5-HT1A mutant flies ($1A^{t/t}$) were generated through the imprecise excision of a P element. A simultaneously generated fly line that contained a precise excision, and thus did not have a lesion in d5-HT1A, served as the background control ($1A^{+/+}$). **$p < 0.01$, by Student's t test; the number of flies tested for each data set was ~30, from two independent experiments. Error bars represent SEM.

shifts in the late night, suggesting nonredundant functions of these two receptors in the circadian system (data not shown). Consistent with the lack of a role in circadian rhythms, transcript levels of d5-HT1A did not vary at different times of day (data not shown).

We then conducted a detailed sleep analysis of d5-HT1A mutant flies. Sleep during the light and dark periods of the day was considered separately for higher resolution. Precise excision lines that did not affect the d5-HT1A gene, as well as heterozygous flies carrying one copy of the deletion, were used as background controls. The circadian profile of activity and sleep indicated that the onset and offset of activity as well as the distribution of sleep were similar in mutant and control flies (Figure 3B). However, the d5-HT1A mutant had significantly reduced total sleep and sleep bout length, as well as an increased number of sleep bouts, indicating that sleep was both reduced and fragmented (Figure 3B). Note that significant changes were noticed only for nighttime sleep in females, since they have limited and poorly consolidated daytime sleep. Although our studies focused largely on females, the gender of choice for sleep studies in *Drosophila* [5, 30], we also examined sleep in male d5-HT1A mutant flies (Figure S2). These also had reduced overall sleep relative to their controls (Figure S2). However, since sleep in *Drosophila* is gender dimorphic, with daytime sleep accounting for a significant proportion of sleep in males [30], the sleep phenotype in d5-HT1A males was evident during the day and night.

We also assayed sleep in constant dark conditions, which typically decrease sleep consolidation. As expected, when flies were transferred from light-dark cycles to constant darkness conditions, total sleep and sleep bout length were reduced, while sleep bout number increased. At the same time, the d5-HT1A mutant showed significantly larger changes in these parameters in response to the absence of light-dark cycles (Figure 3C), suggesting that mechanisms for maintaining sleep stability are impaired in these flies. This was

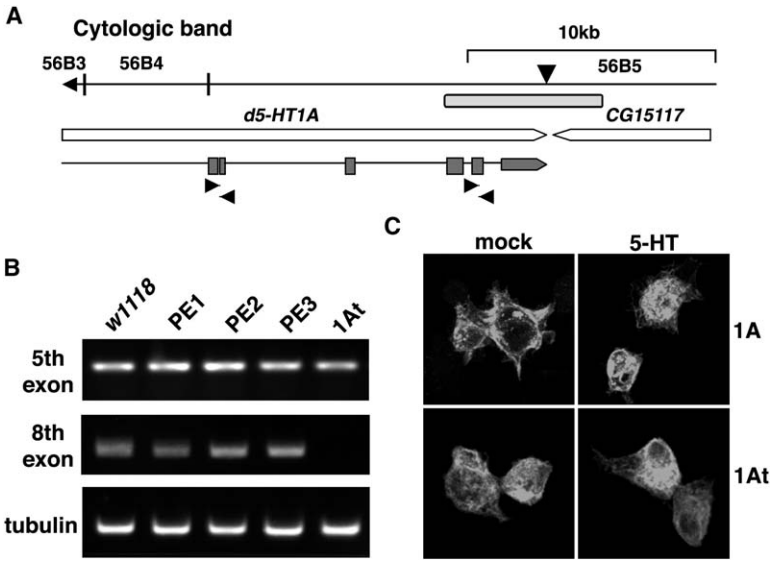

Figure 2. Characterization of the Lesion in the d5-HT1A Gene

(A) Schematic representation of the lesion generated by imprecise excision of a P insertion in the d5-HT1A gene. An arrowhead indicates the P element insertion. The stippled bar represents the extent of the deletion generated by the imprecise excision. Arrows indicate two sets of primers designed to amplify the fifth and eighth exons of d5-HT1A transcripts. The blocks represent exons.

(B) Mutant flies express d5-HT1A transcripts with truncated 3′ ends. Total RNA was extracted from wild-type flies (w^{1118}), three precise excision lines (PE1, 2, 3), and flies with a d5-HT1A deletion ($1A^t/1A^t$). PCR reactions for tubulin transcripts served as controls. Mutant d5-HT1A flies produced d5-HT1A transcripts (indicated by the PCR product of the fifth exon), which were truncated at the 3′ end (indicated by the absence of a PCR product of the eighth exon).

(C) The C-terminal truncated d5-HT1A receptor does not respond to serotonin. Representative projected confocal images of immunostained S2 cells expressing full-length or truncated d5-HT1A receptors are shown. S2 cells transiently transfected with V5 epitope-tagged d5-HT1A receptors were collected after 30 min of mock or serotonin (100 nm) treatment. The cells were fixed and subjected to immunostaining with an anti-V5 antibody. Full-length receptor was expressed mainly on the cell surface and, in ~30% cells, was internalized after serotonin treatment, while the truncated receptor was diffusely distributed in the cytoplasm and failed to respond to serotonin.

also indicated by their reduced homeostatic response to sleep deprivation. We deprived the d5-HT1A mutant and its background control of sleep by subjecting them to mechanical sleep deprivation for 6 hr in the latter half of the night. Compared to the precise excision control line, 1A mutant flies had significantly reduced sleep rebound (Figure S3A).

Mapping of the Sleep Phenotype to the d5-HT1A Locus

To genetically map the phenotype to the lesion in the d5-HT1A gene, we first carried out deficiency-complementation tests with a line carrying a deficiency in the genomic region of d5-HT1A (Df7550) [38]. Flies carrying the d5-HT1A mutant over the deficiency Df7550 had a short and fragmented sleep phenotype similar to that of the d5-HT1A homozygous mutant (data not shown). We also attempted to rescue the mutant sleep phenotype by generating transgenic flies carrying a UAS-d5-HT1A construct [42]. The transgene was introduced into the mutant background and expressed under the control of different drivers. To describe the effect of transgene expression on the sleep phenotype in mutant flies, we focused on total sleep and nighttime sleep bout length and bout number, parameters that were affected in the female d5-HT1A mutant. When the UAS-d5-HT1A transgene was expressed panneuronally, driven by an *elav*-Gal4 driver, sleep levels and consolidation were restored in d5-HT1A mutants: total sleep and nighttime sleep bout length increased while bout number decreased (Figure S4), suggesting that the lesion in d5-HT1A was responsible for the sleep phenotype.

To identify specific brain regions important for the function of d5-HT1A, we first determined its expression pattern through in situ RNA hybridization. In whole-mount adult fly brains, the signal from hybridized d5-HT1A transcripts was observed largely in the mushroom bodies (Figure 4A). Based on this expression pattern, we sought to determine if the sleep phenotype could be rescued through targeted expression of d5-HT1A in adult mushroom bodies. Therefore, we expressed the UAS construct under the control of an RU486-inducible mushroom body Gal4 driver, MB-Switch [43]. d5-HT1A mutant flies carrying the UAS transgene and the inducible Gal4 driver were subjected to sleep tests, with one group treated with RU486 (500 μm, 1% ethanol) for expressing the transgene and another group serving as control (1% ethanol only). Total daily sleep and nighttime sleep bout length were restored to wild-type levels in RU486-treated Gal4-driven transgenic animals but not in uninduced controls (Figure 4B). These results indicate that the effect on sleep of defective d5-HT1A signaling can be rescued by restoring expression of the wild-type gene exclusively in adult mushroom bodies.

Based upon the high degree of sequence homology and similar molecular properties reported previously [22], it is conceivable that d5-HT1A and d5-HT1B act through similar signaling pathways in distinct brain regions and can substitute for each other when expressed in a functionally relevant location. However, despite the similarity, a d5-HT1B transgene expressed in adult mushroom bodies did not rescue the d5-HT1A mutant phenotype (Figure 4B). We also tested for possible genetic interactions between these two receptors by assaying sleep in flies with reduced levels of both. Knocking down the expression of d5-HT1B, by the UAS-1BRNAi transgene, in a 1A heterozygous mutant background did not produce any baseline sleep phenotype (Figure S3B). Together, these data indicate a specific role for d5-HT1A in fly sleep that is carried out in adult mushroom bodies.

Elevating Serotonin Levels in Flies Promotes Sleep

One major piece of evidence supporting a sleep-promoting effect of serotonin in mammals is the effect of

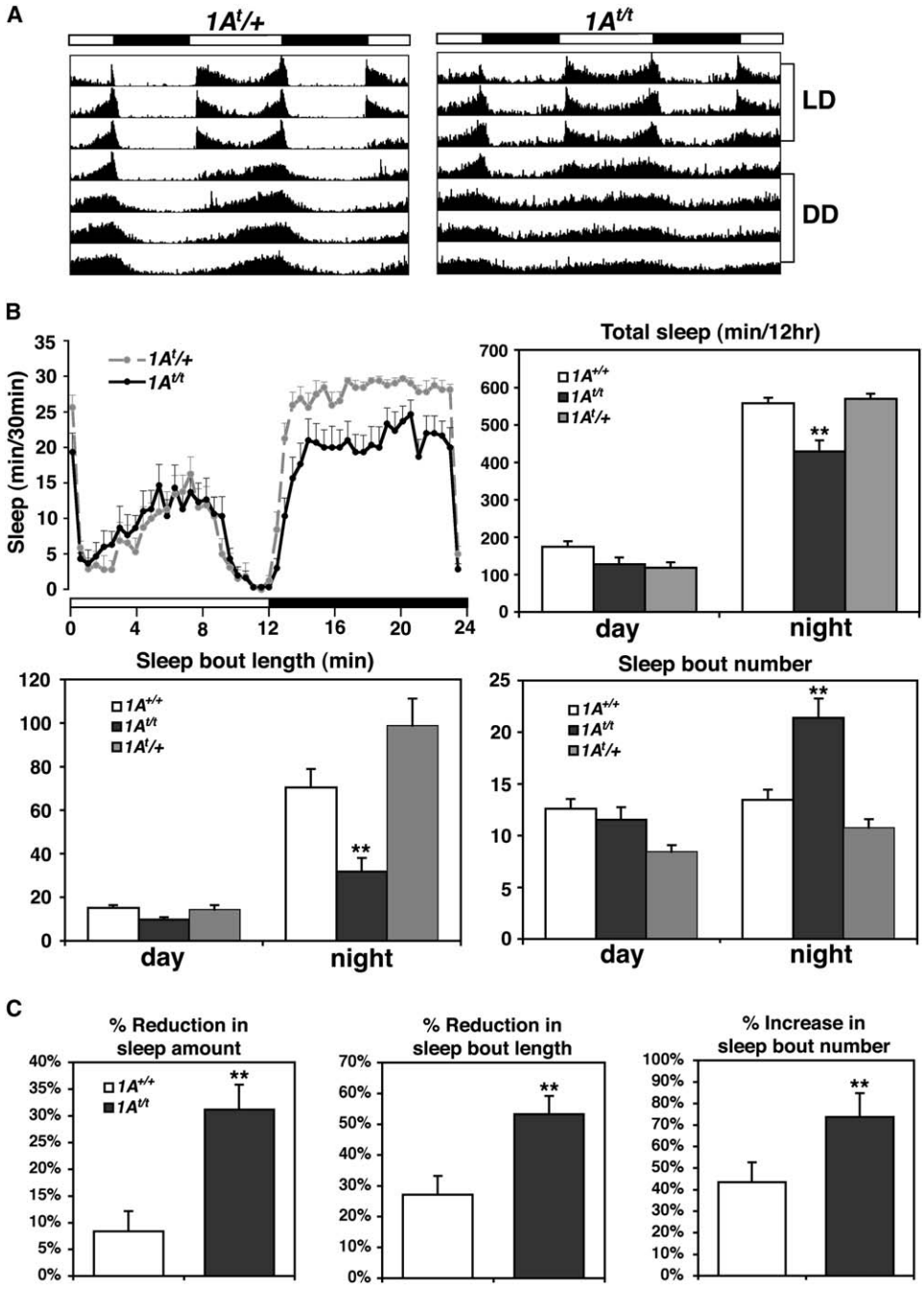

Figure 3. d5-HT1A Mutant Flies Have Short and Fragmented Sleep but Normal Circadian Rhythms

(A) Flies carrying a deletion in the d5-HT1A gene have normal circadian rhythms. Activity records show data averaged from 16 flies. The light-dark cycles and genotypes are indicated. As compared to the heterozygous sibling controls ($1A^{t}/+$), flies carrying the d5-HT1A deletion ($1A^{t/t}$) had normal circadian periods and rhythmicity in DD, but showed increased nighttime activity.

(B) Flies carrying the d5-HT1A deletion have reduced baseline sleep and sleep consolidation. Top left: Daily sleep profile of female d5-HT1A mutant flies ($1A^{t/t}$) versus the heterozygous control ($1A^{t}/+$). Top right: Total sleep in the light and dark periods of the day, plotted for three genotypes: precise excision control ($1A^{+/+}$) (n = 30), the d5-HT1A mutant (n = 31), and the heterozygous sibling control (n = 31). As compared to both controls, the d5-HT1A mutant had significantly reduced nighttime sleep. Bottom: Average sleep bout length (left) and sleep bout number (right) during the light and dark period of the day in female flies. Flies with the d5-HT1A deletion had significantly reduced nighttime sleep bout length and increased nighttime sleep bout number as compared to both controls.

(C) d5-HT1A mutant flies have reduced sleep and sleep consolidation in constant dark conditions. Female flies were entrained in light-dark cycles for 3 days and transferred to constant darkness. The bar graphs show percentage changes effected by constant darkness in total sleep amount, sleep bout length, and sleep bout number. Both precise excision control flies (n = 31) and d5-HT1A mutants (n = 29) responded to the lack of light-dark cycles with reduced overall sleep, reduced bout length, and increased bout number, but the changes were significantly greater in the mutant. **$p < 0.01$, by Student's t test. Error bars represent SEM.

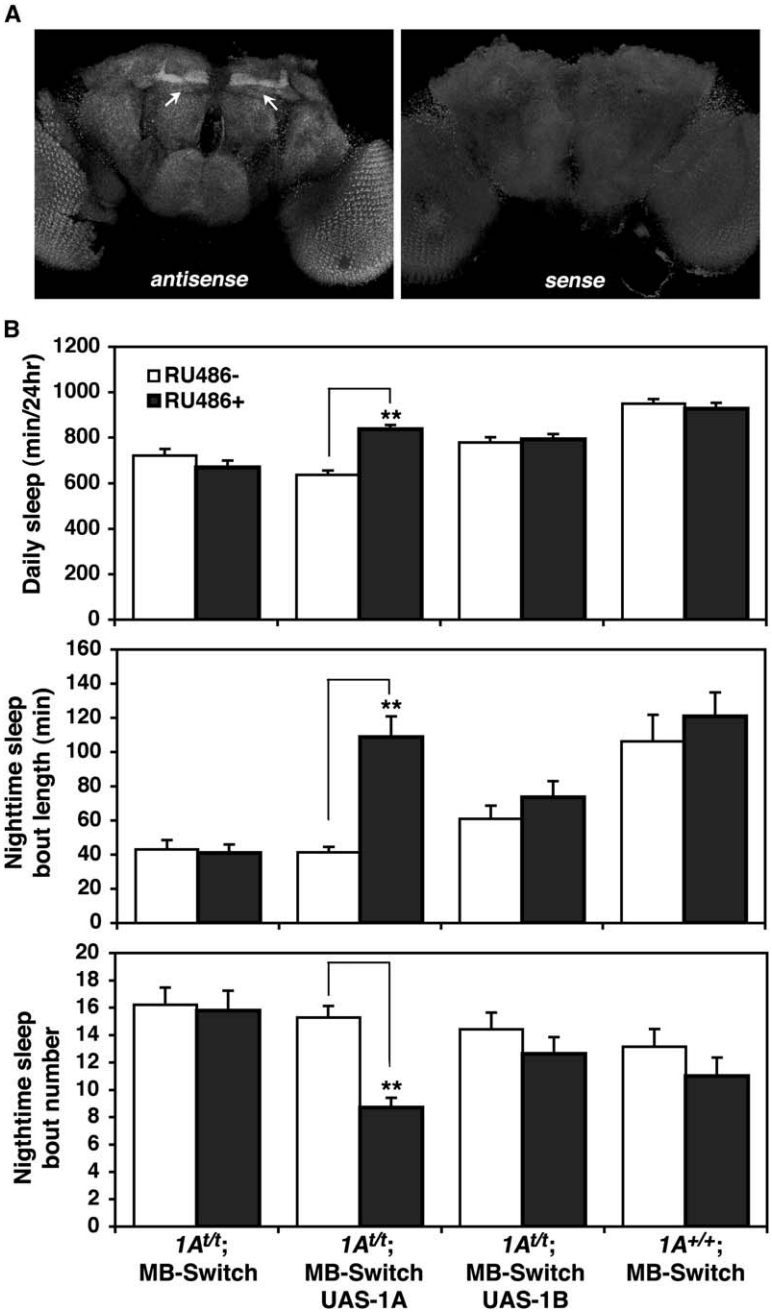

Figure 4. Genetic Mapping of the Sleep Phenotype in d5-HT1A Mutant Flies

(A) The d5-HT1A receptor is expressed in the adult mushroom body. Whole-mount adult fly brains were fixed and subjected to in situ hybridization via a biotin-labeled d5-HT1A RNA probe. Arrows point to the mushroom body (MB) in the fly brain. The antisense probe detected a clear signal in MBs. The sense probe served as control. Similar results were seen in five brain samples.

(B) Rescue of the d5-HT1A mutant phenotype by expressing the wild-type d5-HT1A gene, but not the d5-HT1B gene, in the MB of adult flies. An inducible Gal4 driver, which expresses specifically in the mushroom body (MB-Switch), was introduced into the d5-HT1A mutant background. d5-HT1A mutant flies carrying a single copy of both the MB-Switch driver and either UAS-d5-HT1A (UAS-1A) or UAS-d5-HT1B (UAS-1B) were tested for sleep phenotypes with (RU486+) or without (RU486−). Mutant flies with only the MB-Switch transgene ($1A^{t/t}$; MB-Switch, n = 28 for RU486−; n = 30 for RU486+) and those expressing UAS-d5-HT1B under the control of MB-Switch ($1A^{t/t}$; MB-Switch/UAS-1B, n = 41 for RU486−; n = 43 for RU486+) had no significant response to the drug treatment. However, flies in which UAS-1A expression was driven by RU486-induced MB-Switch ($1A^{t/t}$; MB-Switch/UAS-1A, n = 44) had significantly increased sleep and nighttime sleep bout length and reduced nighttime sleep bout number as compared to controls that were not treated with RU486 (n = 45). Flies with the MB-Switch transgene in the precise excision background ($1A^{+/+}$; MB-Switch, n = 45 for RU486−; n = 43 for RU486+) served as controls with wild-type sleep levels. Although uninduced and induced 1A mutant flies carrying the UAS-1B trangene showed somewhat increased sleep, this was not due to leaky expression of the 1B receptor (data not shown). **p < 0.01, by Student's t test. Error bars represent SEM.

pharmacological agents that modulate serotonin levels. When delivered systemically, the serotonin synthesis inhibitor parachlorophenylalanine (pCPA) causes insomnia, which can be rescued by treatment with the serotonin synthesis precursor 5-hydroxytryptophan (5-HTP) [15]. Previous reports suggested that systemic administration of pCPA does not deplete serotonin in the fly brain [44]. However, 5-HTP treatment increases serotonin levels in the CNS of both mammals and insects [25, 45]. To test whether increasing serotonin levels affects fly sleep, we assayed behavioral responses to chronic treatment with 5-HTP in female wild-type Canton-S flies. Doses of 5-HTP from 1 mg/ml to 5 mg/ml generated similar behavioral responses in Canton-S flies while the response to lower doses varied from fly to fly. Therefore, a 1 mg/ml dose was used in the following experiments. As compared to control flies, flies treated with 5-HTP had significantly increased amounts of sleep (Figure 5A), which was more pronounced during the day (Figure 5A). Because daytime sleep tends to be poorly consolidated, the increased sleep was manifested more as an increase in bout number than bout length (data not shown). The lack of a significant effect upon nighttime sleep was most likely due to a "ceiling" effect in light of the fact that Canton-S female flies have high nighttime sleep. We also observed that 5-HTP treatment reduced locomotor activity in flies (Figure 5A). However, these effects of serotonin on fly locomotion were generally variable and could be dissociated from the effect of serotonin on sleep.

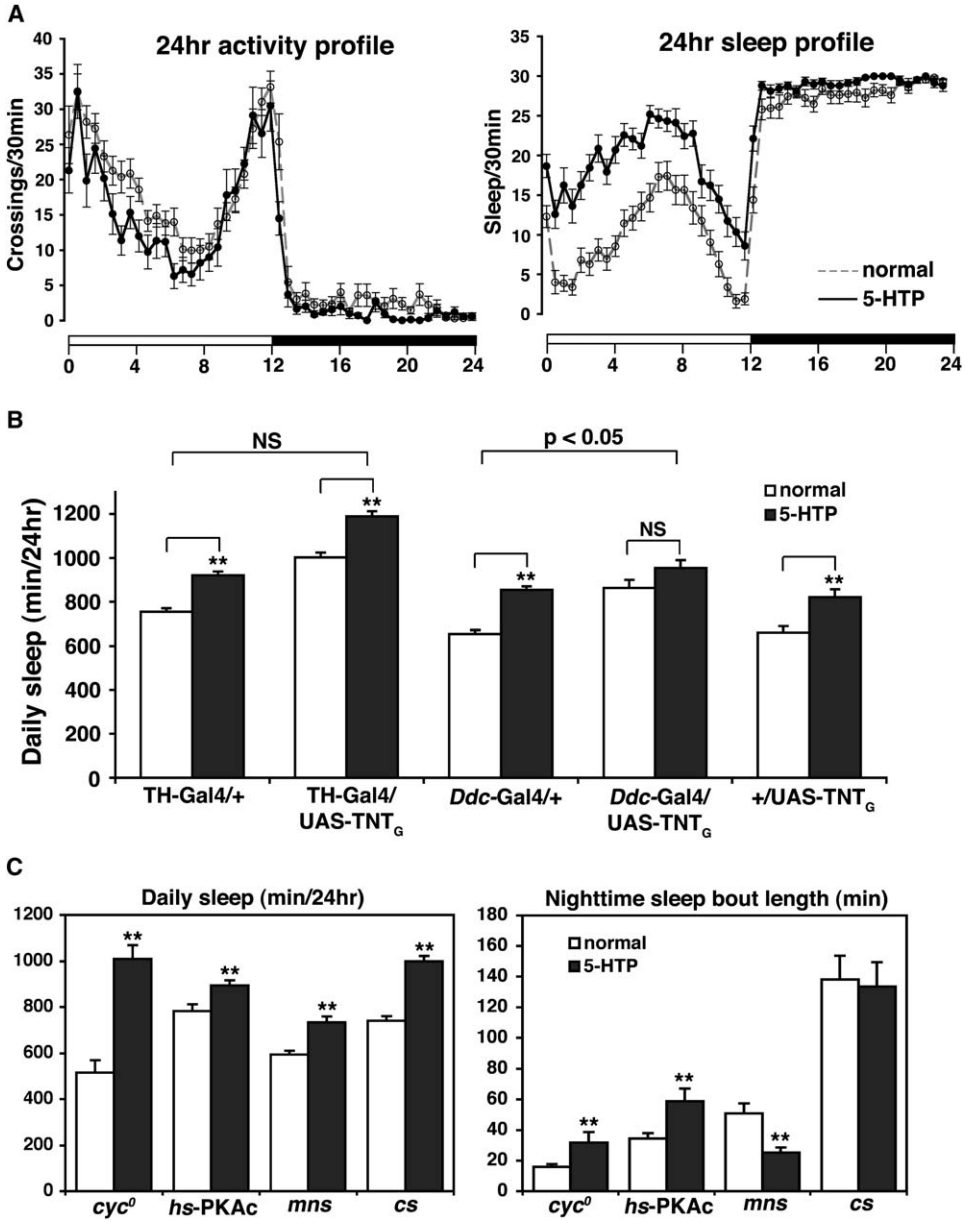

Figure 5. Elevated Serotonin Levels Promote Sleep in *Drosophila*

(A) Baseline sleep is increased in 5-HTP-treated wild-type Canton-S flies. Daily activity (left) and sleep (right) profiles of mock treated (dashed line) versus 5-HTP-treated (solid line) flies. 5-HTP-treated flies had a mild reduction in their locomotion as measured by the number of beam crossings per 30 min in a 24 hr period. The sleep profile shows the time spent in sleep every 30 min over a 24 hr period. Flies treated with 5-HTP had more sleep than the mock treated flies; this was more obvious during the daytime, as nighttime sleep levels are almost saturated in wild-type flies.

(B) The effect of 5-HTP on fly sleep is mediated through elevated extracellular serotonin levels. Flies carrying a UAS-TNT_G transgene were crossed to either a TH-Gal4 or a *Ddc*-Gal4 driver for blocking chemical neurotransmission in dopaminergic cells only or both dopaminergic and serotonergic cells, respectively. The progeny and parental controls were tested for the response to 5-HTP treatment. All the 5-HTP-treated controls and TH-Gal4/UAS-TNT flies had significantly increased sleep compared to their respective untreated controls (**$p < 0.01$, by Student's t test); however, *Ddc*-Gal4/UAS-TNT flies had no significant response. The brackets on top indicate results of ANOVA for a genotype X drug effect in two groups of flies. A significant genotype X drug interaction was indicated for the analysis of *Ddc* neurons ($p < 0.05$, by Student's t test), but not for the TH neurons. Numbers of flies tested for each experimental group was ~30.

(C) Differential response to 5-HTP in known mutants with a short-sleep phenotype. With 5-HTP treatment, daily sleep and nighttime sleep bout length were increased in cyc^{01} mutant flies (n = 14 for mock treated, n = 14 for 5-HTP-treated) and in flies carrying a hs-PKA transgene (n = 44 for mock treated, n = 46 for 5-HTP-treated). A loss-of-function mutant of *shaker*, *minisleep* (*mns*) (n = 29 for mock treated, n = 30 for 5-HTP-treated), responded to the 5-HTP treatment differently, with an increase in daily sleep and a significant reduction in night time sleep bout length. In Canton-S flies (*cs*, n = 31 for mock treated and 5-HTP-treated), daily sleep was significantly increased, but there was no change in nighttime sleep bout length. **$p < 0.01$, by Student's t test. Error bars represent SEM.

To verify that the effect of 5-HTP on fly sleep was mediated through an increase in levels of extracelluar serotonin, we tested the response to 5-HTP in flies in which chemical neurotransmission was blocked in serotonergic cells. Flies carrying a UAS-TNT (tetanus neurotoxin light chain) transgene were crossed either to a *Ddc*-Gal4 driver, which drives expression in dopamine- and serotonin-producing cells, or to a TH-Gal4 driver, which is expressed in dopaminergic cells only. Progeny of each cross were tested in sleep assays with or without 5-HTP treatment and compared to their parental controls. Although daily sleep amount varied among genotypes, all parental control lines responded to the 5-HTP treatment with elevated daily sleep. However, baseline sleep in Ddc-Gal4/UAS-TNT flies did not change significantly in response to 5-HTP treatment (Figure 5B). Further statistical analysis that used ANOVA to test for an interaction between genotype and drug treatment confirmed that the expression of UAS-TNT driven by *Ddc*-Gal4 had a significant effect on the sleep phenotype produced by 5-HTP (Figure 5B). In contrast, TH-Gal4/UAS-TNT flies had elevated sleep, which increased further in the 5-HTP-treated group (Figure 5B). The high baseline sleep phenotype in TH-Gal4/UAS-TNT flies is consistent with published data on the effect of the dopamine system in promoting arousal in flies [8, 46]. At the same time, chemical silencing of the dopaminergic cells did not affect the response to 5-HTP treatment, indicating that the lack of a response to 5-HTP in *Ddc*-Gal4/UAS-TNT flies is due to the loss of serotonergic transmission.

We next determined whether this effect of 5-HTP was universal across fly lines, in particular in lines with short-sleep phenotypes. All control strains tested, including *yw*, *w1118*, and Oregon-R, showed increased sleep in response to 5-HTP treatment (data not shown). Interestingly, d5-HT1A mutants also exhibited a significant increase in sleep after treatment with 5-HTP (Figure S5A), suggesting that increased serotonin levels may compensate for the deficit in d5-HT1A signaling, possibly through activating other unidentified serotonin receptors. Of the short-sleep mutants we tested, flies carrying a loss-of-function mutation in a clock gene (*cycle*) or expressing a constitutively active protein kinase A molecule under the control of a leaky heat-shock promoter [6, 47] showed increased sleep in response to 5-HTP treatment. Since the mutants had decreased sleep at all times, sleep-promoting effects of 5-HTP were visible at night as well as during the day and, in fact, even increased nighttime sleep bout duration was observed (Figure 5C). However, effects on nighttime sleep bout number were different in the two genotypes (Figure S5B). Short-sleep flies mutant for a potassium channel (*Shaker*) [7, 8] slept more in response to 5-HTP, but did not show increased bout duration at night. As a matter of fact, the *Shaker* flies showed reduced consolidation at night by both measures, but particularly in the greatly increased sleep bout number (Figure 5C, Figure S5B). Together, these data suggest that elevated serotonin can counteract the effects of other molecules that affect sleep, although to varying extents. We speculate that the differences in the response of the mutants arise from differences in the mechanism of action of each of the respective mutations.

To further investigate the effect of elevated serotonin on fly sleep, we generated transgenic flies with increased serotonin production. This was achieved by overexpressing an enzyme, tryptophan hydroxylase (TPH), which is responsible for catalyzing the conversion of tryptophan to 5-hydroxytryptamine, the first and the rate-limiting step in serotonin synthesis [44]. As in the mammalian system, there are two TPH isoforms in the *Drosophila* genome, DTPH (*henna*) and DTRH (*CG9122*) [44, 48, 49]. The expression pattern of these two genes, as determined by in situ hybridization at a late embryonic stage, indicated that DTPH is expressed in the fat body, while DTRH is expressed in the central nervous system (BDGP in situ). Thus, DTRH is most likely the TPH isoform that is important for serotonin production in the *Drosophila* CNS.

Overexpression of DTRH was driven by *Ddc*-Gal4 in serotonergic and dopaminergic cells, and the effect on serotonin levels was confirmed by ELISA assays of fly head extracts (Figure 6A). Female flies with elevated serotonin levels were subjected to sleep analysis. As compared to control flies, *Ddc*-Gal4/UAS-DTRH flies had significantly increased total sleep and night sleep bout length, as well as reduced sleep bout number (Figure 6B). Therefore, elevating serotonin levels through both pharmacological and genetic approaches enhances fly sleep.

Discussion

In this report, we utilized *Drosophila* as a model system to study the function of serotonin in sleep. The genetic tools available in *Drosophila* allowed us to study loss-of-function mutants of three serotonin receptors: d5-HT1A, d5-HT1B, and d5-HT2. A significant effect on sleep was observed in flies with a truncated d5-HT1A receptor. These flies had short and fragmented sleep, which was rescued by expressing a d5-HT1A transgene in adult mushroom bodies. Pharmacological studies with a serotonin synthesis precursor achieved a similar effect in flies as in mammals, which is to promote sleep. Our studies provide evidence for serotonergic signaling in the regulation of *Drosophila* sleep, identify a receptor that specifically functions in this process, and suggest that two serotonin receptor subtypes, d5-HT1A and d5-HT1B, mediate effects of serotonin on different behaviors in distinct regions of the brain.

The lesion in the d5-HT1A mutant also affects the neighboring gene CG15117, which codes for a protein of unknown function, possibly involved in carbohydrate metabolism. Since the sleep phenotype was rescued by expression of d5-HT1A alone, we believe that the sleep abnormality of the mutant was caused solely by the disruption of d5-HT1A and not the neighboring gene. While our data are definitive with respect to a role for d5-HT1A in sleep, we acknowledge that at this point we can not completely exclude a role for d5-HT1B and d5-HT2. The loss-of-function mutants we tested for these receptors are still capable of producing small amounts of transcript, although, at least in the d5-HT1B mutant, this transcript expression is too low to conduct its circadian function [27]. In addition, based on the sleep-enhancing effect of 5-HTP treatment in d5-HT1A mutant flies, we

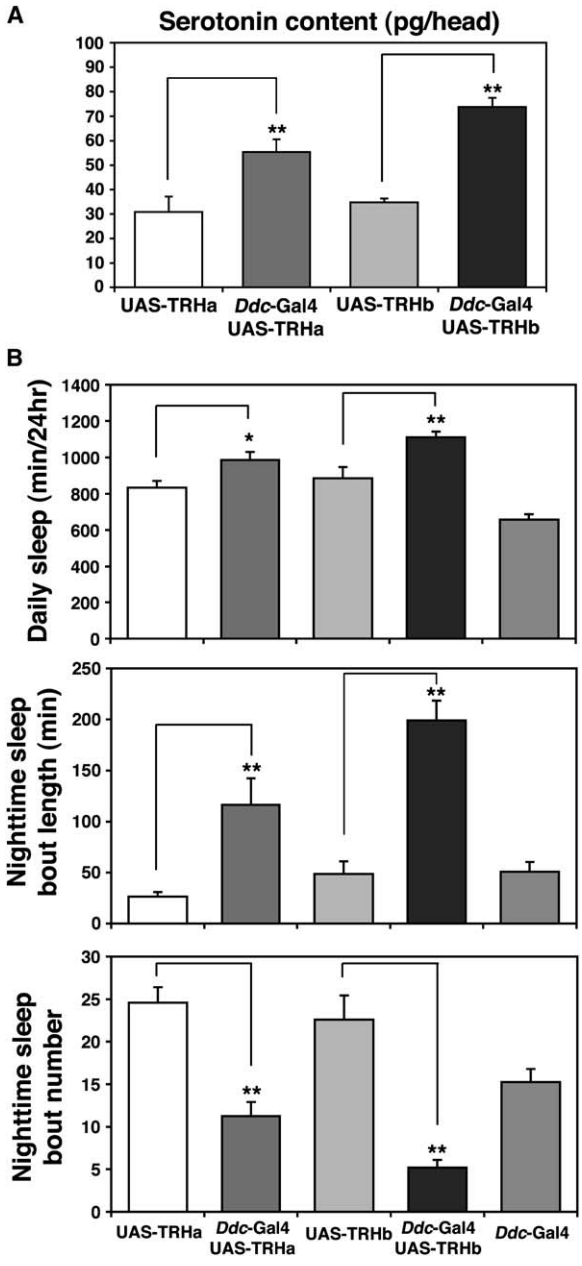

Figure 6. Expression of the DTRH Transgene Increases Serotonin Content in the Fly Head and Enhances Sleep

(A) Increased serotonin levels in flies overexpressing DTRH. Two independent insertion lines for UAS-DTRH transgene (UAS-DTRHa and UAS-DTRHb) were crossed to *Ddc*-Gal4 for expression in serotonergic and dopaminergic cells. Fly heads were collected from indicated genotypes and subjected to quantitative immunoassays for serotonin content. As compared to controls, *Ddc*-Gal4/UAS-TRH flies had significantly increased serotonin levels in head extracts. $^{**}p < 0.01$, by Student's t test. Error bars represent standard deviation.

(B) Flies with genetically elevated serotonin levels have increased total sleep and sleep consolidation. Flies expressing DTRH driven by *Ddc*-Gal4 had significantly increased nighttime sleep bout length and total sleep and reduced nighttime sleep bout number, as compared to parental controls. Number of flies tested for each experimental group was ~16. $^{**}p < 0.01$, $^{*}p < 0.05$, by Student's t test. Error bars represent SEM.

believe other unidentified serotonin receptors function in sleep regulation.

Although they showed no obvious defects, homozygous flies carrying the truncated d5-HT1A receptor were less viable than flies with a single copy of the mutation in the same background. When maintained as heterozygotes, less than 5% of the progeny were homozygous for the d5-HT1A mutation. In homozygous stocks, the short-sleep phenotype weakened, such that sleep increased after several generations. This is consistent with the notion that the sleep phenotype is highly modifiable, especially when it is associated with reduced viability. A similar situation was reported for the *Shaker* mutants where the short-sleep phenotype diminished significantly within some generations in a *w1118* background [7]. For analysis of the d5-HT1A mutant, we avoided this problem by constantly maintaining a heterozygous stock and by employing a homozygous stock for no more than the first two generations. We note, however, that the d5-HT1A mutation was introduced into backgrounds containing different UAS or Gal4 transgenes for the rescue experiment, and it consistently maintained its phenotype. Thus, the effects of modifiers/background do not constitute an insurmountable problem in the analysis of sleep mutants.

We speculate that serotonin has a conserved function in sleep regulation. In mammals, systemic administration of 5-HTP or of an agonist of the mammalian 5-HT1A receptor increases slow-wave sleep (SWS) and reduces sleep latency [13, 14]. However, analysis of the mammalian 5-HT1A receptor has been complicated, in part because of the difficulty of dissociating its pre- and postsynaptic actions and also because of compensatory effects involving upregulation of other receptor subtypes [13, 14, 16–18]. This may account for the lack of a NREM or total sleep phenotype in the 5-HT1A knockout mice [17]. We identified a sleep phenotype in the *Drosophila* 5-HT1A mutant and demonstrate an additional role for this receptor in the maintenance of sleep stability. The phenotype in *Drosophila*, we believe, results from the simpler structure of the fly genome with its less compensated serotonergic system. Also, while sleep in rodents is quite fragmented, flies, like humans, have prolonged sleep episodes. This may facilitate the identification of sleep-consolidating factors such as d5-HT1A.

Associating the effect of serotonin on sleep with a specific receptor subtype provides a means to dissect the underlying circuitry and signaling pathways. Through genetic rescue experiments, we find that d5-HT1A is required in the mushroom bodies to promote sleep stability. Our previous studies demonstrated a role for d5-HT1B in circadian entrainment in clock cells and showed that this receptor is also expressed in the mushroom bodies. However, d5-HT1B could not substitute for the function of d5-HT1A in fly sleep in rescue experiments, suggesting that, in addition to tissue-specific regulation, different downstream signaling is involved in fly behaviors modulated by serotonin. Although the two receptors share more than 80% homology in overall protein sequence, their N termini and third intracellular loops are very different, which could account for their molecular differences. In addition, it is possible that the expression of 1A and 1B in mushroom bodies is

regulated either developmentally or spatially. Our observations demonstrate that it is possible to link the regulatory effect of serotonin on complex behaviors in *Drosophila* with distinct receptor types and with specific neuronal structures.

The localization of d5-HT1A action to the adult mushroom bodies is consistent with recent studies that indicate a sleep-regulating role for mushroom bodies [31]. The mushroom body is an important neuronal structure for control of locomotion and integration of sensory inputs, as well as learning and memory in flies [43, 50, 51]. This study supports the association between sleep and memory consolidation. It is also consistent with the role of serotonin in learning and memory [34].

A link between serotonin and human sleep has been demonstrated in clinical studies. Pathological conditions associated with serotonin deficits are linked to reduced sleep amount and quality. For instance, patients with clinical depression usually have sleep disorders [52, 53]. Antidepressant treatment upregulating serotonergic signaling improves their mental condition as well as their sleep quality [54]. Results of our studies that used pharmacological and genetic approaches suggest that serotonin also promotes sleep in flies. In addition, our studies support the complementary conclusion, that is, flies with defects in serotonin signaling sleep less. Together, our results provide a model for studying the underlying mechanism and neuronal structures involved in the effect of serotonin on sleep.

Conclusions

We show here that serotonin and the d5-HT1A receptor promote sleep in *Drosophila*. Serotonin can even increase sleep and sleep consolidation in some short-sleep mutants, suggesting that it has a central role in the control of sleep. The finding that d5-HT1A acts in the adult mushroom bodies to regulate sleep links sleep and serotonin signaling to learning and memory. Finally, given that effects of serotonin on circadian rhythms are mediated by the d5-HT1B receptor, and not by d5-HT1A, these data indicate nonoverlapping roles for serotonin receptor subtypes in the circadian and homeostatic control of sleep.

Experimental Procedures

Fly Strains and Transgenes

UAS-d5-HT1A and UAS-DTRH (CG9122) transgenic flies were generated as described [55]. Dr. Julian Dow of University of Glasgow provided the transgene construct for UAS-d5-HT1A [42]. The cDNA template for generating the UAS-DTRH transgene was obtained from the DGRC cDNA collection (GH12537). Multiple transformed lines were mapped and balanced in a w^- background. Two independent insertion lines were tested in the behavioral analysis and yielded similar results. Flies carrying UAS-d5-HT1B, UAS-d5-HT1BRNAi, d5-HT1B-Gal4, *tim*-Gal4, *elav*-Gal4, and *Ddc*-Gal4 were as described [27]. Dr. Serge Berman provided TH-Gal4, Dr. Ron Davis provided MB-Switch, and Dr. Chiara Cirelli provided *mns* flies in CS background. Other fly lines were obtained from the Bloomington stock center (Stock number/name: e01363/5HT2RB, 17629/EY09988, 7550/Df7550, 6326/*w1118*).

Behavioral Analysis

For circadian analysis, flies were entrained to 12:12 light-dark (LD) cycles at 25ºC. 3- to 7-day-old flies were used in the behavioral analysis. Locomotor activity of individual flies was monitored in LD followed by constant darkness (DD) conditions as previously described [55]. Activity records were analyzed with Clocklab software (Actimetrics).

For sleep analysis, 7- to 10-day-old flies were monitored in light-dark conditions at 25ºC. Locomotor activity of individual flies was monitored and collected with the DAM systems as previously described [30]. Generally, flies subjected to certain conditions (such as different light:dark conditions or pharmacological treatments) were given a day for adaptation, and data from the second and the third day of the recording were used for analysis. Locomotor activity was collected in 1 min bins and analyzed with a MATLAB-based software written in-house. The total sleep, the average length of sleep bouts, and the number of sleep bouts were calculated based on the sleep definition as a period of 5 or more minutes of behavioral immobility.

Generating d5-HT1A Mutant through Imprecise Excision of a P Element

The fly line, P[EPgy2]EY09988, carrying a P element insertion near the C-terminal end of the d5-HT1A transcript (~2.5 kb downstream of the stop codon of the coding region) was obtained from the Bloomington stock center (stock number 17629) [39]. Mobilization and excision of the P element were as described [56]. ~500 independent excision lines were screened by genomic PCR via primers specific for the eighth exon of d5-HT1A. The imprecise excision line and three precise excision lines were maintained over a second chromosome balancer with the GFP marker (CyO, P[ActGFP]JMR1).

Pharmacological Treatment of Flies

5-HTP and RU486 were obtained from Sigma. For sleep assays, flies were maintained on food containing 1 mg/ml 5-HTP or 500 μm RU486 (treated group) or regular food (mock treated group) for 4 days. Behavioral data from the second and third day were used for analysis.

In Situ RNA Hybridization

Adult fly brains were dissected and fixed in 4% parafomaldehyde/PBS with 0.05% Tween 20 at 4ºC overnight. The tissues were then dehydrated and rehydrated through a methanol gradient (25%, 50%, 75%, 100%), followed by protease K (Roche, ~19 mg/ml) digestion for 10 min at room temperature. After the second fixation (4% PFA/PBS, 0.05% Tween 20, room temperature for 20 min) and prehybridization (50% fomamide, 5×SSC, 5× denhardts solution, 250 μg/ml tRNA, 500 μg/ml ssDNA, 50 μg/ml Heparin, 2.5 mM EDTA, 0.1% Tween 20, at 58ºC for overnight), the tissues were incubated with hybridization solution including probes (~25 ng/μl) at 58ºC for 48 hr. The antisense and sense RNA probes for d5-HT1A (nucleotides 370–726) were synthesized and labeled with a biotin RNA labeling kit (Roche). Signals from hybridized RNA were detected with the Tetramethylrhodamine-conjugated tyramide (Perkin-Elmer). Brains were imaged with a confocal microscope (Leica) and processed with Open-lab and Adobe Photoshop software.

Expression Constructs, Transfection, and Pharmacological Treatments in S2 Cells

Cloning and expression of the full-length and truncated d5-HT1A in the S2 cell culture system were as described [27]. The coding region of d5-HT1A was amplified with the UAS-d5-HT1A construct as template and cloned into pIZ/His-V5 (Invitrogen) [57]. A V5 epitope was tagged to the carboxyl terminus of the receptor. For the receptor internalization assay, S2 cells stably expressing d5-HT1A-V5 were seeded on glass cover slips and serum starved overnight. After treatment with 100 nM serotonin for 30 min, the cover slips were washed with PBS and fixed in 4% PFA/PBS followed by immunostaining with an anti-V5 antibody. The cells were analyzed with a confocal microscope (Leica). The images were processed with Open-lab and Adobe Photoshop software.

Quantitative Immunoassay for Serotonin Levels in Fly Heads

For each sample, ~25 fly heads were homogenized and subjected to acetylation, followed immediately by immunoassay with a serotonin EIA kit as described by the manufacturer (ALPCO). The results were calculated based on a standard curve.

Supplemental Data

Five Supplemental Figures can be found with this article online at http://www.current-biology.com/cgi/content/full/16/11/1051/DC1/.

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