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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