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Serotonin Modulates Circadian Entrainment in *Drosophila*

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Summary

Entrainment of the *Drosophila* circadian clock to light involves the light-induced degradation of the clock protein timeless (TIM). We show here that this entrainment mechanism is inhibited by serotonin, acting through the *Drosophila* serotonin receptor 1B (d5-HT1B). d5-HT1B is expressed in clock neurons, and alterations of its levels affect molecular and behavioral responses of the clock to light. Effects of d5-HT1B are synergistic with a mutation in the circadian photoreceptor cryptochrome (CRY) and are mediated by SHAGGY (SGG), *Drosophila* glycogen synthase kinase 3β (GSK3β), which phosphorylates TIM. Levels of serotonin are decreased in flies maintained in extended constant darkness, suggesting that modulation of the clock by serotonin may vary under different environmental conditions. These data identify a molecular connection between serotonin signaling and the central clock component TIM and suggest a homeostatic mechanism for the regulation of circadian photosensitivity in *Drosophila*.

Introduction

The circadian clock in *Drosophila* consists of interlocked, molecular feedback loops that drive circadian oscillations of gene and protein expression (Williams and Sehgal, 2001). In the major loop, the period (PER) and timeless (TIM) proteins negatively regulate synthesis of their own mRNAs in a rhythmic fashion, resulting in cycling mRNA and protein levels. The cycling of clock proteins also depends upon tightly controlled turnover, which is regulated by the action of several kinases and phosphatases, including casein kinase I and II, protein phosphatase 2A, and glycogen synthase kinase 3β (Akten et al., 2003; Kloss et al., 1998; Lin et al., 2002; Martinek et al., 2001; Sathyanarayanan et al., 2004). Entrainment of the clock to light is mediated, in part, by the light-induced degradation of TIM in response to photic signals transmitted by the circadian photoreceptor CRY (Williams and Sehgal, 2001; Lin et al., 2001).

In addition to CRY, the visual system can entrain the clock, but the mechanisms involved are not known (Helfrich-Forster et al., 2001; Yang et al., 1998). It is clear though that entrainment of the clock is complex and can occur through multiple pathways. Moreover, given that the response of the clock to light is different at different times of day and can also vary based upon environmental conditions, it is likely to be modulated by other systems (Hall, 2000; Refinetti, 2003). In mammals, one such system is that of the neurotransmitter serotonin (5-hydroxytryptamine [5-HT]). Serotonin has an important role in the modulation of various physiological functions and complex behaviors such as locomotion, learning and memory, feeding, addiction, and aggression (Buhot et al., 2000; Gaspar et al., 2003). With respect to the mammalian circadian system, it is implicated in entrainment, particularly in mediating nonphotic phase shifts, and sleep (Glass et al., 2003; Morin, 1999). Consistent with a role for serotonin in circadian modulation, the hypothalamic suprachiasmatic nucleus (SCN), the central circadian oscillator in mammals, receives photic input through the retinohypothalamic tract (RHT), as well as dense serotonergic innervation (Pickard and Rea, 1997). Among others, the mammalian 5-HT1A receptor, the major autoreceptor that regulates the release of serotonin from the dorsal raphe, is associated with the regulation of circadian behavior and sleep (Boutrel et al., 2002; Horikawa et al., 2000; Smart and Biello, 2001). However, despite studies implicating serotonin signaling in the circadian system, its clear role and mechanism of action remain elusive (Morin, 1999).

In insects, serotonin is implicated in visual system function and, to a limited extent, in circadian rhythms (Cymborowski, 1998; Meinertzhagen and Pyza, 1999; Page, 1987; Saifullah and Tomioka, 2002). However, little is known about serotonin signaling in *Drosophila*, the insect most used for molecular analysis of circadian rhythms. In this report, we assayed the effects of serotonin on *Drosophila* circadian rhythms using pharmacological, genetic, and molecular approaches. We focused on one subtype of *Drosophila* serotonin receptors, d5-HT1B, an ortholog of the mammalian 5-HT1A receptor. d5-HT1B is expressed in the clock network and affects circadian light sensitivity by decreasing the activity of GSK3β, which, in turn, produces increased stability of TIM. Our data indicate that serotonin affects the post-translational control of clock proteins and establish a physiological role for it in regulating *Drosophila* circadian photosensitivity.

Results

Serotonin Reduces Circadian Photosensitivity in *Drosophila*

In mammals, serotonergic agents induce phase shifts in the behavioral rhythm similar to those produced by nonphotic stimuli such as locomotor activity (Glass et al., 2003). To address a role for serotonin in entrainment in *Drosophila*, we first subjected adult flies to acute treatment (10 min) with serotonin at different circadian times in constant darkness (DD). Under these conditions, we saw no evidence for a significant phase shift produced by serotonin treatment (data not shown). Extended treatment (24 hr) with agents that increase the level of extracellular serotonin, such as the synthesis

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precursor 5-Hydroxy-L-tryptophan (5-HTP) and the reuptake inhibitor fluoxetine hydrochloride (Prozac), did not produce phase shifts either. However, these treatments had an effect on light-induced phase shifts. Circadian photosensitivity in wild-type flies was measured as the magnitude of the phase shift induced by a short light pulse in the late night. Flies were first entrained to a 12:12 light:dark (LD) cycle, then transferred to DD and pulsed on the first day of DD at circadian time (CT) 20, which is 8 hr into the subjective dark period. The shift in phase was determined by comparing daily activity offsets in pulsed flies and unpulsed controls prior to and after the light pulse. We used light pulses of two different light intensities. As demonstrated by previous studies (Suri et al., 1998; Yang et al., 1998), the magnitude of the phase shift depends upon the dose of the light pulse, and so a larger shift was observed with the more intense pulse. 5-HTP, Prozac, and another reuptake inhibitor, citalopram, reduced light-induced phase shifts significantly, particularly in response to the high intensity light pulse (Figure 1A), suggesting that increased serotonin levels lead to decreased light responses in flies.

To identify possible sites of interaction between the serotonin and circadian systems in *Drosophila*, we determined their relative distribution in the fly brain. Pigment-dispersing factor (PDF) is a marker for the ventral lateral neurons (LNvs), which are a major component of the *Drosophila* central clock (Renn et al., 1999). Adult fly brains expressing green fluorescent protein (GFP) under control of the *Pdf* promoter were stained with an anti-serotonin antibody. As previous described (Lundell and Hirsh, 1994; Valles and White, 1988), cell bodies and projections of serotonergic neurons were observed in distinct areas of the brain. These include a cluster located in the posterior lateral subesophageal ganglion, which will henceforth be called SE5HT-IR. Serotonergic neurons were also observed close to the cell bodies of the LNvs (Figure 1B). Using an mCD8::GFP reporter, we also found that large and small LNvs (l-LNvs and s-LNvs) receive projections from Dopa Decarboxylase (Ddc)-expressing cells (data not shown). Although Ddc is expressed in dopaminergic and serotonergic neurons, in general these observations are consistent with the reported close spatial association of serotonergic systems and clock cells in other organisms (Meinertzhagen and Pyza, 1999; Pickard and Rea, 1997).

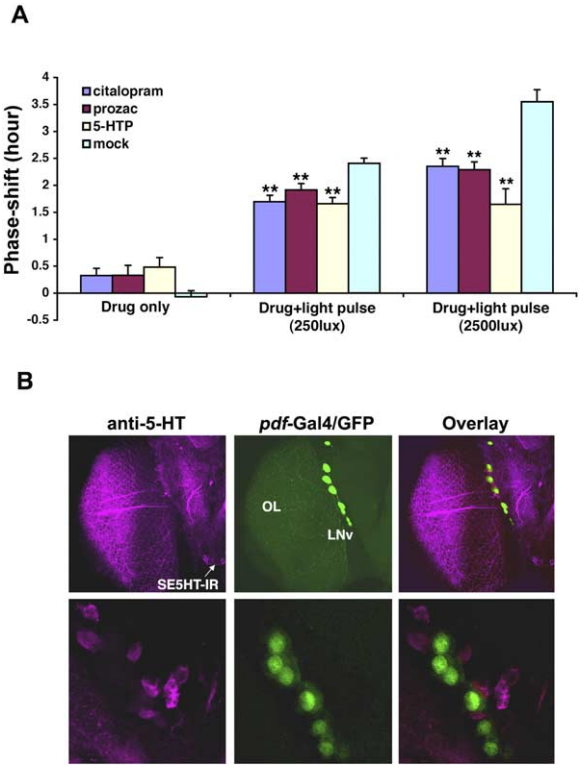


Figure 1. Inhibition of *Drosophila* Circadian Light Sensitivity by Serotonin

(A) Serotonergic agents reduce phase advances induced by a light pulse at CT20. Compared to mock-treated flies (n = 21), flies treated with Prozac (1 mg/ml) (n = 16), 5-HTP (2 mg/ml) (n = 14), and citalopram (1 mg/ml) (n = 26) showed significantly (**p < 0.01, by Student's t test) reduced phase advances in response to a light pulse of ~2500 lux at CT20. Similar results were seen using a low-intensity light pulse of ~250 lux. Each drug-treated group was compared to mock-treated flies. Error bars represent SEM. The drug treatments alone (Prozac, n = 14; 5-HTP, n = 21; citalopram, n = 15; mock, n = 15) produced no significant phase shift and served as control.

(B) Clock cells (ventral lateral neurons) are in close proximity to serotonergic neurons in the adult fly brain. An anti-serotonin antibody (magenta) was used to stain adult brain tissue of *Pdf*-Gal4/UAS-GFP (green) flies. A group of neurons that stained positive for serotonin in the subesophageal ganglion region is labeled as SE5HT-IR and indicated with an arrow. OL, optic lobe; LNv, ventral lateral neuron. (Top panel) Projection of a stack of confocal images (40× magnification). (Bottom panel) A 0.5 μm thick optical section (100× magnification).

Characterization of the d5-HT1B Receptor

The role of the mammalian 5-HT1A receptor in many animal behaviors, including aggression and sleep, was demonstrated through pharmacological treatments and knockout mouse models (Boutrel et al., 2002; Ramboz et al., 1998). However, the involvement of this receptor in circadian regulation is supported only by pharmacological studies and thus is still uncertain (Smart and Biello, 2001). Of the four serotonin receptors identified in the *Drosophila* genome, the closest ortholog of the mammalian 5-HT1A receptor is the *Drosophila* serotonin receptor subtype 1B (d5-HT1B), with 43% sequence similarity between the two receptors (Saudou et al., 1992). To investigate a possible role of this receptor subtype in circadian rhythms, we first assayed the properties of d5-HT1B in an S2 cell culture system. In response to serotonin and to 8-OH-DPAT, a mammalian 5-HT1A receptor specific agonist, the d5-HT1B receptor was internalized in intracellular vesicles and activated downstream mitogen-activated protein kinase (MAPK) (see Figure S1 in the Supplemental Data available with this article online). Thus, this receptor shares molecular and pharmacological properties with its mammalian counterpart (Albert and Tiberi, 2001).

There is virtually no information about the expression pattern or the role of d5-HT1B in adult flies. We initiated our analysis of this receptor by determining its spatial

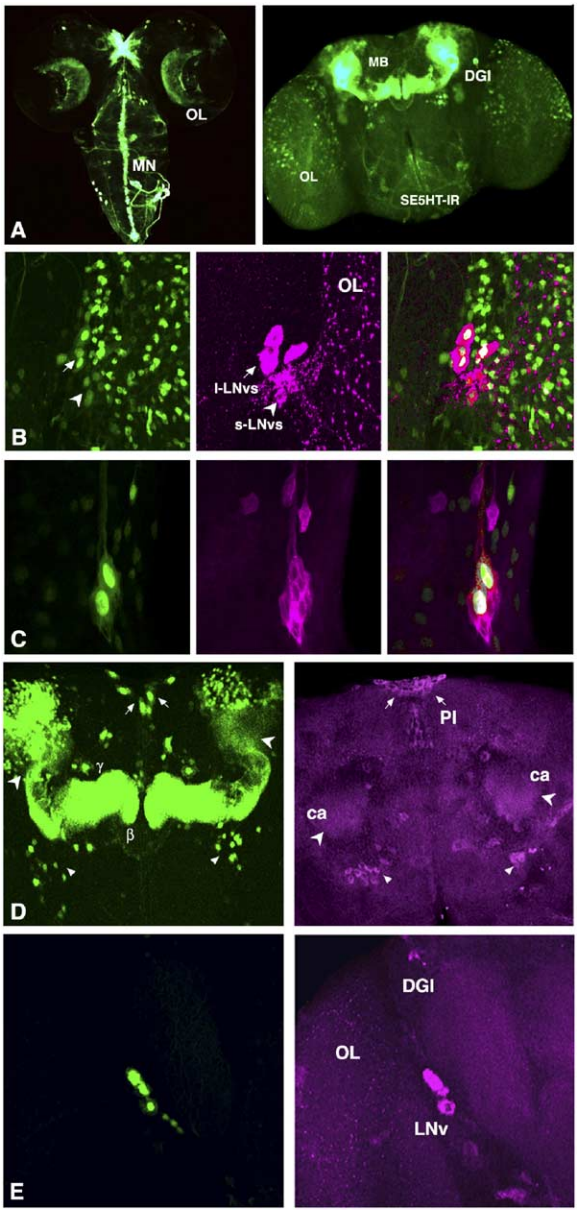


Figure 2. The Spatial Expression Pattern of d5-HT1B in Fly Brains

(A) The d5-HT1B mRNA is expressed in larvae and in adult brains. Flies expressing GFP-NLS under control of d5-HT1B-Gal4 were collected as third instar larvae (left) or as adults (right). MB, mushroom bodies; DGI, dorsal giant interneurons; MN, midline neurons in the ventral ganglion.

(B) The d5-HT1B promoter is expressed in LNvs. (Left) d5-HT1B-Gal4-driven GFP-NLS expression in the LNvs and optic lobes. Large (l-LNvs) and small (s-LNvs) LNvs are indicated by arrows and arrowheads, respectively. (Middle) Anti-PDF staining (magenta) shows the cell bodies and the projections of the LNvs into the optic lobes. (Right) Overlay image.

(C) d5-HT1B is expressed in the SE5HT-IR neurons. (Left) d5-HT1B-Gal4-driven GFP-NLS expression in the SE5HT-IR cells in the lower subesophageal ganglion region (SOG). (Middle) Anti-serotonin staining (magenta). (Right) Overlay image.

(D) The d5-HT1B protein expression pattern in the midbrain region of the adult fly. Frontal views show the expression of GFP-NLS driven by d5-HT1B-Gal4 (left, green) and the pattern detected with an anti-d5-HT1B antibody (right, magenta). Arrows point to the pars intercerebralis neurons (PI). Large arrowheads indicate the calyces (ca) of the mushroom body, while small arrowheads point to a group of cells below the β and γ lobes of the mushroom body.

(E) Expression of d5-HT1B in LNvs. GFP-NLS driven by *Pdf*-Gal4 (green) was used as a marker for LNvs. d5-HT1B signals (magenta) were observed in cell bodies of LNvs.

expression pattern. We generated transgenic flies containing a genomic fragment of the d5-HT1B upstream region fused to GAL4. The d5-HT1B-GAL4 driver was crossed to UAS-GFP transgenic flies, and the expression pattern of the d5-HT1B promoter was then visualized through fluorescence microscopy. The expression of d5-HT1B is initiated in the late embryonic stage and continues through all developmental stages, with abundant expression in both larval and adult central nervous systems. In the adult fly brain, d5-HT1B expression was observed in the mushroom body, the pars intercerebralis (PI) neurons, a subgroup of dorsal neurons, the LNvs, the optic lobes, and SE5HT-IR neurons (Figure 2A). To avoid transactivation caused by insertion sites of the Gal4 driver, multiple transgenic lines carrying independent insertions of the Gal4 driver on different chromosomes were tested, and similar patterns of GFP expression were observed (data not shown).

To determine the relationship of d5-HT1B-expressing neurons to serotonergic neurons and clock neurons, d5-HT1B-Gal4/UAS-GFP fly brains were costained for serotonin and PDF expression. The colocalization of d5-HT1B and PDF in larval (data not shown), as well as adult fly brains (Figure 2B), indicates that the receptor is expressed in LNvs at both stages; in adults it is expressed in large and small LNvs. There was no significant overlap of d5-HT1B and serotonin expression in the midbrain and the optic lobes (data not shown). However, the expression of d5-HT1B in the SE5HT-IR neurons suggests that it might function as an autoreceptor in these cells (Figure 2C).

To study the expression pattern of the d5-HT1B protein, we generated a polyclonal antibody against the third intracellular loop of the receptor. The antibody reacts specifically with a protein of molecular weight ~70 kDa (which matches the predicted size of the d5-HT1B protein) in fly head extracts. No such signal was observed with either preimmune serum from the immunized animal or with antiserum preabsorbed with purified d5-HT1B protein (data not shown). In adult brain whole mounts, immunostaining using this antibody generated signals in the mushroom bodies, LNvs, dorsal neurons, PI neurons, and optic lobes (Figure 2D). As noted above, the same structures were labeled in the d5-HT1B-Gal4/UAS-GFP flies, indicating good agreement between the two methods used to visualize receptor expression. The stronger signals obtained with the antibody in the large LNvs (Figure 2E) and the PI neurons, and the relatively weaker signals in the small LNvs and the mushroom bodies, may be indicative of altered receptor stability in these cells. Sections of adult heads did not indicate any expression of d5-HT1B in the eye (data not shown).

To test possible circadian regulation of the d5-HT1B receptor, we determined the temporal expression pat-

Table 1. Behavioral Phenotype of Flies with Modified d5-HT1B Levels

Genotype	Environmental Conditions	No.	Percent Rhythmic	Period Length (Hour ± SEM)	FFT Relative Power ± SEM
Overexpression					
tim-Gal4/UAS-1B	DD	31	96.8	24.25 ± 0.13	0.149 ± 0.016
UAS-1B	DD	31	100	23.32 ± 0.07	0.212 ± 0.020
tim-Gal4	DD	15	100	24.07 ± 0.10	0.121 ± 0.010
UAS-1B, UAS-SGG10	DD	45	91.1	23.62 ± 0.04	0.123 ± 0.010
tim-Gal4/UAS-SGG10	DD	41	87.8	18.01 ± 0.05	0.079 ± 0.004
tim-Gal4/UAS-1B, UAS-SGG10	DD	21	95.2	19.85 ± 0.13	0.058 ± 0.003
UAS-1B, UAS-SGG10 (A81T)	DD	21	95.2	23.38 ± 0.07	0.085 ± 0.010
tim-Gal4/UAS-SGG10 (A81T)	DD	25	92	23.05 ± 0.06	0.080 ± 0.006
tim-Gal4/UAS-1B, UAS-SGG10 (A81T)	DD	25	88	23.30 ± 0.09	0.068 ± 0.006
tim-Gal4/UAS-1B	LL (2500 lux)	41	21.9	26.0 ± 1.20	0.109 ± 0.018
tim-Gal4/UAS-1B	LL (250 lux)	33	45.5	26.97 ± 0.74	0.123 ± 0.016
UAS-1B	LL (2500 lux)	25	0	—	—
UAS-1B	LL (250 lux)	30	13.3	30 ± 0	0.071 ± 0.017
tim-Gal4	LL (2500 lux)	16	0	—	—
Knockdown					
elav-Gal4/UAS-1BRNAi	LL (<10 lux)	16	100	24.75 ± 0.144	0.188 ± 0.010
elav-Gal4	LL (<10 lux)	16	100	24.16 ± 0.160	0.228 ± 0.025
UAS-1BRNAi	LL (<10 lux)	14	92.9	24.19 ± 0.083	0.085 ± 0.010
elav-Gal4/UAS-1BRNAi	DD	31	100	23.64 ± 0.039	0.176 ± 0.031
elav-Gal4	DD	21	100	23.44 ± 0.098	0.156 ± 0.019
UAS-1BRNAi	DD	14	100	23.85 ± 0.026	0.151 ± 0.010
1B-Gal4/UAS-1BRNAi	LL (<10 lux)	32	93.8	24.78 ± 0.170	0.110 ± 0.010
1B-Gal4	LL (<10 lux)	32	100	24.09 ± 0.070	0.164 ± 0.014
UAS-1BRNAi	LL (<10 lux)	30	93.8	23.73 ± 0.040	0.080 ± 0.010
1B-Gal4/UAS-1BRNAi	DD	16	93.8	23.42 ± 0.046	0.122 ± 0.010
1B-Gal4	DD	16	100	23.38 ± 0.056	0.140 ± 0.012
UAS-1BRNAi	DD	15	93.3	23.49 ± 0.160	0.079 ± 0.010

tern of the d5-HT1B transcript and protein using RNase protection assays and Western blots, respectively. There was no significant circadian variation in RNA or protein levels of d5-HT1B in the presence of LD cycles or in DD (Figure S2A). However, receptor levels were affected in clock mutants. Levels were upregulated in *timeless* (*tim*^{01}) flies and downregulated in *cycle* (*cyc*^{0}) flies (Figure S2B), suggesting possible effects of the circadian system on d5-HT1B protein levels.

Overexpression of d5-HT1B Reduces *Drosophila* Circadian Light Sensitivity

d5-HT1B is expressed in clock cells, where it could mediate the effects of serotonin on the circadian system. We investigated a possible association of d5-HT1B with the inhibitory effect of serotonin on circadian light responses by genetically modifying levels of d5-HT1B. To increase levels of d5-HT1B, we generated transgenic flies carrying a UAS-d5-HT1B construct and used different Gal4 driver lines to drive its expression. The drivers included *tim*-Gal4, which gives high-level expression in neurons associated with circadian function, such as the lateral neurons; GMR-Gal4, which has an eye-specific expression pattern; *elav*-Gal4, which is expressed panneuronally; and d5-HT1B-Gal4, which targets overexpression to cells that normally express d5-HT1B. Increased d5-HT1B protein levels in the overexpression flies were confirmed by Western blots (Figure S3A).

The rest-activity rhythms of d5-HT1B-overexpressing flies in LD cycles and in DD were similar to those of wild-type flies (Table 1). We also tested the effects of overexpressing d5-HT1B on the cycling of the *period* (*per*) clock gene in DD, using a *per*-luciferase reporter (BG-LUC) construct (Stanewsky et al., 1997). Both the cycling and the overall levels of BG-LUC were comparable to those of control flies (data not shown), confirming that overexpressing d5-HT1B does not affect clock function in free-running conditions. To assay the circadian photosensitivity of these flies, we used the behavioral phase shift assay described above. We found that expressing d5-HT1B in clock neurons, using the *tim*-Gal4 driver, significantly reduced the magnitude of the phase shift induced by a short light pulse in the late (CT20) as well as in the early subjective night (CT15) (Figure 3A). Expressing d5-HT1B with a single copy of either *elav*-Gal4 or d5-HT1B-Gal4 also reduced phase shifts, although to a lesser extent (data not shown). The weaker effect of these drivers is most likely because they do not drive expression of the receptor in clock cells to the levels obtained with *tim*-Gal4. In support of this idea, flies carrying two copies of each of the UAS-1B and d5-HT1B-Gal4 transgenes showed reduced phase shifts, similar to those produced when expression of d5-HT1B was driven by *tim*-Gal4 (Figure 3A). Expression of d5-HT1B by GMR-Gal4 had no such effect (data not shown), suggesting that central clock neurons, instead of photoreceptors cells in the eye, are the relevant target cells of this inhibitory effect.

Light-induced behavioral phase shifts in *Drosophila* are associated with the degradation of the clock protein TIM in LNvs (Suri et al., 1998; Yang et al., 1998). To determine if this molecular response was affected in flies overexpressing d5-HT1B, we exposed entrained flies to a light pulse at ZT20 and collected them 1 hr later. Through immunostaining of whole-mount fly brains,

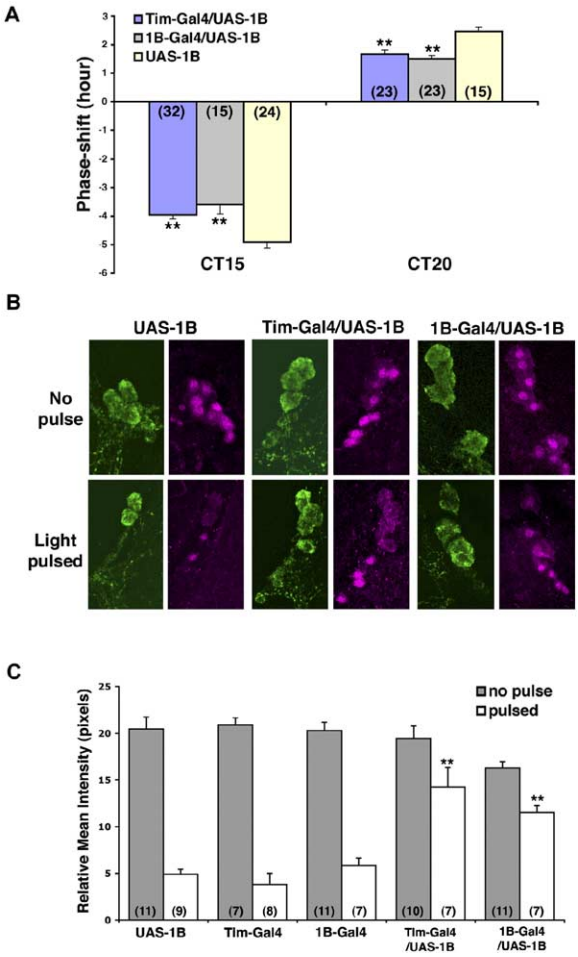


Figure 3. Overexpression of d5-HT1B Levels Decreases Circadian Light Sensitivity

(A) Phase shifts are reduced in d5-HT1B-overexpressing flies. Flies were given a short light pulse (~2500 lux for 2 min) in either the early night (CT15) or the late night (CT20) on the first day of DD. The phase delay (negative values) and advances are plotted. Error bars represent SEM. Genotypes and numbers of flies (in parentheses) used in each data set are indicated. As compared to UAS-d5-HT1B (UAS-1B) control flies, *tim*-Gal4/UAS-d5-HT1B (TG/UAS-1B) and d5-HT1B-Gal4/UAS-d5-HT1B (1B-gal4/UAS-1B, two copies of each transgene) flies showed significantly (**$p < 0.01$, by Student's t test) reduced phase shifts.

(B) Light-induced TIM degradation is reduced in flies overexpressing d5-HT1B. Flies were pulsed with light (~2500 lux, 2 min) at ZT20 and collected 1 hr later, along with unpulsed controls. Fly brains were fixed immediately and subjected to immunostaining. Representative confocal images of whole-mount brains costained with anti-TIM (magenta) and anti-PDF (green) antibodies are shown.

(C) Quantification of the TIM staining in LNvs. The relative mean intensity of the TIM signal is plotted. Error bars represent SEM. After a light pulse, flies overexpressing d5-HT1B had significantly ($p < 0.01$, by Student's t test) higher levels of TIM than control flies.

light-induced TIM degradation in LNvs was measured and quantified. TIM levels after a light pulse were significantly higher in flies expressing d5-HT1B under control of either *tim*-Gal4 or d5-HT1B-Gal4, as compared to the controls (Figures 3B and 3C). The effects were more evident in the small LNvs as compared to the large LNvs (see Discussion).

Thus, behavioral and molecular experiments indicate that flies expressing high levels of the d5-HT1B receptor have reduced circadian light responses, similar to flies treated with serotonergic agents.

RNAi-Mediated Knockdown of d5-HT1B Increases Circadian Photosensitivity and Eliminates the Inhibitory Effect of 5-HTP on Light Responses

To further investigate the role of d5-HT1B in circadian photosensitivity, particularly in the inhibitory effect of serotonin, we knocked down d5-HT1B expression through RNA interference using a UAS-d5-HT1B RNAi transgene. The RNAi construct was expressed using the d5-HT1B-GAL4 driver; this reduced levels of endogenous d5-HT1B by >70% and decreased levels of overexpressed d5-HT1B (Figure 4A). To test the effects of reduced d5-HT1B levels on circadian light sensitivity, we exposed these flies to light pulses of different intensities. d5-HT1B knockdown flies showed significantly increased phase shifts as compared to control flies (Figure 4B). To determine if d5-HT1B is required for the inhibitory effects of serotonin on photosensitivity, we treated the knockdown flies with 5-HTP and assayed light-induced phase shifts. As reported above for wild-type flies, the light response of control flies was inhibited by 5-HTP treatment. However, the same treatment did not have a significant effect on the d5-HT1B knockdown flies (Figure 4C). Thus, flies with reduced levels of d5-HT1B display enhanced light-induced phase shifts that are not inhibited by 5-HTP treatment.

We also tested the specific role of clock cells in the effect of d5-HT1B knockdown by using a *tim*-gal4 driver. Flies in which d5-HT1B was knocked down with the *tim*-Gal4 driver showed significantly increased light-induced phase shifts at low light intensity as compared to control flies. In addition, 5-HTP treatment did not inhibit the light response of these flies (Figure S4). Thus, the effect of d5-HT1B knockdown does appear to be mediated, at least in part, by clock cells, although the *tim*-Gal4 driver had less of an effect than the d5-HT1B-Gal4 driver (see Discussion).

We also measured photosensitivity of the knockdown flies by assaying them under constant dim light conditions (<10 lux), which typically produce long periods in wild-type flies (Konopka et al., 1989). As shown in Figure 4D, expression of the RNAi transgene specifically in cells that normally express d5-HT1B resulted in circadian periods significantly longer than those of controls (see also Table 1), suggesting increased circadian photosensitivity in flies with reduced d5-HT1B levels. Use of the *elav*-Gal4 driver produced similar results (Table 1). Together, these experiments indicate that d5-HT1B is part of a mechanism for modulating circadian photosensitivity and that it mediates inhibitory effects of serotonin on *Drosophila* circadian light responses.

d5-HT1B Overexpression and the cry^b Mutation Have Synergistic Effects on Circadian Photosensitivity

Drosophila circadian light responses are mediated, in part, by the circadian photoreceptor cryptochrome (CRY). Flies carrying a mutation in the *cry* gene (cry^b) have very low light sensitivity. They show almost no phase shift in response to a light pulse at night and are

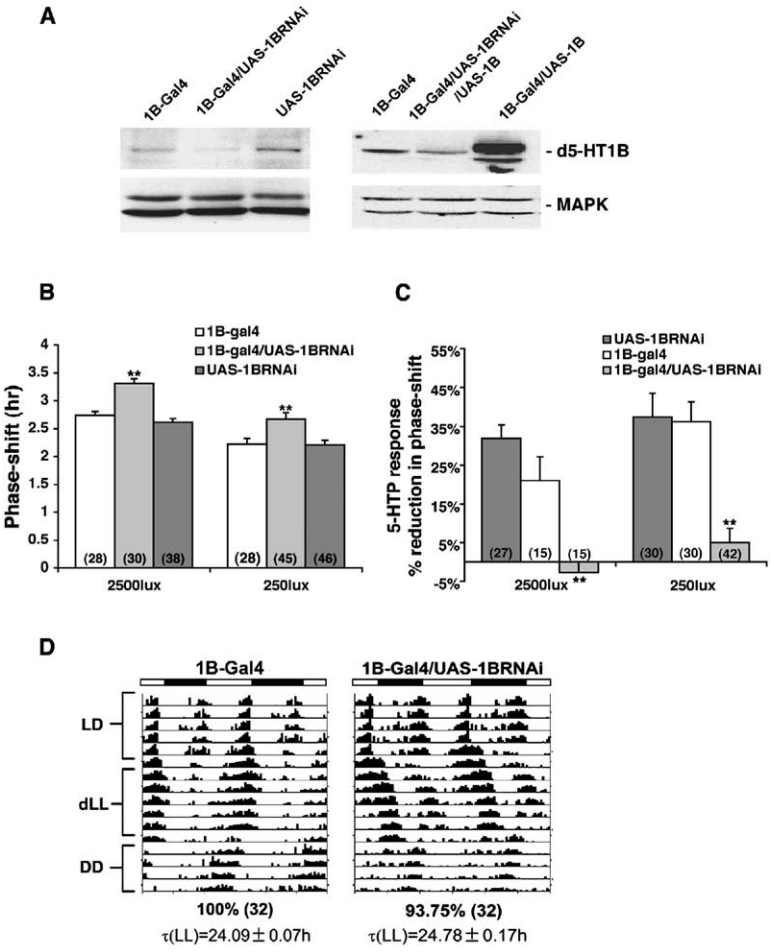


Figure 4. RNAi-Mediated Knockdown of d5-HT1B Increases Circadian Light Sensitivity

(A) Effect of an RNAi transgene on the expression of endogenous and transgenic d5-HT1B. Representative Western blots are shown. (Left) Endogenous d5-HT1B protein levels in flies carrying a UAS-d5-HT1BRNAi transgene driven by the d5-HT1B-Gal4 driver are lower than those in parental control flies. (Right) d5-HT1BRNAi-mediated knockdown of the d5-HT1B overexpression driven by d5-HT1B-Gal4. Total MAPK levels were assayed to control for loading. Similar results were obtained in three independent experiments.

(B) d5-HT1B knockdown flies display enhanced light-induced phase shifts. d5-HT1B knockdown flies and parental controls were subjected to light pulses of two intensities (2500 lux and 250 lux) at CT20. Genotypes and the number of flies (in parentheses) in each data set are indicated. Error bars represent SEM. As compared to parental controls, knockdown flies showed significantly (**$p < 0.01$, by Student's t test) larger phase shifts in response to light pulses of both intensities.

(C) 5-HTP does not inhibit light-induced phase shifts in d5-HT1B knockdown flies. Flies were treated with 5-HTP (2 mg/ml) and subjected to a light pulse at CT20. The response to 5-HTP is depicted as the percent reduction in the light-induced phase shift. Error bars represent SEM. The response to 5-HTP in knockdown flies is significantly (**$p < 0.01$, by Student's t test) different from that in parental controls.

(D) d5-HT1B knockdown flies display longer periods in constant dim light. Flies expressing a UAS-d5-HT1B RNAi transgene driven by d5-HT1B driver were assayed in light:dark (LD) and then in constant dim light (dLL). The number of flies tested (in parentheses) and the average circadian period (τ) in constant dim light conditions are indicated at the bottom. Also see Table 1.

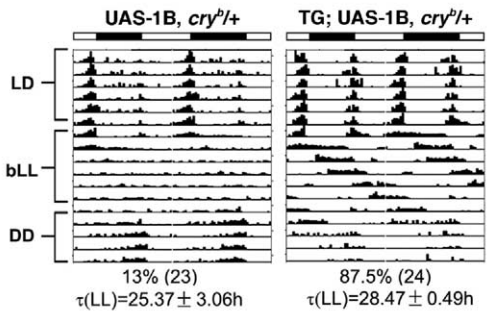
rhythmic in constant bright light conditions that render wild-type flies arrhythmic (Emery et al., 2000; Stanewsky et al., 1998). To determine how the effects of serotonin and d5-HT1B on circadian light sensitivity relate to the CRY signaling pathway, we studied genetic interactions between d5-HT1B and CRY.

We first assayed rhythms of flies overexpressing d5-HT1B in clock cells under constant bright light conditions. For these and other experiments described below in which multiple mutations/transgenes were introduced into the same background, we expressed d5-HT1B with the *tim*-Gal4 driver, since this involved the use of fewer transgenes. As compared to background controls, flies overexpressing d5-HT1B showed increased rhythmicity in constant bright light (2500 lux). The difference was more significant in weaker illumination (250 lux) (Table 1). Overexpression of two other *Drosophila* G protein-coupled receptors (*mth* and d5-HT7) did not have this effect, indicating that it was specific for d5-HT1B (data not shown).

Next, we assayed the effect of expressing transgenic d5-HT1B in a cry^b background. Overexpression of the d5-HT1B receptor in flies homozygous for the cry^b mutation did not have a discernible effect on their light sensitivity due to the strong phenotype produced by the cry^b mutation alone. However, 87% of the cry^b heterozygotes overexpressing d5-HT1B were rhythmic in constant bright light, while only 13% of the control flies were rhythmic under these conditions. Fast Fourier transform (FFT) analysis indicated that cry^b heterozygous flies overexpressing d5-HT1B had very strong behavioral rhythms in constant bright light, although the average period was lengthened to about 28 hr (Figure 5A). This period-lengthening phenotype mimics the response of wild-type flies to constant dim light conditions (Konopka et al., 1989) (also see above) and indicates that the flies are capable of a reduced response to light. The effects of d5-HT1B on circadian photosensitivity were observed with multiple, independent insertions. In addition, reducing d5-HT1B expression with the RNAi transgene, or increasing CRY levels with a UAS-CRY transgene, eliminated this effect, indicating specificity for d5-HT1B and cry^b, respectively (Figure 5B). We infer that overexpressing d5-HT1B reduces circadian photosensitivity and has synergistic effects with the cry^b mutation.

We also examined free-running behavior in cry^b flies overexpressing d5-HT1B. A large number (~43%) of

A



B

Genotype	% rhythmic in bLL	n
tim-gal4; *cry*^{b}/+	10.7%	28
UAS-1B, *cry*^{b}/+	13.0%	23
tim-gal4; UAS-1B, *cry*^{b}/+	87.5%	24
tim-gal4/UAS-CRY; UAS-1B, *cry*^{b}/+	23.4%	48
UAS-CRY; UAS-1B, *cry*^{b}/+	14.8%	27
tim-gal4/UAS-1BRNAi; UAS-1B, *cry*^{b}/+	14.3%	21
tim-gal4/UAS-d5-HT7; *cry*^{b}/+	15.8%	26

C

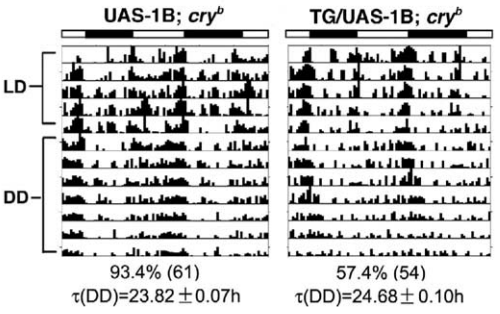


Figure 5. Synergistic Effects of d5-HT1B and the *cry*^{b} Mutation on Circadian Photosensitivity

(A) Representative actograms of individual flies monitored in LD, constant bright light of 2500 lux (bLL), and DD. In a *cry*^{b} heterozygous background, flies overexpressing d5-HT1B under control of *tim*-Gal4 displayed increased rhythmicity in constant bright light. The percentage of flies rhythmic in LL, the number of flies monitored (in parentheses), and the average circadian period (τ) are indicated at the bottom.

(B) Mapping the synergistic effects of *cry*^{b} and d5-HT1B on circadian photosensitivity to the respective mutation/transgene. A summary of the constant light data is shown for flies of different genotypes. Introducing either a UAS-CRY transgene or a UAS-1BRNAi transgene in *cry*^{b}/+ flies overexpressing d5-HT1B eliminates the rhythmicity of these flies in constant bright light. Overexpression of the d5-HT7 receptor with *tim*-Gal4 does not produce a similar phenotype.

(C) d5-HT1B overexpression affects the periodicity and strength of free-running rhythms in *cry*^{b} flies. The percentage of flies rhythmic in DD, the numbers tested (in parentheses), and average periods are indicated.

these flies were arrhythmic in DD as compared to 7% of the control *cry*^{b} flies (Figure 5C). We speculate that this loss of rhythmicity is due either to the deficit in entrainment and/or to decreased synchrony between clock cells (see Discussion).

d5-HT1B Signaling Affects the Phosphorylation of SHAGGY, *Drosophila* GSK3β

To determine the molecular basis of the inhibitory effect of d5-HT1B on *Drosophila* light responses, we looked for possible connections between d5-HT1B and TIM. After a light pulse, as well as during the early hours of the day, TIM levels are higher in d5-HT1B-overexpressing flies as compared to controls (Figure 3 and Figure S3B). The BG-LUC reporter studies mentioned above suggested that d5-HT1B overexpression does not affect the expression of clock genes at the transcriptional level. Therefore, it appeared likely that the protective effect of d5-HT1B on TIM involved posttranslational modifications. In fact, although total levels of TIM were higher in d5-HT1B-overexpressing flies, we observed reduced levels of the low-mobility form (Figure 6A and Figure S3B) that corresponds to phosphorylated TIM (Dissel et al., 2004; Martinek et al., 2001; Wulbeck et al., 2005; Zeng et al., 1996). High-mobility, hypophosphorylated TIM, which is the predominant form in *per*^{0} flies (Martinek et al., 2001), was elevated in flies expressing transgenic d5-HT1B (Figure 6A). However, since d5-HT1B expression was driven by *tim*-Gal4, which is expressed weakly in the eye and also in some brain neurons that do not express endogenous d5-HT1B, these results may include effects of ectopically expressed receptor.

Phosphorylation of TIM by SHAGGY (SGG), the *Drosophila* homolog of glycogen synthase kinase 3β (GSK3β), appears to decrease its stability (Martinek et al., 2001). GSK3β activity is regulated (inhibited) by phosphorylation of an N-terminal pseudosubstrate site (ser9) and thus can be monitored through the use of phospho-specific antibodies directed to this modified form (Doble and Woodgett, 2003; Papadopoulou et al., 2004). An anti-SGG antibody recognizes two major SGG isoforms (SGG39 and SGG10) in control flies and an additional minor form (SGGY) when it is overexpressed in an EP line (Martinek et al., 2001). A phospho-specific antibody, anti-pS9-SGG, recognizes only phosphorylated SGG10 (Papadopoulou et al., 2004).

On a Western blot assay of fly head extracts, we observed increased levels of phosphorylated SGG10 (pS9-SGG) in flies expressing d5-HT1B under control of *tim*-Gal4 (Figure 6B). Endogenous pS9-SGG was sometimes difficult to detect, and it is expressed in many cells that do not express d5-HT1B, making it difficult to reliably measure an effect of the receptor on SGG phosphorylation in assays of whole fly heads. Thus, we also used flies carrying a UAS-SGG10 transgene that can express high levels of total as well as phosphorylated SGG10 (Figure S5A). Coexpression of d5-HT1B further increased the level of phosphorylated SGG10 in these flies (Figure S5A). To examine the effect in clock cells, we stained whole-mount brains with the anti-SGG and anti-pS9-SGG antibodies. The levels of phosphorylated SGG10 in LNs were significantly increased in flies coexpressing d5-HT1B (Figure 6C). Overall levels of SGG were unaffected by d5-HT1B, suggesting that d5-HT1B affects the phosphorylation rather than the level of SGG.

To verify that serotonin signaling affects SGG, we assayed SGG phosphorylation in response to 5-HTP treatment. Lithium, which is known to affect GSK3β activity in many systems (Zhang et al., 2003), was used as a control in these experiments. Levels of phosphorylated SGG10 in fly head extracts increased in response to LiCl, but not in response to NaCl. Flies fed with 2 mg/ml 5-HTP also had increased levels of phosphorylated

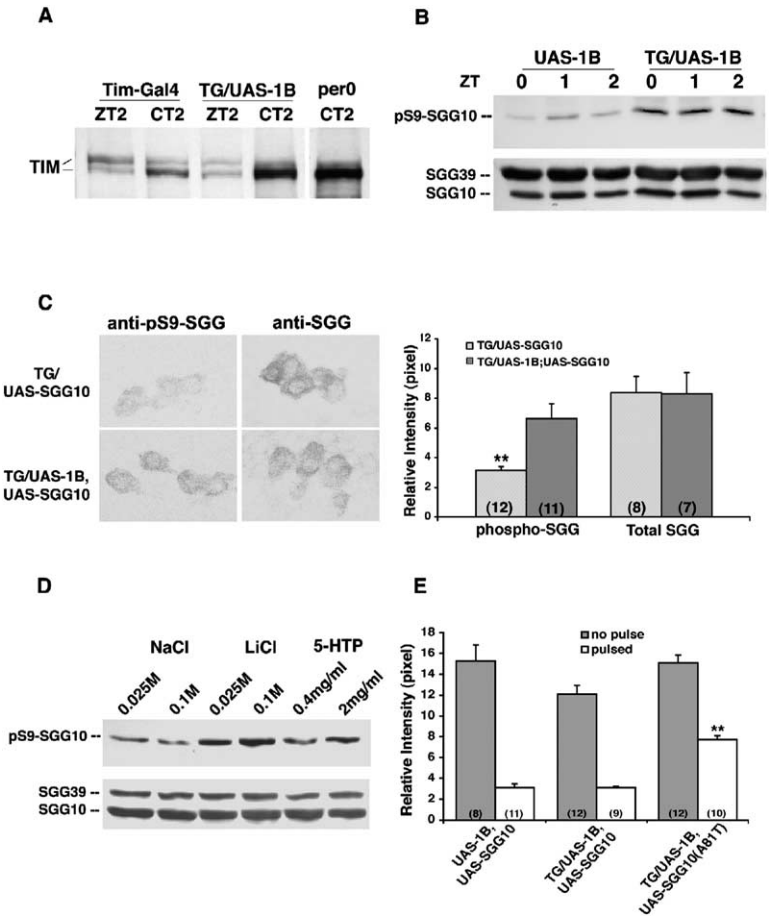


Figure 6. d5-HT1B Increases Phosphorylation of SGG/GSK3β in Adult Fly Heads

(A) Reduced phosphorylation of TIM in d5-HT1B-overexpressing flies. Head extracts from flies overexpressing d5-HT1B under control of *tim*-gal4 (TG/UAS-1B), and from parental controls (*tim*-Gal4), were assayed for TIM expression at different time points. The low-mobility form of TIM is reduced in d5-HT1B-overexpressing flies as well as in per^0 flies.

(B) Increased phospho-SGG10 in d5-HT1B-overexpressing flies. Head extracts from flies expressing d5-HT1B under control of *tim*-gal4 (TG/UAS-1B), and from parental controls (UAS-1B), were assayed by Western blots using anti-pS9-SGG (top) and anti-SGG (bottom) antibodies. Flies overexpressing d5-HT1B have elevated levels of phospho-SGG10 but normal levels of total SGG.

(C) Increased phospho-SGG10 in clock cells of flies coexpressing d5-HT1B and SGG10 under control of *tim*-gal4. (Left) Representative confocal images of LNvs stained with anti-pS9-SGG (phospho-SGG) or anti-SGG (total SGG). (Right) Quantification of anti-pS9-SGG (phospho-SGG) and anti-SGG (total SGG) staining in LNvs. There was a significant difference ($p < 0.01$, by Student's t test) in phospho-SGG10 levels between flies expressing SGG10 and flies coexpressing SGG10 and d5-HT1B. The numbers of samples in each data set are indicated in parentheses. Error bars represent SEM.

(D) Treatment with 5-HTP increases levels of phospho-SGG10 in fly head extracts. Flies overexpressing SGG10 under the control of *tim*-Gal4 were treated for 24 hr with the indicated concentrations of NaCl, LiCl, and 5-HTP. Head extracts were subjected to Western blot assays using anti-pS9-SGG (top) and anti-SGG (bottom) antibodies. While NaCl did not have an effect, LiCl and 5-HTP increased levels of phospho-SGG10 in a dose-dependent manner (quantification in Figure S4A).

(E) Increased SGG levels attenuate the effect of d5-HT1B overexpression on the TIM light response. Flies were pulsed with light at ZT20 and collected 1 hr later, along with unpulsed controls. Fly brains were fixed immediately and subjected to immunostaining. TIM levels in LNvs were quantified. After a light pulse, most of the TIM protein was degraded in control flies carrying the UAS-1B and UAS-SGG10 transgenes (UAS-1B, UAS-SGG10). Flies coexpressing d5-HT1B and SGG10 under the control of *tim*-Gal4 (TG/UAS-1B, UAS-SGG10) showed a light response similar to that of controls. An inactive form of SGG (A81T) failed to rescue the d5-HT1B phenotype, so flies coexpressing d5-HT1B with this form of SGG10 [TG/UAS-1B, UAS-SGG10 (A81T)] had significantly ($p < 0.01$, by Student's t test) higher levels of TIM after the light pulse, as compared to control flies. Error bars represent SEM.

SGG10 (Figure 6D; quantification in Figure S5B). We confirmed the interaction between SGG and d5-HT1B through genetic experiments. Overexpression of two different isoforms of SGG (SGG10, SGGY) in clock cells shortens the free-running period (Martinek et al., 2001). To determine if d5-HT1B affects the function of SGG in a circadian assay, we drove expression of the UAS-SGG10 and UAS-d5-HT1B transgenes with *tim*-Gal4 and assayed circadian behavior under free-running conditions. SGG10 overexpression produced a period of ~18 hr, which is shorter than that reported previously, most likely due to differences in the constructs used (Bourouis, 2002; Martinek et al., 2001). Importantly, the 18 hr period was lengthened to 19.7 hr by coexpression of d5-HT1B (Table 1). An inactive form of SGG10 (A81T), which displays no phenotype in a developmental assay for SGG activity, (Bourouis, 2002), did not produce a short period phenotype. Nor did coexpression of d5-HT1B have a period-lengthening effect in this background (Table 1). These results support the hypothesis that d5-HT1B decreases SGG activity and reduces circadian effects produced by its overexpression. Conversely, increased SGG activity should abolish the protective effect of increased d5-HT1B levels on TIM. To determine if this was the case, we assayed light-induced TIM degradation in clock cells of flies coexpressing d5-HT1B and SGG10. In these flies, TIM degradation in response to a light pulse was comparable to that seen in control flies that express only endogenous d5-HT1B. Overexpression of the inactive form of SGG10 (A81T) did not attenuate the effects of d5-HT1B on circadian photosensitivity, suggesting that increased SGG activity is required for this attenuation (Figure 6E). The reciprocal actions of SGG and d5-HT1B suggest that serotonin signaling modulates circadian light sensitivity by reducing SGG/GSK3β activity, which in turn leads to increased stability of TIM.

Brain Serotonin Levels Are Decreased in Flies Maintained in DD

Our data indicated that serotonin decreases the circadian response to light. To determine whether this mod-

ulation could account for differential light sensitivity at different times of day, we assayed endogenous brain serotonin at different circadian times and also in response to light pulses. There was no acute effect of light, nor any evidence for circadian control of serotonin levels (data not shown). This suggests that the effect of serotonin on circadian light sensitivity is not restricted to a specific time of day. However, we considered the possibility that it was more prevalent under some conditions. For instance, organisms maintained in DD for several days show increased sensitivity to light (Refinetti, 2003; Winfree, 1972). We tested this effect of prolonged DD on circadian light sensitivity by conducting phase shift assays. Wild-type (Canton-S) flies that had periods of 24 hr were maintained in DD for 7 days and then subjected to a light pulse at CT15 or CT20. Light-induced phase shifts were significantly larger in flies maintained in DD than in flies maintained in LD conditions (Figure S6A), confirming dark adaptation of the *Drosophila* circadian clock.

To address a possible role for serotonin in dark adaptation, we assayed serotonin levels in flies after an extended period in DD. Entrained wild-type flies were separated into groups that were maintained in either DD or LD conditions. Fly heads were collected at CT2 (or ZT2 for the flies maintained in LD cycles) after 1, 3, 5, and 7 days, and head homogenates were tested for serotonin content through quantitative enzyme immunoassays (EIA). Serotonin levels in fly heads decreased gradually with increased incubation time in DD such that differences between flies in LD and DD conditions were statistically significant ($p < 0.05$) after 5 days in DD, and more so ($p < 0.01$) after 7 days in DD (Figure 7A). After 7 days, there was an ~25% decrease in serotonin levels in flies kept in DD as compared to those in LD. Similar results were obtained with flies of two different genotypes (Figure 7B). We also examined serotonin expression under these different conditions through immunostaining. We focused on the subesophageal ganglion region that includes the SE5HT-IR neurons and their projections, because this region showed the most discrete anti-serotonin staining. More intense staining was observed in heads collected from flies maintained in LD cycles than in those derived from constant dark conditions (Figure 7C). In higher-magnification images, we observed reduced signals in both cell bodies and projections of brains collected in DD as compared to LD (Figure S6B), indicating that the differences are not due to changes in the transport of serotonin.

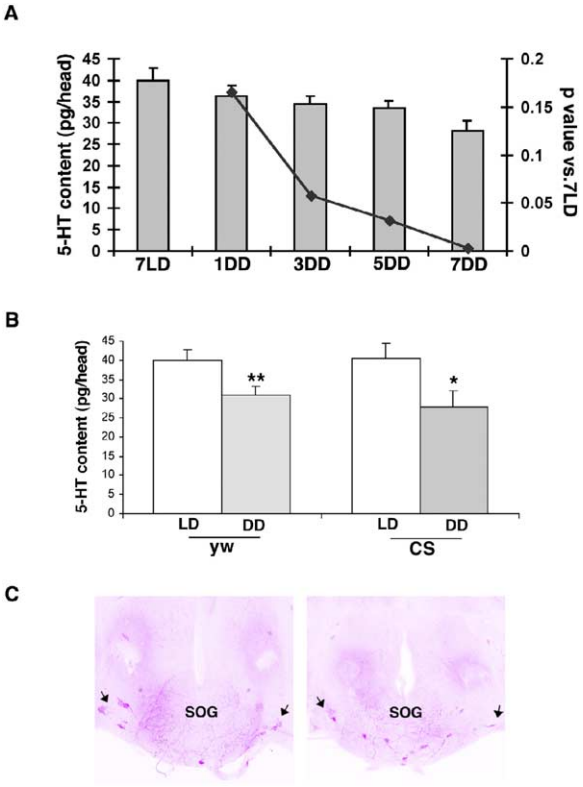


Figure 7. The Effect of Light on Serotonin Levels in the Fly Brain

(A) Serotonin levels decrease in prolonged constant darkness (DD). Flies were kept in LD cycles or in DD for various periods of time, and then collected at ZT or CT2. The bars represent serotonin content, as measured by quantitative immunoassays, per fly head. The line represents the p values (by Student's t test) comparing each sample to a sample collected from LD conditions. The graph represents average results of four independent experiments for *y w* flies. Error bars represent SEM.

(B) Similar effects of prolonged darkness on serotonin levels in flies of two different genotypes. Serotonin levels were assayed in flies maintained in LD or DD conditions for 7 days. Both *y w* and Canton-S flies showed reduced serotonin levels after 7 days in DD as compared to LD samples of the same genotype (**$p < 0.01$, *$p < 0.05$, by Student's t test). Error bars represent SEM.

(C) Serotonin levels are reduced in brains of flies maintained in DD. Representative confocal images of the frontal view of the anterior subesophageal ganglion region (SOG) are shown. Flies were maintained in LD (left) or DD (right) conditions for 7 days and then collected; fly brains were stained with the anti-serotonin antibody. Arrows indicate the cell bodies of the SE5HT-IR neurons. Similar results were obtained with six pairs of brains.

Discussion

We show here that serotonin regulates the entrainment of circadian behavioral rhythms in *Drosophila* by affecting the molecular response to light. By modulating the expression of the d5-HT1B receptor in clock neurons, we established a role of this receptor subtype in the regulation of *Drosophila* circadian photosensitivity. Our data also demonstrate that the molecular connection between d5-HT1B signaling and the clock is GSK3β, which directly phosphorylates the central clock component TIM. We propose that serotonin signaling is a part of the homeostatic regulation that prevents dramatic fluctuations in the phase of the circadian clock. In addition, given the altered levels of serotonin in extended DD, it may confer selectivity on the response of the clock to light under different environmental conditions.

The expression pattern of d5-HT1B, as determined by both UAS-Gal4 experiments and by immunostaining, provides some clues to its functions in *Drosophila*. Besides LNvs and SE5HT-IR neurons, major compartments of the fly brain that express the d5-HT1B receptor include the optic lobes, PI neurons, and mushroom bodies. Interestingly, expression in each of these locations is consistent with functions proposed for serotonin signaling in other organisms. In the housefly, the neuropil of the optic lobes undergoes daily structural

changes regulated possibly by serotonin and PDF (Meinertzhagen and Pyza, 1999). PI neurons are neurosecretory cells that may also participate in the ocellar phototransduction pathway (Helfrich-Forster et al., 2001). The mushroom body is important for olfactory learning and memory in *Drosophila*. Therefore, in addition to its postsynaptic function in the LNvs, d5-HT1B may be involved in other aspects of physiology and behavior.

The effect of d5-HT1B on TIM was especially pronounced in the small LNvs. One of the differences between the large and small LNvs is in the timing of nuclear entry, which is delayed in the small subgroup (Shafer et al., 2002). If delayed nuclear entry accounts for the increased resistance of TIM to light in the small LNvs, it would suggest that d5-HT1B signaling largely affects cytoplasmic TIM (see also below).

In addition to its effect on the light response, d5-HT1B overexpression affected free-running behavioral rhythms of *cry*^{b} flies. We speculate that this is due to the loss of synchrony among LNs. The mutual coupling of oscillators within an organism is important for the generation and synchronization of circadian rhythms, and serotonin is implicated in this process in some insects (Saifullah and Tomioka, 2002). Decreased synchrony may also result from the reduced photosensitivity produced by d5-HT1B overexpression. Interestingly, a significant number of *glass, cry*^{b} double mutants, which lack CRY as well as all visual photoreceptors, are arrhythmic in DD (Helfrich-Forster et al., 2001).

d5-HT1B not only affects circadian photosensitivity when over- or underexpressed, it also appears to be the major receptor subtype required for the inhibitory effects of serotonin on entrainment. Notably, when d5-HT1B was knocked down with the RNAi transgene driven by *tim*-Gal4, the effect on photosensitivity was not as pronounced as with the d5-HT1B-Gal4 driver. This might be due to some background differences in flies carrying the *tim*-Gal4 transgene, or to nonspecific effects produced by expressing the RNAi construct in irrelevant cells. Also, we cannot exclude the possibility that cells other than clock neurons participate in the regulation of light sensitivity via d5-HT1B. However, clock cells clearly have a major role in this effect, in particular since the circadian response to serotonin is eliminated in the *tim*-Gal4/RNAi flies.

Effects of serotonin on circadian photosensitivity were previously demonstrated in other systems (Pickard and Rea, 1997), but the underlying mechanisms were not identified. Our studies in *Drosophila* address this issue by demonstrating an effect of d5-HT1B signaling on the posttranslational modification of TIM via SGG. We show that in d5-HT1B-overexpressing flies, TIM phosphorylation is reduced, and its stability is increased. On the other hand, SGG phosphorylation is increased (i.e., its activity is decreased) in response to elevated levels of d5-HT1B as well as in response to serotonin treatment. Consistent with this effect of d5-HT1B on SGG, increased SGG activity abolishes effects of d5-HT1B overexpression on circadian photosensitivity, while d5-HT1B attenuates the period shortening produced by excess SGG activity. These reciprocal effects in genetic experiments strongly support the regulation of SGG activity by d5-HT1B. Our expression data

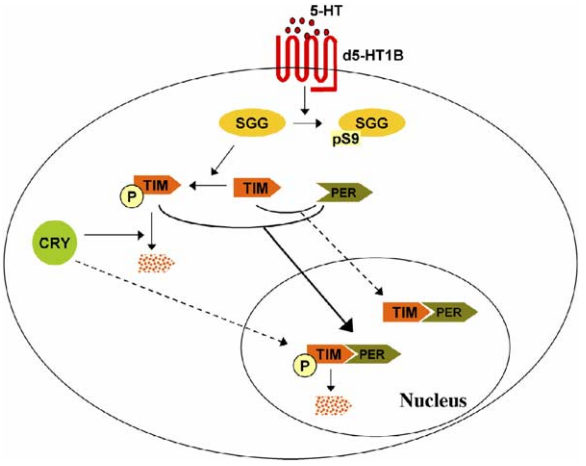


Figure 8. A Model for the Effect of Serotonin Signaling on the Posttranslational Modification of TIM in Clock Cells

SGG phosphorylates TIM in the cytoplasm and promotes TIM-PER translocation into the nucleus. d5-HT1B receptor signaling increases the phosphorylation of SGG, thereby reducing its kinase activity. With increased levels of either d5-HT1B or serotonin, TIM phosphorylation is decreased, and it is less susceptible to light-induced degradation mediated by CRY. This leads to reduced behavioral phase shifts. Solid lines indicate events for which there is experimental evidence. Dashed lines indicate processes that have not yet been experimentally validated. For instance, we do not know if unphosphorylated forms of TIM are transported to the nucleus or if CRY acts directly on nuclear TIM.

indicate that SGG is expressed predominantly in the cytoplasm (Figure 6). The regulation of cytoplasmic SGG by d5-HT1B is predicted to affect the phosphorylation status of TIM mainly in the cytoplasm; SGG-phosphorylated TIM is transported to the nucleus more effectively and is also a better substrate for light-induced degradation (Figure 8; see also below).

d5-HT1B alone did not significantly affect circadian period, suggesting that its effects on SGG are limited. In this context, we note that, while *sgg* hypomorphs have a period of ~26 hr, flies hemizygous for the locus have wild-type periods (Martinek et al., 2001). We infer that small (up to 50%) changes in SGG activity do not alter circadian period but can affect circadian photosensitivity. A role for SGG in circadian photosensitivity was previously suggested by Martinek et al., who found that forms of TIM phosphorylated by SGG were selectively degraded in response to light (Martinek et al., 2001). In fact, phosphorylated TIM is known to be more sensitive to light (Martinek et al., 2001; Rothenfluh et al., 2000; Zeng et al., 1996). While SGG appears to be the primary kinase that increases photic sensitivity of TIM, the actual process of light-induced TIM degradation involves the activity of a tyrosine kinase (Naidoo et al., 1999).

These results provide a new mechanism for circadian regulation by a G protein-coupled signaling pathway. A role for GSK3β in the mammalian circadian system was recently reported (Iwahana et al., 2004). In addition, the mammalian 5-HT1A receptor was recently shown to affect phosphorylation of GSK3β in the mouse brain (Li et al., 2004). It is possible that inhibition of GSK3β activ-

ity is a conserved mechanism in the regulation of circadian entrainment in mammals and insects.

Winfree described slow dark adaptation in *Drosophila*, whereby circadian sensitivity to light increases more than 10-fold over 3 days in DD (Winfree, 1972). Other groups confirmed increased light responsiveness during dark adaptation in rodents, but the mechanism underlying these effects was not addressed (Refinetti, 2003). Elevated responsiveness to light after prolonged exposure to darkness could be due either to a gain in sensitivity in the sensory system or to an increase in sensory output, which may be caused by a reduction in an inhibitory mechanism. In our study, we observed lower serotonin levels in flies maintained in DD. Given that serotonin signaling modulates circadian light sensitivity, it may be the reduction in this inhibitory mechanism that at least partially accounts for the enhanced light response in prolonged DD.

We propose that serotonin signaling, which is itself upregulated by light, is a part of a homeostatic mechanism that regulates circadian light sensitivity. A recent study using human subjects also suggested that serotonin levels in the brain reflect the duration of prior light exposure (Lambert et al., 2002). This change in serotonin levels with light may be relevant to the etiology and treatment of seasonal affective disorder (SAD), a mood disorder related to the reduced hours of sunlight in winter, particularly at northern latitudes. SAD patients respond to antidepression drug treatments, as well as to light therapy, both of which may produce an increase in serotonin (Magnusson and Boivin, 2003). The interplay of serotonin, light, and the circadian system suggests a close relationship between circadian regulation and mental fitness.

Serotonin modulates the entrainment of the circadian system. On the other hand, our results, and studies done in mammalian systems also, suggest circadian effects on serotonin signaling. First, based upon the differences seen in LD versus DD in the fly brain, the level of serotonin is affected by the environmental light cycle. Second, receptor levels are modulated by circadian components, as d5-HT1B levels are altered in fly circadian mutants. In addition, serotonin release and receptor activity are regulated in a circadian fashion in mammals (Dudley et al., 1998; Garabette et al., 2000; Nagayama and Lu, 1997). Mutual regulation of the circadian and serotonin systems may be necessary to maintain the normal physiological functions of both systems.

Experimental Procedures

Immunohistochemistry

Collection and immunostaining of whole-mount brain tissue were performed as described previously (Sathyanarayanan et al., 2004). The tissues were incubated with primary antibodies diluted as follows: 1:1000 for anti-serotonin, 1:1000 for anti-PDF, 1:500 for anti-d5-HT1B, 1:1000 for anti-TIM, 1:10 for anti-pS9-SGG (hybridoma supernatant; gift from Mark Bourouis), 1:500 for anti-SGG. Brains were imaged using a confocal microscope (Leica) and processed with OpenLab and Adobe Photoshop software. Signals in LNs were quantified through densitometry using the Measurement module in OpenLab.

Behavioral Analysis

Flies were entrained to 12:12 LD cycles at 25°C. Three- to seven-day-old flies were used in the behavioral analysis. Locomotor activity of individual flies was monitored and analyzed as previously described (Williams et al., 2001). Circadian phase-resetting experiments were performed as described (Yang et al., 1998). For behavioral assays using transgenic flies, multiple insertion lines were tested.

Pharmacological Treatment of Flies

Fluoxetine, citalopram, and L-5-HTP were obtained from Sigma. Pharmacological treatments consisted of feeding the drugs for 24 hr. The dosage was determined by preliminary trials based on the response of the flies. The drugs were freshly dissolved in water and mixed with regular food (5% sucrose in 1% agarose). Flies kept and monitored in regular food and LD cycles were transferred into activity monitor tubes with drugs at CT0 of the first subjective night and given a light pulse at CT20. Flies were transferred back to regular food at the next CT0.

Fly Strains and Transgenes

Details of the transgenic constructs are in the Supplemental Data. Transgenic flies were generated using the d5-HT1B-GAL4, UAS-d5-HT1B, and UAS-1BRNAi constructs as described (Yang and Sehgal, 2001). Multiple independently transformed lines were mapped and balanced in a *w*– background. Flies carrying UAS-SGG10 transgene (stock number 5361, 5360) were obtained from the Bloomington Stock Center.

Polyclonal Antibody Against 5-HT1B

A polyclonal antibody against d5-HT1B was generated as described (Sathyanarayanan et al., 2004). Briefly, the third intracellular loop of the receptor (T320 to I465) was expressed in bacteria as a maltose binding protein (MBP) fusion protein. The purified protein was used as an antigen for antiserum generation in guinea pigs (Covance Inc). The antiserum was preabsorbed with MBP for Western blots and immunostaining.

Immunoblot

S2 cell lysates and whole-head protein extracts were obtained as described (Sathyanarayanan et al., 2004). Unless otherwise specified in the legend, all fly heads were collected at ZT2. Blots were incubated with various primary antibodies at following dilutions: anti-TIM, 1:2000; anti-d5-HT1B, 1:2000; anti-phosphorylated MAPK (Sigma), 1:2000; anti-MAPK (Sigma), 1:10000; anti-V5 (Invitrogen), 1:2000; anti-pS9-SGG (hybridoma supernatant; gift from Dr. Marc Bourouis), 1:10; anti-SGG (Upstate), 1:1000. Western blots were quantified through densitometry using a Kodak imaging station.

Quantitative Immunoassay for Serotonin Levels in Fly Heads

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

Supplemental Data

The Supplemental Data include Experimental Procedures and six figures and can be found with this article online at http://www.neuron.org/cgi/content/full/47/1/115/DC1/.

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References

Akten, B., Jauch, E., Genova, G.K., Kim, E.Y., Edery, I., Raabe, T., and Jackson, F.R. (2003). A role for CK2 in the Drosophila circadian oscillator. Nat. Neurosci. *6*, 251–257.

Albert, P.R., and Tiberi, M. (2001). Receptor signaling and structure: insights from serotonin-1 receptors. Trends Endocrinol. Metab. *12*, 453–460.

Bourouis, M. (2002). Targeted increase in shaggy activity levels blocks wingless signaling. Genesis *34*, 99–102.

Boutrel, B., Monaca, C., Hen, R., Hamon, M., and Adrien, J. (2002). Involvement of 5-HT1A receptors in homeostatic and stress-induced adaptive regulations of paradoxical sleep: studies in 5-HT1A knock-out mice. J. Neurosci. *22*, 4686–4692.

Buhot, M.C., Martin, S., and Segu, L. (2000). Role of serotonin in memory impairment. Ann. Med. *32*, 210–221.

Cymborowski, B. (1998). Serotonin modulates a photic response in circadian locomotor rhythmicity of adults of the blow fly, Calliphora vicina. Physiol. Entomol. *23*, 25–32.

Dissel, S., Codd, V., Fedic, R., Garner, K.J., Costa, R., Kyriacou, C.P., and Rosato, E. (2004). A constitutively active cryptochrome in Drosophila melanogaster. Nat. Neurosci. *7*, 834–840.

Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci. *116*, 1175–1186.

Dudley, T.E., DiNardo, L.A., and Glass, J.D. (1998). Endogenous regulation of serotonin release in the hamster suprachiasmatic nucleus. J. Neurosci. *18*, 5045–5052.

Emery, P., Stanewsky, R., Helfrich-Forster, C., Emery-Le, M., Hall, J.C., and Rosbash, M. (2000). *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron *26*, 493–504.

Garabette, M.L., Martin, K.F., and Redfern, P.H. (2000). Circadian variation in the activity of the 5-HT(1B) autoreceptor in the region of the suprachiasmatic nucleus, measured by microdialysis in the conscious freely-moving rat. Br. J. Pharmacol. *131*, 1569–1576.

Gaspar, P., Cases, O., and Maroteaux, L. (2003). The developmental role of serotonin: news from mouse molecular genetics. Nat. Rev. Neurosci. *4*, 1002–1012.

Glass, J.D., Grossman, G.H., Farnbauch, L., and DiNardo, L. (2003). Midbrain raphe modulation of nonphotic circadian clock resetting and 5-HT release in the mammalian suprachiasmatic nucleus. J. Neurosci. *23*, 7451–7460.

Hall, J.C. (2000). Cryptochromes: sensory reception, transduction, and clock functions subserving circadian systems. Curr. Opin. Neurobiol. *10*, 456–466.

Helfrich-Forster, C., Winter, C., Hofbauer, A., Hall, J.C., and Stanewsky, R. (2001). The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron *30*, 249–261.

Horikawa, K., Yokota, S., Fuji, K., Akiyama, M., Moriya, T., Okamura, H., and Shibata, S. (2000). Nonphotic entrainment by 5-HT1A/7 receptor agonists accompanied by reduced Per1 and Per2 mRNA levels in the suprachiasmatic nuclei. J. Neurosci. *20*, 5867–5873.

Iwahana, E., Akiyama, M., Miyakawa, K., Uchida, A., Kasahara, J., Fukunaga, K., Hamada, T., and Shibata, S. (2004). Effect of lithium on the circadian rhythms of locomotor activity and glycogen synthase kinase-3 protein expression in the mouse suprachiasmatic nuclei. Eur. J. Neurosci. *19*, 2281–2287.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase Iε. Cell *94*, 97–107.

Konopka, R.J., Pittendrigh, C., and Orr, D. (1989). Reciprocal behaviour associated with altered homeostasis and photosensitivity of Drosophila clock mutants. J. Neurogenet. *6*, 1–10.

Lambert, G.W., Reid, C., Kaye, D.M., Jennings, G.L., and Esler, M.D. (2002). Effect of sunlight and season on serotonin turnover in the brain. Lancet *360*, 1840–1842.

Li, X., Zhu, W., Roh, M., Friedman, A., Rosborough, K., and Jope, R. (2004). In vivo regulation of glycogen synthase kinase-3β (GSK3β) by serotonergic activity in mouse brain. Neuropsychopharmacology *29*, 1426–1431.

Lin, F.J., Song, W., Meyer-Bernstein, E., Naidoo, N., and Sehgal, A. (2001). Photic signaling by cryptochrome in the Drosophila circadian system. Mol. Cell. Biol. *21*, 7287–7294.

Lin, J.M., Kilman, V.L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M., and Allada, R. (2002). A role for casein kinase 2α in the Drosophila circadian clock. Nature *420*, 816–820.

Lundell, M.J., and Hirsh, J. (1994). Temporal and spatial development of serotonin and dopamine neurons in the Drosophila CNS. Dev. Biol. *165*, 385–396.

Magnusson, A., and Boivin, D. (2003). Seasonal affective disorder: an overview. Chronobiol. Int. *20*, 189–207.

Martinek, S., Inonog, S., Manoukian, A.S., and Young, M.W. (2001). A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. Cell *105*, 769–779.

Meinertzhagen, I.A., and Pyza, E. (1999). Neurotransmitter regulation of circadian structural changes in the fly's visual system. Microsc. Res. Tech. *45*, 96–105.

Morin, L.P. (1999). Serotonin and the regulation of mammalian circadian rhythmicity. Ann. Med. *31*, 12–33.

Nagayama, H., and Lu, J.Q. (1997). Circadian rhythm in the response to intracerebroventricular administration of 8-OH-DPAT. Brain Res. *756*, 92–95.

Naidoo, N., Song, W., Hunter-Ensor, M., and Sehgal, A. (1999). A role for the proteasome in the light response of the timeless clock protein. Science *285*, 1737–1741.

Page, T.L. (1987). Serotonin phase-shifts the circadian rhythm of locomotor activity in the cockroach. J. Biol. Rhythms *2*, 23–34.

Papadopoulou, D., Bianchi, M.W., and Bourouis, M. (2004). Functional studies of shaggy/glycogen synthase kinase 3 phosphorylation sites in Drosophila melanogaster. Mol. Cell. Biol. *24*, 4909–4919.

Pickard, G.E., and Rea, M.A. (1997). Serotonergic innervation of the hypothalamic suprachiasmatic nucleus and photic regulation of circadian rhythms. Biol. Cell. *89*, 513–523.

Ramboz, S., Oosting, R., Amara, D.A., Kung, H.F., Blier, P., Mendelsohn, M., Mann, J.J., Brunner, D., and Hen, R. (1998). Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc. Natl. Acad. Sci. USA *95*, 14476–14481.

Refinetti, R. (2003). Effects of prolonged exposure to darkness on circadian photic responsiveness in the mouse. Chronobiol. Int. *20*, 417–440.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell *99*, 791–802.

Rothenfluh, A., Young, M.W., and Saez, L. (2000). A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. Neuron *26*, 505–514.

Saifullah, A.S., and Tomioka, K. (2002). Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket Gryllus bimaculatus. J. Exp. Biol. *205*, 1305–1314.

Sathyanarayanan, S., Zheng, X., Xiao, R., and Sehgal, A. (2004). Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. Cell *116*, 603–615.

Saudou, F., Boschert, U., Amlaiky, N., Plassat, J.L., and Hen, R. (1992). A family of Drosophila serotonin receptors with distinct intracellular signalling properties and expression patterns. EMBO J. *11*, 7–17.

Shafer, O.T., Rosbash, M., and Truman, J.W. (2002). Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of Drosophila melanogaster. J. Neurosci. *22*, 5946–5954.

Smart, C.M., and Biello, S.M. (2001). WAY-100635, a specific 5-HT1A antagonist, can increase the responsiveness of the mammalian circadian pacemaker to photic stimuli. Neurosci. Lett. *305*, 33–36.

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the Drosophila clock gene period and a lacZ reporter: mapping elements of the PER protein involved in circadian cycling. J. Neurosci. *17*, 676–696.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M., and Hall, J.C. (1998). The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell *95*, 681–692.

Suri, V., Qian, Z., Hall, J.C., and Rosbash, M. (1998). Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster*. Neuron *21*, 225–234.

Valles, A.M., and White, K. (1988). Serotonin-containing neurons in Drosophila melanogaster: development and distribution. J. Comp. Neurol. *268*, 414–428.

Williams, J.A., and Sehgal, A. (2001). Molecular components of the circadian system in Drosophila. Annu. Rev. Physiol. *63*, 729–755.

Williams, J.A., Su, H.S., Bernards, A., Field, J., and Sehgal, A. (2001). A circadian output in Drosophila mediated by neurofibromatosis-1 and Ras/MAPK. Science *293*, 2251–2256.

Winfree, A.T. (1972). Slow dark adaptation in Drosophila's circadian clock. J. Comp. Physiol. *77*, 428–434.

Wulbeck, C., Szabo, G., Shafer, O.T., Helfrich-Forster, C., and Stanewsky, R. (2005). The novel Drosophila tim(blind) mutation affects behavioral rhythms but not periodic eclosion. Genetics *169*, 751–766.

Yang, Z., and Sehgal, A. (2001). Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. Neuron *29*, 453–467.

Yang, Z., Emerson, M., Su, H.S., and Sehgal, A. (1998). Response of the timeless protein to light correlates with behavioral entrainment and suggests a nonvisual pathway for circadian photoreception. Neuron *21*, 215–223.

Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light-entrainment mechanism for the Drosophila circadian clock. Nature *380*, 129–135.

Zhang, F., Phiel, C.J., Spece, L., Gurvich, N., and Klein, P.S. (2003). Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. J. Biol. Chem. *278*, 33067–33077.