Published in final edited form as:

Neuroscience. 2009 April 10; 159(4): 1430–1440. doi:10.1016/j.neuroscience.2009.02.016.

BRIEF CONSTANT LIGHT ACCELERATES SEROTONERGIC RE-ENTRAINMENT TO LARGE SHIFTS OF THE DAILY LIGHT-DARK CYCLE

Gagandeep Kaur, Raja Thind, and J. David Glass*

Department of Biological Sciences, Kent State University, Kent, OH 44242, USA

Abstract

Brief (~2 day) constant light exposure (LL_b) in hamsters dramatically enhances circadian phase-resetting induced by the serotonin (5-HT) receptor agonist, 8-OH-DPAT and other nonphotic stimuli. The present study was undertaken to determine if LL_b can also amplify phase-resetting responses to endogenous 5-HT and accelerate re-entrainment to large-magnitude advance and delay shifts of the light-dark (LD) cycle. First, central serotonergic activity was increased by i.p. injection of L-tryptophan +/- the 5-HT reuptake inhibitor fluoxetine. Hamsters under LD or exposed to LL_b received vehicle or drugs during the early morning, and phase-shifts of the locomotor activity rhythm were measured after release to constant darkness. Neither drug phase-shifted animals not exposed to LL_b ($p > 0.5$ vs. vehicle); however in animals receiving LL_b, L-tryptophan with and without fluoxetine produced large phase-advance shifts (means = 2.5 ± 0.4 h and 2.6 ± 0.2 h, respectively; both $p < 0.035$ vs. vehicle). Next, the effects of LL_b combined with 8-OH-DPAT or L-tryptophan+fluoxetine on serotonergic re-entrainment to 10 h phase-advance and phase-delay shifts of the LD cycle were assessed. In groups not exposed to LL_b, vehicle controls re-entrained slowly to the advance and delay shifts (means = 16 ± 1 days and 24 ± 4 days, respectively), but those treated with 8-OH-DPAT re-entrained faster (means = 11 ± 2 days and 9 ± 2 days, respectively; both $p < 0.05$ vs. vehicle). In groups exposed to LL_b, vehicle controls re-entrained slowly to the advance and delay shifts (means = 15 ± 2 and 25 ± 3 days, respectively); however those receiving 8-OH-DPAT rapidly re-entrained to the delay and advance shifts, with the majority (75%) requiring only 1–2 days (means = 2 ± 1 days and 4 ± 2 days, respectively; both $p < 0.05$ vs. vehicle). Animals exposed to LL_b and treated with L-tryptophan +fluoxetine also exhibited accelerated re-entrainment to a 10 h advance shift (mean = 5 ± 2 days; $p < 0.05$ vs. vehicle). Thus through enhancing serotonergic phase-resetting, LL_b facilitates rapid re-entrainment to large shifts of the LD cycle which offers a potential approach for treating circadian-related desynchronies.

Keywords

Syrian hamster; suprachiasmatic nucleus; tryptophan; fluoxetine

*Corresponding author: voice: (330) 672-2934, fax: (330) 672-3713, e-mail: jglass@kent.edu.

Section Editor: Systems Neuroscience, Dr. Miles Herkenham, MS. No.: NSC-08-1498

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INTRODUCTION

The master circadian clock in mammals is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Inouye and Kawamura, 1979; Klein et al., 1991; Rusak, 1979; Stephan and Zucker, 1972). The SCN clock is entrained by photic input received directly from the retina via the retinohypothalamic tract (RHT; Hendrickson et al., 1972; Moore and Lenn, 1972; Pickard, 1982; Youngstrom and Nunez, 1986; Johnson et al., 1988), and indirectly via a projection from the intergeniculate leaflet (IGL), the geniculohypothalamic tract (GHT; Card and Moore, 1982; Johnson et al., 1989). The IGL also provides nonphotic (behavior-related) entraining input to the SCN mediated by neuropeptide Y (NPY) release from GHT terminals (Albers and Ferris, 1984; Biello et al., 1994; Marchant et al., 1997). Serotonergic input from the midbrain raphe nuclei is also believed to be another important source of nonphotic signaling to the SCN (Glass et al., 2003; Meyer-Bernstein and Morin, 1996). It is now apparent that nonphotic influences, in the form of behavioral manipulations (eg. sleep deprivation, social interaction, cage changing, or novel wheel exposure) or pharmacological interventions (eg. benzodiazepines or serotonin [5-HT] agonists), can be as potent as photic cues for resetting the clock (~1–2 h; Ellis et al., 1982; Mistlberger et al., 2000; Mrosovsky, 1988),

Recently we showed that the nonphotic phase-resetting effects of various stimuli, including treatment with the 5-HT_{1A,7} receptor agonist, 8-OH-DPAT, can be dramatically increased by brief prior exposure to constant light (1–2 days [LL_b]) Knoch et al., 2004). Notably, immediate phase-advance and phase -delay shifts of ~12 h are produced using this combined treatment. The neurologic mechanism underlying the potentiating effects of LL_b on 8-OH-DPAT-induced phase-resetting is not known, but possibly could involve an up-regulation of 5-HT mediated postsynaptic responses centered in the SCN clock, as phase-advance shifts caused by intra-SCN microinjection of 8-OH-DPAT are potentiated by LL_b (Knoch et al., 2006). It should be noted that while the circadian phase-resetting effects of serotonergic agonists including 8-OH-DPAT are well documented (summarized in Ehlen et al., 2001; Mistlberger et al., 2000; Morin, 1999; Sprouse et al., 2005), the role for 5-HT as a player in nonphotic phase resetting remains controversial, and it is therefore uncertain how the potentiated phase-resetting action of LL_b on behavior-induced shifting may be linked to (endogenous) 5-HT signaling.

This uncertainty is related in part to observations that raphe lesions (Meyer-Bernstein and Morin, 1998), 5-HT antagonist treatments (Antle et al., 1998) and SCN 5-HT depletion (Bobrzynska et al., 1996) do not block activity-induced phase-shifts and that the phase-resetting effect of direct injection of 8-OH-DPAT into the SCN is absent or small (Challet et al., 1998; Ehlen et al., 2001; Mintz et al., 1997). Also, approaches used to increase endogenous serotonergic activity have yielded inconsistent phase-resetting results. For example, while electrical stimulation of the midbrain raphe nucleus which acutely enhances 5-HT release in the SCN (Dudley et al., 1999) induces phase-advances comparable to those induced by behavioral activation (Glass et al., 2000; Meyer-Bernstein and Morin, 1999), acute administration of 5-HT reuptake blockers (including fluoxetine and clomipramine), which also increases central extracellular 5-HT levels, has weak or no circadian phase-resetting effects *in vivo* (Klemfuss and Kripke, 1994; Yannielli et al., 1998) or *in vitro* (Sprouse et al., 2006) (but does attenuate photic phase-shifts [Gannon and Millan, 2007]).

The present study was thus undertaken to exploit the potentiating effect of LL_b on nonphotic clock resetting as a means to assess the potential actions of 5-HT in nonphotic clock-resetting and rhythm re-entrainment. Two experiments were undertaken to: 1) explore the *in vivo* circadian phase-shifting effects of increased endogenous 5-HT activity stimulated by the 5-HT precursor, L-tryptophan, and/or fluoxetine under LL_b-sensitized phase-shifting conditions; and 2) determine if the large serotonergic (8-OH-DPAT- and L-tryptophan-induced) phase-shifts potentiated by LL_b exposure could accelerate rhythm re-entrainment to simulated jet-lag

involving large (10 h) advance and delay shifts of the light-dark cycle. Results from these experiments would strengthen the case for participation of endogenous serotonergic in nonphotic phase-resetting, and also could help in the design of therapeutic strategies for more efficient resynchronization of the circadian clock during periods of circadian phase disruption.

EXPERIMENTAL PROCEDURES

Animals

Adult male Syrian hamsters obtained from Harlan (Indianapolis, IN) were housed in a light- and temperature-controlled (22°C) environmental chamber. Animals were individually housed in polystyrene cages and kept under a 14 h light:10 h dark photocycle (LD) with light intensity of approximately 250 lux. Food (Prolab 3000, PMI Feeds; St. Louis, MO) and water were provided *ad libitum*. The experiments were conducted using the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Constant Light Protocol and Phase-Shift Analyses

The method for administering the LL_b regimen is the same as that described by Knoch et al., 2004. This photic treatment begins at Zeitgeber time 12 (ZT 12; designated as the time of lights off under an LD cycle), and is maintained over 2 consecutive days by keeping the ambient room lighting [~250 lux] on, thus eliminating two dark-phase periods (Fig. 1). This constituted 50 h of continuous light extending from the last dark phase for treatments delivered at ZT 0. In the L-tryptophan/fluoxetine phase-resetting trials, the animals were released to constant darkness (DD) immediately after drug injection for a minimum of 14 days to assess phase-resetting response using a modified Aschoff type II procedure (Aschoff, 1965). For clarity of data presentation, ZT rather than the circadian time (CT) convention for free-running conditions with no zeitgeber, was used to designate the circadian phase of treatments delivered during the ~2 day LL_b exposures. There were no perceptible changes in phase or period of the free-running circadian activity rhythm during these brief exposures, so under these conditions ZT is considered equivalent to CT as a phase marker.

For all experiments, the circadian rhythm of general locomotor activity was recorded using overhead infrared sensors interfaced with a computerized data acquisition system (ClockLab; Coulbourn Instruments, Allentown, PA). Phase-shifts were calculated as follows: A line based on general locomotor activity onsets for the 7 preceding days of LD was extrapolated to the day of treatment under LL_b. Then a regression line based on days 3–14 post-treatment activity onsets was back-extrapolated to the same day of treatment. The difference between these two extrapolated lines on the day of treatment was considered the phase-shift. Activity onset was defined as the first bout of activity sustained for at least thirty minutes. Rates of re-entrainment to 10 h advance or delay shifts of the LD cycle were measured by counting the number of days required for the stable adjustment of the locomotor activity rhythm to the new LD cycle. Stable adjustment was characterized as the first 10 days of activity onset temporally aligned with the beginning of dark phase of the new LD cycle, with $\tau = 24$ h.

Experimental Protocols

Effects of brief constant light on L-tryptophan/fluoxetine phase-resetting—

Potential endogenous 5-HT phase-resetting effects were explored using LL_b in combination with various treatments to enhance serotonergic activity, including L-tryptophan loading (50 mg/kg i.p.), fluoxetine (10 mg/kg i.p.) and L-tryptophan+fluoxetine. Controls received i.p. vehicle injection (DMSO; $n = 5$ –6/treatment group). Drugs were administered at ZT 0, the phase of the 8-OH-DPAT phase-response curve (PRC) when robust phase-advance shifts to 8-OH-DPAT (>10 h) occur in LL_b-treated animals (Knoch et al., 2004). All hamsters were initially housed under LD with general locomotor activity sensors and were exposed to LL_b or

maintained under LD prior to drug or vehicle treatment. Immediately after each treatment, the animals were released into DD for 3 wk to assess phase-resetting responses using the Aschoff type II procedure.

Effects of brief constant light together with 8-OH-DPAT or L-tryptophan and/or fluoxetine on re-entrainment to new LD cycles—Hamsters exposed to LL_b exhibit large and immediate phase-advance and phase-delay shifts to 8-OH-DPAT treatment at ZTs 0 and 21 (~10 h and ~11 h, respectively; Knoch et al., 2004). Based on these observations, it was hypothesized that such treatments should promote accelerated re-entrainment to large simulated jet-lag advance and delay shifts of the LD cycle. The protocol for this experiment is presented in Fig. 2. For the phase-advance trial, animals whose activity rhythms were recorded for 7 days under LD were exposed to LL_b for 50 h, eliminating 2 dark-phase periods. At the end of this photic treatment the animals received i.p. injection of either 8-OH-DPAT (5 mg/kg), L-tryptophan (50 mg/kg), fluoxetine (10 mg/kg) or L-tryptophan and fluoxetine (same dosages) at ZT 0 and then were released into the initial dark-phase of a 10 h advanced LD cycle. The extra 2 h of darkness from ZT 0 -ZT 2 following drug treatment was necessary to avoid the blocking effect of light on the phase-shifting action of the drugs. A separate group maintained under LD (LD controls) received vehicle or L-tryptophan and fluoxetine. The general circadian locomotor rhythm was measured for up to 35 days thereafter to measure the rate of re-entrainment to the 10 h LD phase-shift.

A similar protocol was used for the phase-delay trial, except that the animals were exposed to LL_b for 47 h to eliminate 2 dark-phase periods with the LD phase-delay of 10 h. At the end of the LL_b treatment the animals received 8-OH-DPAT injection at ZT 21, and then were released into the first dark-phase of the 10 h delayed LD cycle. The additional 1 h of darkness from ZT 21 – ZT 22 following drug treatment was necessary to avoid the blocking effect of light on the phase-shifting action of 8-OH-DPAT.

Drugs

Drugs used in this study were $\pm$ 8-OH-DPAT, fluoxetine and L-tryptophan (Tocris, Ellisville, MI)

Statistical Analysis

Data from the L-tryptophan/fluoxetine LL_b trials were analyzed using a one-way ANOVA followed by the Student-Newman-Keuls post hoc test to compare drug phase-shifting effects under LD and LL_b. Re-entrainment effects of the LL_b + 8-OH-DPAT treatments were analyzed using a two-way ANOVA followed by the Student-Newman-Keuls post hoc test to compare drug and photoperiod treatment effects separately for the phase-advance and phase-delay trials. The level for statistical significance was set at $p < 0.05$.

RESULTS

Brief constant light potentiates endogenous serotonergic phase-resetting

Shown in Figs. 3&4 is the marked overall potentiating effect of LL_b on phase-resetting responses to serotonergic treatments compared to similar treatments administered under LD ($F_{1,3} = 91.6$; $p < 0.0001$). Within-photoperiod group analysis revealed a pronounced serotonergic shifting effect in the LL_b-exposed animals ($F_{2,14} = 4.2$; $p < 0.04$), with L-tryptophan and L-tryptophan+fluoxetine treatments producing significantly greater phase-advance shifts compared to vehicle treatment (2.6 ± 0.2 h and 2.5 ± 0.4 h vs. 1.5 ± 0.3 h respectively; both $p < 0.04$). Fluoxetine did not significantly enhance L-tryptophan shifting ($p > 0.20$ vs. L-tryptophan alone), and it did not have any phase-resetting effect on its own ($p > 0.29$ vs. vehicle controls). In animals maintained under LD, there was no significant phase-resetting effect of

any drug treatment ($F_{3,19}=1.53$; $p>0.24$). For all experimental groups there were no post-treatment differences in rhythm period under DD which could have influenced calculated phase-shifting effects.

Brief constant light-potentiated serotonergic phase-resetting facilitates rapid re-entrainment to 10 h phase-advance and phase-delay shifts of the LD cycle

Phase-delay re-entrainment—Exposure to LL_b in combination with i.p. 8-OH-DPAT injection at ZT 21 significantly accelerated re-entrainment to a 10 h phase-delay of the light-dark cycle (Figs. 5,6), with 75% of the 8-OH-DPAT-treated animals (4/6) showing immediate re-entrainment to the delay shift (within one LD cycle; mean = 2.0 ± 0.5 days). Vehicle controls of the LL_b group took significantly longer to re-entrain (mean = 24.5 ± 3.4 days; $F_{1,10} = 42.5$; $p<0.0001$ vs. 8-OH-DPAT). In animals not exposed to LL_b , re-entrainment was more rapid with 8-OH-DPAT treatment compared to vehicle (means = 9.0 ± 1.9 days vs. 24.0 ± 3.9 days, respectively; $F_{1,7} = 10.0$; $p<0.02$), but was significantly slower than for those treated with 8-OH-DPAT and exposed to LL_b ($F_{1,8} = 18.1$; $p<0.003$). Also, there was no difference in re-entrainment rates between the LL_b and non- LL_b vehicle control groups ($F_{1,9} = 0.01$; $p>0.9$).

Phase-advance re-entrainment—Similar to the phase-delay trials above, exposure to LL_b in combination with i.p. 8-OH-DPAT injection at ZT 0 significantly accelerated re-entrainment to a 10 h phase-advance of the light-dark cycle, with the majority of 8-OH-DPAT-treated animals (8/12) undergoing immediate re-entrainment to the phase-advance shift (Figs. 5,6). There was some variability in effect, with four animals requiring 9–13 days to re-entrain (group mean = 4.2 ± 1.8 days). Vehicle controls of the LL_b group took significantly longer to re-entrain (mean = 14.7 ± 1.6 days; $F_{1,21} = 19.2$; $p<0.001$ vs. 8-OH-DPAT). In animals not exposed to LL_b , re-entrainment was more rapid with 8-OH-DPAT treatment compared to vehicle controls (means = 11.2 ± 1.7 days vs. 16.2 ± 0.7 days, respectively; $F_{1,9} = 7.8$; $p<0.02$), but was significantly slower than for those treated with 8-OH-DPAT and exposed to LL_b ($F_{1,15} = 5.6$; $p<0.03$). Also, there was no difference in the rate of re-entrainment between the LL_b and non- LL_b vehicle control groups ($F_{1,15} = 0.4$; $P>0.5$).

Exposure to LL_b in combination with i.p. L-tryptophan and L-tryptophan+fluoxetine injection at ZT 0 also significantly accelerated re-entrainment to a 10 h phase-advance of the light-dark cycle, with the animals re-entraining significantly faster than vehicle controls (means = 5.2 ± 2.1 , 5.4 ± 1.6 days vs. 14.7 ± 1.6 days, respectively; $F_{3,22} = 7.52$; $p<0.002$; Figs 7,8). Fluoxetine by itself did not significantly enhance re-entraining than vehicle controls (mean = 10.6 ± 0.6 days). In animals not exposed to LL_b , treatment with L-tryptophan and fluoxetine also significantly increased the rate of re-entrainment compared to vehicle controls (means = 13.3 ± 0.3 days vs. 16.2 ± 0.7 days, respectively; $F_{1,10} = 11.9$; $p<0.01$).

DISCUSSION

Exposure to LL_b markedly alters the magnitude and shape of the 8-OH-DPAT phase-response curve (PRC), facilitating large-magnitude (Type 0) phase-advances and phase-delays during early morning and late night, respectively (Duncan et al., 2005; Knoch et al., 2004 & 2006). Similarly, LL_b treatment strongly potentiates behavioral (sleep deprivation- and wheel-running-induced) phase-resetting (Knoch et al., 2004). Based upon the potentiating effects of LL_b on serotonergic (8-OH-DPAT) shifting, and past observations that behavioral activation induces SCN 5-HT release (Dudley et al., 1998), it is thought that the large-magnitude behavioral shifts potentiated by LL_b could, at least in part, be mediated through the enhancement of 5-HT release associated with behavioral stimulation. This is a reasonable hypothesis; however there is no direct evidence linking the potentiating effect of LL_b to (endogenous) 5-HT. Moreover, the issue is clouded by the overall speculative nature of the

proposed physiological role for 5-HT in mediating non-photoc phase-resetting responses *in vivo* (reviewed in Mistlberger et al., 2000). In extending our previous studies, we report here that animals receiving LL_b exposure also exhibit significant phase-advances to tryptophan loading, which confirms the existence of an *in vivo* circadian clock-resetting mechanism that is responsive to endogenous 5-HT. In addition, the present results reveal that LL_b potentiated exogenous (8-OH-DPAT) and endogenous (L-tryptophan+fluoxetine-stimulated) serotonergic phase-resetting responses can promote rapid re-entrainment to large-magnitude advances and/or delays of the LD cycle.

Previously we showed that LL_b strongly potentiates the phase-resetting actions of a variety of nonphotoc stimuli in the Syrian hamster, including sleep deprivation, novel wheel exposure, i.p. or intra-SCN injection of 8-OH-DPAT and intra-SCN injection of NPY (Duncan et al., 2005; Knoch et al., 2004 & 2006). The mechanism underlying the potentiating effects of LL_b is not clear, but its effect appears to be selective for nonphotoc shifting responses, as it does not enhance photic-type shifts mimicked by NMDA in the SCN (Landry and Mistlberger, 2005). It is noteworthy in this regard that other types of interventions besides LL_b can also induce strong nonphotoc phase-resetting responses in this species. For example, phase-advances >12 h have been reported in hamsters group housed under LD with no running wheel, then transferred individually during the light phase to a cage with a wheel under DD (Gannon and Rea, 1995). The large shifts were mostly associated with continuous running in excess of 4 h. Large phase-advance shifts also have been reported in hamsters which were able to re-entrain quickly (<2 days) to an 8 h advance of the LD cycle by being confined to a running wheel for 3 h (Mrosovsky and Salmon, 1987). The large shifting responses seen in these and in our LL_b studies are of similar magnitude and phase, suggesting that they may share some common pathway associated with behavioral activation (i.e. exercise, sleep disruption and/or general arousal). The identity of this pathway is uncertain, however the midbrain raphe serotonergic system is a good candidate, because: 1) wheel-running and sleep deprivation significantly elevate 5-HT release in the SCN and IGL (Dudley et al., 1998; Grossman et al., 2004); 2) this raphe system supplies all 5-HT to circadian-related areas including the SCN and IGL (Meyer-Bernstein and Morin, 1996); 3) electrical stimulation of the midbrain raphe at midday induces phase-advance shifts (Glass et al., 2000; Meyer-Bernstein and Morin, 1999); 4) the 5-HT_{1A} antagonist, (-)-pindolol abolishes the large behavior (sleep deprivation)-induced shifts produced by LL_b exposure (Knoch et al., 2006); and 5) these large LL_b-potentiated behavior-induced shifts are mimicked by serotonergic treatments (8-OH-DPAT (Knoch et al., 2004) and to a lesser extent L-tryptophan loading reported here. It should be noted in this latter context that the potentiated phase-advancing response to L-tryptophan is smaller than that of 8-OH-DPAT, possibly due to a greater binding affinity and selectivity of 8-OH-DPAT for the 5-HT_{1A} and 5-HT₇ receptors implicated in serotonergic circadian phase-advancing. Also, based on observations that both sleep deprivation and L-tryptophan loading at 150 mg/kg increase extracellular 5-HT levels by ~200% (Glass et al., 1995; Grossman et al., 2000), the 50 mg/kg dose of L-tryptophan used in the present study may not have optimally stimulated 5-HT for phase-resetting.

A central question pertaining to the potentiating action of LL_b exposure on L-tryptophan- and 8-OH-DPAT-induced phase resetting concerns the mechanism underlying serotonergic hypersensitivity. It is hypothesized that the capacity to respond robustly to serotonergic phase-advancing influences could depend upon the degree of postsynaptic sensitivity to 5-HT, which could be modulated by experimental, and possibly endogenous changes in serotonergic tonus. For example, in the deafferented rat and mouse SCN brain slice, where 5-HT release is presumably reduced, direct application of 8-OH-DPAT elicits phase-advances (~4 h) that are considerably larger than those seen *in vivo* (~1 h; Prosser, 2003; Prosser and Gillette, 1989; Shibata et al., 1992; Sprouse et al., 2005). Correspondingly, pretreatment of the SCN slice with 8-OH-DPAT desensitizes the phase-resetting response to subsequently applied 8-OH-DPAT

(Prosser et al., 2006). Also, phase-advances to intra-SCN 8-OH-DPAT perfusion are significantly larger in animals treated with the 5-HT synthesis inhibitor, parachlorophenylalanine, compared to vehicle treated controls (Ehlen et al., 2001). Along similar lines, the LL_b suppression of nocturnal *in vivo* 5-HT release measured in the SCN (Knoch et al., 2004) could produce a depletion-induced hypersensitization of postsynaptic serotonergic response the following day, enhancing phase-shifting responses to 8-OH-DPAT. This is supported by the finding in the same study that reestablishing near-normal nighttime levels of SCN 5-HT during LL_b by reverse microdialysis perfusion with 5-HT dampens the potentiating effect of LL_b on 8-OH-DPAT phase-advances, presumably by decreasing hypersensitization of postsynaptic serotonergic response.

In considering endogenous sensitization of 5-HT post-synaptic response, it is possible that an animal's recent behavioral history (ie. being more or less active during the night), could proportionately decrease or increase serotonergic tonus at that time. Such changes could presumably result in subsequent up- or down-regulation of 5-HT postsynaptic receptor-mediated phase-resetting the next day. This idea is supported in part by the observation of a daily variation in responsiveness of central 5-HT_{1A} receptors (Lu and Nagayama, 1996). Notably, the finding that 5-HT_{1A}, 5-HT₇ and 5-HT_{1B} receptor binding in the SCN is not affected by LL_b suggests that the hypersensitization of postsynaptic response may involve changes in 5-HT receptor-mediated action that occur at some signal transduction step downstream from ligand binding. Possibly this may involve effects on clock gene (*Per 1*) and neuropeptide (arginine vasopressin [AVP]) expression in the SCN, since the mRNAs for these moieties are significantly suppressed during LL_b exposure (Duncan et al., 2005). With further reference to behavioral history affecting serotonergic response, it is evident from the actograms presented in Fig. 4 that the L-tryptophan and L-tryptophan+fluoxetine treatments at ZT 0 significantly inhibited locomotor activity during the following subjective night. It is thus possible that this inhibition could have contributed to the larger phase-shift magnitude under LL_b. This is unlikely however as the same activity suppression is apparent in the LD controls which did not exhibit significant phase-shift responses to these serotonergic treatments. The reason for the behavioral inhibition is unclear, but possibly could be due to a prolonged sedative effect of the 50 mg/kg dose of L-tryptophan.

Another mechanism for the LL_b potentiation of nonphotic phase-resetting is proposed from limit cycle theory, where a suppression of circadian oscillator amplitude could theoretically render the clock more sensitive to a phase-shifting stimulus, producing strong phase-resetting (Jewett et al, 1991; Kronauer, 1990; Winfree, 1970). Such attenuation of circadian pacemaker amplitude is evidenced by the generalized attenuating effect of LL_b exposure on circadian functions, including locomotor activity, SCN 5-HT release (Knoch et al., 2004), SCN *Per* and AVP mRNAs (Duncan et al., 2005; Sudo et al., 2003) and SCN electrical activity (Shibata et al., 1984). For oscillator systems like the SCN, amplitude is considered to be an important determinant of pacemaker response, and if made sufficiently small (as reflected by these attenuations), stimuli that would, under normal conditions produce small (Type 1) shifts could produce larger (Type 0) shifts. Such an effect has been documented in humans (Jewett et al., 1991), and in the present study, the action of LL_b on pacemaker amplitude could produce the large phase-resetting effects of 8-OH-DPAT, as well as the significant phase-advance shifts induced by the L-tryptophan +/- fluoxetine treatments. It must be noted that the sensitizing effects of smaller pacemaker amplitude should be effective in potentiating all forms of phase-resetting stimuli. Observations that shifts induced by photic (NMDA) stimulation (Landry and Mistlberger, 2005), as well as melatonin (Glass, unpublished observations) are not potentiated by LL_b argue against the limit cycle amplitude hypothesis. On the other hand, it is conceivable that limit cycle effects could be constrained for stimuli such as these due to the numerous neurophysiological effects of the LL_b treatment that could possibly down-regulate pathways for their phase-resetting action.

Circadian rhythm desynchronization is an issue that has received considerable attention, as it is both causally and symptomatically related to a variety of debilitating chronopathological disorders. Principal among these are depressive syndromes associated with circadian clock phase-advance or phase-delay disruptions (Duncan, 1996; Healy and Waterhouse, 1995; Lewy et al., 2006; Millan, 2006; Murray et al., 2005), and shift work- and jet lag-related disorders resultant from disrupted sleep-wake schedules and LD cycles. Strategies for resynchronizing clock phase are of use in treating such conditions; however phase-shifts induced by applications of most natural (i.e. behavioral, photic etc.) or pharmacological stimuli are relatively small (1–2 h), and for this reason their benefits, especially those pertaining to jet lag malaise, are generally limited. One potential approach to this problem is to exploit manipulations like LL_b that produce very rapid and large adjustments of circadian phase. As discussed above, the phase of the clock can be immediately (without transients) advanced or delayed by as much as 12 h by appropriately timed co-application of LL_b and serotonergic (8-OH-DPAT) stimulation (Knoch et al., 2004). In theory this type of treatment could be used to rapidly accelerate re-entrainment to large delay (westward) or advance (eastward) jet lag shifts of the LD cycle. This is borne out (at least for nocturnal hamsters), by the LL_b + 8-OH-DPAT treatment producing nearly instantaneous re-entrainment in the majority of animals (75%) subjected to large-magnitude (10 h) simulated east *and* west jet lag shifts. Notably, it was also shown that combined LL_b L-tryptophan and/or fluoxetine treatment can significantly speed re-entrainment to a 10 h LD phase-advance, albeit at a slower rate than that of 8-OH-DPAT (due likely to its less potent phase-resetting action). A similar action of L-tryptophan and/or fluoxetine on phase-delay shifting was not explored, as their accelerating effects on phase-advance shifting adequately tested our hypothesis that endogenous 5-HT can help re-entrain the clock. From these results it is apparent that the rapid phase-resetting response to 5-HT receptor agonists potentiated by LL_b could offer an important approach for therapeutically advancing or delaying circadian clock phase for the treatment of chronopathological conditions.

A number of other nonphotic behavioral and pharmacological interventions that elicit large phase-shifts in animal models also offer potential approaches for therapeutic clock resetting. For example, the re-entrainment rate of hamsters to an 8 h advance of the LD cycle is more than doubled by simply increasing activity (running wheel confinement) for a 3 h period beginning shortly after the advanced onset of darkness (Mrosovsky and Salmon, 1987). Also, pharmacological agents that stimulate activity, such as the benzodiazepine, triazolam, can significantly accelerate re-entrainment. For instance, a single injection of triazolam in hamsters subjected to an 8 h phase advance can shorten the time for re-entrainment by ~50% (Van Reeth and Turek, 1987; but see Mrosovsky and Salmon, 1990). In rats, treatment with vitamin B₁₂ in the drinking water (which doubles wheel-running activity during the re-entrainment period) can accelerate re-entrainment to a reversed LD cycle by ~30% (Tsujimaru et al., 1992). Serotonergic intervention (other than that in the present study) also could be potentially effective, as treatment of hamsters with the 5-HT_{1A} mixed agonist/antagonists (BMY 7378 and S 15535) prior to a light pulse during the late night elicits large (~5 h) phase-advance shifts (Gannon, 2003; Gannon and Millan, 2006). From these results, this class of drugs was proposed to be beneficial in treating circadian phase-delay syndromes associated with depression and Alzheimer's disease. Possibly they could offer relief with shift work and jet lag conditions as well. It is interesting to note that the re-entrainment experiment here is the only one to show acceleration in the absence of a running wheel. It is possible that the presence of a wheel (possibly inducing more nocturnal 5-HT release) could contribute to 5-HT sensitization during the LL_b suppression of activity, producing an even more enhanced rate of re-entrainment.

In summary, the LL_b procedure, mediating a switch from Type 1 to Type 0 phase-resetting, offers a gateway for strong serotonergic and behavioral resetting of the mammalian circadian clock. The mechanism underlying the hypersensitizing effect of LL_b exposure is not clear. Nevertheless, it has potential importance in understanding the basis of nonphotic clock

resetting, and for exploring the role of 5-HT in circadian nonphotic phase regulation. In addition, it could help in the development of strategies for enhancing circadian clock resetting in humans.

Acknowledgements

This research was supported by National Institutes of Health Grant NS35229 to J.D.G.

Abbreviations

5-HT	serotonin
8-OH-DPAT	(±)-2-dipropyl-amino-8-hydroxyl-1,2,3,4-tetrahydronaphthalene
DD	constant darkness
GHT	geniculohypothalamic tract
IGL	intergeniculate leaflet
LD	Light:dark cycle
LL	constant light
LL_b	brief constant light
NPY	neuropeptide Y
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
ZT	zeitgeber time

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Figure 1.

Protocol for assessing the effects of 2 days of brief constant light (LL_b) exposure on serotonergic (L-tryptophan +/- fluoxetine) phase-resetting responses. Shown is a treatment delivered at zeitgeber time 0 on the second day of LL exposure. Animals are released from LL_b to constant darkness (DD) at the outset of the drug treatment. The top black bar represents the dark phase of the initial 14 h light: 10 h dark photocycle (LD).

Figure 2.

Protocol for assessing the effects of 2 days of brief constant light (LL_b) exposure on serotonergic (8-OH-DPAT or L-tryptophan +/- fluoxetine) mediation of re-entrainment to a 10 h phase-advance (left panel) or phase-delay (right panel) of the 14 h light: 10 h dark photocycle (LD). Drugs or vehicle were delivered at zeitgeber time 0 (for the phase-advance shift trials) or zeitgeber time 21 (for the phase-delay shift trial). In both cases, animals were released into darkness at the beginning of a drug or vehicle treatment. LD controls did not receive LL_b exposure.

Figure 3.

Potentiating effect of 2 days of brief constant light exposure (LL_b; upper panel) on the phase-advancing responses to i.p. Injection of vehicle (Veh), L-tryptophan and fluoxetine (Trypt +Fluox), L-tryptophan (Trypt) or fluoxetine (Fluox). Numbers in bars represent the number of animals in each group. Within a photoperiod group (LL_b or LD [no LL_b exposure]), bars with different letters are significantly different ($p < 0.04$). Within a drug or vehicle treatment group, “*” designates photoperiod effect ($p < 0.01$).

Figure 4.

Representative double-plotted actograms of general locomotor activity showing the phase-resetting responses to showing the phase-resetting responses to L-tryptophan (A,E), L-tryptophan and fluoxetine (B,F), fluoxetine alone (D,H) or vehicle (C,G) injected i.p. at ZT 0 (arrows) in animals receiving 2 days of brief constant light exposure (LL_b; left panels; shading represents the period of LL_b exposure) or no LL_b exposure (right panels). Note suppression of activity under the LL_b exposure. All animals are released to DD at the time of injection.

Figure 5. Potentiating effect of 2 days of brief constant light exposure (LL_b; left panel) on re-entrainment to 10 h phase-delay (upper panel) or phase-advance (lower panel) shifts of the LD cycle following i.p. injection of 8-OH-DPAT or vehicle (Veh) injection compared to no LL_b exposure (LD). Numbers in bars represent the number of animals in each group. Within a photoperiod group (LL_b or LD), bars with different letters are significantly different (p < 0.05). Within a drug or vehicle treatment group, bars with "*" are significantly different (p < 0.03).

Figure 6.

Representative double-plotted actograms of general locomotor activity showing re-entrainment responses to 8-OH-DPAT (**A,C,E,G**) or vehicle (**B,D,F,H**) injected i.p. at ZT 0 for phase-advance shift (upper panels) or ZT 21.0 for phase-delay shift (lower panels) in animals receiving 2 days of brief constant light exposure (left panels [shading represents period of LL_b exposure]) or no LL_b exposure (right panels). Arrows denote time of injection. Panel D shows animal advancing to new LD re-entrainment.

Figure 7.

Potentiating effect of 2 days of brief constant light exposure (LL_b; left panel) or no LL_b (LD; right panel) on re-entrainment to a 10 h phase-advance shift of the LD cycle following i.p. injection of vehicle (Veh), L-tryptophan and fluoxetine (Trypt+Fluox), L-tryptophan (Trypt) or fluoxetine (Fluox). Numbers in bars represent the number of animals in each group. Within a photoperiod group, bars with different letters are significantly different ($p < 0.05$). Within a drug or vehicle group, bars with “*” are significantly different ($p < 0.01$).

Figure 8.

Representative double-plotted actograms of general locomotor activity showing re-entrainment responses of animals receiving LL_b (top four panels) or no LL_b (bottom two panels) to L-tryptophan+fluoxetine (**B,F**), L-tryptophan (**C**), fluoxetine (**D**) or vehicle (**A,E**) injected i.p. at ZT 0 prior to a 10 h phase-advance shift after exposure to brief constant light (for LL_b panels, shading represents the period of LL_b exposure). Arrows denote time of injection.