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ALTERED PHOTIC AND NON-PHOTIC PHASE SHIFTS IN 5-HT$_{1A}$ RECEPTOR KNOCKOUT MICE

V. M. SMITH,[a] R. STERNICZUK,[a] C. I. PHILLIPS[a] AND M. C. ANTLE[a,b,c]*

[a]*Department of Psychology, University of Calgary, 2500 University Drive Northwest, Calgary, Alberta, Canada T2N 1N4*

[b]*Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Alberta, Canada T2N 4N1*

[c]*Pharmacology and Therapeutics, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Alberta, Canada T2N 4N1*

Abstract—The mammalian circadian clock located in the suprachiasmatic nucleus (SCN) is thought to be modulated by 5-HT. 5-HT is though to inhibit photic phase shifts by inhibiting the release of glutamate from retinal terminals, as well as by decreasing the responsiveness of retinorecipient cells in the SCN. Furthermore, there is also evidence that 5-HT may underlie, in part, non-photic phase shifts of the circadian system. Understanding the mechanism by which 5-HT accomplishes these goals is complicated by the wide variety of 5-HT receptors found in the SCN, the heterogeneous organization of both the circadian clock and the location of 5-HT receptors, and by a lack of sufficiently selective pharmacological agents for the 5-HT receptors of interest. Genetically modified animals engineered to lack a specific 5-HT receptor present an alternative avenue of investigation to understand how 5-HT regulates the circadian system. Here we examine behavioral and molecular responses to both photic and non-photic stimuli in mice lacking the 5-HT$_{1A}$ receptor. When compared with wild-type controls, these mice exhibit larger phase advances to a short late-night light pulse and larger delays to long 12 h light pulses that span the whole subjective night. Fos and *mPer1* expression in the retinorecipient SCN is significantly attenuated following late-night light pulses in the 5-HT$_{1A}$ knockout animals. Finally, non-photic phase shifts to (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT) are lost in the knockout animals, while attenuation of the phase shift to the long light pulse due to rebound activity following a wheel lock is unaffected. These findings suggest that the 5-HT$_{1A}$ receptor plays an inhibitory role in behavioral phase shifts, a facilitatory role in light-induced gene expression, a necessary role in phase shifts to 8-OH-DPAT, and is not necessary for activity-induced phase advances that oppose photic phase shifts to long light pulses.

*Correspondence to: M. C. Antle, Department of Psychology, University of Calgary, 2500 University Drive Northwest, Calgary, AB, Canada T2N 1N4. Tel: +1-403-220-2574; fax: +1-403-282-8249.
E-mail address: antlem@ucalgary.ca (M. C. Antle).

Abbreviations: ANOVA, analysis of variance; DD, constant darkness; DIG, digoxigenin; GRP, gastrin-releasing peptide; ICC, immunocytochemistry; IR, immunoreactive; KO, knockout; LD, light/dark cycle; LP, light pulse; PBS, phosphate-buffered saline; PBSx, phosphate-buffered saline with 0.1% Triton X-100; RHT, retinohypothalamic tract; ROD, relative optical density; SCN, suprachiasmatic nucleus; VP, vasopressin; WL, wheel lock; WT, wild type; 8-OH-DPAT, (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide.

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The mammalian circadian clock, located in the suprachiasmatic nucleus (SCN), regulates daily oscillations in physiology and behavior (Antle and Silver, 2005). These endogenously generated rhythms are entrained to the daily environmental cycles through exposure to light (Daan and Pittendrigh, 1976; Rusak and Zucker, 1979) as well as various non-photic cues such as activity (Mrosovsky, 1996), dark pulses (Boulos and Rusak, 1982), and sleep deprivation (Antle and Mistlberger, 2000). The neurotransmitter 5-HT has been implicated in mediating and modulating both photic and non-photic phase shifts of the circadian system.

Photic information is relayed from the retina to the SCN by way of the glutamatergic retinohypothalamic tract (RHT, Mintz and Albers, 1997; Mintz et al., 1999). In hamsters, serotonergic innervation from the median raphe nucleus (Meyer-Bernstein and Morin, 1996; Morin and Meyer-Bernstein, 1999) is thought to modulate the release of glutamate from the RHT (Rea and Pickard, 2000), as well as the postsynaptic response to glutamate by cells in the SCN (Ying and Rusak, 1994, 1997; Rea and Pickard, 2000). These responses are thought to be mediated by 5-HT$_{1B}$ receptors on RHT terminals (Pickard et al., 1996,1999; Pickard and Rea, 1997; Rea and Pickard, 2000), as well as 5-HT$_{1A,\ 1B,\ \text{and}\ 7}$ receptors on SCN cells (Ying and Rusak, 1994, 1997; Rea and Pickard, 2000; Belenky and Pickard, 2001). Blocking the release and/or postsynaptic actions of 5-HT appears to enhance phase shifts both to light (Rea et al., 1995; Smart and Biello, 2001; Gannon, 2003) and to treatments that mimic light by activating the photic pathway downstream of glutamate release (Sterniczuk et al., 2008).

In rats, agonists for the 5-HT$_{2A/2C}$ receptor produce photic-like phase shifts of locomotor, temperature and melatonin rhythms (Kennaway et al., 1996; Kennaway and Moyer, 1998; Kalkowski and Wollnik, 1999; Kohler et al., 1999) and induce expression of the immediate early gene c-Fos and the clock genes *Per1* and *Per2* in the SCN (Moyer et al., 1997; Kennaway and Moyer, 1999; Kohler et al., 1999; Ferguson and Kennaway, 2000; Varcoe et al., 2003). These results suggest that, for this species, 5-HT may help to mediate photic phase shifts. Recent findings suggest that this effect may be mediated by 5-HT$_3$ receptors on RHT terminals (Kennaway and Moyer, 1999; Graff et al., 2005, 2007). It has been hypothesized that activation of the 5-HT$_3$ receptors on the RHT terminals stimulates or enhances glutamate release, thereby eliciting a photic response (Graff et al., 2007).

5-HT has also been suggested to underlie non-photic phase shifts of the circadian clock. 5-HT release is associated with behavioral state (Jacobs and Fornal, 1995, 1997, 1999), with high levels of 5-HT release observed during locomotor activity. In hamsters, 5-HT release in the SCN is at its lowest during the day (Dudley et al., 1998), but output increases dramatically at this time if hamsters are kept awake (Grossman et al., 2000) or run in a wheel (Dudley et al., 1998). Electrical stimulation of the median raphe elicits 5-HT release at the SCN and produces phase shifts consistent with those elicited by non-photic stimuli (Meyer-Bernstein and Morin, 1999; Glass et al., 2000). When the 5-$HT_{1A/7}$ agonist (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT) is given systemically to hamsters (Tominaga et al., 1992; Antle et al., 2003) or *in vitro* to rat or mouse SCN slice cultures (Prosser, 2000, 2003; Sprouse et al., 2004b, 2005; Guscott et al., 2005), phase advances can be elicited during the daytime. Systemic application of 8-OH-DPAT was initially reported to have no phase shifting properties in mice (Antle et al., 2003), however more recent studies suggest that 8-OH-DPAT can phase shift the mouse circadian system when applied 6 h before activity onset (Horikawa and Shibata, 2004; Gardani and Biello, 2008), a phase not examined in the original study.

Understanding how 5-HT influences both photic and non-photic phase shifts has been complicated by a number of factors: the different species and techniques employed to investigate the system; the wide range of 5-HT receptors, each with specific distributions in both the brain and the synapse; and a limited range of selective agonists and antagonists for the receptors of interest. Of particular interest to the serotonergic modulation of both photic and non-photic regulation of circadian rhythmicity is the 5-HT_{1A} receptor. The 5-HT_{1A} receptor serves not only as a postsynaptic receptor (Kia et al., 1996; Riad et al., 2000), but also as a somatodendritic autoreceptor in the raphe nuclei (Verge et al., 1986; Sotelo et al., 1990), that decreases 5-HT output when activated (Dudley et al., 1999). Thus systemic application of 5-HT_{1A} agonists could conceivably activate postsynaptic receptors while simultaneously inhibiting 5-HT output, thereby decreasing activation of all other 5-HT receptors. Conversely, systemic application of 5-HT_{1A} antagonists could conceivably block all postsynaptic 5-HT_{1A} receptors, while simultaneously blocking the 5-HT_{1A} autoreceptors, resulting in an increase in 5-HT output, effectively increasing the activation of all other 5-HT receptors. Furthermore, while early studies implicated the 5-HT_7 receptor in regulation of non-photic phase shifts to 8-OH-DPAT *in vitro* (Lovenberg et al., 1993; Prosser, 2000), recent studies employing 5-HT_7 knockout (KO) mice suggest that the 5-HT_7 receptor is only partially responsible for phase shifts to 8-OH-DPAT (Guscott et al., 2005; Sprouse et al., 2005). In these studies the phase shifts were only fully blocked in 5-HT_7 KO mice when a 5-HT_{1A} receptor antagonist was included. Given the problematic pharmacology involved in studying serotonergic regulation of circadian rhythmicity, specific 5-HT receptor KO mice present a novel tool for understanding the role played by those particular receptors. We examine here how circadian responses to both photic and non-photic treatments are altered in 5-HT_{1A} KO mice.

EXPERIMENTAL PROCEDURES

Animals and housing

A total of 46 male mice were used, consisting of 13 C57BL/6J mice obtained from the University of Calgary Biological Sciences breeding colony (Calgary, AB, Canada), as well as 24 5-HT_{1A} receptor KO mice and nine 5-HT_{1A} receptor wild-type (WT) mice. The KO mice were generated from a breeding colony established in our laboratory from mice originally developed by Dr. Thomas Shenk (Princeton University, Princeton, NJ, USA), provided by Dr. Miklos Toth (Cornell University Medical College, New York, NY, USA) and bred on the C57BL/6J background. For information regarding the generation of the 5-HT_{1A} receptor KO mice see Parks et al. (1998). Animals from our breeding colony were genotyped using a pair of KO primers (amplifying a 400 base pair product that included a portion of the neomycin cassette that replaced the start codon of the 5-HT_{1A} receptor gene) as well as a pair of WT primers (amplifying a 238 base pair product that included a portion of the 5-HT_{1A} receptor gene replaced by the neomycin cassette in the KO animals) to identify the presence or absence of each allele of the 5-HT_{1A} receptor gene (KO up primer: CTT TAC GGT ATC GCC GCT CCC GAT TC; KO down primer: TGC AGG ATG GAC GAA GTG CAG CAC A; WT up primer: AGT GCA GGC AGG CAT GGA TAT GTT; WT down primer: CCG ATG AGA TAG TTG GCA ACA TTC TGA). Mice were individually housed in Nalgene Type L clear polycarbonate cages (30.3 cm long×20.6 cm wide×26 cm high; Nalg Nunc International, Rochester, NY, USA), equipped with a stainless steel running wheel (diameter of 24.2 cm). Animals were housed in a temperature- (21±1 °C) and humidity-controlled room, with *ad libitum* access to food and water. Cages were changed at least every 14 days (7–10 days prior to, and following a manipulation). All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary, and adhered to the Canadian Council on Animal Care guidelines for the ethical use of animals. Every attempt was made to minimize both the number and the suffering of animals used in these experiments.

Activity rhythms

Wheel-running activity was continuously monitored using magnetic switches mounted on the running wheel, and connected to a computer running the Clocklab data collection software package (Coulbourn Instruments, Allentown, PA, USA). Actograms (graphical representations of wheel-running behavior) were generated and analyzed by Clocklab analysis software. Mice were allowed to entrain to the 1500 lux light–0 lux dark (LD) cycle or free-run in constant 0 lux darkness (DD) for a minimum of 7 days prior to the start of manipulations. Onset of running wheel activity for manipulation day was predicted using a regression line fit to the activity onsets for the 7–10 days prior to the testing day.

Experiment 1: Phase shifts to short duration light

Experiment 1 examined the effects of a brief exposure to light on behavioral phase shifts of the activity rhythm. Animals were housed in DD for the duration of this experiment. The light pulse (LP) was administered using a light-sealed box with a light bulb partially covered in aluminum foil such that the light intensity at cage level was measured as 40 lux. As an enhanced response in the KO animals was hypothesized, this light intensity was selected to avoid a ceiling effect that might result from saturating the circadian system with a brighter LP. Mice (n=12, 6 KO and 6 WT) were exposed to a 15-min LP at two different phases in their circadian activity rhythm, either 4 h or 10 h after their predicted activity onset