Published in final edited form as:

Chronobiol Int. 2011 February ; 28(1): 31–38. doi:10.3109/07420528.2010.532894.

Circadian Periods of Sensitivity for Ramelteon on the onset of Running-wheel Activity and the Peak of Suprachiasmatic Nucleus Neuronal Firing Rhythms in C3H/HeN Mice

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Abstract

Ramelteon, an MT₁/MT₂ melatonin receptor agonist, is used for the treatment of sleep-onset insomnia and circadian sleep disorders. Ramelteon phase shifts circadian rhythms in rodents and humans when given at the end of the subjective day; however, its efficacy at other circadian times is not known. Here, the authors determined in C3H/HeN mice the maximal circadian sensitivity for ramelteon in vivo on the onset of circadian running-wheel activity rhythms, and in vitro on the peak of circadian rhythm of neuronal firing in suprachiasmatic nucleus (SCN) brain slices. The phase response curve (PRC) for ramelteon (90 µg/mouse, subcutaneous [sc]) on circadian wheel-activity rhythms shows maximal sensitivity during the late mid to end of the subjective day, between CT8 and CT12 (phase advance), and late subjective night and early subjective day, between CT20 and CT2 (phase delay), using a 3-day-pulse treatment regimen in C3H/HeN mice. The PRC for ramelteon resembles that for melatonin in C3H/HeN mice, showing the same magnitude of maximal shifts at CT10 and CT2, except that the range of sensitivity for ramelteon (CT8–CT12) during the subjective day is broader. Furthermore, in SCN brain slices in vitro, ramelteon (10 pM) administered at CT10 phase advances (5.6 ± 0.29 h, $n = 3$) and at CT2 phase delays (-3.2 ± 0.12 h, $n = 6$) the peak of circadian rhythm of neuronal firing, with the shifts being

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Part of the data included in this article had been presented as a poster at the Society for Research in Biological Rhythms, May 17–21, 2008, Sundestin, Florida.

significantly larger than those induced by melatonin (10 pM) at the same circadian times (CT10: 2.7 ± 0.15 h, $n = 4$, $p < .05$; CT2: -1.13 ± 0.08 h, $n = 6$, $p < .001$, respectively). The phase shifts induced by both melatonin and ramelteon in the SCN brain slice at either CT10 or CT2 corresponded with the period of sensitivity observed in vivo. In conclusion, melatonin and ramelteon showed identical periods of circadian sensitivity at CT10 (advance) and CT2 (delay) to shift the onset of circadian activity rhythms in vivo and the peak of SCN neuronal firing rhythms in vitro.

Keywords

Circadian rhythms; Melatonin; Melatonin receptors; Phase response curve; Ramelteon; Suprachiasmatic nucleus

INTRODUCTION

Ramelteon is a high-affinity MT₁/MT₂ melatonin receptor agonist with high specificity for melatonin receptors (Buysse et al., 2005; Kato et al., 2005). Ramelteon, as does melatonin, promotes sleep in cats, nonhuman primates, and humans (Miyamoto et al., 2004; Roth et al., 2005; Simon et al., 2008; Wurtman, 2006; Yukuhiro et al., 2004) and phase shifts circadian rhythms in both rodents and humans (Cajochen et al., 2004; Hirai et al., 2005; Richardson et al., 2008). Furthermore, ramelteon can accelerate the rate of re-entrainment following an abrupt 8-h phase advance in the light:dark cycle in rats (Hirai et al., 2005). In humans, ramelteon at 1, 2, and 4 mg, but not 8 mg, was shown to significantly enhance, in a time-dependent manner, re-entrainment of the dim-light saliva melatonin offset rhythm after a 5-h advance in the sleep-wake cycle (Richardson et al., 2008). In the above-mentioned studies, the time of ramelteon administration was selected based on the well-established periods of circadian sensitivity to melatonin (Benloucif & Dubocovich, 1996; Burgess et al., 2008; Lewy et al., 1992, 1998).

The efficacy and periods of sensitivity for ramelteon in mediating phase shifts of circadian rhythms as compared to melatonin are not known. Generation of phase response curves (PRCs) is a standard method for characterizing the effects of both photic and nonphotic stimuli on the circadian pacemaker to assess periods of sensitivity and represent the relation between phase changes by stimuli of choice at different circadian times (De Coursey, 1961). Direct activation of receptors for neurotransmitters or neurohormones expressed by the master biological clock, the suprachiasmatic nucleus (SCN), can induce phase shifts (reviewed by McArthur et al., 1997; Miller, 1993; Smith et al., 1992). To date, five melatonin PRCs have been generated in humans for oral (Burgess et al., 2008, 2010; Lewy et al., 1992, 1998) or intravenous (Zaidan et al., 1994) administration of melatonin. The 3-day (single-dose) melatonin PRCs generated in C3H/NeH mice show periods of sensitivity for melatonin at similar circadian times as that for humans (Benloucif & Dubocovich, 1996; Burgess et al., 2008). Furthermore, the direction of melatonin-induced phase shifts (advance or delay) depending on time of treatment in vivo is similar in C3H/HeN mice and humans, despite differences in their activity patterns, nocturnal and diurnal, respectively.

We generated the PRC to ramelteon, a melatonin receptor agonist, to establish the periods of circadian sensitivity to increase its effectiveness in treating circadian disturbances. The PRC to ramelteon was generated using the onset of running-wheel activity rhythms as a phase marker over the 24-h (circadian) cycle in C3H/HeN mice, as used to establish the first melatonin PRC. The C3H/HeN mouse strain expresses melatonin receptors in the SCN (Dubocovich et al., 1996; Siuciak et al., 1990) and is one of the few strains of laboratory mice that produce melatonin in the pineal gland (Ebihara et al., 1986; Goto et al., 1989; von Gall et al., 2000). Complementary to the in vivo PRC to ramelteon based on running-wheel activity, we also assessed the phase-shifting effects of both melatonin and ramelteon in vitro on the circadian rhythm of neuronal firing when administered at either CT10 or CT2. We demonstrated that the periods of sensitivity for ramelteon to phase shift the onset of circadian rhythms of running-wheel activity in vivo and the peak of neuronal firing rate in the SCN brain slice in vitro occur at the same circadian time as for melatonin.

MATERIALS AND METHODS

Animals and Housing

Animals used in this study were male C3H/HeN wild-type mice (4–6 months old) raised in our colony at Northwestern University (Chicago, IL), and at the University at Buffalo (Buffalo, NY). Animals were group housed in a temperature ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and relative humidity controlled environment under 12 h:12 h light: dark (LD) cycles. Food and water were provided ad libitum. Mice were handled according to institutional guidelines set forth by the National Institute of Health standards and approved by the Institutional Animals Care and Use Committee. Further, all experiments, research protocols, and methods conformed to the standards described by Portaluppi et al. (2010).

Phase Response Curve to Ramelteon

All mice used to generate the in vivo PRC to ramelteon were housed individually in cages equipped with a running wheel (18 cm $\times$ 30 cm $\times$ 12 cm) in climate-controlled cabinets for at least 2 wks under a fixed LD cycle, with 12 h of lights-on (300 lux on top of the cages) and 12 h of lights-off prior to any further manipulations. Every running wheel was connected to a computer-controlled, data-acquisition program.

Running-wheel activity was monitored in male wild-type C3H/HeN mice for the initial 2 wks under 12 h:12 h LD cycles followed by free-running in constant dark (DD) conditions. Animals were maintained for 2–3 wks in DD until stabilized free-running rhythms were confirmed. Mice were then treated with either 0.1 ml vehicle (20% ethanol in saline, subcutaneous [sc]) or 0.1 ml ramelteon (90 $\mu\text{g}/\text{mouse}$ in vehicle, sc) at CT0, CT2, CT4, CT6, CT8, CT10, CT12, CT14, CT16, CT18, and CT20 (CT: circadian time; CT12: onset of activity in nocturnal species). The chosen dose for ramelteon was based on the previously effective dose of melatonin used for the 3-day-pulse melatonin PRC in C3H/HeN mice (Benloucif & Dubocovich, 1996). Administration of vehicle and ramelteon was repeated for 3 consecutive days (three-pulse treatment protocol) at indicated circadian times (Benloucif & Dubocovich, 1996). Treatments were administered using a dim-red light source (15 watts, Kodak 1A filter) with a luminescence of <3 lux.

Extracellular Recordings From the C3H/HeN Mouse SCN Brain Slice

C3H/HeN mice (3–6 months of age) maintained in 12 h: 12 h LD cycles were sacrificed by decapitation 2 h before in vitro treatment with vehicle or drugs. Brains were quickly dissected and the area containing the SCN was cut into a block. Coronal brain slices (400 μ m thick) comprising the SCN were prepared, placed in a recording chamber, and continuously perfused with Earle's balanced salt solution supplemented with 24.6 mM glucose, 26.2 mM sodium bicarbonate, and 5 mg/L gentamicin and saturated with 95% O₂–5% CO₂ at 35°C. Extracellular recordings were performed following procedures previously described (Dubocovich et al., 2005; Hunt et al., 2001). A glass microelectrode filled with 5 M NaCl was used for single-unit recordings throughout the SCN. Firing cells were monitored for 4 min, the action potentials were grouped into 10-s bins, and the mean firing rate determined for each cell. Mean firing rates were grouped into 2-h bins and smoothed by plotting the data as 15-min running averages. Data were collected using customized software (National Instruments, Austin, TX). The peak of the circadian rhythm of neuronal firing was visually determined. Brain slices were stimulated at CT2 or CT10 with either vehicle, melatonin (10 pM), or ramelteon (10 pM) applied by a 1- μ L microdrop for 10 min. At this time, the medium was removed and the perfusion was resumed.

Data Collection and Data Analysis

Running-wheel activity data as well as lighting conditions were controlled, collected, and analyzed with Clocklab (Actimetrics, Evanston, IL) on an external Pentium II computer system. Predictions for circadian times when pulses of either vehicle or ramelteon were to be administered were based on the circadian period (τ) of the stable preinjection activity profile (Benloucif & Dubocovich, 1996). The circadian times of treatments for the second and third days were predicted based on the same preinjection 24-h activity profile.

In this study, only 7 out of a total of 193 data points were excluded due to either change in the τ of the 24-h activity rhythm posttreatment or disturbances in the actograms, making accurate τ and phase determinations difficult. Phase advances and delays were calculated based on least squares linear regression lines for pre- and postinjections. The τ was calculated using chi-square analysis of periodograms for 8–10 days of stable pre- and postinjection activity records using Clocklab (Actimetrics). One-way analysis of variance followed by the Newman-Keuls post hoc test (GraphPad Software) were used for statistical comparison between vehicle and ramelteon treatment groups for each CT (GraphPad Software). Statistical significance was set to $p < .05$.

RESULTS

Phase Shifts of Circadian Activity Rhythms Onset Following Repeated Daily Pulses of Ramelteon

Figure 1 shows representative single-plotted actograms of running-wheel activity from C3H/HeN mice free-running in DD and treated with either vehicle (sc) or ramelteon (90 μ g/mouse, sc) for 3 consecutive days at CT2, CT10, and CT16. Vehicle administration did not affect the onset of circadian rhythm of running-wheel activity when given at either circadian time (Figure 1A, C, E); however, ramelteon phase delayed at CT2 (-0.94 ± 0.14 h), phase

advanced at CT10 (1.13 ± 0.14 h), and was without effect on the phase-onset of running-wheel activity at CT16 (-0.12 ± 0.16 h) (Figure 1B, D, F).

Figure 2 shows the PRC for vehicle and ramelteon generated in the C3H/HeN mice using as phase marker the onset of the circadian rhythm of wheel activity. The shape of the PRC for melatonin, generated under identical conditions, is also showed for comparison (Benloucif & Dubocovich, 1996). Phase shift data were pooled in 2-h bins to generate the PRCs (Figure 2). Vehicle administration on 3 consecutive days at the indicated circadian times did not phase shift the onset of circadian activity rhythms. Further, no statistical differences on the phase shift of the onset of circadian activity rhythms in response to handling and/or vehicle injections were observed along the 24-h period. In contrast, administration of ramelteon (90 μ g/mouse, sc) for 3 consecutive days to individual mice at each circadian time resulted in phase-dependent shifts of the onset of circadian activity rhythms (Figure 2) when compared with vehicle-treated mice. Ramelteon-mediated phase shifts were particularly robust between CT8 and CT12 and between CT20 and CT2. Figure 2 shows the PRC to ramelteon, demonstrating the phase shift of the onset of circadian rhythms of activity, with significant phase advances between CT8 to CT12 (CT8: 0.85 ± 0.16 h [n = 8], $p < .005$; CT10: 1.13 ± 0.14 h [n = 13], $p < .001$; CT12: 1.0 ± 0.24 h, $p < .005$ [n = 10]) and significant phase delays between CT 20 and CT2 (CT20: -0.45 ± 0.16 h [n = 8], $p > .05$; CT22: -0.52 ± 0.1 h [n = 8], $p < .05$ [n = 8]; CT2: -0.94 ± 0.14 h, $p < .05$ [n = 10]) when compared with phase shifts induced by vehicle administration, respectively (CT8: -0.11 ± 0.15 h [n = 7]; CT10: -0.07 ± 0.08 h [n = 7]; CT12: 0.09 ± 0.04 h [n = 9], CT20: -0.05 ± 0.17 h [n = 7]; CT22: -0.15 ± 0.09 h [n = 8]; CT2: -0.19 ± 0.12 h [n = 8]).

The resulting PRC for ramelteon (Figure 2) revealed a wider peak of sensitivity in the advance region and a more prolonged delay region of sensitivity as compared to the previously published 3-day-pulse melatonin PRC using the same mouse strain (C3H/HeN) (Benloucif & Dubocovich, 1996). Both the phase advancing (CT8–CT12) and delaying (CT20–CT2) regions of the ramelteon PRC were statistically different from phase shifts induced by vehicle at the corresponding circadian times CT8–CT12 ($p < .05$) and CT20–CT2 ($p < .05$).

Phase Shift of the Peak of Neuronal Firing Rhythms by Melatonin or Ramelteon in SCN Brain Slices

A direct effect of melatonin or ramelteon on melatonin receptors within the master circadian clock was assessed by determining the phase shift of the peak of circadian rhythms of neuronal firing in SCN brain slices. Phase shifts by the agonist was determined at either CT2 or CT10, which are the times when melatonin (90 μ g/ mouse, sc) (Benloucif & Dubocovich, 1996; Figure 2) and ramelteon (90 μ g/mouse, sc) (present data) induced maximal phase delays and phase advances of the onset of running-wheel activity, respectively. Extracellular single-unit recordings from C3H/HeN mice SCN brain slices showed a circadian rhythm of neuronal firing rate, with a peak at CT 6.1 ± 0.08 (n = 7). The position of this peak was identical in SCN slices treated with vehicle at either CT10 or CT2. Therefore, the data obtained after vehicle treatment at CT2 and CT10 were pooled (Figure 3A and E). Melatonin (10 pM for 10 min) and ramelteon (10 pM for 10 min) applied, using the

microdrop technique, at CT10 phase advanced the peak of SCN neuronal firing to CT 3.4 $\pm$ 0.15 (n = 4) ($p < .001$) (Figure 3B) and CT 1.2 $\pm$ 0.73 (n = 4) ($p < .001$) (Figure 3C), respectively. Both the phase advance (CT10) and phase delay (CT2) for the peak of the circadian rhythm of neuronal firing were significantly larger when melatonin receptors were activated by ramelteon (advance: 4.9 $\pm$ 0.73 h, n = 4; delay: -3.2 $\pm$ 0.12 h, n = 6) than melatonin (advance: 2.7 $\pm$ 0.15 h, n = 4, $p < .05$; delay: -1.13 $\pm$ 0.08 h, n = 6, $p < .001$) (Figure 3D, H).

DISCUSSION

Here, we demonstrated for the first time the ramelteon-mediated phase shift on the onset of circadian running-wheel activity rhythms and on the peak of the circadian rhythm of SCN neuronal firing in brain slices of C3H/HeN mice with a distinct pattern of circadian sensitivity. The PRC for ramelteon on the onset of circadian activity rhythms shows robust phase advances between CT8 and CT12 and phase delays between CT20 and CT2, as previously reported for melatonin (Benloucif & Dubocovich, 1996). Further, we demonstrated that the maximal circadian phase shifts induced by ramelteon (this study) or melatonin (Benloucif & Dubocovich, 1996) on overt circadian rhythms in vivo correlate with robust phase advances (CT10) and phase delays (CT2) on the peak of neuronal firing in SCN brain slices. Ramelteon applied at either CT10 or CT2 induces significantly larger phase shifts in the SCN neuronal firing peak than melatonin. Together, these results suggest that phase shifts in the onset of circadian activity rhythms induced by ramelteon (current study) or melatonin (Benloucif & Dubocovich, 1996) in the C3H/HeN mice are likely mediated through activation of melatonin receptors in the SCN (Dubocovich, 2007; Dubocovich et al., 2005).

Ramelteon and melatonin phase shift circadian rhythms in mice and humans following a PRC with similar periods of circadian sensitivity for phase advances and delays (current study; Benloucif & Dubocovich, 1996; Burgess et al., 2008, 2010; Lewy et al., 1998). In C3H/HeN mice, using the onset of running-wheel activity as a circadian marker, ramelteon administered for 3 consecutive days shows robust phase advances and delays spanning from CT8 to CT12 and CT20 to CT2, respectively, although of similar maximal magnitude (1 h) for both CT10 (maximal advance) and CT2 (maximal delay). The PRC for ramelteon generated using the same circadian marker and the three-pulse treatment protocol resembles closely the PRC for melatonin (Benloucif & Dubocovich, 1996) (Figure 2). The increased range of sensitivity of the circadian system to ramelteon in vivo when compared with melatonin, resulting in phase advances and delays over a wider time spectrum, may be attributed to a combination of its higher affinity for the MT₁ melatonin receptor involved in the phase shift of overt circadian activity rhythms in C3H/HeN mice (Dubocovich et al., 2005; Kato et al., 2005) and the pharmacokinetics of ramelteon when compared with melatonin (Karim et al., 2006; Pandi-Perumal et al., 2007). Ramelteon reaches peak serum concentrations of 5700 pg/mL within 0.5–1.5 h (Karim et al., 2006) as compared to melatonin of equal dose that decreases to 1000 pg/mL from its peak value by 1.5 h (Hartter et al., 2000). This may explain why ramelteon if compared to the three-pulse melatonin PRC (Benloucif & Dubocovich, 1996) reveals a wider range of sensitivity, in particular, during the subjective day.

In humans, a PRC based on the dim light melatonin onset (DLMO) has been reported using a 4-day 0.5-mg oral-dose protocol of melatonin administration in subjects kept in dim light, with maximal advances (1 h) at CT9–CT11 and delays (0.5 h) at CT2 (Lewy et al., 1992, 1998). Furthermore, phase advances and phase delays of similar magnitude as those reported by Lewy and colleagues (1992, 1998) were observed by a 3-h infusion of physiological doses of melatonin in the late subjective day or subjective night, respectively (Zaidan et al., 1994). Burgess et al. (2008) reported maximal phase advances (CT10) of 1.8 h and phase delays (CT2) of 1.3 h, respectively, using an oral dose of 3 mg melatonin in human subjects kept in constant dim light for the duration of the experiment. The shape of these curves resembles the melatonin PRC of C3H/HeN mice, as maximal phase advances and phase delays are observed at exactly the same circadian time (Benloucif & Dubocovich, 1996; Burgess et al., 2008). In a recent paper, Burgess et al. (2010) reported melatonin PRCs in humans to two doses of melatonin (0.5 and 3 mg), with similar maximal phase shifts, but at different circadian times. In summary, there is a good correlation between multiple consecutive doses of melatonin or ramelteon on the shape of the PRC between mice and human.

PRCs generated by a single dose of melatonin administration in vivo produces phase shifts of smaller magnitude. In C3H/HeN mice, phase advances at CT10 and phase delays at CT2 induced by a single pulse of melatonin were significantly smaller in magnitude and less robust than shifts induced using a 3 consecutive day pulse protocol with treatments at the same circadian times (Benloucif & Dubocovich, 1996). In C3H/HeN mice and Syrian hamsters, a single pharmacological dose (2.5–25 mg/kg, intraperitoneal [ip]) of the melatonin agonist S-20098 phase advances circadian activity rhythms at CT9–CT11 and phase delays at the end of the subjective night, but with phase shifts of reduced magnitude (Van Reeth et al., 1997). In humans, induction of robust phase advances and delays by melatonin also requires between 3 and 4 consecutive days of pulse treatments (Burgess et al., 2008; Lewy et al., 1992, 1998), with no reported phase-delay effects observed using a single melatonin pulse during the late subjective night (Wirz-Justice et al., 2002). The need for multiple treatments to generate robust PRCs to melatonin or ramelteon in vivo is not known at the present time. It is likely, however, that cumulative small phase shifts are necessary to induce the robust change in phase observed after a 3–4-day treatment.

In rats, a single treatment with melatonin at the end of the subjective day (CT10) advances the phase onset of running-wheel activity; however, no phase advances or delays at other circadian times were observed (Armstrong, 1989; Armstrong et al., 1993; Warren et al., 1993). The effect of multiple pulse treatments with melatonin on phase shifts of overt circadian rhythms has not been reported in the rat. McArthur and colleagues (1997) generated a PRC to a single melatonin (1 nM) treatment/timepoint in rat SCN brain slices using as a phase marker the phase of the peak of circadian rhythm of neuronal firing. This PRC shows two distinct periods of sensitivity; however, both induce phase advances, i.e., at CT8–CT12 and at CT23–CT24, with no phase shift at CT2. The lack of correlation between phase shifts by melatonin in vitro and in vivo led to questioning of the use of the peak of the circadian rhythm of neuronal firing rate as a phase marker in the SCN brain slice to assess periods of sensitivity to this hormone.

Here, we described for the first time that in mice melatonin and ramelteon show periods of circadian sensitivity for phase advance (CT8–CT12) and delay (CT22–CT2) of overt circadian rhythms which correlate with corresponding circadian phase shifts of the peak of the circadian rhythm of neuronal firing in SCN brain slices. Indeed, in the SCN brain slice from C3H/HeN mice, melatonin (10 pM) and ramelteon (10 pM) applied at CT10 or CT2 induced statistically significant phase advances and phase delays, respectively, of the peak of the circadian rhythm of neuronal firing (Figure 3A, B, C and Figure 3E, F, G). The magnitude of phase shifts of the peak of SCN neuronal firing rhythms by ramelteon (10 pM) was significantly larger, both for advances at CT10 (5.6 ± 0.29 h, $n = 3$) and delays at CT2 (-3.2 ± 0.12 h, $n = 6$) than those induced by an equimolar concentration of melatonin (CT10: 2.7 ± 0.15 h, $n = 4$, $p < .05$; CT2: -1.13 ± 0.08 h, $n = 6$, $p < .001$, respectively) (Figure 3D and H). Similarly, the melatonin agonist 2-iodomelatonin at 1 nM phase advanced the peak of the circadian rhythm of neuronal firing in the rat SCN brain slices by almost 7 h (McArthur et al., 1997). The mechanism(s) by which ramelteon and 2-iodomelatonin induced larger phase shifts of the peak of neuronal firing rhythms than melatonin is not known at the present time, but it could well be related to their higher affinity and/or activation of MT₁ melatonin receptors in the SCN, which may contribute to the phase shift induced by activation of MT₂ receptors (Dubocovich et al., 2005; Liu et al., 1997). Together, our results strongly suggest that activation of melatonin receptors by either melatonin or ramelteon in the SCN of C3H/HeN mice mediates the phase shift of running-wheel activity in vivo.

The shape of any PRC is characteristic for each given substance/drug, and can have similar periods of sensitivity across mammalian species (Turek, 1987). The similarities in shape and periods of sensitivity of the PRC to melatonin and ramelteon in mice and humans make the mouse an ideal animal model to infer the temporal efficacy of new drugs in humans (Benloucif & Dubocovich, 1996; Burguess et al., 2008, 2010; Lewy et al., 1998). It follows that the PRC to ramelteon in mice should predict, in principle, the periods of sensitivity for ramelteon in humans. In summary, the sensitivity of the circadian system to ramelteon at both dusk and dawn is similar to that of melatonin both in vivo and in vitro.

Acknowledgments

This research was supported by an investigator-initiated grant from Takeda Pharmaceutical North America (Takeda 06-016R) to Northwestern University, with M.L. D. as principal investigator. M.L.D. has received research support through an investigator-initiated research grant from Takeda Pharmaceuticals North America, has served as consultant and/or speaker for Takeda Pharmaceuticals North America, Takeda Pharmaceutical Company Limited, and has solicited unrestricted educational grants awarded to Northwestern University from Takeda Pharmaceuticals North America. We thank Elizabeth A. Marshall for editorial assistance.

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FIGURE 1.

Representative actograms for mice treated with either ramelteon or vehicle at three circadian time points (CT2, CT10, and CT16). The actograms represent running-wheel activity rhythms of mice maintained in constant darkness (DD) pulse-treated with vehicle (ethanol/saline, sc) or ramelteon (90 $\mu\text{g}/\text{mouse}$, sc), for 3 consecutive days at CT2, CT10, or CT16. Phase shifts on the onset of running-wheel activity rhythms induced by vehicle (VEH: open circle) or ramelteon (RAM: square) administration were at CT2 (VEH: 0.19 h; RAM: -1.9 h) (A, B), at CT10 (VEH: 0.09 h; RAM: 1.52 h) (C, D), and at CT16 (VEH: -0.16 h; RAM: 0.13 h) (E, F).

FIGURE 2.

Three-day-pulse phase response curve for ramelteon on the onset of running-wheel activity rhythms in C3H/HeN mice. The curves show the magnitude of phase shift (advances, delays, or no shift) induced by vehicle (ethanol/saline, sc; circles) or ramelteon (90 µg/mouse, sc; squares) administration for 3 consecutive days at CT2, CT4, CT6, CT8, CT10, CT12, CT14, CT16, CT18, CT20, CT22, and CT24. The PRC for melatonin (90 µg/mouse, sc) using the onset of running-wheel activity rhythms in C3H/HeN mice generated under identical experimental conditions is reproduced here for comparison (Benloucif & Dubocovich, 1996). Each point represents mean $\pm$ SEM. * $p < .05$, when compared with vehicle (one-way analysis of variance followed by the Newman-Keuls post hoc test).

FIGURE 3.

Melatonin- and ramelteon-mediated phase advances (CT10) and phase delays (CT2) of the peak of neuronal firing in SCN brain slices from C3H/HeN mice. Shown are representative rhythms of neuronal firing assessed by single-unit extracellular recordings in SCN brain slices of C3H/HeN mice. Vertical arrows represent application of vehicle (A, E), melatonin (10 pM for 10 min) (B, F), or ramelteon (10 pM for 10 min) (C, G) applied at either CT10 or CT2, respectively. Mean single-unit firing rates from individual cells in SCN brain slices treated with vehicle (A, E; open circle), melatonin (B, F; closed circle), or ramelteon (C, G; square) are represented as running averages. Panels D (gray) and H (black) represent (mean $\pm$ SEM) total phase advances or delays, respectively, of the peak of the circadian rhythm of

neuronal firing in response to either melatonin (B, F) or ramelteon (C, G) application with respect to control peaks in panels A and E. $*p < .05$; $**p < .001$ when comparing phase shifts by ramelteon to phase shifts by melatonin (Student *t* test). VEH: vehicle; MLT: melatonin; RAM: ramelteon.

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