The Sleep-Promoting Action of Ramelteon (TAK-375) in Freely Moving Cats

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Introduction: Ramelteon (TAK-375) is an MT_1/MT_2 receptor agonist being studied for the treatment of insomnia and circadian rhythm sleep disorders. We compared the behavioral effects of ramelteon and exogenous melatonin in freely moving cats.

Methods: Ramelteon and melatonin were each suspended in a 0.5% (weight per volume) methylcellulose solution and administered orally to freely moving cats. In the control trial, each cat was given vehicle. Each dose of ramelteon or melatonin was compared with the vehicle control in a crossover design. Electroencephalogram, electromyogram, and electrooculogram recordings were assessed.

Results: Ramelteon significantly decreased wakefulness at doses of 0.001, 0.01, and 0.1 mg/kg, increased slow-wave sleep at doses of 0.001, 0.01, and 0.1 mg/kg, and increased rapid eye movement sleep at a dose of 0.1 mg/kg, compared with the vehicle controls, as assessed by analysis of variance. The effects of ramelteon lasted for up to 6 hours when evaluated by reduction of wakefulness. Exogenous melatonin (0.01-1 mg/kg) significantly increased slow-wave sleep, but the effect was weaker than that of ramelteon and lasted for only 2 hours. The lowest doses of ramelteon (0.0001 mg/kg) and melatonin (0.001 mg/kg) had no significant effect on sleep-wakefulness stage.

Conclusions: Ramelteon was more effective than exogenous melatonin in promoting and maintaining sleep in freely moving cats. Based on its unique mechanism of action, ramelteon should be studied further to evaluate its potential for the treatment of sleep disorders.

Key Words: ramelteon (TAK-375), melatonin, sleep, cat, electroencephalogram, slow-wave sleep, rapid eye movement, insomnia

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INTRODUCTION

PRODUCTION OF THE HORMONE MELATONIN IN HUMANS IS CONCURRENT WITH NOCTURNAL SLEEP, AND THE EVENING INCREASE IN MELATONIN LEVELS CORRESPONDS WITH THE ONSET OF SELF-REPORTED EVENING SLEEP PROPENSITY. Inhibition of nighttime melatonin production by β-adrenergic receptor-blocking agents or by exposure to bright light attenuates the usual nighttime decrease in body temperature and reduces sleep propensity; these effects are reversed by the administration of melatonin.[1]

Melatonin exerts its action through membrane-associated receptors, and 2-[^{125}I]-melatonin is the radioligand of putative melatonin receptors in neuronal and nonneuronal tissues from vertebrate species. In human, 2 subtypes of high-affinity melatonin receptors have been cloned and designated as MT_1 (also designated as Mel_{1a})[2] and MT_2 (Mel_{1b})[3] by the International Union of Pharmacology. [4] The MT_1 and MT_2 receptors in the suprachiasmatic nucleus (SCN) may be involved in the effect of melatonin on circadian rhythms.[5,6] In chick brain, both Mel_{1a} (80% amino acid identity to the human MT_1) and Mel_{1c} (distinct from the human MT_1 and MT_2) receptors were cloned. To date, Mel_{1c} receptors have not been found in mammals.

Melatonin exerts its effects through the high-affinity MT_1 and MT_2 receptors in signal transduction pathways involving an inhibitory G protein, leading to inhibition of intracellular adenyl cyclase and decreased intracellular cAMP levels.[5,7] In addition to these high-affinity MT_1 and MT_2 receptors, it has been reported that there exists a nanomolar binding site (designated as *MT_3* by the International Union of Pharmacology) in hamster brain and kidney.[8-10] Recently, the *MT_3* binding site has been characterized as a melatonin-sensitive form of quinone reductase 2 (QR_2),[11] an enzyme related to the detoxifying enzyme NAD(P)H: quinone oxidoreductase 1 (QR_1, EC 1.6.99.2), and it shows wide tissue distribution, including in the kidney, liver, brain, heart, and skeletal muscle in mammals. The profile of the *MT_3* binding site is completely different from those of the high-affinity MT_1 and MT_2 receptors.[12]

Exogenous administration of melatonin has been shown to decrease the time to sleep onset, but it has no consistent effect on sleep efficiency and total sleep,[13-15] which may be attributed to its short half-life. A controlled-release melatonin has been shown to improve initiation of sleep and to increase sleep efficiency and total sleep time in clinical trials in elderly people with insomnia.[16,17] This suggests that a high-affinity MT_1/MT_2 receptor agonist with a longer half-life than that of melatonin might be a useful therapy for sleep disorders in this population.

Until specific information on the function of the *MT_3* binding sites is available, a specific MT_1/MT_2 receptor agonist without an affinity for *MT_3* binding sites is desirable. With this in mind, we have found that *(S)-N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-*b*]furan-8-yl)ethyl]propionamide (ramelteon, TAK-375, Figure 1) has a high affinity for human MT_1 and MT_2 receptors, while

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showing negligible affinity for the MT_3 binding site in the hamster brain.[18] Here, we report in vivo preclinical pharmacology of ramelteon in freely moving cats.

METHODS

Subjects

Male and female cats were obtained from KEARI (Hashimoto, Japan) and housed individually in a room maintained at 22°C to 27°C with a 12-hour light-dark cycle (lights on at 7:00 AM). They were fed once daily (9:00 AM) and water was available ad libitum. On the experiment day, however, they were fed after completion of the experiment. The methods for care and use of the animals and the experimental protocols were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Ltd. (Osaka, Japan).

Measurement of Sleep and Wakefulness in Freely Moving Cats

Fourteen adult cats (5 males and 9 females) weighing 2.5 to 6.1 kg at time of surgery were used. Cats were placed on a stereotaxic apparatus under pentobarbital (40 mg/kg, intraperitoneal) anesthesia. Electrodes for electroencephalogram (EEG) recording were implanted bilaterally in the frontal and parietal cortexes and hippocampus according to the cat brain atlas of Snider and Niemer.[19] Stainless-steel screws were used as cortical electrodes. The depth bipolar recording electrode consisted of twisted stainless-steel wires (0.3 mm in diameter) insulated except at the tips (0.5 mm). Two insulated stainless electrodes were implanted into the back cervical muscles to record electromyogram (EMG) data. Stainless-steel screws were fixed over the bony orbit to record electrooculogram (EOG) data.

The cats were allowed to recover from surgery for at least 7 days before habituation to the test chamber and EEG recording. The test chamber (65 × 35 × 45 cm), constructed of metal with 1 Plexiglas wall, was located in a constantly illuminated, ventilated, soundproofed, and electrically shielded room. Cats were well accustomed to the test chamber and EEG-recording procedure before testing. Each cat was transferred to the test chamber in the experimental room, attached to the cables, and EEG, EOG, and electromyogram (EMG) findings were recorded for 9 hours (from 1 hour before treatment to 8 hours after treatment with vehicle, ramelteon, or melatonin). Ramelteon (0.0001, 0.001, 0.01, and 0.1 mg/kg), melatonin (0.001, 0.01, 0.1, and 1.0 mg/kg), or vehicle was orally administered between 9:30 AM and 10:30 AM. During recording, each cat was observed from outside the experimental room using a video monitor, and behavioral and postural changes were recorded continuously throughout the experiment. Each potential, amplified and filtered with a polygraph (Nihondenki-Sanei, Osaka, Japan), was recorded using a magnetic pen recorder. The EEG power spectral analysis was also performed continuously by means of a fast Fourier transform system equipped with a personal computer (PC-9821, NEC, Tokyo, Japan); these data were recorded on a magnetic optical disc.

The sleep-wakefulness stage for each cat throughout the monitoring period was classified as 1 of 3 stages using the following criteria: (1) wakefulness (marked tonic EMG activity, low-voltage fast cortical EEG with a low power delta wave spectrum, a regular hippocampal theta rhythm, and slow EOG activity); (2) slow-wave sleep (SWS, markedly reduced EMG activity, spindles and slow waves of high-voltage cortical EEG with a high power delta wave spectrum, and reduced EOG activity); or (3) rapid eye movement (REM) sleep (almost complete absence of EMG activity, low-voltage fast cortical EEG with a low power delta wave spectrum, an extremely regular hippocampal theta rhythm, and frequently observed high-voltage EOG due to rapid eye movement).

Drugs and Drug Administration

(S)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno-[5,4-*b*]furan-8-yl)ethyl]propionamide (ramelteon, TAK-375, Figure 1) was synthesized at Takeda Pharmaceutical Company Ltd. (Osaka, Japan). Melatonin was purchased from Sigma Chemical Co. (St. Louis, MO). Ramelteon and melatonin were each suspended in 0.5% (weight per volume) methylcellulose solution. Ramelteon or melatonin solution was administered orally to each cat in a gelatin capsule. In the control trial, each cat was given a capsule-containing vehicle. Each dose of ramelteon or melatonin was compared with vehicle control in a crossover design. The interval between the trials was more than 7 days in order to avoid the carry-over effects from the previous trial.

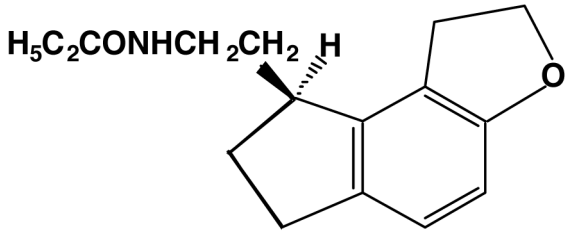
Data and Statistical Analyses

For statistical analysis, 1-way and 2-way analyses of variance (ANOVA) and paired *t* tests were used. In posthoc tests, Holm correction was utilized for the multiple comparisons.

RESULTS

Effects of Ramelteon on Time Spent in Sleep Stages

The effects of ramelteon on sleep and wakefulness in freely moving cats are shown in Figure 2. Results are presented as the mean percentage of time spent in each sleep-wakefulness stage (ie, wakefulness, SWS, and REM sleep) at each 2-hour period after administration. Ramelteon (0.1 mg/kg, PO) significantly decreased the percentage of wakefulness [$F_{1,7} = 47.8$, $P < .01$] and increased the percentage of SWS [$F_{1,7} = 31.4$, $P < .01$] and REM sleep [$F_{1,7} = 8.85$, $P < .05$]. Similarly, lower doses of ramelteon (0.001 and 0.01 mg/kg, PO) significantly decreased the percentages of wakefulness [$F_{1,7} = 10.0$, $P < .05$ and $F_{1,7} = 27.6$, $P < .01$, respectively], and increased the percentages of SWS [$F_{1,7} = 6.96$, $P < .05$ and $F_{1,7} = 21.9$, $P < .01$, respectively], although there were no significant differences in the percentages of REM sleep. The lowest dose of ramelteon (0.0001 mg/kg, PO)



$C_{16}H_{21}NO_2$ (MW: 259.34)

Figure 1—Chemical structure of ramelteon (TAK-375).

had no significant effect on the sleep-wakefulness stage in freely moving cats.

Effects of Ramelteon on Duration of Sleep

The duration of sleep-promoting action of ramelteon was evaluated using the posthoc test on the reduction of wakefulness. Significant reductions in wakefulness were observed at 2, 4, and 6 hours with the highest doses (0.1 and 0.01 mg/kg) of ramelteon ($P < .01$, paired t test with Holm correction). Treatment with 0.001 mg/kg of ramelteon resulted in significant decreases in the stage of wakefulness at 6 hours after administration.

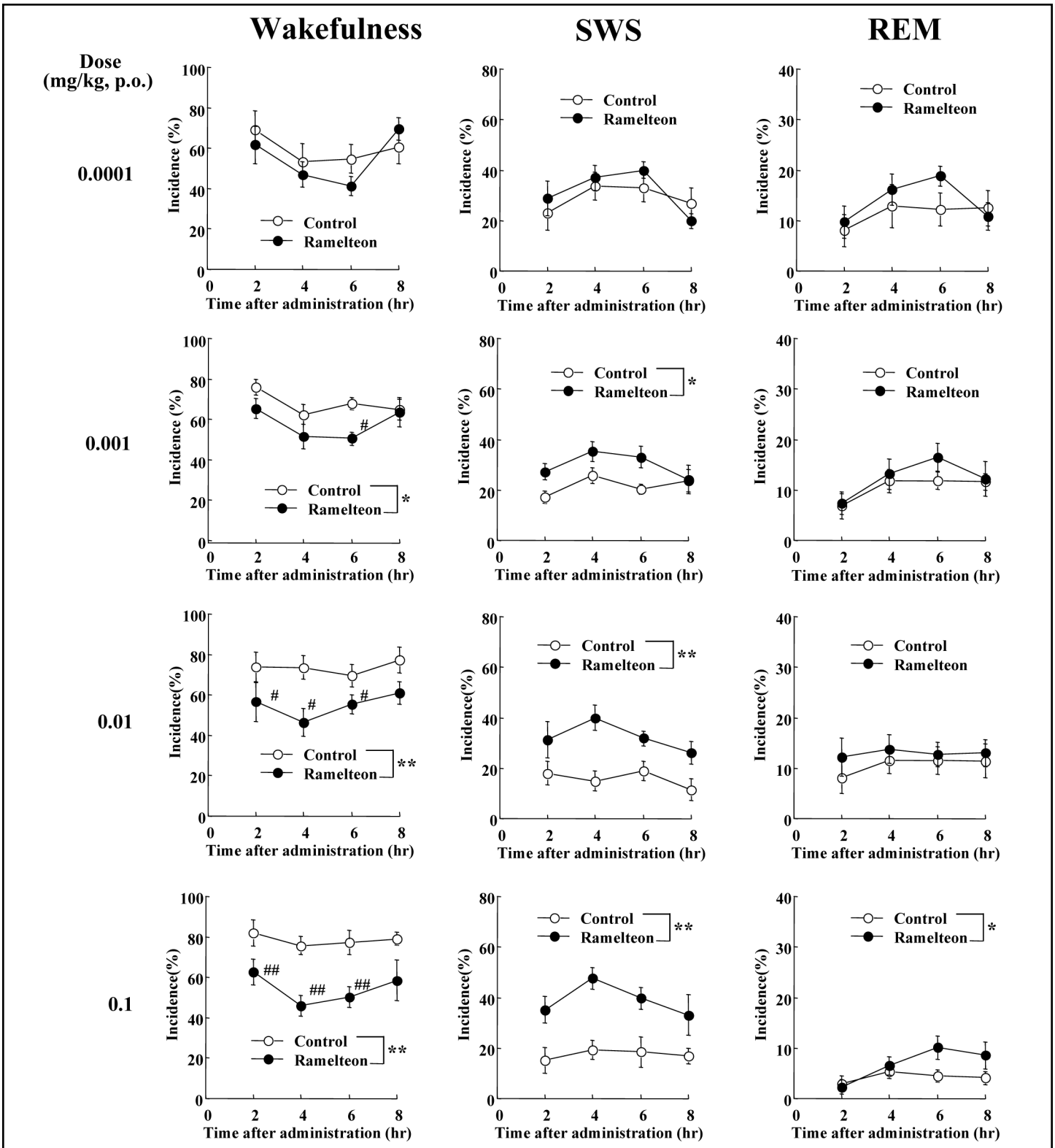


Figure 2—Effects of ramelteon on sleep and wakefulness in freely moving cats. Each value shows the mean percentage of time spent in the stages of wakefulness, slow-wave sleep (SWS), or rapid eye movement (REM) sleep during each block of 2 hours after drug administration with SEM. Eight of 14 cats were randomly used in each dose group. *$P < .05$, **$P < .01$, compared with the vehicle-treated control (analysis of variance). #$P < .05$, ##$P < .01$, compared with the vehicle-treated control (paired t test with Holm correction).

Effects of Ramelteon on Sleep Latency

Sleep latency (time to first SWS lasting more than 1 minute) was also measured in each group (Figure 3). At the highest dose of ramelteon (0.1 mg/kg, PO), median sleep latency was 24 minutes in the ramelteon group compared with 60 minutes in the vehicle-treated group, but the difference was not statistically significant [$F_{1,6} = 5.28$, crossover ANOVA design, $P = .06$] because of large individual variance. Lower doses of ramelteon also showed a tendency to reduce sleep latencies compared with respective controls; median latencies were 17 minutes with ramelteon 0.0001 mg/kg (vs 41 minutes with control, $F_{1,6} = 2.74$, $P = .15$); 23 minutes with 0.001 mg/kg (control: 36.5 minutes, $F_{1,6} = 1.69$, $P = .24$) and 9 minutes with 0.01 mg/kg (control: 38 minutes, $F_{1,6} = 3.09$, $P = .13$). Thus, ramelteon reduced the sleep latency, but the effect was not statistically significant.

Effects of Melatonin on Time Spent in Sleep Stages

The effects of melatonin on sleep and wakefulness in freely moving cats are shown in Figure 4. The highest dose of melatonin (1 mg/kg, PO) reduced the percentage of wakefulness [$F_{1,7} = 54.8$, $P < .01$] and increased SWS [$F_{1,7} = 98.4$, $P < .01$] compared with the vehicle-treated control, although it had no significant effect on REM sleep. Lower doses of melatonin (0.01 or 0.1 mg/kg, PO) significantly increased SWS sleep compared to control [$F_{1,7} = 5.93$, $P < .05$ and $F_{1,7} = 6.07$, $P < .05$, respectively]. The lowest dose of melatonin did not affect sleep or wakefulness in freely moving cats.

Effects of Melatonin on Sleep Duration

The duration of the sleep-promoting action of melatonin was shorter than that of ramelteon, with a significant reduction of wakefulness at 2 hours only for the highest dose of melatonin (1 mg/kg) ($P < .01$, paired t test with Holm correction). There were no significant differences between lower doses of melatonin and vehicle.

Effects of Melatonin on Sleep Latency

Treatment with melatonin (0.001 to 1 mg/kg, PO) showed no significant effect on sleep latency (Figure 5), and an apparent tendency to decrease the latency was not observed even at the highest dose of melatonin. The median sleep latencies were 23.0 minutes for melatonin 1 mg/kg and 23.5 minutes for the vehicle-treated group.

DISCUSSION

Ramelteon exhibited a sleep-promoting action in freely moving cats studied during the daytime. Based on the minimum effective dose, ramelteon was about 10 times more potent than exogenous melatonin. This finding is consistent with the demonstrated in vitro affinity of ramelteon and melatonin for high-affinity MT_1 and MT_2 receptors, Ki values of ramelteon and melatonin for MT_1, MT_2 receptors using 2-[^{125}I]-melatonin binding being 14.0 and 80.7 pmol for the MT_1 receptor, and 112 and 383 pmol for the MT_2 receptor, respectively.[18] Since in vitro studies indicate that ramelteon has a low affinity for the MT_3 binding site, the Ki value of ramelteon for the MT_3 receptor being 2,650 nmol, or other receptors and does not inhibit any enzyme tested,[18,20] the sleep-promoting action of ramelteon can be attributed to its agonistic action at high affinity melatonin receptors. The sleep-promoting effect of ramelteon also lasted longer than the effect of melatonin, as indicated by the significant reduction in the stage of wakefulness observed for 6 hours after the administration of ramelteon (0.001, 0.01, and 0.1 mg/kg) and for 2 hours after administration of the highest dose of melatonin (1 mg/kg). The exact mechanisms of the long-lasting action of ramelteon, however, require further studies.

The effects of melatonin on sleep have been studied in nocturnal and crepuscular species. In rats, some studies have found that high pharmacologic doses of melatonin (2.5-10 mg/kg, intraperitoneal) increase SWS and REM sleep[21,22]; however, other investigators studying rats and hamsters found that melatonin did not promote sleep but, rather, promoted wakefulness.[23,24] These inconsistent findings may derive from a crucial difference in temporal patterns of melatonin secretion relative to sleep and wakefulness in rodents.

In cats, the sleep-promoting effects of melatonin may be more stable. Microinjection of high doses of melatonin (15-30 μg) into the preoptic area or nucleus centralis medialis of the hypothalamus (but not the reticular formation) produced sleep lasting 2 to 3 hours,[25] and melatonin injected into the third ventricle induced non-REM sleep with REM sleep suppression.[26] In the present study, oral administration of melatonin and ramelteon at low doses produced marked SWS and REM sleep in freely moving cats. Taken together, these data indicate that the cat model is useful for the study of the sleep-promoting action of melatonin or melatonin-receptor agonists.

Recently, Zhdanova et al[27] (using actigraphic recording of motor activity the evaluate sleep and wakefulness) showed that the oral administration of low doses of melatonin (0.005-0.32 mg/kg) promoted sleep in 3 species of monkeys, suggesting that monkeys are also an adequate animal model for studying melatonin's action on sleep. We have shown by polysomnographic recordings that melatonin and ramelteon, administered orally in monkeys just before the nighttime, reduced sleep latency and that ramelteon, but not melatonin, increased total sleep time.[28]

In humans, benzodiazepine-receptor agonists have been shown to decrease the duration of REM sleep after a single or repeated

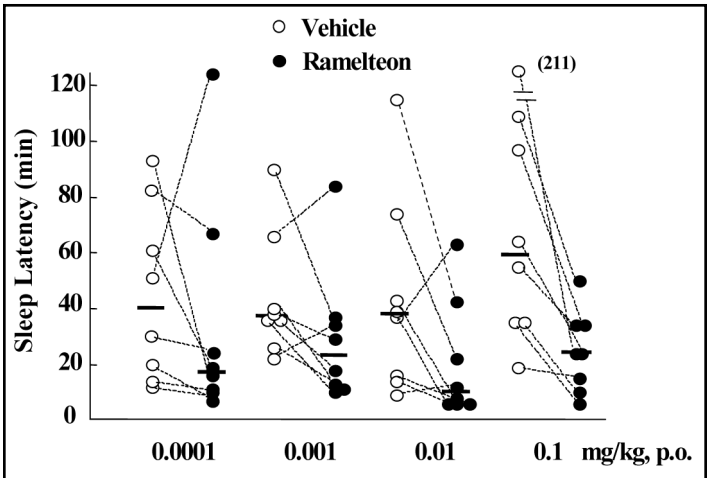


Figure 3—Effects of ramelteon on sleep latency in freely moving cats. Sleep latency (time to first slow-wave sleep epoch lasting more than 1 minute) for each dose of ramelteon compared with the vehicle control. Each bar indicates the median sleep latency of each group.

administration,[29,30] and the decrease of REM sleep, especially in the first half of the night, may be related to the effect of these agents on cognition.[31] Newer benzodiazepine-receptor agonists, including zopiclone, zaleplon, and zolpidem, also have been shown to decrease REM sleep or REM density in the first half of the night[32,33]; however, zolpidem appears to have the least effect of the 3 on sleep architecture,[34] and the cognitive impairment associated with zolpidem seems milder than that found with the use of other γ-aminobutyric acid (GABA)ergic agonists.[35] However, it has been reported that a higher dose of zolpidem (15

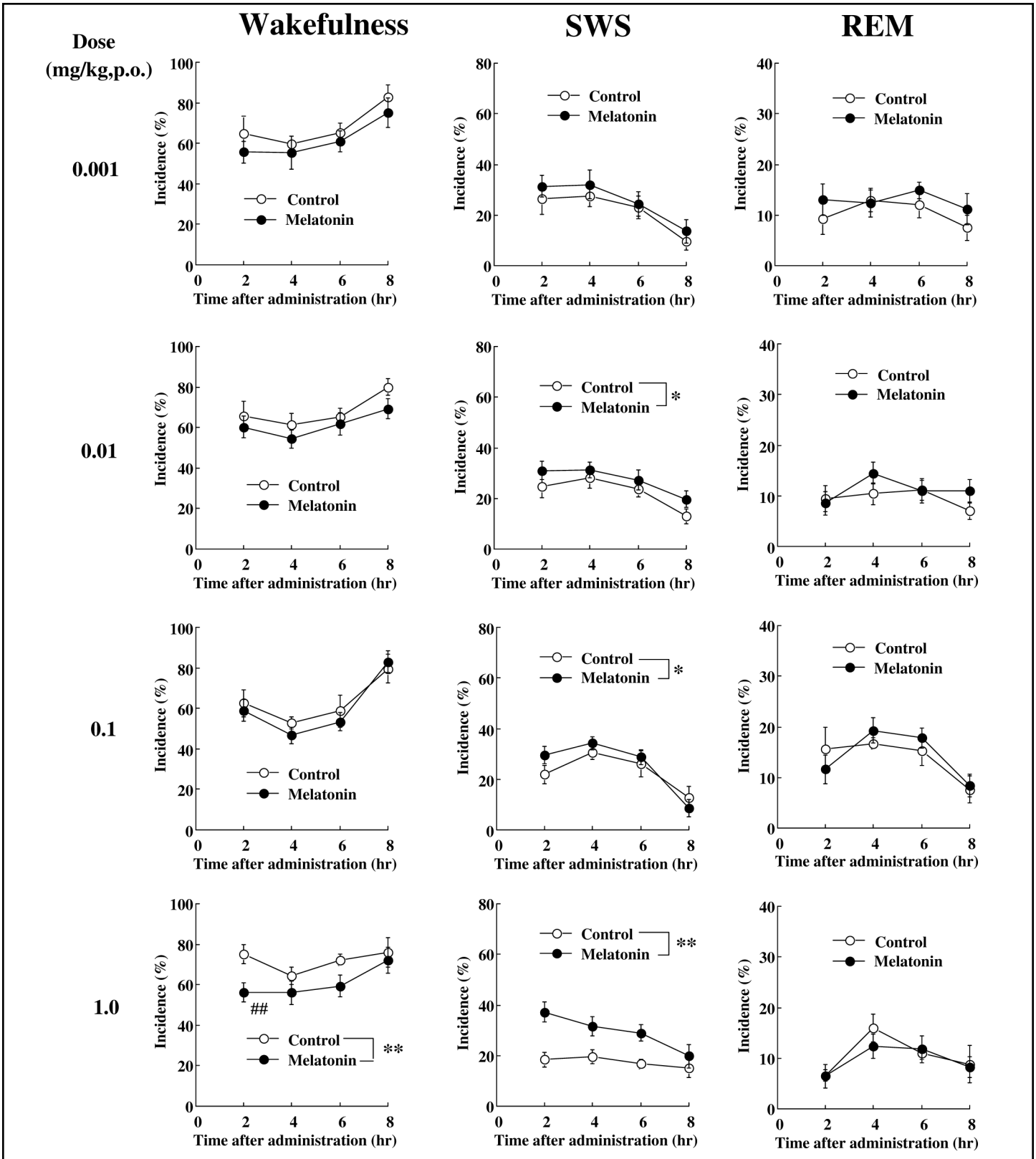


Figure 4—Effects of melatonin on sleep and wakefulness in freely moving cats. Each value shows the mean percentage of time spent in the stages of wakefulness, slow-wave sleep (SWS), or rapid eye movement (REM) sleep during each block of 2 hours after drug administration with SEM. Eight of 14 cats were randomly used in each dose group. *$P < .05$, **$P < .01$, compared with the vehicle-treated control (analysis of variance). ##$P < .01$, compared with the vehicle-treated control (paired t test with Holm correction).

mg) still decreases REM sleep.[36] In this study of sleep in cats, ramelteon and melatonin did not decrease REM sleep, but, rather, ramelteon increased both REM and SWS. We have previously shown that ramelteon does not impair learning and memory in rats.[37] In clinical trials, it has been shown that melatonin induces qualitatively good sleep and increases REM sleep[15,38] without affecting cognitive function.[39]

In humans, the circadian rhythm for the release of melatonin from the pineal gland is closely synchronized with the habitual hours of sleep. Melatonin has been suggested to have a dual effect on sleep, a sleep-promoting action and a phase-shifting property. Exogenous melatonin shifts the human circadian rhythm according to a phase-response curve. In the rat SCN brain slice, bath-applied melatonin induces concentration-dependent advances of the neuronal firing rate,[40] which is circadian time-dependent of application. The targeted deletion of the MT_1 receptor did not significantly alter the melatonin-induced phase shifting effect in a study of C57BL/6J mice; however, the melatonin-mediated acute inhibition of neuronal firing rate in the SCN was abolished.[41] The suppression of the neuronal activity by melatonin might be important in defining the SCN sensitivity to entraining stimuli, and, in diurnal species, may contribute to the regulation of sleep.[6] Conversely, Dubocovich et al[42,43] reported that MT_2 melatonin-receptor antagonists block melatonin-mediated phase advances of circadian rhythms. These findings imply an essential role of MT_2 receptors in the phase-shifting action of melatonin.

In spite of all this research, the mechanisms behind the sleep-promoting action of melatonin are still unknown. $GABA_A$ receptors are widely distributed in the SCN and may serve both presynaptic and postsynaptic roles in controlling the mammalian circadian rhythms.[44,45] Wang et al[21] have shown that bicuculline, a specific antagonist of the $GABA_A$ receptor, abolishes the melatonin-induced increase in SWS and REM sleep and the decrease in wakefulness in rats. Also, exogenous melatonin has been shown to increase in GABA contents in certain regions of the brain, including the hypothalamus and the pineal gland in rats,[46] and to potentiate $GABA_A$ receptor-mediated current in cultured neurons of chick spinal cord.[47] Furthermore, GABA and muscimol, a $GABA_A$ receptor agonist, inhibited neuronal discharge of the SCN during both subjective day and subjective night in a concentration-dependent manner in rat SCN slices, the effect being reversed by $GABA_A$-receptor antagonists.[48] Melatonin also inhibited the neuronal firing of the SCN, possibly due to activation of MT_1 receptors. Thus, sleep-promoting action of melatonin and ramelteon might be derived from activation of GABAergic system in the SCN, in which MT_1 receptors may be involved. However, flumazenil, a benzodiazepine-receptor antagonist, did not block the sleep effect of melatonin in human[49] or in zebrafish,[50] suggesting that the action of melatonin may not be derived from activation of the benzodiazepine receptor. Ultimately, species-specific differences in the mechanisms of sleep promotion by melatonin or melatonin-receptor agonists cannot be excluded. Sleep induced by high doses of melatonin in rats may be qualitatively different from that observed in humans. Thus, further studies are required.

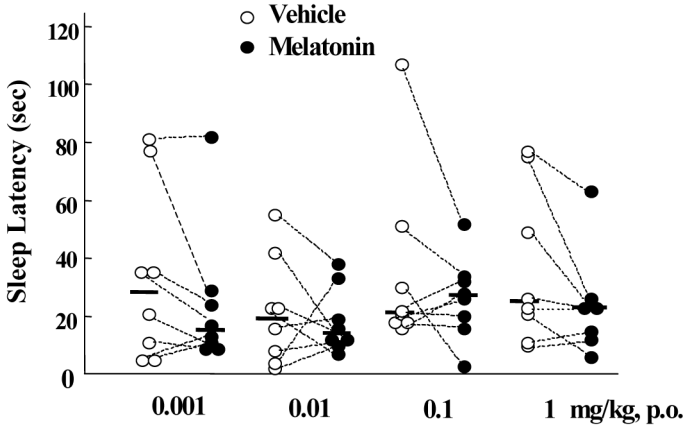


Figure 5—Effects of melatonin on sleep latency in freely moving cats. Sleep latency (time to first slow-wave sleep epoch lasting more than 1 minute) for each dose of melatonin compared with the vehicle control. Each bar indicates the median sleep latency of each group.

Benzodiazepine-receptor agonists are commonly used for the short-term treatment of insomnia despite evidence of altered sleep architecture and their association with varying degrees of memory impairment, cognitive impairment, motor impairment, incoordination, daytime sleepiness, rebound insomnia, tolerance, and dependence. Thus far in animals, ramelteon has been shown to promote sleep (in this study in cats) and has not been associated with impairment of learning behavior or motor function, or with rewarding properties in other animal models.[37] In addition, a recent Phase II clinical trial found that ramelteon decreased latency to persistent sleep and increased total sleep time and sleep efficiency (as measured by polysomnography) in subjects with primary chronic insomnia.[51] Further investigations are needed to fully explore the potential of this novel agent for the treatment of sleep disorders.

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