Feeding melatonin enhances the phase shifting response to triazolam in both young and old golden hamsters

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As both humans and laboratory animals age, their circadian timing systems undergo profound changes. There are changes in the free-running period of circadian rhythms, a decrease in amplitude of several output rhythms, and changes in sensitivity to both photic and nonphotic stimuli. Disruption of circadian rhythmicity by a variety of means can alter phase relationships between organs (47) and can even increase the risk of heart failure in both humans and animals (12, 23). The age-related changes in circadian rhythmicity may induce similar disruptions and could have serious consequences for the health and well-being of the aging animal as a whole. Although there has been much research into the mechanisms underlying these age-related changes, there have been few agents that have been found that can reverse or attenuate the effects of advanced age on the circadian clock.

The circadian timing system is composed of three parts: a central pacemaker that generates the rhythm, inputs to this pacemaker that affect entrainment, and output mechanisms that convey information of circadian timing to target organs. In advanced age, there are changes to all three components of the circadian timing system. The age-related decline in the ability to respond to external stimuli reflects a decline in the functioning of either the input pathway or the response of the clock to input signals. Circadian clocks are set by daily adjustments that shift the phase of the central pacemaker; this adjusts the mismatch between the period of the external light-dark (LD) cycle (usually 24 h) and the period of the animal’s endogenous pacemaker, τ, which is usually a few minutes less than or greater than 24 h. Old hamsters show a decreased sensitivity to both photic and nonphotic inputs to the circadian clock. For example, old animals show smaller phase shifts in response to pulses of light than young hamsters (49), and old hamsters fail to show a phase-shifting response to the benzodiazepine triazolam (37, 43). Old hamsters also fail to respond to serotonergic stimuli that can phase shift the circadian clock of young hamsters (24). This decreased responsiveness to both photic and nonphotic stimuli may contribute to the altered entrainment patterns that are observed in old animals.

Aging also changes the endogenous period of measured rhythms and dampens the amplitude of many rhythmic outputs of the circadian clock; these effects are thought to be due to changes in the functioning of the central circadian pacemaker itself (see Ref. 14 for a more thorough discussion of age-related changes in the central pacemaker). Finally, there are changes in many of the peripheral rhythms driven by the circadian clock. In addition to the effects of age on the
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timing of these output rhythms, which can be explained by a change in the functioning of the circadian pacemaker, at least some of the effects of age on rhythmic behavior and physiological processes can be explained by changes in the functioning of the organs that generate these behaviors and secretions. For example, old animals run less (39, 40) and also have lower levels of pineal and serum melatonin (22, 28), a hormone whose production is regulated by the circadian clock. The decrease in daily magnitude of both wheel-running activity and pineal melatonin is probably due, in part, to the diminished functional capacities of skeletal muscles and the pineal glands of old animals and may be independent of the effects of age on the timing of such outputs.

Besides being an output of the circadian clock, melatonin can also act as an input to the clock (31, 41, 48). In both nocturnal and diurnal animals, melatonin is produced exclusively at night (27). Melatonin receptors are expressed in the suprachiasmatic nuclei (SCN) of the hypothalamus, the location of the master circadian pacemaker in mammals (25, 29). Although melatonin can entrain the pacemaker of neonatal golden hamsters (9), adult hamsters show little or no phase shifts in response to melatonin (3, 9, 41). Nonetheless, the circadian timing system of adult golden hamsters remains responsive to melatonin. 125I-Melatonin binds to the adult hamster SCN (44), and melatonin 1a receptors are expressed in the SCN of adult hamsters (6, 7, 17, 36). Acute application of melatonin to SCN neurons in vitro alters their firing rate (31, 48). Furthermore, exogenous melatonin speeds reentrainment of hamster locomotor and body temperature rhythms to a shift in the LD cycle (8). Taken as a whole, the data suggest that melatonin has access to the hamster circadian timing system and modulates the responses of this system to other inputs. It may have its effect by altering the sensitivity of pacemaking cells to other inputs (8).

Old hamsters produce less melatonin, and the age-related changes in circadian rhythmicity, including the decreased sensitivity to entraining agents, may be exacerbated by improperly timed or decreased melatonin feedback onto the clock, which is itself brought about by aging of the circadian timing system and the pineal gland. Koster-van Hoffen et al. (15) found that daily injection with a melatonin agonist attenuated the age-related decline in the amplitude of the circadian rhythm of body temperature in rats. Recently, Van Reeth and colleagues (42, 45) reported that old hamsters treated with a melatonin receptor agonist reentrain to a shift in the LD cycle faster than old control animals and show larger phase shifts to a dark pulse when presented on a background of constant light. Together, these data suggest that increasing the strength of the input signals to the mammalian circadian pacemaker may have beneficial effects on circadian rhythmicity. We sought to determine if augmenting the amplitude of the rhythm of the endogenous molecule melatonin would augment the response of the circadian timing system of the hamster to a nonphotic phase-shifting agent, triazolam.

MATERIALS AND METHODS

Animals and experimental manipulations. All experiments were conducted at the University of Wisconsin-Parkside. Experimental protocols had been approved by the Animal Care and Use Committees of both Northwestern University and the University of Wisconsin-Parkside. Young (<2 mo old) male golden hamsters (Mesocricetus auratus) were purchased from Charles River Laboratories (Kingston, NY). Young animals were allowed to aclimate to the facility for 2 wk before the start of an experiment. Retired breeders were purchased at 6 mo of age and maintained in individual cages under a 14:10-h LD cycle until the beginning of the experiment, when they were 18 mo of age. All animals were individually housed in cages equipped with a running wheel and connected to a personal computer to record activity levels (Chronobiology Kit, Stanford Software Systems, Stanford, CA). During the experiment, the cages were placed inside light-tight wooden cabinets equipped with fans and fluorescent lights (40 W). The animals were provided with food and water ad libitum throughout the experiment. All reagents were purchased from Sigma Chemical (St. Louis, MO) except as noted.

All animals were fed a diet of standard chow at the beginning of the experiment. Two weeks later, one-half of the animals from each age group were assigned to receive a melatonin-supplemented diet, and one-half of the animals from each age group were assigned to receive a control diet. For the melatonin-supplemented diet, 750 g of powdered food (either Harlan-Teklad LM-485 meal or Purina 5001 meal) was mixed with 500 ml H2O, 250 g peanut butter, and 100 ml melatonin solution (9 μg/ml in 95% ethanol). The final concentration of melatonin was 563 ng/g of food. The control food was made in an identical fashion except that 95% ethanol was substituted for the melatonin solution. Food was stored at 4°C and made fresh every 3–4 days. The standard chow was removed from the cages for the remainder of the experiment. Animals were given ~30 g of the appropriate food daily; pilot experiments had indicated that this species routinely eats 10–15 g of food/day (E. Challet and F. W. Turek, unpublished observations). At each feeding, food that had not been consumed was removed from the cages. Animals were fed near the end of the light phase while under an LD cycle. To avoid possible entrainment to the feeding, they were not fed at the same time every day during exposure to constant darkness. No attempt was made to coordinate the time of feeding with circadian patterns of activity when animals were housed in constant darkness.

Experiment 1. Animals were fed their assigned diets for 2 mo under a 14:10-h LD cycle. They were then transferred to constant darkness by extension of the dark phase. Daily feeding and animal care were accomplished with the aid of a dim red safelight (15-W bulb, Kodak filter 1A). After 10 days in constant darkness, animals were randomly assigned to receive an intraperitoneal injection of either triazolam (2 mg/kg) or vehicle (50% DMSO in saline) at circadian time (CT) 8. CT12 is defined as the onset of activity and is a marker of circadian phase in the absence of external time cues; thus CT8 is 4 h before the predicted onset of activity. The dose of triazolam was chosen to induce submaximal phase shifts in hamsters on the basis of the results of previous experiments (38). Approximately 1 mo after the first injection, each animal received an injection of the other solution in a crossover design.
**Experiment 2.** To determine the effect that feeding exogenous melatonin may have on serum melatonin levels, additional groups of young and old hamsters were fed the melatonin and control diets for 1 mo. Animals were maintained in the 14:10-h LD cycle for the duration of the experiment. After 1 mo on the melatonin and control diets, the hamsters were anesthetized with isoflurane, and blood was collected via cardiac puncture 9.5 h after lights off [zeitgeber time (ZT) 21.5; by convention ZT212 is the time of lights off] and again 2 h after lights on (ZT0). Ten days later, the animals were anesthetized again, and blood was collected 2 h after lights off (ZT14) and again 5 h after lights off (ZT17). Samples were centrifuged, and serum was stored at −80°C until assayed.

**Melatonin RIA.** Serum (150 μl) was extracted with 2 ml dichloromethane. Samples were vortexed 15 s and centrifuged 6 min at 500 g at 4°C in a refrigerated centrifuge (Beckman J-B6, Beckman-Coulter, Fullerton, CA). The protein layer was gently removed with a cotton swab, and 1,800 μl of the lower (organic) phase was transferred to a new tube. Samples were evaporated in a RapidVap (Labconco, Kansas City, MO) for 20 min at 30°C. The pellet was resuspended in 200 μl assay buffer [0.1% gelatin (wt/vol), 0.1 M tricine, 0.0075% thimerosal, 0.9% NaCl, pH 8.0] at 4°C overnight.

The next day, 50 μl of primary antibody (rabbit anti-melatonin, Stockland, Surrey, UK; diluted 1:5,600 with 0.5% normal rabbit serum in assay buffer) was added to each sample. After a 1-h incubation at room temperature, 50 μl of 2-[125I]iodomelatonin [diluted to 1 × 10^5 counts per minute (cpm/ml; New England Nuclear, Boston, MA) was added and incubated 20–24 h at 4°C. Fifty microliters of secondary antibody (goat anti-rabbit IgG, diluted 1:14 in assay buffer) was added and incubated 20–24 h at 4°C. One milliliter of assay buffer was added to each sample; they were then centrifuged for 30 min at 2,000 g at 4°C. The supernatant was removed, and the pellet was counted. The lower limit of detection in this assay was 2.6 pg/ml; the upper limit was 293 pg/ml. All samples were run in the same assay; the intra-assay coefficient of variation averaged 16.3% for values <10 pg/ml, 13.0% for values 10–30 pg/ml, and 7.2% for values >100 pg/ml.

Specificity of the assay was assessed by examining serial dilutions of serum pools to which known concentrations of melatonin (9.3, 49, and 133 pg/ml) of melatonin had been added. Duplicate aliquots (50, 75, 100, and 150 μl) of each pool were added to appropriate volumes of charcoal-stripped hamster serum (100, 75, 50, or 0 μl) to bring the samples to a volume of 150 μl. These samples were then extracted and subjected to RIA using the methods described above. Samples derived from the low pool exhibited a slightly flatter slope than the standard curve, consistent with the small amounts of hormone present; however, dilution series made from the medium and high pools were parallel to the standard curve (Fig. 1).

**Data analysis and statistics.** Phase shifts were determined by eye-fitting lines through onsets of activity before and after each injection. The magnitude of the phase shift was assessed by two independent observers who were blind to the animals’ age, diet, and injectate. Data were compared with a three-way ANOVA (age × diet × injectate) with repeated measures using NCSS (Number Cruncher Statistical Systems, Kaysville, UT). The distributions of RIA data were tested for normality with NCSS. Log-transformed RIA data were compared with three-way (age × diet × time of day) ANOVA, with repeated measures. Differences between groups were considered significant at P < 0.05. Post hoc comparisons were made with Duncan’s multiple comparison test.

**RESULTS**

**Experiment 1.** Chronic treatment with melatonin enhanced the phase-shifting effects of triazolam in both young and old hamsters. The ANOVA showed that there was a significant main effect of drug (P < 0.001), indicating that animals showed larger phase shifts to triazolam than to vehicle. There was a significant age × drug × food interaction effect (P < 0.001). As shown in Fig. 2, the phase shifts induced by triazolam in old animals fed the control diet were not significantly different from the phase shifts induced by the vehicle (P > 0.05, Duncan’s test). However, old animals fed the melatonin diet showed greater responses to triazolam than to vehicle (P < 0.05). Interestingly, young hamsters fed melatonin also showed significantly larger phase shifts to triazolam than young hamsters fed the control diet (food × drug interaction effect, P < 0.001).

**Experiment 2.** To determine if the feeding regimen delivered a physiological or pharmacological dose of melatonin, we examined plasma melatonin levels from a second group of animals maintained on an identical diet. The results of this experiment are shown in Fig. 3. The ANOVA on all the time points examined showed that melatonin levels vary over the course of the day and that old animals have less melatonin than young animals (main effect of age, P < 0.05; main effect of time of day, P < 0.0001). Additionally, animals fed melatonin had higher serum melatonin levels than control-fed animals (main effect of food, P < 0.01). Young animals showed a robust diurnal melatonin rhythm, whereas old control-fed animals showed a relatively flat melatonin rhythm (Fig. 3). Feeding melatonin to young animals did not significantly raise
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Fig. 2. Phase shifts induced by triazolam or vehicle in young or old control- or melatonin-fed hamsters. Triazolam or vehicle was administered by intraperitoneal injection at circadian time (CT) 8; n = 6–7 old animals on each diet and n = 12 young animals on each diet. Both young and old hamsters on the melatonin diet had significantly larger responses to triazolam than animals on the control diet.

their serum melatonin levels over those of young control-fed animals. However, old melatonin-fed animals had significantly higher melatonin levels than old animals fed the control diet; this difference is most evident at ZT17, the approximate time when endogenous melatonin levels peak in this species (28) (P < 0.05). The serum melatonin levels of old animals fed melatonin were similar to those of young animals on either diet (Ps > 0.05).

DISCUSSION

These results demonstrate that chronic treatment with melatonin enhances the sensitivity of the circadian timing system to a nonphotic phase-shifting stimulus, triazolam, and that this enhancement of sensitivity persists in old animals. Our results are consistent with previous reports, which have shown that in old rodents chronic treatment with either melatonin or the melatonin receptor agonist S-20098 can enhance the phase-shifting response to another nonphotic stimulus, a dark pulse, and can speed reentrainment to a shift in the LD cycle (8, 34, 42, 45). Whereas a recent study showed that chronic treatment with either melatonin or S-20098 enhanced the phase-shifting response to a dark pulse in old hamsters but not in young control hamsters (42), we detected enhanced phase shifts in both young and old animals treated with melatonin (see Fig. 2). The enhancement observed in young animals may be due to our use of a subsaturating dose of the phase-shifting agent; in contrast, the previous study used a saturating stimulus (dark pulse) that may have induced a maximal response in young animals regardless of other treatments (4, 26, 38, 42). Taken together, the results of our experiments with those previously published show that chronic treatment with melatonin or an agonist can affect the response of the circadian timing system to both photic and nonphotic phase-shifting stimuli.

How might the chronic melatonin feeding be having its effect? Melatonin is an output of the circadian timing system and can also influence the activity of SCN neurons (31, 48). A recent model of the circadian clock suggests that outputs of the clock, which also feed back onto it, can strengthen rhythmicity (30). These clock-driven entraining agents, or zeitnehmers (literally, time takers), not only prevent rhythms from damping in constant conditions but also may tune the phase sensitivity of the circadian system (30). We hypothesize that melatonin may be such a zeitnehmer, as it is a clock-driven output that also feeds back on to the circadian oscillator. By feeding hamsters melatonin, we may have increased the input to the circadian system, thereby modulating its ability to respond to other phase-shifting stimuli, such as triazolam. In fact, the exogenous activation of the melatonin receptors may increase the responsiveness of the circadian system generally; hamsters treated with either S-20098 or melatonin also show more rapid reentrainment to shifts in the LD cycle (8, 45). Melatonin receptors are expressed in the SCN nuclei of golden hamsters, and melatonin has been shown to alter the firing rate of SCN neurons and to speed reentrainment to a change in the LD cycle in this species (6, 8, 31, 48). These effects could be due to the effect of melatonin on the expression of one or more of the recently discovered circadian clock genes (see Ref. 13 for a review), although at the present time, there is no evidence that melatonin influences circadian clock gene expression in the SCN. One recent limited study found no effect of melatonin on the expression of Per1 in the hamster SCN (19). Although acute changes in the abundance of Per1 and Per2 mRNA seem to be an integral part of the phase-shifting responses to light and certain nonphotic stimuli (1, 2, 11, 18, 32, 33, 35, 50), the relationship between melatonin and circadian clock gene expression in the SCN remains unknown. A complete understanding of the mechanism through which feeding melatonin enhances the magnitude of phase shifts awaits further investigation.

Fig. 3. Serum melatonin levels across the day and night in young or old control- or melatonin-fed hamsters. Lights were off from zeitgeber time (ZT) 12 to ZT22, as indicated by the black horizontal bar. Samples were collected by cardiac puncture on 2 days. Serum melatonin concentration was determined by RIA. Old control-fed animals had significantly lower melatonin levels than animals in any of the other 3 groups 5 h after lights off; n = 13 old control-fed, n = 7 old melatonin-fed, and n = 10–11 young animals on each diet.

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elucidation of the responses of circadian clock genes to nonphotic phase-shifting stimuli, as well as the mechanism by which melatonin modulates the pacemaking or entrainment mechanisms in the SCN.

Curiously, we found that feeding melatonin raised serum melatonin levels in old, but not young, hamsters. The increase in serum melatonin levels seen in old animals may be the result of better-consolidated circadian rhythms or may be due to a direct effect of the feeding of melatonin on serum levels. Our assay does not distinguish between endogenous and exogenous melatonin, and thus we cannot tell the source of the increased serum melatonin levels from the data. If the decrease in serum melatonin levels seen in old animals is due to changes in the timing of melatonin synthesis and release, then peak concentrations of melatonin may be higher after interventions that consolidate circadian rhythmicity. However, in young hamsters, which already have well-consolidated rhythms, feeding melatonin would do little to improve consolidation, thereby leaving peak melatonin levels unchanged. The increase in serum melatonin concentrations in old but not young melatonin-fed hamsters may also be due to the age-related decrease in the activity of liver enzymes responsible for the metabolism of melatonin (see, for example, Refs. 5, 10, 20). It is possible that feeding melatonin to young hamsters did in fact increase serum melatonin concentrations, but the relatively sparse sampling schedule and rapid clearance of melatonin from the serum of young hamsters resulted in a failure to detect this transient increase. Future studies should examine the relationship between the intake of dietary melatonin and the appearance of melatonin in the serum, as well as the effect of age on this phenomenon.

Perspectives

Labyak et al. (16) found that increasing the intensity of the light phase of an LD cycle can attenuate some of the effects of aging in middle-aged hamsters. Specifically, increasing the light intensity decreased fragmentation of the daily activity rhythm and increased total activity (16). Similarly, increasing the light intensity in the home cage increased the day-night differences in the daily sleep-wake cycle of old rats, perhaps by inhibiting inappropriately timed awakenings (46). Both of these studies suggest that increasing the strength of the input signal modulates the circadian timing system so that the behavior of old animals is organized in a temporal fashion that more closely resembles that of young animals. Naylor et al. (21) recently reported that social and physical activity timed to occur repeatedly at the same time of day both increased slow-wave sleep and improved performance on cognitive tests in old people. The authors of that study hypothesized that the improvements may be due to augmentation of an entraining signal. However, it remains unclear whether the improvements in sleep and cognitive performance were due to the direct effects of moderate exercise or to alterations to the circadian timing system.

Together with the results of previous studies on the effects of increasing the strength of entraining signals to the aging circadian clock (16, 21, 46), the present results suggest that increasing the amplitude of the inputs to the clock improves the functioning of the circadian timing system as a whole. Our data also show that administering melatonin in the food is an effective mode of restoring the diurnal rhythm of serum melatonin in old animals (see Fig. 3). Melatonin now represents one of just a handful of interventions that augment the response of the circadian clock to entraining stimuli. We hypothesize that increasing the strength of inputs to the circadian timing system through use of either pharmacological agents (such as melatonin) or nonpharmacological agents (such as increasing the light intensity or giving other environmental timing cues) may make it possible to enhance the responsibility of the circadian timing systems of old humans. This could have important consequences not only for the circadian organization of behavior but also for the integrity of many of the physiological systems regulated by the circadian clock.

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