Responsiveness to melatonin and its receptor expression in the aging circadian clock of mice

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Benloucif, Susan, Monica I. Masana, and Margarita L. Dubocovich. Responsiveness to melatonin and its receptor expression in the aging circadian clock of mice. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1855–R1860, 1997.—This study determined the effect of age on the efficacy of melatonin treatment to phase shift circadian activity rhythms and on melatonin receptor expression in the suprachiasmatic nucleus (SCN) and paraventricular nucleus of the thalamus (PVNT) of C3H/HeN mice. The circadian rhythm of 2-[125I]iodomelatonin binding, assessed at three times of the day [circadian times (CT) 2, 10, and 18], showed a modest age-related decrease in the SCN but not the PVNT of old C3H/HeN mice (24 mo). There was a tendency for age to reduce Mel1α, melatonin receptor mRNA expression in the suprachiasmatic nucleus during the day, but not during the night. The magnitude of phase shifts of circadian activity rhythms (advances or delays) induced by administration of melatonin at CT 10 or CT 2 was identical in young and old C3H/HeN mice. Together, these results suggest that the decrease in melatonin receptor expression in the SCN had little effect on melatonin-induced phase shifts of circadian activity rhythms. We conclude that the responsiveness of the circadian timing system to melatonin administration does not decrease with age.

2-[125I]iodomelatonin binding; Mel1α mRNA; suprachiasmatic nucleus; old

It is estimated that ~50% of Americans over the age of 65 have sleep disturbances. The high prevalence of chronic sleep disruptions in older adults indicates a need to understand the etiology of and develop effective therapies for sleep disorders. Evidence suggests that age-related changes in the circadian timing system contribute to the impairment of sleep in older adults (3, 14, 27). Treatments for sleep disturbances that are based on adjusting the circadian timing of older adults utilize scheduled daily exposure to bright light or exercise (4, 26). Animal models suggest, however, that the effectiveness of these treatments may be limited by age-related changes in responsiveness of the biological clock to these stimuli (2, 23, 25, 30).

The responsiveness of the circadian clock to external stimuli is commonly assessed by exposing animals to a stimulus at different phases of the circadian cycle (24). Exposure to a variety of stimuli induces steady-state shifts in the phase of the rhythm in animals housed under constant environmental conditions. The magnitude and direction of the phase shift is dependent on the circadian time (CT) of stimulus exposure, and the resultant phase advances and delays are plotted to generate a phase-response curve (24). In addition, exposure to light, but not nonphotic stimuli, during the subjective night induces the expression of a set of immediate-early genes in the suprachiasmatic nucleus (SCN) (9, 13, 18). In old rodents, light-induced phase shifts of circadian activity rhythms and immediate-early gene expression in the SCN are decreased (2, 23, 30). Similarly, phase shifts resulting from activity-inducing stimuli are reduced in aged hamsters (25). Together, these results suggest a reduced responsiveness to synchronizing stimuli (zeitgebers) by the aging circadian timing system.

Recent studies suggest that insomnia in older adults might also be treated by nightly administration of the hormone melatonin (8). At present it is not clear whether the effects of melatonin on sleep are due to somnogenic or circadian actions of the hormone, as melatonin induces sleep when administered in the day or early evening and also shifts the phase of circadian rhythms according to a phase-response curve (5, 6, 11, 16). The response of the aging circadian timing system to this zeitgeber is not known. However, reductions in the density of melatonin receptors in the SCN of old rats suggest that age might also decrease responsiveness of the clock to melatonin (28).

This study sought to determine whether age alters melatonin-induced phase shifts of circadian activity rhythms, melatonin receptor density, and melatonin receptor (Mel1α) mRNA expression in the SCN of C3H/HeN mice (1, 12). Old mice of this strain were previously found to exhibit decreases in both phase shifts of circadian activity rhythms and immediate-early gene expression in the SCN after exposure to light (2). The present study found modest reductions in melatonin receptor expression in the SCN. There was little effect of age on the functional response to melatonin, as phase shifts of circadian activity rhythms were of similar magnitude in both young and old C3H/HeN mice after melatonin administration.

METHODS

Animals and general protocol. Young (4–5 wk, n = 17) and old (8–9 mo; retired breeders, n = 23) male C3H/HeN mice were purchased from Harlan (Indianapolis, IN) and were maintained in temperature (22 ± 1°C)- and humidity-controlled rooms under a 14:10-h light-dark cycle until they reached 4 or 16 mo of age, respectively. Mice were transferred to constant and complete darkness (DD) in individual cages (18 × 30 × 12 cm) equipped with activity wheels. Cages were placed six per shelf in a light-tight ventilated cabinet. Food and water were provided ad libitum. These conditions were maintained for the 6-mo experimental period. Four old mice died during the course of the experiment.

One month after the last treatment with either melatonin or vehicle, animals were killed by decapitation at CT 2, 10, or 18 to determine 2-[125I]iodomelatonin-binding sites and Mel1α mRNA expression in the SCN (12). Brains were removed,
embedded in OCT compound (Miles, Elkhart, IN), and quickly frozen in 2-methylbutane on dry ice. Brains were stored at -80°C until processing.

Melatonin-induced phase shifts of circadian activity rhythms. Mice were maintained under DD for 3 wk to stabilize free-running activity rhythms and then treated every 3-4 wk. Vehicle (3% ethanol-saline sc) or melatonin (90 µg in 3% ethanol-saline sc) was administered for 3 consecutive days at the same clock time based on the estimated CT (CT 2, 4, 8, 10, 12, 16, 20, 24) for the second day of treatment. Melatonin administered according to this protocol phase shifts circadian activity rhythms of young C3H/HeN mice (1). Treatments were carried out under a dim red safelight (15 W, Kodak 1A filter) with illuminance of <3 lx. The CTS of treatments were selected from times previously shown to induce shifts in the phase of circadian activity rhythms, e.g., CT 10 and CT 24-2 (1). CT 12 was defined as the onset of running wheel activity, and the exact CT of administration was determined after treatment from the steady-state activity onset on the days of melatonin administration. Each mouse received three melatonin and three vehicle treatments over the 6-mo experimental period. Treatments were counterbalanced across both groups and individual mice at each CT. Circadian activity rhythms were measured with a magnetic microswitch, which detected revolutions of the running wheel and was on-line with an IBM-compatible computer. Data were collected using the Dataquest III hardware and software package and analyzed with TAU software (Mini-Mitter, Sunriver, OR). The prepulse and postpulse periods (tau) were assessed by eye-fitting the TAU guide through activity onsets for 7–10 days before treatment and for 14 days after treatment. The 3 days after drug administration were not included in the postpulse analysis because of transient shifts in activity onset immediately following phase shifting stimuli. The magnitude of the phase shift was calculated as the difference between the steady-state prepulse and the steady-state postpulse activity onset. Differences between treatments (melatonin or vehicle) and groups (young or old) were assessed by two-way analysis of variance (ANOVA), followed by pairwise comparisons when indicated (level for statistical significance set at P < 0.05). Curves were fitted by Cubic Splines (GraphPad Prism, San Diego, CA).

2-[125]Iodomelatonin binding and Mel1a melatonin receptor mRNA expression. Serial coronal brain sections (20 µm) comprising the SCN were cut on a Reichert-Jung cryostat (Tris·HCl) buffer (50 mM with 4 mM CaCl2, pH 7.4) with 2-[125]Iodomelatonin binding was determined by the methods of Siuciak et al. (22). Slide-mounted sections were air dried for 15 min, then incubated with 2-[125]Iodomelatonin at a concentration of 60 or 240 pM, as described below.

2-[125]Iodomelatonin binding was determined by the methods of Siuciak et al. (22). Slide-mounted sections were air dried for 15 min, then incubated with 2-[125]Iodomelatonin (60 or 240 pM) in tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer (50 mM with 4 mM CaCl2, pH 7.4) with (nonspecific binding) or without (total binding) 1 µM melatonin for 1 h at 25°C. These concentrations of 2-[125]Iodomelatonin were selected based on results of previous studies showing saturation at 240 pM with a total number of binding sites of 25.1 ± 1.2 fmol/mg protein and an affinity of 26.1 ± 6.8 pM in the C3H/HeN mouse brain (7). Slides were rinsed in ice-cold Tris·HCl buffer (2 × 10 min) followed by a rapid rinse in ice-cold distilled water to remove buffer salts. Labeled sections were apposed to Kodak SB X-ray film for 7 days.

In situ hybridization histochemistry was performed with an antisense or sense 35S-labeled riboprobe and examined for mRNA expression by quantitative densitometric analysis of autoradiograms. Mouse Mel1a melatonin receptor cDNA was obtained by reverse transcription-polymerase chain reaction using C3H/HeN mouse brain mRNA as a template. Degenerate primers for Mel1a melatonin receptors were designed based on conserved sequences in the second intracellular loop (containing the NRY amino acid motif characteristic of the melatonin receptor family) and in the VI transmembrane domain. The 402-bp cDNA obtained by polymerase chain reaction was cloned into pGEMT plasmid and then sequenced to confirm the identity as the Mel1a melatonin receptor (19). Both antisense and sense riboprophbes were synthesized from linearized plasmid by in vitro transcription in the presence of UTP [α-35S] as previously described (13). The integrity of the riboprophbes was determined using urea gel electrophoresis.

Sections were processed for in situ hybridization histochemistry according to a modified protocol of Masana et al. (13). Slide-mounted sections were air dried for 10 min at room temperature, fixed for 10 min in buffered 4% paraformaldehyde, rinsed twice in 0.1 M phosphate-buffered saline, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated, and deparaffinized. Hybridization was carried out at 53°C for 18 h in hybridization buffer [4× SSC (0.15 M NaCl·0.025 M sodium citrate), 50% formamide, 1× Denhardt’s solution, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, 0.5 mg/ml poly(A), 10% dextran sulfate, 100 µM 1,4-dithiothreitol] and 10,000 counts/min · μl-1 35S-labeled probe. After hybridization, the slides were washed three times in 2× SSC, 50% formamide, 0.1% 2-mercaptoethanol (25°C, 15 min), incubated with 20 µg/ml ribonuclease A (Promega, Madison, WI) for 30 min at 37°C, and washed twice with 2× SSC, 50% formamide, 0.1% 2-mercaptoethanol (25°C, 15 min), once with 1× SSC, 1% 2-mercaptoethanol (40°C, 15 min), and twice with 1× SSC, 1% 2-mercaptoethanol (53°C, 30 min). The sections were dehydrated, air dried, and apposed to Kodak SB X-ray film for 21 days.

Quantitative analysis of autoradiograms was performed using an imaging analysis system (Bioquant System IV; R & M Biometrics, Nashville, TN). Total 2-[125]Iodomelatonin binding, expressed in femtomole per milligram protein, was determined from optical density (OD) measurements taken from the ventral SCN or paraventricular nucleus of the thalamus (PVN). Measurements from the PVN were restricted to the anterior portion of the nucleus, which contains both afferent and efferent connections with the SCN (15). Specific binding was determined by subtracting the value for nonspecific binding obtained from adjacent sections incubated with 1 µM melatonin. The expression of melatonin receptor mRNA was determined from OD measurements of hybridization of the Mel1a riboprobe in the SCN. Background OD, measured in the adjacent hypothalamus, was subtracted from the value obtained in the ventral nucleus. Differences between young and old mice were compared by two-factor ANOVA [group (young and old) and time of death (CT 2, 10, 18)], followed by pairwise comparisons where indicated.

RESULTS

Melatonin-induced phase shifts of circadian activity rhythms. Activity records in Fig. 1 illustrate phase shifts of circadian rhythms of wheel running in young and old mice treated with melatonin. The double-
plotted actograms show delays in the phase of circadian activity rhythms following melatonin administration for 3 days at the end of the subjective night (CT 2) and advances in the phase of circadian activity rhythms following melatonin administration 2 h before the onset of nocturnal activity (CT 10) in both young and old mice. Phase shifts of circadian activity rhythms following administration of vehicle or melatonin at various CTs (2, 4, 8, 10, 12, 16, 20, or 24) are shown as a phase-response curve in Fig. 2. Compared with the sporadic shifts in phase observed after 3 days of control injections (Fig. 2A), administration of melatonin (Fig. 2B) induced reliable delays in the phase of circadian activity rhythms when administered at CT 2 (F$_{1,22}$ = 7.90, P < 0.01) and advances in phase when administered at CT 10 (F$_{1,32}$ = 19.04, P < 0.001). The magnitude of the phase shifts was similar in both young and old mice at these two times. Melatonin administered at CT 8 resulted in a significant advance in the phase of circadian activity rhythms in young mice (P < 0.05). One-half of the old mice exhibited phase shifts of circadian activity rhythms following melatonin administration at CT 8. However, the average phase shifts were not significantly different from either young mice treated with melatonin nor from old mice administered vehicle at this time. In addition, melatonin administration induced phase advances of circadian activity rhythms in 50% of old mice treated at CT 16 (P < 0.05), a time when young mice did not shift phase.

2-[125I]-iodomelatonin binding and Mel$_{1a}$ melatonin receptor mRNA expression. Figure 3 shows discrete localization of 2-[125I]-iodomelatonin binding to the SCN and PVNT in young and old C3H/HeN mice. A gradient in the specific binding of 2-[125I]-iodomelatonin was observed across the SCN, with maximum levels of binding obtained in sections from the mid-anterior SCN.

An overall age-related reduction in specific binding of a saturating concentration of 2-[125I]-iodomelatonin (240
copy
pM) was observed in the mid-anterior SCN at all CTs (2, 10, or 18; Fig. 4A, F₁,₂₅ = 4.70, P < 0.05; n = 4–6). To verify that this difference was not due to sampling from different levels of the SCN, we assessed the effect of age on melatonin receptor density at four different rostral-caudal levels of the nucleus. Specific binding of 2-[¹²⁵I]iodomelatonin was uniformly decreased across all levels of the SCN in the old mice (Fig. 4B; F₁,₁₁₄ = 5.72, P < 0.05). There were no age-related differences in the specific binding of a lower concentration of 2-[¹²⁵I]iodomelatonin (60 pM, data not shown). Age did not affect the specific binding of 2-[¹²⁵I]iodomelatonin in the anterior PVNT of mice killed at CT 2, 10, or 18 (data not shown).

To determine whether age affected melatonin receptor mRNA expression in the SCN, we performed in situ hybridization using a riboprobe for the Mel₁α melatonin receptor in mice killed at CT 2, 10, and 18. Figure 5 shows the selective hybridization of the riboprobe to the SCN of young and old C3H/HeN mice. Quantification of the autoradiograms (Fig. 6) showed a significant circadian rhythm of Mel₁α melatonin receptor mRNA expression in both young and old mice that peaked at CT 18 (F₂,₂₆ = 8.81, P < 0.001, n = 4–6). A differential effect of age on Mel₁α melatonin receptor mRNA expression at the different CT was also apparent from examination of the data presented in Fig. 6. However, the power was inadequate to detect significant differences (interaction between age and CT: P = 0.07).

**DISCUSSION**

These studies demonstrate that age decreases the density of melatonin receptors in the SCN but does not impair the magnitude of phase shifts of circadian activity rhythms in C3H/HeN mice induced by melatonin administration at the peak periods of sensitivity. These results contrast with the age-related deficits in phase shifts of circadian activity rhythms in rodents after stimulation with either light or activity (2, 25, 30), suggesting that age does not decrease responsiveness of the biological clock to all zeitgebers.

Melatonin-induced phase shifts in circadian activity rhythms were similar to those previously observed in young C3H/HeN mice and to those observed in humans (1, 11, 29). Melatonin administration at subjective dusk (CT 10) induced robust advances in the phase of circadian activity rhythms, and melatonin administration at subjective dawn (CT 2) induced delays in phase in both young and old mice. Phase shifts in response to melatonin administration at times near the peak periods of sensitivity (CT 2 and CT 10) differed slightly from results obtained previously in young C3H/HeN mice (1), e.g., the young mice in the current study exhibited sensitivity to melatonin administered at CT 8, but not at CT 24. In addition, melatonin induced phase advances of circadian activity rhythms in one-half of the old mice when administered at CT 16, a time when melatonin does not affect the clock of young mice. This finding is similar to the unusual phase shifts observed in old hamsters following exposure to light at CT 16 and is consistent with the suggestion that the
clock of older animals is unstable in transitional zones occurring between periods of advances and delays (20). Slight but significant decreases in the density of melatonin receptors were observed in the SCN of old C3H/HeN mice. Reduced densities of melatonin receptors were observed in the SCN of old rats exhibiting disruptions in diurnal drinking rhythms but not in old rats retaining robust rhythms of drinking (28). In contrast with the relationship between the density of melatonin receptors in the SCN and circadian function observed by Whealin et al. (28), we found no correlation between the magnitude of melatonin-induced phase shifts of circadian activity rhythms and the density of melatonin receptors in the SCN in individual mice. Furthermore, the reduction in melatonin receptor density in the old mice was not associated with a reduction in melatonin-induced phase shifts of circadian activity rhythms, suggesting that the maximal response can be elicited by activation by only a fraction of melatonin receptors.

The age-related decrease in melatonin receptor density observed in the present report may be a consequence of a generalized deterioration of the aging SCN. In the rat, the density of melatonin receptors in the SCN remains constant across age when controlled by overall protein content (10). In contrast with the dramatic effect of an age-related reduction in signal transduction following photic stimulation (2, 23, 30), the functional impact of a loss of cell number in the aging SCN is uncertain (17, 21). In the study reported here, the magnitude of phase shifts of circadian activity rhythms induced by exogenous melatonin administration at CT 10 and CT 2 was not affected by age. However, endogenous melatonin levels decrease with age (8), and it is not known whether the response to low levels of melatonin would be affected by a decrease in melatonin receptor density. Therefore, with the caveat that the response to low levels of stimulation is not known, these results indicate that the effect of melatonin receptor activation in target areas involved in the regulation of circadian rhythms is not impaired by age. Melatonin receptor mRNA expression in the SCN exhibited a robust circadian rhythm with maximal mRNA expression in both young and old mice killed during the subjective night (CT 18) (12). Specific 2-[125I]iodomelatonin binding in the SCN of 3- to 6-mo-old C3H/HeN mice follows a modest circadian rhythm (Ref. 12, unpublished data). However, a circadian rhythm of specific 2-[125I]iodomelatonin binding was not observed in the SCN of the 10- and 24-mo-old mice in the present report. A lack of correlation between Mel1a mRNA and its protein product was also observed with respect to the factor of age. Specific 2-[125I]iodomelatonin binding was decreased in the SCN of old mice at all CTs (2, 10, and 18, Fig. 4). In contrast, age affected Mel1a mRNA expression detected by in situ hybridization histochemistry in the SCN of young (A) and old (B) C3H/HeN mice. Coronal brain sections (20 µm) were hybridized with a 35S-labeled riboprobe for the Mel1a receptor. Adjacent control sections were processed with a sense probe (A’ and B’).
expression differently according to the CT, with a tendency for Mel1a mRNA expression to be reduced at CT 2 and CT 10 and increased at CT 18 (Fig. 6). The lack of correlation between the circadian variation in Mel1a mRNA expression and its protein product may be due to the binding of 2-[125I]iodomelatonin to more than one receptor subtype.

In summary, in old C3H/HeN mice exhibiting slight reductions in the expression of melatonin receptors in the SCN, melatonin-induced phase shifts of circadian activity rhythms are not impaired. The responsiveness of the aging circadian clock to this internal zeitgeber is in marked contrast with the age-related reduction in responsiveness to light and activity-inducing stimuli (2, 23, 25, 30), indicating that the aging circadian pacemaker is not less responsive to all zeitgebers. The finding that melatonin retains its ability to synchronize the aging circadian timing system supports the clinical use of melatonin for treatment of circadian disruptions in the elderly (8).

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