Melatonin Receptor 1 Deficiency Affects Feeding Dynamics and Pro-Opiomelanocortin Expression in the Arcuate Nucleus and Pituitary of Mice

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Key Words

Pro-opiomelanocortin · Melatonin receptor 1 · Arcuate nucleus · Melatonin · Neuropeptide Y

Abstract

Background/Methods: Melatonin, the neurohormone for darkness, mediates photoperiod-dependent changes in physiology and behavior by targeting specific membrane-bound receptors (MT1 and MT2). In the present study, we investigated the impact of MT1 receptor deficiency on feeding behavior, locomotor activity and mRNA expression levels encoding for the polypeptide pro-opiomelanocortin (*Pomc*) and neuropeptide Y (*Npy*) in the hypothalamic arcuate nucleus (ARC) and the adenohypophysis [pars distalis (PD) and pars intermedia (PI)] in a comparison between wild-type (WT) and MT1-deficient (MT1^{-/-}) mice. **Results:** The MT1^{-/-} mice spent significantly more time feeding than the WT mice, while the general locomotor behavior, body weight and the total amount of food consumed did not differ between both genotypes. The nocturnal expression levels of *Pomc* in the ARC and PD were significantly higher in WT as compared to MT1^{-/-} mice and exogenous melatonin administered during

the light phase stimulated *Pomc* expression in WT mice only. No differences were found between WT and MT1^{-/-} mice with regard to *Pomc* expression levels in the PI. **Conclusion:** Thus, the MT1-mediated signaling stimulates *Pomc* expression in a region-specific pattern. Since the MT1-mediated changes in *Pomc* expression do not elicit direct orexigenic or anorexigenic effects, such effects are obviously mediated by regulatory systems downstream of the *Pomc* mRNA (e.g. cleavage and release of POMC derivatives), which are independent of MT1 signaling.

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Introduction

Metabolic functions follow circadian and seasonal rhythms. Melatonin, the neurohormone of darkness produced in and secreted from the pineal organ, is a key messenger for the phase and the length of the night and trans-

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duces photoperiodic information [1] influencing the functional activity of the mediobasal hypothalamus and the adenohipophysis [2–4]. In general, melatonin can act upon two high-affinity melatonin receptors, MT1 and MT2 (formerly Mel1a and Mel1b) [5–8]. However, several studies suggest that it is mainly the MT1 receptor which mediates melatonin signaling to the hypothalamo-hypophyseal axis [9–14].

The arcuate nucleus (ARC) plays a major role in the regulation of appetite and food intake [15, 16]. The pathways of feeding regulation are modulated by the opposing actions of appetite-promoting (orexigenic) neuropeptide Y (NPY)/agouti-related neuropeptide neurons and appetite-inhibiting (anorexigenic) pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript neurons, which project from the ARC to the paraventricular nucleus [17–21]. Thus, activation of POMC neurons triggers α -melanocyte-stimulating hormone release from POMC axon terminals leading to the suppression of food intake [21, 22].

There is strong evidence for diurnal and seasonal variation of *Pomc* mRNA expression in the hypothalamus of rodents [23, 24]. Furthermore, in nocturnal animals, POMC derivatives, e.g. adrenocorticotropin and β -endorphin (β -END), are not released constitutively, but periodically, resulting in high concentrations of glucocorticoids in the early night [25, 26]. Thus, in the ARC of rats, *Pomc* expression follows a diurnal rhythm [27] as well as a circadian rhythm with peak levels at mid-subjective night (CT18) [28], which closely follows the reported pattern of the daily rhythmic activity in β -END-containing neurons [29]. In rodents, *Pomc* expression in ARC varies between day and night, and a stimulatory effect of melatonin on *Pomc* expression in ARC was observed in rats [30] and mice [31]. However, the pathways and messengers that control rhythmic *Pomc* expression in the hypothalamus are not known in detail.

In order to determine the receptor type through which melatonin controls hypothalamic and hypophyseal *Pomc* expression, we analyzed *Pomc* expression by means of in situ hybridization in wild-type (WT) and MT1-deficient (MT1 $^{-/-}$) mice at two different time points: mid-subjective day (CT6) and night (CT18). To correlate putative effects of the melatonergic system on *Pomc* expression with behavioral data we investigated the feeding pattern, measured the body weight, and compared the latter with the general locomotor activity in both mouse strains. Furthermore, the acute effect of melatonin on *Pomc* expression was analyzed in WT and MT1-deficient mice after a single melatonin injection at midday.

Materials and Methods

Animals

All experiments (Ref. No. F6/19) reported here were conducted in accordance with accepted standards of humane animal care and were consistent with Federal Guidelines and Directive of the European Union (2010/63/EU). Mice with a targeted deletion of the MT1 gene (MT1 $^{-/-}$) and the corresponding WT were bred onto melatonin-proficient C3H/HeN background for at least 10 generations [14, 32, 33]. Experiments were performed with adult (10- to 18-week-old) male littermates obtained by heterozygous breeding. Animals were adapted to 12-hour light:12-hour dark (12:12 LD; light phase: 230 μ W/cm²; dark phase: dim red light <5 μ W/cm²; >680 nm) standard photoperiod for at least 2 weeks with food and water ad libitum. Lights-off was defined as zeitgeber time (ZT) 12. Two days before tissue collection, animals were transferred to constant darkness. Circadian time 0 was defined as light on at the previous LD schedule. Animals were sacrificed at two different time points (CT6 and CT18).

Recording of Food Intake and Locomotor Activity

Animals were single-housed in standard cages placed in a light-tight ventilated, temperature controlled cabinet under 12:12 LD conditions (lights on from 07:00 to 19:00 h). A digital supervision camera connected to a computer was mounted along the side of the cage and focused on the food container. All mice had ad libitum access to water and pelleted standard mouse chow (Sniff, Soest, Germany). The supervision camera detected movements anywhere in the cage. Images were recorded automatically every minute. Feeding behavior was determined by analyzing the time spent in contact with the food container (CTFC) within 1-hour intervals comparing successive images visually. The total amount of individual food intake per day was assessed by difference in weight of food pellets in the food container of each single cage between the onset and the end of recording divided by the number of days ($n = 6$). The body weight of each mouse was determined after the end of the recording. During the entire time of recording the light-tight cabinet remained closed. The total number of animals used in this experiment was 16 (8 mice/genotype).

In addition, spontaneous locomotor activity was recorded from 3 mice of each genotype. The mice were kept in individual cages equipped with infrared movement detectors linked to an automated recording system (Mouse-E-Motion, Hamburg, Germany). Locomotor activity was continuously recorded during the entire experiment in 10-min intervals. Actograms and activity profiles were created using Clocklab software (Actimetrics, Wilmette, Ill., USA) as described [34, 35]. The daytime activity was expressed in percent of the total/daily (24 h) activity as described [34, 36].

Melatonin Injection

Melatonin injections were performed according to the protocol described in Unfried et al. [32]. WT mice were subcutaneously injected once either with 10 μ g melatonin (Sigma-Aldrich, Schnellendorf, Germany) dissolved in 100 μ l of 5% ethanol and 0.9% NaCl or with vehicle in the middle of the subjective day (CT6). Four animals per experimental group were sacrificed by decapitation 2.5 h after the single injection at CT8.5. Brains were immediately frozen in 2-methylbutane (Fluka, Schnellendorf, Germany) placed on dry ice. Pituitaries were dissected out of the sella turcica,

Fig. 1. Analysis of total locomotor activity and daytime locomotor activity in a standard photoperiod. **a** Double plotted actograms of spontaneous locomotor activity in 12:12 LD conditions from representative WT (left) and MT1^{-/-} (right) mice. Gray shading indicates periods of darkness. **b** Bar plot of overall locomotor activity of WT and MT1^{-/-} mice. **c** Bar plot of locomotor activity during the light phase of WT and MT1^{-/-} mice. Data are expressed as the mean $\pm$ SEM of 3 animals per genotype.

embedded in Tissue-Tek (Sakura, Alphen aan de Rijn, Netherlands) using vinyl specimen molds (Sakura) and then frozen on dry ice. Tissues were stored at -80°C until cryosectioning.

Cryosectioning

Tissues embedded in Tissue-Tek were cut into 20- μm -thick frozen sections at -20°C using a cryostat. Brains were cut in the coronal plane and sections were arranged from the rostral to caudal plane on 8 serial SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). Pituitaries were cut in the coronal plane on 6 serial slides. Prior to the in situ hybridization, the slides were stored at -80°C .

In situ Hybridization

Radioactive in situ hybridization was carried out with brain and pituitary cryosections of WT and MT1^{-/-} mice (4 mice/genotype and time point) as previously described [32]. The following antisense or the corresponding sense 45-mer probes for *Pomc* and *Npy* mRNA expression were used (*Pomc* sense: 5'-CCA GAG CCG AGT CCA CGC GAG GGC AAG GCG TCC TAC TCC ATG GAG-3'; *Pomc* antisense: 5'-CTC CAT GGA GTA GGA GCG CTT GCC CTC GCG TGG ACT CGG CTC TGG-3'; *Npy* sense: 5'-GGG TGT CCC ACC AAT GCA TGC CAC CAC TAG GCT GGA CTC CGC CCC-3'; *Npy* antisense: 5'-GGG GCG GAG TCC AGC CTA GTG GTG GCA TGC ATT GGT GGG ACA GGC-3'). Probes were labeled with [^{32}P]dATP (PerkinElmer, Rodgau, Germany) using a terminal deoxynucleotidyl transferase kit (Invitro-

gen, Karlsruhe, Germany). Following the in situ hybridization procedure, sections were air-dried and exposed to a Kodak Biomax-MR X-ray film (Sigma-Aldrich). Exposure time for the detection of *Npy* was 10 days and for the detection of *Pomc* 1 day (pituitaries) or 7 days (brains), respectively. Expression levels were determined by optical density of the autoradiographic signal using ImageJ software and converted into the radioactive value (nCi) by use of ^{14}C microscales (GE Healthcare Bioscience, Piscataway, N.J., USA) which were included in each film cassette. Three sections were analyzed per mouse and condition and averaged to obtain a single value for each animal. The tissue-specific autoradiographic signals were assessed as gray-scale units above background (unstained neuropil) within an area of 22,500 μm^2 in the ARC, of 40,000 μm^2 in the pars distalis (PD) or of 16,500 μm^2 in the pars intermedia (PI).

Statistical Analysis

All data were statistically analyzed using Graph Pad Prism 5 software (San Diego, Calif., USA) by calculating the mean and the corresponding SEM from the entered data. Genotypes and day/night differences were compared by two-way ANOVA followed by a Bonferroni's comparison of all pairs of columns to assess differences between the genotypes and time points examined. For analysis of locomotor activity rhythms, total food intake and body weight, an unpaired t test was employed after confirming that variances were not significantly different. Values were considered significantly different at $p < 0.05$.

Fig. 2. Feeding behavior, total food intake and body weight of MT1^{-/-} mice and the corresponding WT mice kept under 12:12 LD conditions under ad libitum feeding. **a** Feeding behavior is expressed as CTFC in 1-hour intervals plotted against ZT from mice with a targeted deletion of the MT1 receptor (MT1^{-/-}) and the corresponding WT littermates. Two-way ANOVA revealed a significant effect of genotype ($p < 0.001$) and two-way ANOVA followed by Bonferroni's post hoc test revealed a significantly higher peak at ZT16 in feeding behavior of MT1^{-/-} as compared to WT mice. White bar indicates light phase, black bar indicates dark phase. Values are expressed as the mean $\pm$ SEM of 8 animals per genotype and time point. * $p < 0.05$; two-way ANOVA with Bonferroni post hoc test. **b** Bar plot of food intake as a measure of the amount of consumed food pellets. Data are expressed as the mean $\pm$ SEM of 8 animals per genotype. **c** Bar plot of body weight between WT and MT1^{-/-} mice during the whole recording time of 6 successive days.

Results

WT and MT1^{-/-} mice kept under an LD cycle displayed a clear 24-hour rhythm with expected increased locomotor activity during darkness (fig. 1a). In both genotypes, the activity onset started with the beginning of the

dark phase (fig. 1a). The overall spontaneous locomotor activity (mean counts per 10 min) did not differ significantly between WT and MT1^{-/-} mice (fig. 1b). Also day-time activity was not significantly different between both genotypes (fig. 1c).

Both genotypes spent very little time in CTFC during the light phase; the main feeding episode occurred in the dark (fig. 2a). Although their activity onsets did not differ, the time patterns of CTFC were significantly different between the two genotypes (two-way ANOVA, effect of genotype, $p < 0.001$): the WT mice spent significantly less time in CTFC than the MT1^{-/-} mice ($p < 0.05$; two-way ANOVA followed by the Bonferroni post hoc test, fig. 2a), but there was no significant difference in the total amount of consumed food ($p = 0.138$) between MT1^{-/-} mice (4.81 g per day) and WT mice (4.11 g per day) (fig. 2b) or in the body weight ($p = 0.33$) between WT (29.78 g) and MT1-deficient (28.85 g) mice (fig. 2c).

To analyze the effects of MT1 receptor-mediated signaling on the hypothalamic POMC/NPY system, *Pomc* and *Npy* mRNA expression values were analyzed in the hypothalamic ARC of WT and MT1^{-/-} mice during mid-subjective day (CT6, low levels of endogenous melatonin and minimum CTFC) and mid-subjective night (CT18, high levels of endogenous melatonin and maximal CTFC) by in situ hybridization (fig. 3). Specific hybridization signals for *Pomc* (fig. 3b, d) and *Npy* (fig. 3a, d) mRNA expression were detected in the ARC of the hypothalamus; the sense controls (fig. 3d) generated no signal. *Npy* hybridization signals in the ARC did not show a significant difference between mid-subjective night (CT18) and mid-subjective day (CT6) in both WT mice and MT1-deficient mice (fig. 3a). In the ARC of WT mice, *Pomc* hybridization signals were significantly higher at mid-subjective night (CT18) as compared to mid-subjective day (CT6) ($p < 0.001$; two-way ANOVA followed by the Bonferroni post hoc test, fig. 3b). In contrast, in the ARC of MT1^{-/-} mice, no significant difference in the *Pomc* hybridization signals between CT6 and CT18 could be observed and the hybridization signals were significantly lower in MT1^{-/-} mice as compared to WT mice at both time points (fig. 3b).

A specific *Pomc* hybridization signal was also found in the adenohypophysis (fig. 3c, d). In the PD, *Pomc* hybridization signals were significantly different between CT6 and CT18 in WT but not in MT1^{-/-} mice ($p < 0.001$; fig. 3c). Moreover, *Pomc* values were significantly lower in MT1^{-/-} mice than in WT mice at both time points ($p < 0.001$; fig. 3c). Notably, in the PI there was no significant difference in *Pomc* hybridization signals between CT6 and CT18 in both genotypes (fig. 3c).

(For legend see next page.)

Fig. 4. Effect of exogenous melatonin on *Pomc* expression in the hypothalamus and pituitary of WT and MT1^{-/-} mice. **a** Representative autoradiographs and quantification of *Pomc* hybridization signals in the ARC of WT and MT1^{-/-} mice 2.5 h after a single melatonin (Mel) or vehicle (Veh) injection at mid-day (CT8.5). Exogenous melatonin applied at mid-subjective day results in an increased *Pomc* expression in the ARC in WT mice but not in MT1^{-/-} mice. **b** Representative autoradiographs and quantifica-

tion of *Pomc* hybridization signals in the PD of the pituitary of WT and MT1^{-/-} mice 2.5 h after a Mel or Veh injection at mid-day. In the PD *Pomc* expression was higher after melatonin treatment in WT mice but not in MT1^{-/-} mice. Scale bar = 500 μ m. Values are expressed as the mean $\pm$ SEM of 4 animals per genotype and time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA with Bonferroni post hoc test.

Fig. 3. *Npy* and *Pomc* expression in the hypothalamus and pituitary of WT and MT1^{-/-} mice at mid-subjective day (CT6) and night (CT18). **a** Representative autoradiographs of *Npy* in situ hybridization and quantification in the ARC of WT and MT1^{-/-} mice. *Npy* hybridization signals are equally high independent of genotype and time point. Scale bar = 200 μ m. **b** Representative autoradiographs and quantitative analysis of *Pomc* in situ hybridization in the ARC of WT and MT1^{-/-} mice. In the ARC of WT mice *Pomc* expression is higher at night than at day. In MT1^{-/-} mice, *Pomc* expression is not different between day and night and lower as in WT mice. Scale bar = 200 μ m. **c** Representative autoradiographs and quantitative analysis of *Pomc* hybridization signals in

the PD (upper graph) and PI (lower graph) of the pituitary of WT and MT1^{-/-} mice during day and night. In the PD of WT mice, *Pomc* expression is higher at night than at day. In MT1^{-/-} mice, *Pomc* expression is not different between day and night and lower as in WT mice. In contrast, *Pomc* expression in the PI is not different between day and night independent of genotype. Scale bar = 500 μ m. **d** Representative autoradiographs of *Pomc* and *Npy* anti-sense and sense hybridization signals in coronal brain and transversal pituitary sections of a WT mouse. Scale bar brain = 2 mm; scale bar pituitary = 500 μ m. Values are expressed as the mean $\pm$ SEM of 3–4 animals per genotype and time point. *** $p < 0.001$; two-way ANOVA with Bonferroni post hoc test.

To analyze acute effects of melatonin on *Pomc* expression in the ARC and adenohypophysis, WT and MT1^{-/-} mice received a single melatonin injection at mid-subjective day (CT6) (fig. 4). In both the ARC and the PD of WT mice, hybridization signals of *Pomc* expression were significantly increased 2.5 h after an acute single melatonin injection as revealed by two-way ANOVA followed by the Bonferroni post hoc test (ARC: $p < 0.01$, fig. 4a; PD: $p < 0.001$, fig. 4b). In contrast, in MT1^{-/-} mice, the single melatonin injection did not affect *Pomc* hybridization signals in either the ARC or the PD (fig. 4a, b). These data suggest that the melatonin-induced stimulation of *Pomc* mRNA expression in both regions is mediated by the MT1 receptor. Again, hybridization signals of *Pomc* were significantly lower in MT1^{-/-} as compared to WT mice (fig. 4a, b). In both genotypes, melatonin injection did not affect hybridization signals of *Pomc* in the PI.

Discussion

The comparison between WT and MT1^{-/-} mice presented here demonstrates that MT1-mediated signaling influences feeding behavior. The MT1^{-/-} mice spent significantly more time feeding than the WT mice, while the general locomotor behavior (see also Refinetti [37]), body weight and the total amount of food consumed did not differ between both genotypes. Furthermore, MT1-mediated signaling stimulates *Pomc* mRNA expression levels in the hypothalamic ARC and the hypophysial PD, but not in the PI. Thus, the MT1-mediated signaling stimulates *Pomc* expression in a region-specific pattern. Since MT1-dependent increases in *Pomc* expression levels in the ARC and PD did not elicit direct orexigenic or anorexigenic effects, it is conceivable that MT1-dependent signaling acting on the availability and day/night fluctuations of *Pomc* expression ‘opens the window’ for other regulatory processes that occur downstream of *Pomc* mRNA expression and are independent of MT1.

We found effects of melatonin and the MT1 receptor on the statics and dynamics of the POMC system: the baseline levels of *Pomc* expression in the ARC were clearly reduced in MT1^{-/-} mice as compared to WT mice. Additionally, *Pomc* expression of MT1^{-/-} mice does not show day/night differences, whereas *Pomc* expression levels are significantly increased at night in the ARC of WT mice. This suggests a stimulation of *Pomc* expression by melatonin acting through the MT1 receptor. Consistently, exogenous melatonin injected during daytime led to an increase in *Pomc* expression in WT mice but not in

MT1^{-/-} mice. These findings are in line with data obtained in rat [28, 30] and mice [31].

Our observation that, despite a reduction in *Pomc* expression levels, food intake is not significantly elevated in C3H MT1^{-/-} mice does not support the notion that hypothalamic POMC neurons generally and exclusively elicit appetite-inhibiting, anorexigenic effects [38]. In this regard, it should be kept in mind that both appetite-promoting, orexigenic (e.g. β -END) and anorexigenic (e.g. α -melanocyte-stimulating hormone) signal peptides may be generated from POMC under the influence of various other regulatory factors, such as endocannabinoids [39], and the cannabinoid 1 (CB1) receptor activation in the hypothalamus increases the release of the orexigenic POMC derivative β -END in the paraventricular nucleus [40–42]. Hence, it is suggested that the impact of MT1-mediated signal transduction pathway on *Pomc* expression in the ARC probably is not an orexigenic, or anorexigenic signal per se. In accordance with this interpretation, we observed only moderate effects of MT1 deficiency on the food intake. The only significant difference concerned the dynamics of feeding and not the amount of food consumed: MT1^{-/-} mice are ‘slow eaters’, while the WT mice prefer ‘fast food’. This finding is in line with the study of Adamah-Biassi et al. [43] showing that melatonin and the MT1 receptor do not have a major influence on the amount of food ingested but affect the temporal dynamics of food intake and various other behaviors. Also the *Pomc* expression in the PD was found to be under the stimulatory control of the MT1-signaling pathway, because only WT, but not MT1^{-/-} mice, show nocturnal elevated *Pomc* expression, and exogenous melatonin injected during daytime stimulates *Pomc* expression in the PD of WT mice but not of MT1^{-/-} mice.

In contrast to the results obtained in the PD and ARC, the *Pomc* expression profile of the PI is not significantly regulated by day/night differences or melatonin. Accordingly, our melatonin injection showed no acute effect of exogenously applied melatonin on *Pomc* expression in this tissue (data not shown). Compared to the clear results in the ARC and PD, it can be suggested that melatonin has no acute effect on *Pomc* expression in the PI.

An important question relates to the location of the MT1 receptors that mediate *Pomc* expression in ARC and PD. One possibility may be that the MT1 receptors are located in the ARC itself, since *Mt1* mRNA and ¹²⁵I-melatonin binding sites have been demonstrated in the ARC of rodents (mouse and rat) [44–46]. On the other hand, a recent study using antibodies against MT1 and MT2 failed to detect these receptors in the rat ARC [47] and

also in the sheep ARC, no *Mt1* mRNA has been found [48].

Another possibility may be that the melatonin signal affecting the POMC system and thus the *Pomc* expression in the ARC and PD is decoded in the hypophysial pars tuberalis which contains a high density of MT1 receptors [8] and conveys the MT1-mediated melatonin signal retrogradely via thyrotropin to the hypothalamus including the ARC and anterogradely via endocannabinoids to the PD [14, 32, 49–52].

The possibility that MT1 receptors that control *Pomc* expression in the PD are located in the PD itself can be ruled out. In the rodent PD (rat and hamster), melatonin binding sites are expressed in the fetus and decline after birth [53, 54]. Thus, the observed effect of melatonin on *Pomc* expression could be indirectly mediated by endocannabinoid signals originating from the pars tuberalis [55], as the CB1 receptor is present in the rodent PD [52].

Surprisingly, the expression levels of the gene encoding for the orexigenic peptide NPY in the ARC did not show a day/night difference in WT mice and also no differences between WT and MT1^{–/–} mice. Thus, the NPY-ergic orexigenic system in the ARC is not influenced by MT1-dependent signaling in mice. This conforms to observations in Siberian hamsters showing that *Npy* expres-

sion did not change with the photoperiod, i.e., the length of the melatonin [24, 56, 57]. However, other species such as sheep [58] or Spargue-Dawley rats [59] do show a modulation of *Npy* expression by the photoperiod and the light/dark cycle. Thus, the melatonin-dependent photoperiodic regulation of the orexigenic *Npy* mRNA appears to be species-specific.

In summary, the present study shows that MT1 receptor deficiency alters the temporal feeding pattern but not the amount of food ingested, and melatonin signaling mediated through the MT1 receptor stimulates *Pomc* expression in both the ARC and PD of mice.

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Disclosure Statement

The authors have nothing to disclose.

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