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Novel non-indolic melatonin receptor agonists differentially entrain endogenous melatonin rhythm and increase its amplitude

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Abstract

In this study we have examined the ability of melatonin and four synthetic melatonin receptor agonists to entrain endogenous melatonin secretion in rats, free running in constant darkness. The circadian melatonin profile was measured by trans-pineal microdialysis, which not only reveals the time of onset and end of production (phase), but also the amplitude of the rhythm. Exogenous melatonin given at the onset of subjective darkness (clock time 12 h) was effective to entrain endogenous melatonin production. Only one agonist, 2-chloroacetamido-8-methoxytetralin (AH-017), mimicked this action. Two other agonists, 4-methoxy-2-(methylene propylamide)indan (GG-012) and *N*-[2-[2,3,7,8-tetrahydro-1*H*-furo(2,3-*g*)indol-1-yl]ethyl]acetamide (GR196429), induced a phase-delay under free running conditions, possibly by increasing tau (τ) period. One agonist, 2-acetamido-8-methoxytetralin (AH-001) did not show any phase effect on the free running rhythm. Unexpectedly, all melatonin receptor agonists increased the amplitude of melatonin secretion. The amount of the increase varied from just below the level of significance (AH-001) to an approximately 2-fold increase (GG-012 and GR196429). This is in clear contrast to entrainment with melatonin, which significantly decreased the amplitude. It is hypothesized that entrainment and effects on amplitude of melatonin secretion are mediated by different mechanisms which can be differentially modulated using specific ligands.

Keywords: Microdialysis; Entrainment; Melatonin receptor agonist; Pineal gland; Amplitude

1. Introduction

Melatonin, the primary hormone of the pineal gland, has been reported to be a regulatory factor in a variety of physiological, immunological and behavioural processes in the body. Of particular interest are both its putative role as an internal Zeitgeber (time giver) for the circadian clock in the suprachiasmatic nuclei and its neuroendocrine effects, such as regulation of reproductive function in seasonal breeding mammals (Arendt, 1995). Melatonin is known to exert some of its effects through specific membrane-bound G protein-coupled receptors which can be labeled with high picomolar affinity by the radioligand, 2-[^{125}I]iodomelatonin, in several tissues, including rat and human suprachiasmatic nuclei, chicken and rabbit retina (Morgan et al., 1994). Melatonin is reported to exert feedback on the suprachiasmatic nuclei, most clearly demonstrated by its effects on circadian rhythms (Armstrong, 1989; Cassone, 1990; Cassone et al., 1993, etc.). These effects include phase-shifts, entrainment, and reversal of the direction of re-entrainment following a phase shift of the light–dark cycle. This feedback effect on the suprachiasmatic nuclei is an important mechanism which has led to the development of new series of melatonin receptor agonists which could be used as drugs acting on the biological clock (Redman et al., 1983; Warren et al., 1993).

Situations of disturbed circadian rhythms, such as certain sleeping disorders, seasonal affective disorders, shift-work syndrome and jet-lag, are possible therapeutic targets for these compounds. Reinforcement of the circadian rhythms might improve the sleep/wake cycle. It is there-

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fore likely that the ability of melatonin receptor agonists to entrain circadian rhythms is of crucial importance to the clinical development of these compounds. To test the entrainment capacity of melatonin receptor agonists, a variety of techniques have been used (Redman et al., 1983; Warren et al., 1993). In most cases, animal behaviour is recorded for long periods of time under free running conditions. The parameter measured can be locomotor activity, drinking/eating behavior, wheel-running activity, etc. Daily injections with melatonin or a melatonin receptor agonist at the start of the subjective night results in entrainment of these rhythms to the time of administration (Redman et al., 1983). Although such studies are crucial for providing information about the ability of a compound to entrain circadian rhythms, behavioural measurements can be masked, and do not provide information on underlying physiological rhythms.

Recently, measurement of the endogenous melatonin secretion profile by means of microdialysis was reported upon (Drijfhout et al. 1993; Kanematsu et al. 1994). Microdialysis of the pineal gland is a suitable technique to monitor the circadian rhythm of the production of melatonin in vivo in various species. The clean samples allow direct analysis by on-line injection into a high-performance liquid chromatography (HPLC) system. We have described an HPLC system with fluorescence detection with a very high level of sensitivity (up to 1 pg/sample) (Drijfhout et al., 1993). In a previous study, it was shown that this method not only provides information on both phase markers of the rhythm, i.e., onset and stop of melatonin production, but also on the amplitude of its rhythm (estimated as the peak pineal concentration of melatonin), which appeared to be reduced after 4 weeks of melatonin treatment (Drijfhout et al. 1996a).

The present report describes the use of trans-pineal microdialysis in an entrainment study with newly developed non-indolic melatonin receptor agonists. These compounds, 2-acetamido-8-methoxytetralin (AH-001), 4-methoxy-2-(methylene propylamide)indan (GG-012) and 2-chloroacetamido-8-methoxytetralin (AH-017) and *N*-[2-[2,3,7,8-tetrahydro-1*H*-furo(2,3-*g*)indol-1-yl]ethyl]acetamide (GR196429) were synthesized in our departments (Copinga et al. 1993; Gruppen and Grol, 1995; Beresford et al. 1998) and are examples of semi-rigid structures with affinity for melatonin receptors. Their abilities to entrain free-running melatonin rhythms in rats are compared with the effects of saline and melatonin, following 2 or 4 weeks of treatment.

2. Materials and methods

2.1. Animals

Male albino rats (Wistar, Harlan, The Netherlands) weighing 180–200 g were used. Animals arrived in groups of 10 animals, each group being assigned to a certain treatment (melatonin, saline or a melatonin receptor agonist). The rats were housed in plastic cages ($70 \times 40 \times 20$ cm) and kept under light–dark 12:12 h lighting regiment (lights on from 0100 until 1300 h) for at least 2 weeks. Light was provided by a set of fluorescent tubes, resulting in a light intensity of 100–300 lux at cage level. Temperature was maintained at 21 ± 2°C. Water and food were continuously available. After the adaptation period, rats were placed into constant darkness and housed individually in plastic cages ($40 \times 25 \times 16$ cm). From this moment on, with the exception of the control group, the rats were injected daily with saline or the experimental drug (see below). The experimental protocols were approved by the Ethical Committee of the Faculty of Mathematics and Natural Sciences, State University of Groningen, The Netherlands.

2.2. Experimental procedures

A total of eight groups of rats, each group receiving a different treatment, was used. One group received daily melatonin injections at a dose of 0.5 mg/kg for 4 weeks and one group received saline injections for 4 weeks. Five groups received treatment with melatonin receptor agonists. These treatments were AH-001 (10 mg/kg, 2 weeks), AH-017 (2 mg/kg, 2 weeks), GG-012 (5 mg/kg, 2 weeks) and GR196429 (0.5 and 5 mg/kg, 4 weeks). One group did not receive any treatment and was measured under light darkness conditions. This group is referred to as the control group. Injections took place between 1245 and 1315 h, i.e., circadian time $12 - 15$ and $12 + 15$ min. All injections were given subcutaneously (s.c.). After the treatment period, the rats were operated on and received their final injection. They were allowed to recover from surgery until the next day, when the microdialysis experiments took place. The day after the experiment, the animals were killed by anaesthesia with choral hydrate (1000 mg/kg, i.p.) and the tissues were fixed by intracardial perfusion with paraformaldehyde (4%). After removal of the skull, the location of the probe was determined visually.

2.3. Surgery and dialysis

All operations took place between clock time 7 and 12 and lasted about 1.5 h. Surgery on animals in the dark phase took place under dim red light conditions. Implantation was performed as reported previously (Drijfhout et al.,1993). A dialysis tube of saponified cellulose ester (i.d. 0.22 mm, o.d. 0.27 mm), bearing a tungsten wire with a sharpened point, was fastened in a transverse position to a holder mounted on the right bar of a stereotaxic apparatus (David Kopf Instruments). One hole was drilled on each side of the temporal bone (co-ordinates A 0.8, V 2.5) according to the atlas of Paxinos and Watson (1982). The tube was inserted transversally through the pineal gland

and the tungsten wire was removed. A blunted needle was glued to the tube, after which both inlet and outlet of the tube were fixed on the skull in a vertical position. Surgery was performed under chloral hydrate anaesthesia (400 mg/kg, i.p.). The rats were allowed to recover from the surgery for at least 16 h.

During experiments, the inlet and outlet of the probe were connected to a CMA/100 microinjection pump (CMA/Microdialysis, Stockholm, Sweden) and to the valve (Valco) of the HPLC system, respectively, using microbore PEEK tubing (i.d. 0.005″, o.d. 0.02″). The use of a quartz dual channel swivel (Instech, Plymouth, PA, USA) prevented the tubing from becoming tangled and allowed the experiments to run for 20–24 h. Perfusion was performed with Ringer's solution (NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1.0 mM) at a flow rate of 3.0 μl/min. Samples were collected in the loop (50 μl) of the valve for periods of 20 min and were then automatically injected onto the column (see below). In order to average the values from several experiments, the times of injection were synchronised across experiments. The duration of sample collection and time of injection were computer-controlled.

2.4. Chemical assay

Microdialysates were assayed on-line for melatonin using two identical HPLC systems with fluorescence detection. A Waters 610 pump was used in conjunction with a Waters M470 fluorescence detector (Ex 280 nm, Em 345 nm). Samples were separated on a reversed phase C_{18} column (Supelco, 250 × 4.6 mm), kept at a constant temperature of 30 ± 1°C using a column heater controlled by a Waters 600 System Controller. The mobile phase consisted of 10 mM sodium acetate, adjusted to a pH of 4.5 with concentrated acetic acid, 0.01 mM Na_2–EDTA and 200 ml/l acetonitrile. Using a flow of 1.0 ml/min resulted in a pressure of approximately 1400 psi. Peak identification and quantification were based on retention time and peak areas by comparison to those of standard solutions. The detection limit of the assay was 5 fmol/injection, signal to noise ratio 2:1. Automated control of the HPLC system as well as handling and storage of the chromatograms were done by an external computer with Millennium 2010 chromatography software (Millipore, Milford, MA, USA).

2.5. Chemicals

Melatonin was obtained from Sigma (St. Louis, MO, USA); AH-001 and AH-017 were synthesised by Dr. S. Copinga (Copinga et al. 1993). GG-012 was synthesised by G. Gruppen (Gruppen and Grol, 1995). GR196429 (GR196429 · HCl was provided by Glaxo Wellcome Research and Development (Stevenage, UK) (Beresford et al. 1998). Structural formulae are shown in Fig. 1.

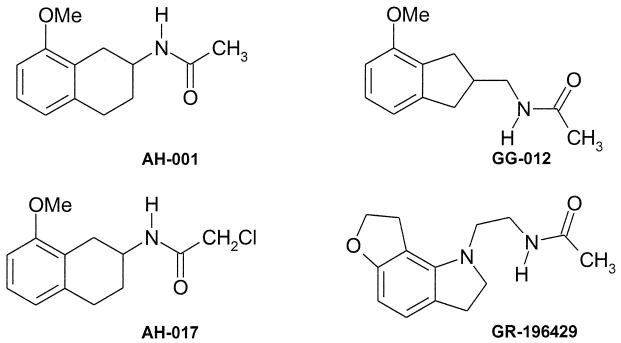

Fig. 1. Synthetic non-indolic melatonin receptor agonists.

Saline was prepared by dissolving 9 g NaCl in 1 l of distilled water. Melatonin solutions were prepared by dissolving melatonin in ethanol and diluting with saline, resulting in a final melatonin concentration of 0.25 mg/ml (1% ethanolic saline). AH-001 was dissolved in equal amounts of ethanol/polyethyleneglycol and diluted with saline to a final concentration of 5 mg/ml (20% ethanol, 20% polyethyleneglycol, 60% saline). AH-017 and GG-012 were dissolved in Solutol® (BASF, Switzerland) and diluted with saline to a final concentration of 1 and 2.5 mg/ml, respectively (40% Solutol®, 60% saline). Solutions were prepared once in amounts sufficient for the experiments. The compounds were stable (thin layer chromatography) in their solutions for the duration of the experiments.

GR196429 was dissolved in saline in concentrations of 0.288 and 2.88 mg/ml, resulting in free base concentrations of 0.25 and 2.5 mg/ml, respectively. The animals received 2.0 ml injection fluid per kg body weight. Solutions of GR196429 were prepared freshly every 3–4 days. All solutions were kept in the dark and refrigerated (4°C).

2.6. Data analysis

All data are expressed as the relative melatonin concentration compared to the melatonin concentrations during subjective day. This corrects for differences in absolute melatonin output due to variations in placement and recovery of the probes. Data from several animals within the same group were averaged and expressed as the means ± S.E.M. In order to make a comparison of the phase of the various rhythms measured, two phase-markers (onset and stop) of the melatonin rhythm were calculated as described previously (Drijfhout et al. 1996a). Two sigmoïdal curves were fitted through the data points of each individual experiment, describing both increase and decrease of melatonin levels. Based on these curves, the time at which 50% of the maximal increase in melatonin levels was reached was calculated and referred to as IT_{50}. Similarly, the time at which 50% of the decrease in melatonin levels was reached was calculated and referred to as DT_{50}. By definition IT_{50} is measured with clock time 12 h as the origin,

while the DT_{50} has clock time 0 as the origin. For the maximal increase in melatonin, defined as amplitude, a set of data points was created from each individual experiment, consisting of all data between $IT_{50} + 1.5$ h and $DT_{50} - 1$ h, which were then averaged. Data sets from all experiments within one group were averaged and tested statistically.

Statistical analyses of IT_{50} and DT_{50} values and amplitudes were performed by one-way analysis of variance followed by Newman–Keuls pairwise multiple comparison tests. Entrainment was considered to be achieved when the IT_{50} of the experimental group was not significantly different from the IT_{50} of the control group ($P < 0.05$).

3. Results

3.1. Control

Fig. 2 shows the day–night rhythm of melatonin production in a 12:12 h light–dark cycle. Basal melatonin levels in daytime were 6.0 ± 2.9 pg/sample. Melatonin levels started to increase approximately 2 h after the start of darkness ($IT_{50} = 3.2 \pm 0.1$ h). Maximal values, which were about 15-fold higher than the average daytime values (amplitude $= 1563 \pm 85\%$), were reached at approximately clock time 16 h. This high level of melatonin production was maintained throughout the dark period up to about 1 h before the end of darkness. At this point a steep decline in melatonin levels was observed ($DT_{50} = -1.2 \pm 0.1$ h). Before the lights went on, melatonin levels had returned to their basal daytime values. The data from this control experiment correlate well with those from previous studies (Drijfhout et al. 1996a). In all subsequent figures, this control curve is shown as a dashed curve for comparison.

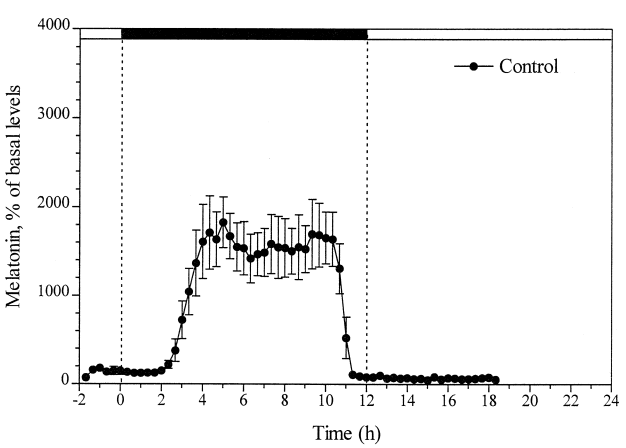

Fig. 2. The day–night rhythm in melatonin production measured in a light darkness cycle (12/12 h). Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 6$). The black bar on the top indicates the period of darkness. Clock time 12 is defined as $t = 0$.

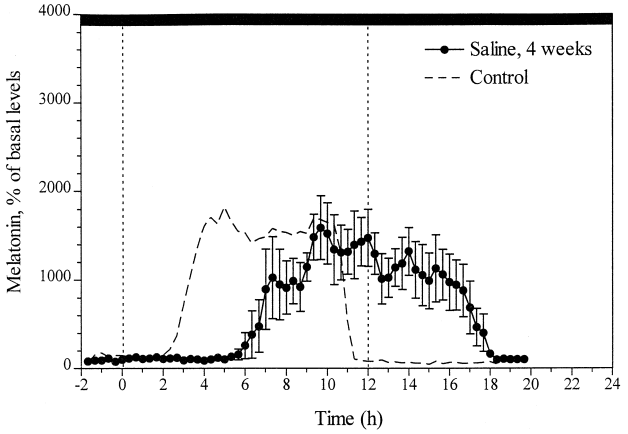

Fig. 3. The day–night rhythm in melatonin production measured in constant darkness after 4 weeks of daily melatonin injections (0.5 mg/kg, s.c.). Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 7$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

3.2. Free running experiments

Saline treatment for 4 weeks in total darkness resulted in a significant phase shift that was proportional to the duration of treatment, when compared to findings of a previous study (Drijfhout et al. 1996a), in which free running was observed following 2 weeks' saline treatment. Four weeks' saline treatment (Fig. 3) resulted in a phase delay of approximately 5 h ($IT_{50} = 8.0 \pm 0.4$ h; $DT_{50} = 5.2 \pm 0.2$ h). The 2-week saline treatment from the previous study resulted in a phase delay of approximately 2.5 h. The amplitude after 4 weeks of saline ($1338 \pm 54\%$) was not

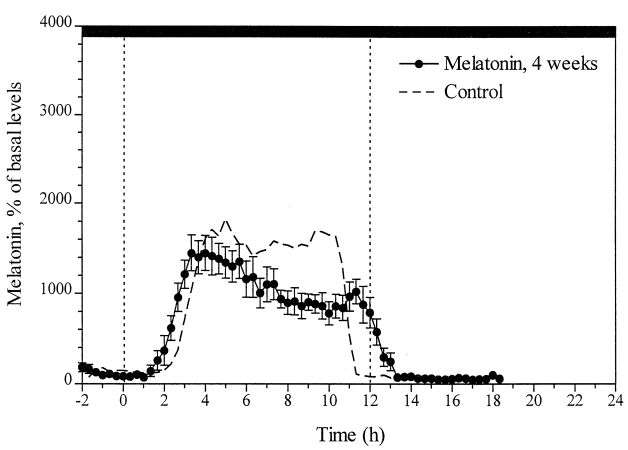

Fig. 4. The day–night rhythm in melatonin production measured in constant darkness after 4 weeks of daily saline injections (s.c.). Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 5$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

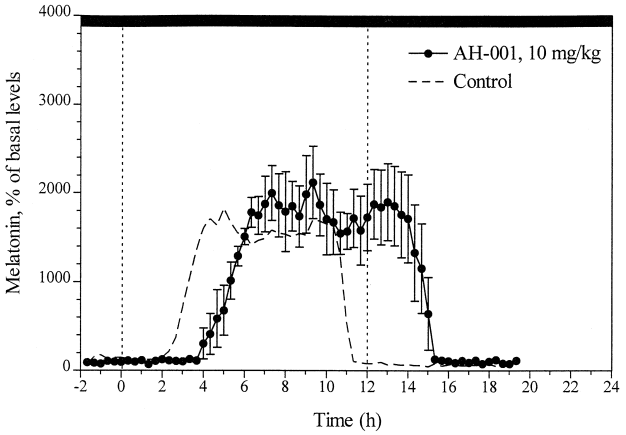

Fig. 5. The effect of daily AH-001 injections (10 mg/kg, s.c.) for 2 weeks on pineal melatonin production in constant darkness. Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 5$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

significantly different from that under control conditions. A small number of animals (two out of eight) were entrained to the injection procedure. Data from these animals were discarded.

3.3. Effects of melatonin

All animals were entrained after melatonin treatment (0.5 mg/kg) for 4 weeks in total darkness (Fig. 4). This entrainment was both qualitatively and quantitatively similar to that in previous experiments (Drijfhout et al. 1996b). Both IT_{50} (2.4 ± 0.2 h) and DT_{50} (0.5 ± 0.2 h) were in the same range as reported earlier and again, the DT_{50} was significantly phase-delayed, while the IT_{50} was entrained. A remarkable finding from the previous study was also observed in this study: the amplitude following melatonin treatment (1090 ± 40%) was significantly lower than that under control conditions (1563 ± 85%).

3.4. Effects of AH-001

At a dose of 10 mg/kg, the amidotetralin compound, AH-001, did not appear to entrain the melatonin rhythm (Fig. 5). After 2 weeks of dosing, the free-running melatonin profile was clearly phase-delayed in five out of seven animals. This resulted in an IT_{50} of 5.1 ± 0.4 h and a DT_{50} of 2.5 ± 0.3 h. Two animals entrained to the injection procedure. An unexpected finding in this group was a tendency for the amplitude to be somewhat increased (1811 ± 70%) compared to that under control conditions (1563 ± 85%). Although this effect did not reach significance ($P > 0.05$), the finding was nevertheless intriguing, especially since melatonin itself reduced the amplitude.

3.5. Effects of AH-017

AH-017, the most potent analogue from the amidotetralin-based series of melatonin receptor agonists (Table 1) was effective to entrain the endogenous melatonin profile in constant darkness (Fig. 6). After 2 weeks of dosing, both IT_{50} (2.7 ± 0.3 h) and DT_{50} (−0.4 ± 0.2 h) were similar to those under control light–dark conditions in all animals. The small, insignificant phase-delay of DT_{50} compared to control light–dark conditions was similar to the melatonin induced entrainment. The amplitude of the rhythm following AH-017 treatment was significantly increased (2471 ± 76%), compared to that for saline conditions (1563 ± 85%).

3.6. Effects of GG-012

GG-012, the lead structure of an amidoindane based series of synthetic melatonin receptor agonists, did not entrain the endogenous melatonin profile at a concentration of 5 mg/kg (Fig. 7). A significant phase-delay was obtained after 2 weeks of daily dosing, resulting in an IT_{50} of 8.0 ± 1.1 h and a DT_{50} of 4.9 ± 1.0 h. Remarkably, this phase shift of 5 h was approximately twice that seen in

Table 1
In vitro pharmacological data for the synthetic melatonin receptor agonists compounds tested

Tissue	Chicken retina	Human	Human	Rabbit retina	
Receptor subtype	mel_{1A}/MT_2	mt_1	MT_2	ML_1	
	pK_i[a]	pK_i[b]	pK_i[b]	IC_{50} (nM)[c]	
Melatonin	9.24	9.48	9.74	0.017	agonist
AH-001	7.34	7.33	8.39	1.4	agonist
AH-017	8.34	8.27	9.16	0.063	agonist
GG-012	8.02[d]	N.T.	N.T.	0.48[e]	partial agonist (50%)
GR196429	9.38[b]	9.85[b]	9.79[b]	0.03[b]	agonist

[a] Inhibition of 2-[^{125}I]iodomelatonin binding to chicken retina (Copinga et al., 1993).
[b] (Dubocovich et al., 1997; Beresford et al., 1998).
[c] Inhibition of Ca^{2+}-dependent dopamine release from rabbit retina (Copinga et al., 1993; Dubocovich, 1983).
[d] Personal communication, R.M. Hagan.
[e] Personal communication, M. Dubocovich.

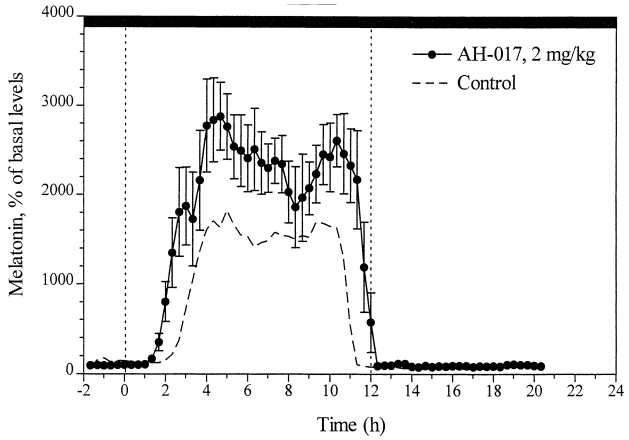

Fig. 6. The effect of daily AH-017 injections (2 mg/kg, s.c.) for 2 weeks on pineal melatonin production in constant darkness. Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 6$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

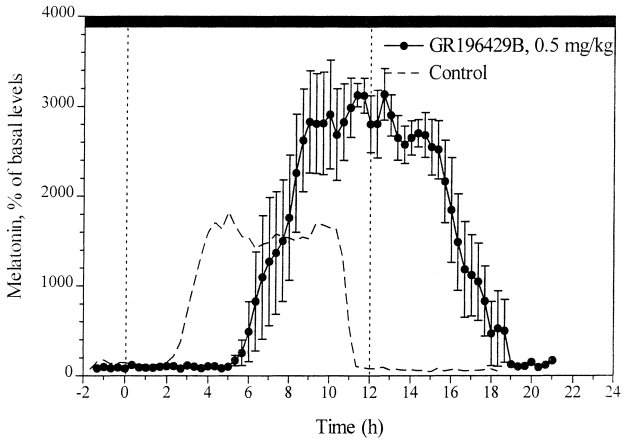

Fig. 8. The effect of daily GR196429 injections (0.5 mg/kg, s.c.) for 4 weeks on pineal melatonin production in constant darkness. Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 6$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

animals treated for 2 weeks with saline, or in AH-001-treated animals. Again, the amplitude was significantly increased (3089 ± 99%) compared to that under control conditions (1563 ± 85%).

3.7. Effects of GR196429

GR196429 was administered for 4 weeks at doses of 0.5 mg/kg (Fig. 8) and 5 mg/kg (Fig. 9). Full entrainment of the melatonin profile did not occur at either dose. At the lower dose, the phase shifts in IT_{50} (8.5 ± 0.8 h) and DT_{50} (6.0 ± 0.9 h) were significantly different from those of the controls and in the same range as with saline treatment for 4 weeks. Also at the higher dose, the phase shift was significantly different from that in the controls. In addition, IT_{50} (13.4 ± 0.9 h) and DT_{50} (8.9 ± 1.1 h) were approximately 3–5 h later than at the lower dose. Thus, the higher dose resulted in a larger phase-delay of the melatonin profile. At both doses, a small number of animals entrained to the injection procedure (lower dose: three out of ten animals, higher dose: three out of seven animals). At both doses, the curves were broadened because of averaging individual curves that were shifted to different extents. The amplitude was significantly increased at both doses. The increase of the amplitude, compared to control (1563 ± 85%), at the low dose (2868 ± 66%) was similar to the increase at high dose (2367 ± 66%), indicating that the amplitude effect was independent from entraining properties.

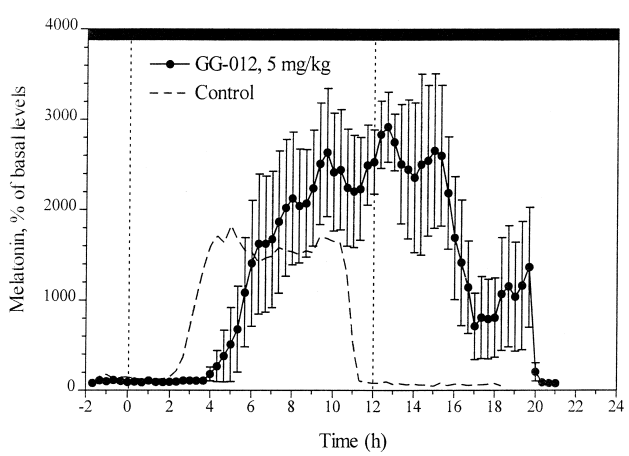

Fig. 7. The effect of daily GG-012 injections (5 mg/kg, s.c.) for 2 weeks on pineal melatonin production in constant darkness. Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 7$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

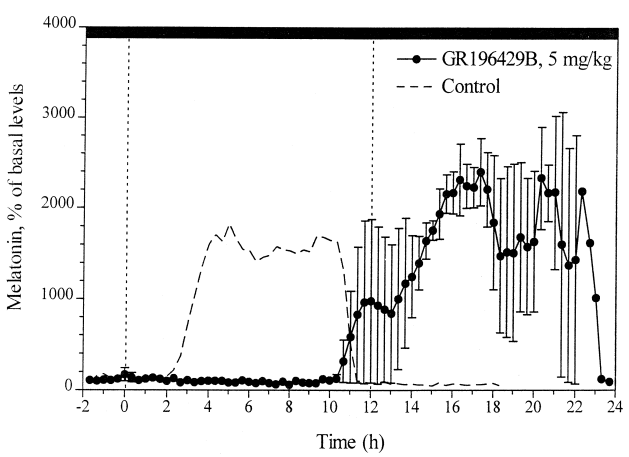

Fig. 9. The effect of daily GR196429 injections (5 mg/kg, s.c.) for 4 weeks on pineal melatonin production in constant darkness. Data are expressed as relative values compared to daytime levels and plotted as the means ± S.E.M. ($n = 4$). The dashed curve represents the melatonin profile in light darkness (Fig. 2). Clock time 12 h is defined as $t = 0$. Vertical dotted lines represent onset and end of the subjective night.

3.8. General comments on entrainment and amplitude changes

The ratio of entrained to non-entrained animals in a group is important for the conclusion as to the entraining ability of a certain treatment. Usually, under control conditions, about 20%–30% of the animals remain entrained, even though they are under constant dark conditions and are injected only with saline. This is a common phenomenon in such experiments and can be considered a false positive result. There were no significant differences in the absolute subjective daytime levels of melatonin in animals treated with melatonin or melatonin receptor analogues. Therefore changes in amplitude of melatonin release are not caused by changes in absolute basal daytime output but by increased night-time melatonin production.

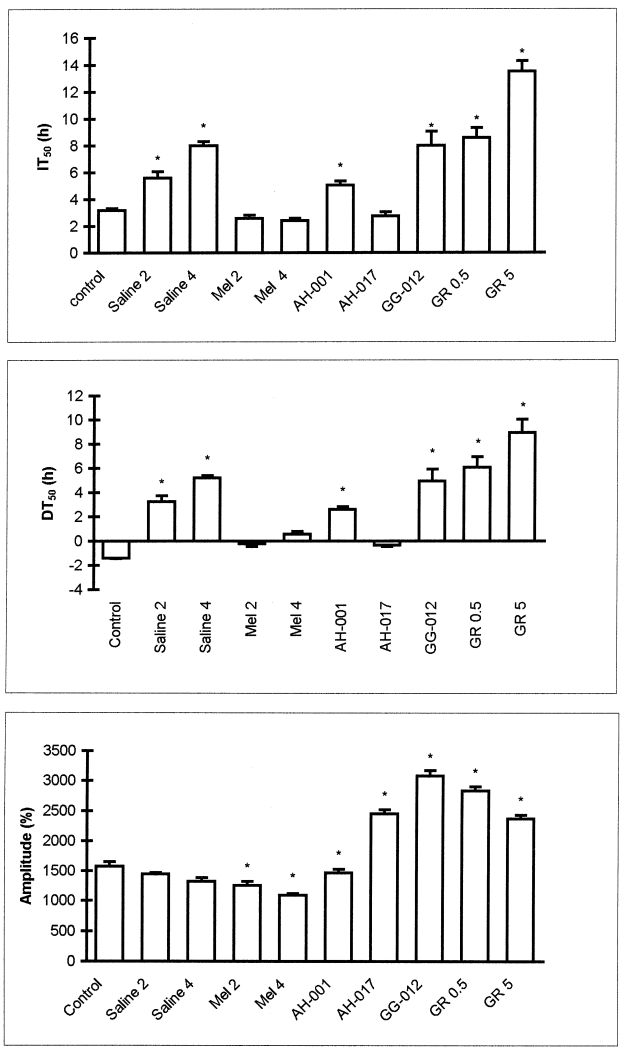

Fig. 10. Graphical representation of IT_{50} (A), DT_{50} (B) and amplitude (C) data from all experiments described. The columns represent data under control light darkness conditions (Control) and under free running conditions following treatment with saline for 2 (Saline 2) and 4 (Saline 4) weeks, with melatonin for 2 (Mel 2) and 4 (Mel 4) weeks, with agonists for 2 weeks (AH-001, AH-017 and GG-012) and with GR196429 for 4 weeks in doses of 0.5 mg/kg (GR 0.5) and 5 mg/kg (GR 5). The data for Saline 2 and Mel 2 were taken from a previous study (Drijfhout et al. 1996b). Asterisks (*) indicate statistically significant differences from control ($P < 0.05$).

Fig. 10 shows a graphic representation of IT_{50} (A) and DT_{50} (B) values and amplitude (C) of melatonin rhythm under the various experimental conditions. Data following 2 weeks of saline and melatonin treatment are from a previous publication (Drijfhout et al. 1996a).

4. Discussion

In many aspects, the present data confirm findings from previous experiments in which melatonin was used as an entraining agent (Drijfhout et al. 1996a). Exogenously administered melatonin entrained endogenous melatonin and decreased the amplitude of its production at a dose of 0.5 mg/kg. The phase shift following 4 weeks of saline treatment (5 h) was roughly twice the shift seen previously after 2 weeks of dosing (2.5 h). Non-specific entrainment to the injection procedure was similar to that observed previously. In an environment of constant darkness, animals will entrain more readily to any kind of 'Zeitgeber', for example the injection procedure. Based on these findings, the reproducibility of the data indicate that this model can be used to test melatonin receptor agonists agents.

4.1. Entrainment with melatonin receptor agonists

The compounds used in this study are shown in Fig. 1, while their in vitro pharmacology is summarized in Table 1. Three different cDNAs encoding putative 7-transmembrane domain high-affinity melatonin receptors have been cloned. To date, only two of these have been found in mammalian tissues, the mt_1 (previously called Mel_{1A}) is expressed in the human and rat suprachiasmatic nuclei, a result that implicates the receptor in circadian and reproductive effects of melatonin. The second G protein-coupled melatonin receptor, MT_2, refers to native receptors with pharmacological characteristics similar to those of the recombinant receptor mt_2 (formerly called the Mel_{1B}). It is expressed in retina and brain and the pharmacological profile is similar to that of the functional presynaptic ML_1 heteroreceptor of rabbit retina (Reppert et al. 1995). MT_3 refers to the pharmacologically defined melatonin receptor subtype, previously referred to as ML_2 (Beresford et al. 1998).

In contrast to melatonin, our compounds—except GR196429—all had a great difference in selectivity for the cloned mt_1 and MT_2 receptors. Based on the binding data (Copinga et al. 1993; Dubocovich et al. 1997) for compounds AH-001 and AH-017, with a higher affinity for the melatonin MT_2 receptor than for the mt_1, it can be expected that the amidoindan is likely to be more selective for the melatonin MT_2 receptor subtype.

Considering the chemical structure of the synthetic compounds, their interaction with many other G-protein coupled receptors will be minimal. The receptors for biogenic amines posses an essential aspartic residue in helix III for an interaction with a positive charged amine in the ligand. As the amidotetralins studied here lack an amine moiety, interaction with G-protein coupled receptors is unlikely. The selectivity of GR196429 for melatonin receptors has been confirmed by extensive receptor profiling (Beresford et al. 1998).

While melatonin is effective as an entraining agent at relatively low doses, entrainment of the endogenous melatonin rhythm with the synthetic melatonin receptor agonists seems to be more difficult to achieve. AH-001 has a low potency compared to that of melatonin. Therefore, a much higher dose was used in the experiments. However, the poor solubility of the compound limited the maximal dose to 10 mg/kg. The low solubility may also have caused precipitation of the compound on the injection site, resulting in blood levels too low to be effective and thus caused the failure of this compound to entrain. A similar cause may explain the failure of GG-012 to entrain melatonin levels. Alternatively, it may be because GG-012 has lower efficacy than melatonin, as demonstrated by its partial agonism compared to melatonin in rabbit retina (Table 1).

In the case of AH-017, the situation was quite different. Although its potency at the in vitro melatonin ML_1 receptor, modulating Ca^{2+}-dependent dopamine release, is about 25% of that of melatonin, the dose used was 4-fold higher than that of melatonin, presumably resulting in complete compensation for the lower activity. Its effectiveness in entraining the melatonin profile may therefore not be surprising.

The compound GR196429 was developed as a highly potent melatonin receptor agonist. In vitro, the compound was as potent as melatonin to inhibit dopamine release from rabbit retina (see Table 1). The finding that, even at a relatively high dose, GR196429 did not entrain was unexpected as the compound appears to be very effective in the phase shift model (Beresford et al. 1997). One of the properties of melatonin, often used in the characterization of melatonin receptor agonists, is its ability to reverse the direction of re-entrainment after an 8-h phase-advance shift (Redman et al., 1983, 1995). Normally animals respond by a phase-delay to the new light darkness cycle. When melatonin is administered daily at the new start of darkness, animals respond to the new situation by a phase advance instead of a phase delay. GR196429B, at a dose of 5 mg/kg caused five out of six animals to phase-advance to the new light darkness cycle, whereas vehicle injections caused all six animals to phase-delay. At first glance, these data seem to be contradictory. However, there are important differences in the models used.

In the phase-shift model, animals remain under light darkness conditions. Because light is an important external 'Zeitgeber', it may have an effect additional to the effect of the compound itself. Because the entrainment experiments reported here were carried out in constant darkness, such an additional effect is lacking, which may have resulted in poor entrainment. Another difference was the duration of the experiment. Generally, in phase-shift experiments the compound is given for several days, after which full re-entrainment has occurred. In the present experiments, chronic dosing was applied for 4 weeks. During such long periods, additional effects may be expected, such as desensitization of the system. It is emphasized that the basic principle of the two test systems is different. In the phase-shift model the animals remain entrained, since they remain in light darkness. Only the direction of entrainment is the object of the study. Such an effect may be achieved shortly after the phase shift, maybe even during the first day. In the entrainment studies, entrainment must be imposed by the melatonin receptor agonist for the duration of the experiment. If the drug entrained free-running rhythms but also desensitized the system, the drug would appear to be ineffective.

Although GR196429 did not entrain the melatonin profile, the higher dose resulted in a much larger phase shift than did the lower dose, suggesting that the compound indeed interacted with the circadian system. That this interaction did not result in entrainment, could possibly be caused by an increase in the tau (τ) period. A similar effect was seen with GG-012, which resulted in a phase shift in 2 weeks that was similar to the phase shift after saline treatment for 4 weeks.

4.2. Amplitude variations

Data on changes in amplitude of circadian rhythms are scarce. Locomotor activity provides only poor information about amplitude. *N*-acetyltransferase activity is related to pineal melatonin production, but a quantitative correlation has not been proved and has even been called into question (Delgado et al. 1993). Plasma concentrations and excreted 6-sulphatoxy-melatonin show substantial variability and the possibility of interfering with extra-pineal production of melatonin is still a matter of debate (Yaga et al., 1993). Based on the data presented here, clear changes in the amplitude of pineal melatonin production can be monitored. All melatonin-treated animals showed a decrease in amplitude, while melatonin analogues generally increased the amplitude.

When the effects on amplitude are considered, two important questions arise. The first is about the mechanism behind the amplitude changes. The second regards the difference between melatonin on one hand and the synthetic receptor agonist on the other. All the synthetic analogues caused an increase in endogenous melatonin secretion, varying from almost significant to highly significant, while melatonin reduced the amplitude, an effect that appeared to be highly reproducible. Generally, less specific endogenous compounds elicit a wider variety of effects

than do more specific agonists. In this respect it is speculated that melatonin exerts both an inhibitory and a facilitating effect, of which the inhibitory dominates, whereas in the case of the synthetic agonists the inhibitory effect is either not present, or at least less pronounced, resulting in increased amplitude. Both possible mechanisms, that behind amplitude regulation and that accounting for the differences between agonists and melatonin are discussed below.

Melatonin is suggested to entrain rhythms by a direct action on the suprachiasmatic nuclei. One of the explanations for its amplitude effects could be a negative effect on the amplitude of suprachiasmatic nuclei rhythmicity, apart from an entraining effect on its phase. In this case, the differences between melatonin and the synthetic agonists could be based on differences in specificity for receptor subtypes. There are clear indications that various subtypes of the melatonin receptor do exist (Dubocovich, 1995; Dubocovich et al. 1997), with the mt_1 receptor, being present in the suprachiasmatic nuclei. An additional problem with direct action on the suprachiasmatic nuclei arises from the nature of signal transduction between suprachiasmatic nuclei and pineal gland. When the noradrenaline release profile was monitored in the pineal gland, it appeared to behave as a switch (Drijfhout et al. 1996c). It was proposed that there is a relay station between the suprachiasmatic nuclei and the pineal gland, which would allow signals from the suprachiasmatic nuclei to reach the pineal gland only after they reach a certain threshold level. With such a system, it would be difficult to propose a mechanism that would cause changes in amplitude, other than at, or downstream from, this relay station. These arguments make it unlikely that an amplitude effect is caused by a direct interaction with suprachiasmatic nuclei rhythmicity.

If the amplitude effects are receptor-mediated, a different interaction site is much more likely. In the pituitary, especially the pars tuberalis, a high melatonin receptor density has been reported for almost all mammals (Stankov et al., 1991). Because this area plays a major role in the endocrine system, interactions at this site could result in manipulation of plasma levels of a variety of hormones. Such a regulatory role in the endocrine system has been described repeatedly, including the stimulation of growth hormone production (Valcavi et al. 1993), the suppression of follicle stimulating hormone and luteinizing hormone (Wun et al. 1986; Vanecek and Klein, 1992), the inhibition of vasopressin and oxytocin (Yasin et al. 1993) and a delay of the seasonal rise in prolactin (Kaplan et al., 1991; Rozell and Mead, 1993). To explain the effects on amplitude, not only is an effect on plasma levels of various hormones necessary, but an effect of these hormones on pineal metabolism is a prerequisite. Very little is known about this, but interactions of the endocrine system with pineal melatonin production may be very interesting and need further research.

It is possible that the amplitude effects are not receptor-mediated. Intrapineal effects could explain the results found. Direct effects on the enzymes involved in the biosynthesis of melatonin, such as *N*-acetyltransferase, or hydroxy-indole-*O*-methyltransferase may occur. Clear data on this point are lacking, but if an intrapineal effect of melatonin did exist, *N*-acetyltransferase seems to be the most likely candidate as the target site, based on the crucial role this enzyme plays in most regulatory mechanisms involved in melatonin biosynthesis.

4.3. Clinical implications

Despite the difficulties involved in explaining the amplitude effects of the melatonin receptor agonists, it is a feature that may well be interesting from a clinical point of view. Many clinical developments are directed towards the use of these agonists in elderly people with sleeping problems. It is known that in elderly people, the endogenous production of melatonin is greatly reduced compared to that in young individuals. An increase in the amplitude of the melatonin profile might therefore be as beneficial, if not better than, as an entrainment of the phase. Several studies are underway on replacement of endogenous melatonin by controlled release of exogenous melatonin (Nelson et al., 1994; Haimov and Lavie, 1995; Benes et al. 1997). Special slow-release preparations are therefore used to mimic the normal profile. Based on the present data, a melatonin receptor agonist may be able to induce similar effects.

In conclusion, chronobiology often directs its attention towards the phase of rhythms. Because the circadian system itself is frequently the object subject of research, this will yield useful data. However, for the development of melatonin receptor agonists agents, while circadian data are very important, they must be accompanied by data on other physiological effects, such as the amplitude of melatonin production. The trans-pineal microdialysis technique offers unique possibilities to study both aspects of the new class of ‘‘circadian drugs’’ simultaneously.

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