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Studies on the vasoconstrictor action of melatonin and putative melatonin receptor ligands in the tail artery of juvenile Wistar rats

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1 In this study we compared the vasoconstrictor activity of melatonin in rat isolated tail artery using two different recording systems, the Halpern pressure myograph and the Halpern-Mulvany wire myograph, with the view to determining a reliable method for obtaining pharmacological data on vascular melatonin receptors. In addition, we characterized the melatonin receptor in this preparation, using analogues of melatonin, and examined the activity of various naphthalenic derivatives with biological activity in non-vascular models of melatonin receptors.

2 Using the Halpern pressure myograph, cumulative addition of melatonin (0.1 nM to 1 μM) produced direct vasoconstriction ($19.3 \pm 6.4\%$ reduction in lumen diameter, $n=5$) in five of 11 pressurized segments, with pEC_{50} of 9.14 ± 0.17. Similarly, non-cumulative application of melatonin caused vasoconstriction ($19.7 \pm 4.6\%$ reduction in lumen diameter, $n=7$) in seven of 20 preparations examined with pEC_{50} of 8.74 ± 0.26. The selective alpha$_2$-adrenoceptor agonist, UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate), produced vasoconstriction in all 'melatonin-insensitive' preparations.

3 Melatonin (0.1 nM to 1 μM) failed to elicit isometric contractions of tail artery segments in the Halpern wire myograph, but produced concentration-dependent potentiation of electrically-evoked, isometric contractions (maximum effect of 150–200% enhancement) when applied either non-cumulatively (seven of seven preparations) or cumulatively (four of seven preparations). The pEC_{50} value of melatonin (non-cumulative) was 8.50 ± 0.10 ($n=7$) which was not different from that obtained in the pressure myograph. All further experiments were conducted using a non-cumulative protocol against electrically-evoked, isometric contractions.

4 Based on the pEC_{50} values for the melatonin analogues examined, the pharmacological profile for the enhancement of electrically-evoked contractions was 2-iodomelatonin > 6-chloromelatonin $\geqslant$ (−)-AMMTC $\geqslant$ S21634 $\geqslant$ melatonin $\geqslant$ S20098 > S20242 $\geqslant$ S20304 > 6- hydroxymelatonin > S20932 > (+)-AMMTC > *N*-acetyl-5-HT. Our data suggests the vascular receptor belongs to the MEL$_1$-like subtype. All the indole-based analogues of melatonin, 2-iodomelatonin, (−)-AMMTC, (+)-AMMTC, S20932, 6-chloromelatonin, 6-hydroxymelatonin and *N*-acetyl-5-HT, behaved as full agonists. All the naphthalenic derivatives examined, S21634, S20098, S20242 and S20304 behaved as partial agonists relative to melatonin.

5 The naphthalenic-based antagonists, S20928 and S20929, did not modify electrically-evoked, isometric contractions of the tail artery, but produced a parallel, rightward displacement of the melatonin concentration-response curve. Based upon the effect of 1 μM S20928 and S20929, the estimated pK_B values for these antagonists were 7.18 ± 0.25 ($n=4$) and 7.17 ± 0.25 ($n=5$), respectively.

6 We demonstrated that enhancement of electrically-evoked, isometric contractions of the rat isolated tail artery (using the Halpern-Mulvany wire myograph) is a simple and reproducible model for assessing the activity of putative agonists, partial agonists and antagonists at vascular melatonin receptors. Pharmacological characterization of the receptor suggests the presence of a MEL$_1$-like subtype.

Keywords: Melatonin; 2-iodomelatonin; melatonin analogues; melatonin MEL$_1$-like receptors; vasoconstriction; rat tail artery; noradrenergic contractions

Introduction

Melatonin has been suggested to have multifunctional roles in influencing many major organs, eg. the immune system, endocrine glands and the cardiovascular system (Karsch *et al*., 1984; Nelson *et al*., 1995; Bertuglia *et al*., 1996). Based upon radioligand binding studies on membranes from various tissues, this action appears to be mediated through two pharmacologically distinct groups of receptors, MEL$_1$ and MEL$_2$ subtypes, which have been characterized largely on the basis of the rank order of affinity of melatonin and various indole-based derivatives (Dubocovich, 1991; Krause & Dubocovich, 1991).

In the case of the cardiovascular system, recent evidence has raised the possibility that part of the effect of melatonin may be exerted at the level of the vasculature, in addition to inputs from the central nervous system. First, specific binding sites (MEL$_1$-like) have been detected in cerebral arteries of rats and primates (Viswanathan *et al*., 1990; Stankov *et al*., 1992; Capsoni *et al*., 1994) and the tail artery of the rat (Viswanathan *et al*., 1990). The localization of these binding sites is discrete (there is no evidence for similar binding sites on either coronary artery, carotid artery or aorta of the rat) and this has been taken as evidence for a thermoregulatory role of the pineal hormone (Viswanathan *et al*., 1990; Saarela & Reiter, 1993). In support of this proposal, near physiological levels of melatonin (0.3 nM to 1 nM) have been reported to increase vascular tone in both isolated cerebral

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vessels (Geary *et al*., 1995; Mahle *et al*., 1995), the cerebral vascular bed (Capsoni *et al*., 1995) and the isolated tail artery (Evans *et al*., 1992). However, the precise nature of the vasoconstriction appears to be a function of the recording technique employed. In the case of the tail artery maintained under isometric tension, melatonin is devoid of direct vasoconstriction, but is able to potentiate contractions involving exogenous and endogenous (electrical field stimulation of noradrenaline release) activation of alpha-adrenoceptors (Viswanathan *et al*., 1990; Krause *et al*., 1995). In marked contrast, direct vasoconstriction to melatonin has been reported in both the tail (Evans *et al*., 1992) and cerebral arteries (Geary *et al*., 1995; Mahle *et al*., 1995) of the rat when vessels are pressurized and vascular tone measured as changes in lumen diameter. At present the basis of the difference between the two techniques is not known, but both appear to offer a relatively simple method for studying the vascular action of melatonin and characterizing the receptor involved.

Thus, the aims of the present study are twofold. First, to compare the direct vasoconstrictor activity of the melatonin in pressurized segments of the caudal artery from juvenile Wistar rats with the effect against electrically-evoked, isometric contractions in the preparation. Second, to characterize the vascular melatonin receptor using various indole-based analogues of melatonin and then to assess the activity of a range of naphthalenic derivatives which have been shown to possess agonist and antagonist activity in several non-vascular models for melatonin (see Depreux *et al*., 1994; Le Gouic *et al*., 1996; Ying *et al*., 1996).

Some of these results have been presented to the British Pharmacological Society (Ting *et al*., 1996, 1997).

Methods

Tissue preparation

Male juvenile (3–4 weeks old; 55–100 g weight) Wistar rats were housed (strain BKW, from colony maintained at Queen's Medical Centre, Nottingham, U.K.) in a 12 h light dark cycle (lights on at 8 am; lights off at 8 pm). Rats were usually killed 1–2 h after lights on by decapitation. The ventral artery of the tail was dissected and placed onto a dissecting disc immersed in gassed, modified Krebs-Henseleit (K-H) solution. The blood vessel was carefully cleaned from fat and connective tissues with the aid of a dissecting microscope (Nikon SMZ-2B, Japan), and divided into ring segments of 2–3 mm in length. Ring segments were either mounted on glass cannulae of the pressure myograph (Evans *et al*., 1992) or suspended between two supporting jaws in a stainless steel chamber of a Mulvany-Halpern wire-myograph and allowed to equilibrate for 30 min. Each preparation was bathed in K-H solution maintained at $34 \pm 1^{\circ}C$ and gassed with 95% O_2/5% CO_2. For the pressure myograph, one of the cannulae was connected to the pressure-servo system and the vessel slowly pressurised and maintained at 60 mmHg. Any change in length of the segment resulting from pressurization was adjusted with the rotating head micrometer attached to the pressure cannula to remove any buckling (Halpern *et al*., 1984). In the case of the wire myograph, tension was applied by adjusting the micrometer connected to one of the supporting jaws, while force was detected by an isometric transducer connected to the other jaw. The initial resting tension was 0.2–0.5 g weight and preparations allowed to relax to 0.1–0.4 g weight. Changes in either vessel diameter or isometric tension were recorded by a MacLab and displayed on a Macintosh computer.

Experimental procedure

The distal segment of the tail artery was examined in the pressure myograph. After 60 min of equilibration the pressurized segment was exposed to melatonin in one log unit increment from concentrations 0.1 nM to 1 μM by either cumulative or non-cumulative addition. In the case of the cumulative application of melatonin a minimum of 10 min was allowed between increments in concentration. For non-cumulative application of melatonin each concentration was examined for 7–10 min and the solution exchanged for K-H solution. A minimum of 40 min was allowed between periods of exposure to melatonin. In preparations that failed to respond to melatonin ('unresponsive' segments), UK14304, an α_2-adrenoceptors agonist (1 μM), was added into the bath to check the viability of the preparations.

In an earlier study we reported there was no difference between the responsiveness of either the proximal or distal end of the tail artery mounted onto the wire myograph (Ting *et al*., 1996). Therefore, the proximal segment of the tail artery was chosen for all subsequent studies. Vessels were contracted with KCl (60 mM) to assess tissue viability and provide a reference contracture for subsequent data analysis. A D330-Multisystem stimulator (Digitimer, U.K.) was used to deliver 5 s train of electrical pulses (10–20 V; 0.3 ms pulse width) at a frequency of 2–3 Hz every 4–5 min. The voltage and frequency of the electrical field stimulation were modified in the beginning of each set of experiments to obtain a contraction sized between 0.1 and 0.25 g weight (20–35% to KCl responses). Upon obtaining constant, 'baseline' neurogenic responses, cumulative and non-cumulative concentration-response curves (CRC) were constructed for melatonin. When the effects of various melatonin analogues were compared to that of melatonin, however, only a non-cumulative approach was adopted. This involved exposing the tissues to increasing concentrations (0.5 log unit increments) of the agonist until a maximum response was attained. The vessel was exposed to one concentration of any drug for a period of 20 min, and was left for 30 min between two washouts and the next addition of drug to prevent any desensitisation and to re-establish the stable, neurogenic contractions. During the course of some experiments there was a tendency for the neurogenic responses to decline slightly. If the reduction in these responses exceeded 20% of the 'baseline' value (prior to exposure to an agonist), then the voltage for electrical field stimulation was increased to re-establish the 'baseline' neurogenic responses. On each experimental day, two dual channel wire myographs were employed, with one segment of the tail artery from each animal used to construct the melatonin CRC while other (three) segments were exposed to a range of melatonin analogues.

For experiments involving putative antagonists, these agents were added at least 20 min before the construction of non-cumulative CRC of melatonin, and readded after the final washout between non-cumulative application of melatonin (20 min before the next addition of the agonist).

Data analysis

In the pressurized arterial segments, changes in the lumen diameter have been expressed as a percentage of the resting lumen diameter and are shown as mean $\pm$ s.e.mean. For studies involving isometric tension recordings responses have been expressed as percentage of the enhancement to the predrug, neurogenic contractions. The sensitivity of the preparations to the agonists examined was assessed as the negative logarithm of the concentration required to produce 50% of the maximum response (pEC_{50}) after the agonist concentration-effect (E/[A]) data were fitted to this formula:

$$E = \frac{\alpha[A]^n}{[A]^n + [A_{50}]^n}$$

where E is the response, α is the asymptote, [A] is the agonist concentration, n is the gradient of the E/[A] curve and $[A_{50}]$ is the mid-point of E/[A] curve (Black *et al*., 1985). $[A_{50}]$ values

represent agonist concentration giving 50% of the maximum responses and are shown as the negative logarithm (pEC_{50}). The maximum response of each analogue was expressed as a ratio of the maximum response to melatonin (E_{max}) obtained in segments of the artery from the same animal. For the antagonist experiments, the agonist concentration-ratio (CR) was determined in each experiment. The CR is the ratio of EC_{50} values of melatonin in the presence and absence of antagonist. The negative logarithm of the dissociation constant for the antagonist (pK_B) value was determined by the method of Furchgott (1972).

Differences between mean values have been compared using either paired or unpaired Student's *t* test (two-tailed) and were considered statistically significant if $P<0.05$.

Solutions and drugs

The composition of the K-H solution was (in mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. The following compounds were used: UK14304 [5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline bitartrate] (Pfizer); tetrodotoxin (Sigma); prazosin HCl (Sigma); L-phenylephrine HCl (Sigma); 6-hydroxymelatonin (Sigma); 2-iodomelatonin (RBI); KCl (Fisons); *N*-acetyl-5HT (Sigma); 6-chloromelatonin (ICN); (−) and (+) enantiomers of AMMTC (*N*-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydroarbazole) (synthesized by D. Sugden and colleagues, King's College London). In addition the following compounds were obtained from the Institut de Recherches Internationales, Servier France: S20098 (*N*-[2-(7-methoxy napth-1-yl)-ethyl]-acetamide); S20304 (*N*-[2-(7-methoxy napth-1-yl)-ethyl]-cyclopropane carboxamide); S20242 (*N*-[2-(7-methoxy napth-1-yl)-ethyl]-propionamide); S20932 (*N*-[2-(5-methoxy indol-3-yl)ethyl]*N*′-propyl urea); S21634 (*N*-[2-(3-ethyl-7-methoxynaphtyl)ethyl]-acetamide); S20928 (*N*-[2-napth-1-yl-ethyl]-cyclobutyl carboxamide) and S20929 (*N*-[2-napht-1-yl-ethyl]-cyclopropyl carboxamide). All drugs were dissolved in distilled water with the exception of melatonin, 6-hydroxymelatonin, 6-chloromelatonin, 2-iodomelatonin, *N*-acetyl-serotonin and all the Servier compounds (each prepared as 10 mM aliquots in 100% dimethylsuphoxide (DMSO)) and (−)- and (+)-AMMTC (prepared as 10 mM aliquots in 100% ethanol) and stored at −20°C until required. Further dilutions were freshly prepared each day with distilled water (except for the first dilution for the Servier drugs, which were prepared (1 mM) in 100% DMSO). With the exception of N-acetyl-5-HT the maximum concentration of the solvent in the organ bath never exceeded 0.1% v/v.

Results

Effect of melatonin in pressurized distal segments of isolated tail artery from juvenile rats

Cumulative application of melatonin produced a concentration-dependent vasoconstriction in five of the 11 tail arteries from juvenile Wistar rats (Figure 1). Responses were observed at 0.1 nM melatonin and the maximum response usually attained at 10 nM, with a pEC_{50} of 9.14 ± 0.17 ($n=5$). The maximum reduction in lumen diameter measured was $19.3\pm6.4\%$ of the resting lumen diameter (364.8 ± 30.8 μm). In all other 'melatonin-insensitive' preparations, UK14304 (1 μM) reduced the lumen diameter by $54.1\pm9.1\%$ ($n=6$) of the resting diameter (336 ± 19.1 μm). Non-cumulative addition of melatonin produced vasoconstriction in approximately 30% of preparations examined (seven of 20) (Figure 1). The maximum response ($19.7\pm4.6\%$, $n=7$) and the potency ($pEC_{50}=8.74\pm0.26$) were similar to that observed in the preparations exposed to cumulative application of melatonin. In view of the low success rate of the pressure myograph for detecting vasoconstrictor responses to melatonin, this method was not used for further experiments.

Effect of melatonin against electrically-evoked contractions in isolated proximal arterial segments of the juvenile rat tail artery

Electrical field stimulation (2–3 Hz, 0.3 ms, 10–20 V, 5 s) of the rat tail artery every 4–5 min caused a reproducible, transient contraction equivalent to $36.3\pm4.2\%$ of the response to 60 mM KCl (0.70 ± 0.08 g weight, $n=7$). Tetrodotoxin (300 nM) and prazosin (100 nM), an α_1-adrenoceptor antagonist, abolished electrically-evoked contractions of the rat isolated tail artery ($n=3$), indicating that these responses were mediated by the neuronal release of noradrenaline.

Non-cumulative addition of melatonin (0.1 nM to 1 μM) produced a concentration-dependent enhancement of the neurogenic response in seven of seven preparations (Figure 2), without causing direct vasoconstriction (<5% of the response to 60 mM KCl). Responses to low concentrations of melatonin (0.1–1 nM) were sustained but became transient at higher concentrations (3–100 nM). The largest enhancement of the neurogenic response at each concentration was used for generating the concentration–response curve for melatonin (Figure 3). The maximum effect by melatonin was a $178\pm30.3\%$

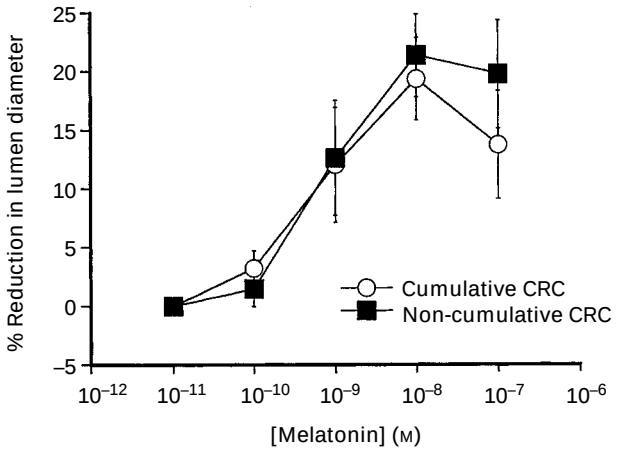

Figure 1 Cumulative ($n=5$) and non-cumulative ($n=7$) concentration–response curves (CRC) of melatonin in pressurized segments of the isolated caudal artery from juvenile Wistar rats. Vasoconstrictor responses (reduction in diameter) have been expressed as the percentage reduction in the resting lumen diameter and are shown as the mean±s.e.mean (vertical lines).

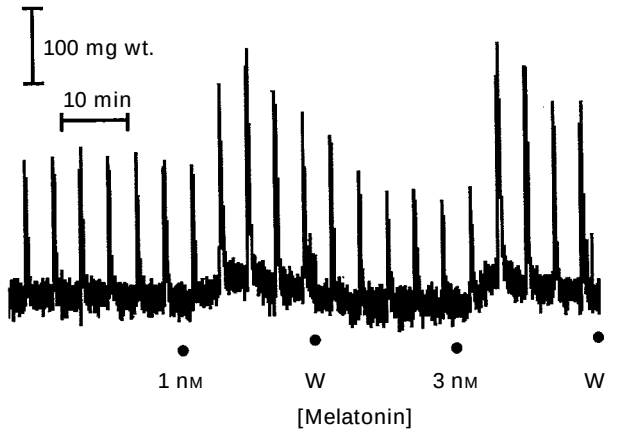

Figure 2 Representative trace recording of non-cumulative application of melatonin against electrically-evoked contractions (2–3 Hz, 5 s, 0.3 ms pulse width, 10–20 V) of isolated tail arteries from juvenile rats. W indicates washout (twice) between exposure to melatonin.

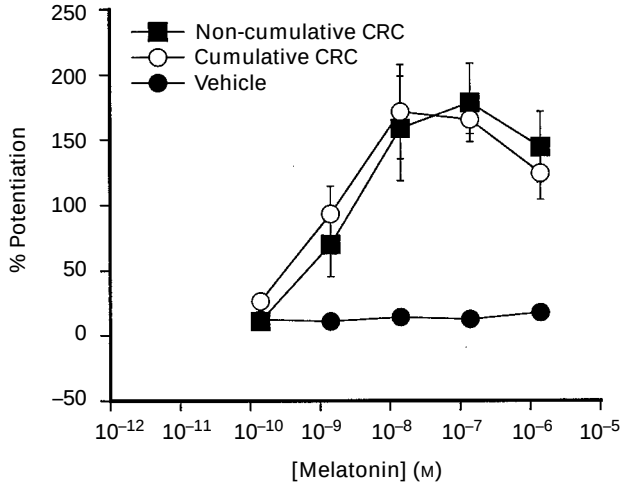

Figure 3 Effects of melatonin and vehicle ($n=10$) against electrically-evoked contractions of isolated tail arteries from juvenile rats. Cumulative ($n=4$) and non-cumulative ($n=7$) CRCs to melatonin in (7) preparations: four of seven preparations responded to the cumulative application of melatonin while all (seven of seven) preparations responded to the non-cumulative application of melatonin. Responses have been expressed as the percentage enhancement of the electrically-evoked contractions (measured prior to exposure to melatonin) and are shown as the mean ± s.e.mean (vertical lines). For the vehicle control preparations the s.e.mean were less than the size of the symbol.

($n=7$) potentiation of neurogenic responses, with a pEC_{50} value of 8.50 ± 0.10 ($n=7$). In marked contrast, cumulative addition of melatonin produced concentration-dependent enhancement of electrically-evoked responses in only four of seven preparations. However, the maximum response ($171 \pm 35.3\%$ potentiation of neurogenic responses, $n=4$) and the potency ($pEC_{50} = 8.70 \pm 0.22$, $n=4$) of melatonin was comparable to that observed with non-cumulative application of the hormone. Thus, a non-cumulative protocol for melatonin and various melatonin analogues was used in all further studies; Figure 3 also shows the lack of effect of the vehicle on the neurogenic responses using the non-cumulative protocol.

Pharmacological characterization of melatonin receptor on the rat tail artery

Figure 4a and b show 2-iodomelatonin, *N*-acetyl-5HT, (−)-AMMTC and (+)-AMMTC caused a concentration-dependent enhancement of neurogenic contractions qualitatively similar to that produced by melatonin. 2-Iodomelatonin was approximately 5 to 10-fold more potent than melatonin, which was equipotent with (−)-AMMTC, while (+)-AMMTC and *N*-acetyl-5HT possessed 1/150th and 1/3000th the potency of melatonin, respectively. Table 1 summarizes the results obtained with various melatonin analogues that mimicked the action of melatonin against neurogenic contractions. Based upon the pEC_{50} values the rank order of potency of all the agonists was 2-iodomelatonin > 6-chloromelatonin ⩾ (−) AMMTC ⩾ S21634 ⩾ melatonin ⩾ S20098 > S20242 ⩾ S20304 > 6-hydroxymelatonin > S20932 > (+) AMMTC > *N*-acetyl-5HT. A characteristic feature of the action of (−) and (+) AMMTC was that the concentration–response curves were bell-shaped, but they exhibited a 400-fold difference in potency (Figure 4b). 6-Chloromelatonin, 6-hydroxymelatonin and S20932 also behaved as full agonists (Table 1). In contrast, the maximum response produced by S20098 and S20304 (Figure 5), S21634 and S20242 (Table 1) were significantly less than that observed for melatonin and is taken as evidence that they behave as partial agonists. Qualitatively the characteristics of the enhancement of electrically-evoked responses produced by these agents were similar to that elicited by melatonin; the effect of submaximally-effective concentrations was sustained while

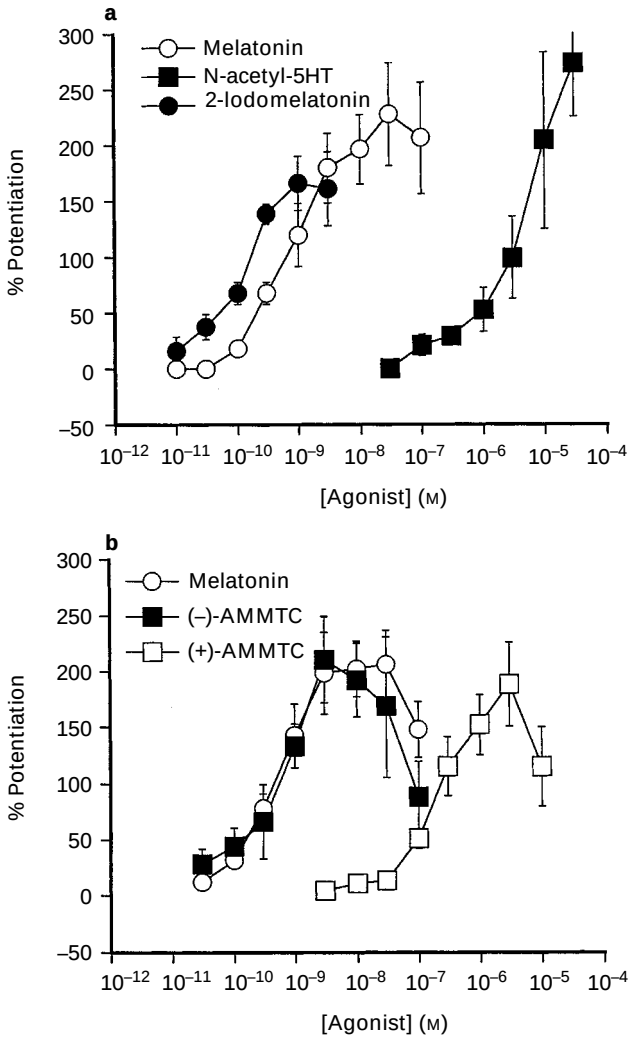

Figure 4 Effects of (a) melatonin ($n=10$), 2-iodomelatonin ($n=6$) and *N*-acetyl-5-HT ($n=4$); (b) melatonin ($n=7$), (+)-AMMTC ($n=6$) and (−)-AMMTC ($n=6$) on electrically-evoked contractions of the isolated tail arteries from juvenile rats. The control CRC to melatonin is the mean of data for separate experiments, each with a different degree of potentiation. The maximum potentiation values for each set of experiments are (a) melatonin ($172 \pm 27\%$) vs 2-iodomelatonin ($165 \pm 24\%$); melatonin ($296 \pm 42\%$) vs *N*-acetyl-5-HT ($273 \pm 48\%$) and (b) melatonin ($219 \pm 32\%$) vs (+)-AMMTC ($188 \pm 38\%$); melatonin ($212 \pm 35\%$) vs (−)-AMMTC ($210 \pm 38\%$). Responses have been expressed as the percentage enhancement of the electrically-evoked contractions (measured prior to exposure to the agonist) and are shown as the mean ± s.e.mean (vertical lines).

those to maximally-effective concentrations were subject to 'fade'. S20928 and S20929 (1–100 nM) did not significantly ($\pm 20\%$) modify per se neurogenic contractions ($n=3$).

Effects of S20928 and S20929 against melatonin-induced enhancement of neurogenic contractions

S20928 and S20929 were examined in greater detail as potential receptor antagonists. The concentrations employed were chosen on the basis of the IC_{50} values from competitive binding assays of the ovine pars tuberalis (Delagrange *et al*., 1995). As shown in Figure 6a, 1 and 10 μM S20928 caused a rightward displacement of the CRCs for melatonin with no significant change in the maximum response. Another putative antagonist, S20929 (1 μM), also produced a parallel rightward shift of the melatonin CRC without altering the maximum response (Figure 6b). The estimated pK_B values for S20928 (7.18 ± 0.25, $n=4$) and S20929 (7.17 ± 0.25, $n=5$), based on the effect of 1 μM of both agents, were similar.

Discussion

Vascular responses to melatonin: methodological considerations

In an earlier report from this laboratory melatonin, and the selective alpha$_2$-adrenoceptor agonist, UK-14304, were shown to produce concentration-dependent contractions in 'pressurized' segments of the rat isolated tail artery examined (Evans *et al*., 1992). We argued that the Halpern pressure myograph represented a better method for the study of vascular melatonin receptors compared to the more conventional isometric preparations, since the use of the latter required that the preparation be preconstricted to uncover vascular responses to melatonin (see Viswanathan *et al*., 1990). In the present study, however, cumulative application of melatonin (0.1 – 100 nM) produced direct vasoconstriction in only 50% of preparations examined with the Halpern pressure myograph. The viability of the 'melatonin-unresponsive' preparations was assured by the ability of UK-14304 to elicit pronounced vasoconstriction. Significantly, the percentage of 'melatonin-responsive' preparations was not increased by adopting a non-cumulative protocol (as used by Evans *et al*., 1992), thereby discounting receptor desensitization as an explanation for the low success rate. Thus, in contradiction of our earlier study (Evans *et al*., 1992), the findings herein suggest the Halpern pressure myograph may not be optimal, at least under the current conditions, for examining the pharmacological characteristics of melatonin receptors on the rat tail artery.

On the other hand, we noted that while the non-cumulative application of melatonin failed to elicit direct vasoconstriction of the tail artery maintained under isometric tension (using the Halpern-Mulvany wire myograph), it caused a concentration-dependent enhancement of noradrenergic contractions in 100% of preparations examined. These observations provide confirmation of the earlier findings of Krause *et al*. (1995) and

Table 1 Pharmacological profile of melatonin receptor in the rat tail artery including the mean pEC_{50} and relative E_{max} (compared to melatonin) values of various putative melatonin agonists

Agonist	*pEC_{50}*	*Relative E_{max}*	*Relative potency*
2-Iodomelatonin ($n=6$)	9.70±0.12	0.96	0.1
6-Chloromelatonin ($n=5$)	9.27±0.22	0.89	0.9
(−)-AMMTC ($n=6$)	9.23±0.15	1.02	0.4
S21634 ($n=8$)	9.06±0.19	0.67*	0.6
Melatonin ($n=30$)	8.89±0.07 (8.15−9.61)	1	1
S20098 ($n=7$)	8.84±0.26	0.68*	1
S20242 ($n=7$)	8.11±0.13	0.65*	6
S20304 ($n=8$)	7.90±0.05	0.49*	7
6-Hydroxymelatonin ($n=5$)	7.59±0.16	0.92	15
S20932 ($n=8$)	6.95±0.12	0.88	75
(+)-AMMTC ($n=6$)	6.64±0.16	0.91	150
N-acetyl-5-HT ($n=4$)	5.72±0.12	0.93	3000

Upper and lower limits of the mean pEC_{50} values for melatonin from several series of experiments are shown. The EC_{50} of each agonist has been expressed relative to the corresponding EC_{50} value of melatonin (relative potency). The number of observations are shown in parentheses. *$P<0.05$, agonist vs melatonin (by two-tailed paired Student's *t* test).

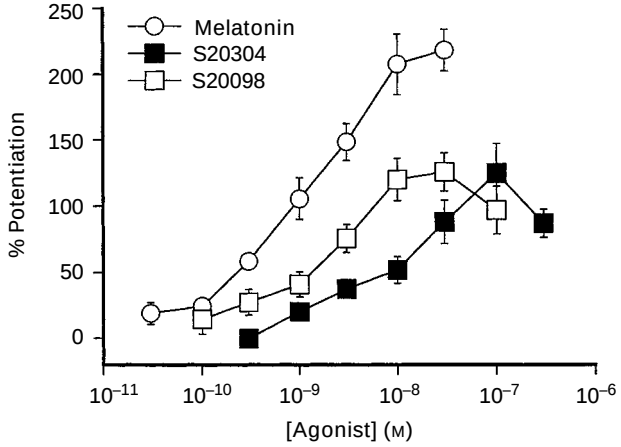

Figure 5 Effects of melatonin ($n=15$), S20098 ($n=7$) and S20304 ($n=8$) on electrically-evoked contractions of the isolated tail arteries from juvenile rats. The control CRC to melatonin is the mean of data for separate experiments, each with a different degree of potentiation. The maximum potentiation values for each set of experiments are melatonin (182±19%) vs S20098 (125±14%) and melatonin (254±17%) vs S20304 (124±22%). Responses have been expressed as the percentage enhancement of the electrically-evoked contractions (measured prior to exposure to the agonist) and are shown as the mean±s.e.mean (vertical lines).

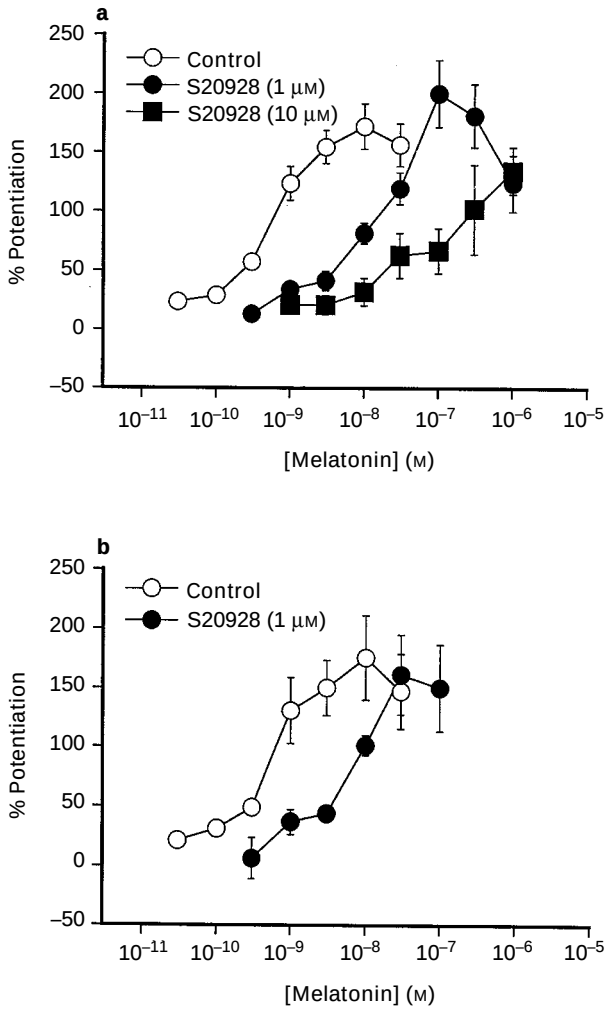

Figure 6 Effects of putative melatonin antagonists. (a) S20928 (1 μM; $n=4$), (10 μM; $n=4$) and melatonin ($n=8$). (b) S20929 (1 μM; $n=5$) and melatonin ($n=5$) on electrically-evoked contractions of juvenile rat tail arteries. Responses have been expressed as the percentage enhancement of the electrically-evoked contractions (measured prior to exposure to melatonin) and are shown as the mean±s.e.mean (vertical lines).

indicate that this is a reliable method for assessing vascular melatonin receptor function. A characteristic feature of the action of melatonin was that low concentrations (0.1–1 nM) produced a sustained potentiation over 15–20 min, but for higher concentrations (>3 nM) this effect was transient, with the maximum effect occurring 5–10 min after exposure. The non-sustained nature of responses to maximal and supramaximal concentrations of melatonin may be a function of receptor desensitization, with too little time allowed between exposures. In this regard, it is noteworthy that cumulative application of melatonin was associated with only 50–60% success rate against electrically-evoked contractions (Figure 3) and phenylephrine-induced tone (Ting *et al*., 1996). Alternatively, it is possible that the effect of high concentrations of melatonin in the tail artery may be the result of the vasoconstrictor action and a receptor or non-receptor-mediated vasodilator effect, as has been observed in the rabbit aorta (Satake *et al*., 1991), rat aorta (Weekley, 1995) and marginal mesenteric artery of the pig colon (unpublished results).

Krause *et al*. (1995) attributed the effect of melatonin on neurogenic responses to a postjunctional action as equivalent responses to noradrenaline were similarly affected. If this is the case, the discrepancy between the findings on the Halpern-Mulvany wire myograph and the Halpern pressure myograph noted above may indicate that direct vasoconstriction and enhancement of neurogenic responses by melatonin involve different (postjunctional) mechanisms. This possibility is supported by preliminary observations, using segments of the tail artery from the same animal, where zero of four preparations in the pressure myograph responded to melatonin while neurogenic contractions in four of four preparations in the Halpern-Mulvany wire myograph were increased by melatonin (unpublished results). Additional studies are clearly warranted to elucidate the mechanism(s) involved.

Vascular responses to melatonin: pharmacological considerations

Radioligand binding studies using [^{125}I]-2-iodomelatonin to label membranes of various tissues, and a range of indole-based analogues of melatonin, have revealed the presence of two major subtypes of melatonin binding sites (for a review, see Morgan *et al*., 1994). At the putative MEL_1 subtype the rank order of potency of key ligands is 2-iodomelatonin > 6-chloromelatonin ≥ melatonin > 6-hydroxymelatonin > > *N*-acetyl-5-HT, while at the putative MEL_2 subtype the rank for the same ligands is 6-chloromelatonin ≥ 2-iodomelatonin > *N*-acetyl-5HT ≥ melatonin ≥ 6-hydroxymelatonin (see also Duncan *et al*., 1988; Dubocovich, 1995). Recently it has been reported that the human cloned melatonin receptor subtypes, MEL_{1A} and MEL_{1B}, can be distinguished using a range of melatonin antagonists and partial agonists (Dubocovich *et al*., 1997). All of those melatonin analogues tested were shown to have higher selectivity for the MEL_{1B} melatonin receptor subtype (Dubocovich *et al*., 1997). However, there are no reports of melatonin agonists with high selectivity for either subtype.

All of the indole-based analogues tested in the present study behaved as full agonists in potentiating neurogenic contractions in rat tail artery, (without altering basal tone). The rank order of potency clearly reveals that the melatonin receptor involved in this response belongs in the MEL_1 subgroup. Of particular note is that 2-iodomelatonin is the most potent agonist, followed by 6-chloromelatonin and melatonin, which are roughly equipotent. 6-Hydroxymelatonin, a melatonin metabolite, is less potent than melatonin while the potency of the melatonin precursor, *N*-acetyl-5-HT, is roughly three orders of magnitude lower. This pharmacological profile, 2-iodomelatonin > 6-chloromelatonin ≥ melatonin ≤ 6-hydroxymelatonin > *N*-acetyl-5-HT, is similar to that found for [^{125}I]-2-iodomelatonin binding sites in the tail artery (Viswanathan *et al*., 1990), amphibian melanophores (Sugden, 1991), chicken retina (Dubocovich & Takahashi, 1987) and ovine pars tuberalis (Caignard *et al*., 1995), preparations with MEL_1-like melatonin binding sites/receptors. Our findings clearly support the earlier study of Krause *et al*. (1995) on the pharmacological characteristics of melatonin receptors that enhance neurogenic contractions of the rat tail artery, which was based on a less extensive range of indole-based melatonin analogues. Another important observation is that the (−) and (+) isomers of AMMTC behaved as full agonists but exhibited a 400-fold difference in potency. (−)-AMMTC has been reported to be about 130-fold and 230-fold more potent than (+)-AMMTC against [^{125}I]-2-iodomelatonin binding to chick brain membranes and pigment aggregation of *Xenopus* melanophores, respectively (Sugden *et al*., 1995). Thus, these enantiomeric isomers underline the essential similarity of vascular and non-vascular MEL_1-like receptors, and highlight the potential advantage they offer for identifying functional melatonin receptors.

The Servier compounds, S20098, S21634, S20242, S20304 and S20932, enhanced neurogenic contractions of the tail artery without modifying basal vascular tone. S21634 and S20098 exhibited similar potency to melatonin, while S20242 and S20304 were approximately one-fifth as potent as melatonin. S20932 was the least potent derivative (1/70th the potency of melatonin). These findings are consistent with earlier reports that S20098 (Redman *et al*., 1995; Martinet *et al*., 1996; Ying *et al*., 1996), S20242 (Koster-Van Hoffen *et al*., 1993) and S20304 (Guardiola-Lemaitre & Delagrange, 1995) are agonists at melatonin receptors. With the exception of S20932, which, interestingly, is an indole-based derivative, none of the above agents behaved as a full agonist. This raises the intriguing possibility that for S20098, S21643, S20242 and S20304, the inclusion of a naphthalenic group to replace the indole moiety, which increases the biological half-life of these compounds (Depreux *et al*., 1994), may result in reduced efficacy at vascular melatonin receptors. This observation stands in marked contrast to other functional models, e.g. inhibition of forskolin-stimulated cyclic AMP accumulation of the ovine par tuberalis (see Depreux *et al*., 1994), where many of these agents appears to behave as full agonists.

The naphthalenic derivatives, S20928 and S20929, are highly specific for melatonin binding sites and have been characterized as antagonists by their ability to inhibit (1) melatonin-induced inhibition of forskolin-stimulated cyclic AMP production in culture ovine pars tuberalis cells, and (2) melatonin-induced aggregation of pigment granules in the cultured melanophores (for references, see Delagrange *et al*., 1995). In addition, S20928 has been shown to block the effect of melatonin in suppressing the neuronal firing activity of suprachiasmatic nucleus *in vivo* (Ying *et al*., 1996) and prevent body weight gain induced by short photoperiods, a model thought to involve physiological regulation of melatonin levels (Guardiola-Lemaitre & Delagrange, 1995; Le Gouic *et al*., 1996). Neither agent enhanced neurogenic contractions of the rat isolated tail artery but (at a concentration of 1 μM) were capable of producing a competitive antagonism of the effect of melatonin. Higher concentrations of S20928 produced a further rightward displacement of the melatonin concentration response curve but, unlike that reported in the hamster suprachiasmatic nucleus (Ying *et al*., 1996), failed to exhibit any evidence of mixed agonism/antagonism activity. Based upon the estimated pK_B values, S20928 and S20929 were equipotent antagonists (7.18 and 7.17, respectively) at vascular melatonin receptors in the rat. Further studies with these agents are clearly warranted to establish whether they also antagonise melatonin-induced (direct) vasoconstriction of the tail artery (Evans *et al*., 1992; see also Figure 1) or middle cerebral artery (Geary *et al*., 1995) of the rat.

Taken together, the findings of the present study underline the value of the present model for discriminating between full agonists, partial agonists and antagonists at vascular melatonin receptors, and for quantitatively assessing their potency. It is noteworthy that most functional models for melatonin receptors do not permit a detailed comparison of concentra-

tion–response curves (Morgan *et al*., 1989; Depreux *et al*., 1994). Furthermore, biological demonstration of antagonism at melatonin receptors is usually limited to the use of single concentration of an agonist and a putative antagonist and is, therefore, semi-quantitative (see Morgan *et al*., 1994).

In conclusion, we have shown that the biological activity of melatonin in the rat tail artery is most easily quantified by examining the effect of the hormone against neurogenic contractions, rather than by assessing the direct vasoconstrictor activity. Furthermore, using a range of indole-based analogues of melatonin, the pharmacological characteristics of the receptor appear to belong to the MEL_1-like group of receptors, which is also sensitive to naphthalenic-derivatives that possess agonist and antagonist activity.

References

BERTUGLIA, S., MARCHIAFAVA, P.L. & COLANTUONI, A. (1996). Melatonin prevents ischaemia reperfusion injury in hamster cheek pouch microcirculation. *Cardiovasc. Res*., **31,** 947–952.

BLACK, J.W., LEFF, P. & SHANKLEY, N.P. (1985). An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br. J. Pharmacol*., **84,** 561–571.

CAIGNARD, D., LESIEUR, D., DEPREUX, P., RENARD, P., DELAGRANGE, P. & GUARDIOLA-LEMAITRE, B. (1995). Structure-activity relationships of melatonin analogues. *Eur. J. Med. Chem*., **30,** S637–S642.

CAPSONI, S., STANKOV, B.M. & FRASCHINI, F. (1995). Reduction of regional cerebral blood flow by melatonin in young rats. *Neuroendocrinology*, **6,** 1346–1348.

CAPSONI, S., VISWANATHAN, M., DE OLIVERIA, A.M. & SAAVEDRA, J.M. (1994). Characterization of melatonin receptors and signal transduction system in rat arteries forming the circle of willis. *Endocrinology*, **135,** 373–378.

DELAGRANGE, P., RENARD, P., CAIGNARD, D.H. & GUARDIOLA-LEMAITRE, B. (1995). Development of melatonin analogs. In *The Pineal Gland and its Hormone*. ed. Fraschini, F. pp. 139–153. New York: Plenum Press.

DEPREUX, P., LESIEUR, D., MANSOUR, H.A., MORGAN, P., HOWELL, H.E., RENARD, P., CAIGNARD, D.-H., PFEIFFER, B., DELAGRANGE, P., GUARDIOLA, B., YOUS, S., DEMARQUE, A., ADAM, G. & ANDRIEUX, J. (1994). Synthesis and structure-activity relationships of novel naphthalenic and bioisosteric related amidic derivatives as melatonin receptor ligands. *J. Med. Chem*., **37,** 3231–3239.

DUBOCOVICH, M.L. (1991). Pharmacological characterization of melatonin binding sites. *Adv. Pineal Res*., **5,** 167–173.

DUBOCOVICH, M.L. (1995). Melatonin receptors: Are there multiple subtypes? *Trends Pharmacol*., **16,** 50–56.

DUBOCOVICH, M.L. & TAKAHASHI, J.S. (1987). Use of 2-[125]iodomelatonin to characterize melatonin binding sites in chicken retina. *Proc. Natl. Acad. Sci. U.S.A*., **84,** 3916–3920.

DUBOCOVICH, M.L., MASANA, M.I., IACOB, S. & SAURI, D.M. (1997). Melatonin receptor antagonists that differentiate between the human Mel_{1a} and Mel_{1b} recombinant subtypes are used to assess the pharmacological profile of the rabbit retina ML_1 presynaptic heteroceptor. *Naunyn-Schmiedeberg's Arch. Pharmacol*., **355,** 365–375.

DUNCAN, M.J., TAKAHASHI, J.S. & DUBOCOVICH, M.L. (1988). 2-[125]Iodomelatonin binding sites in hamster brain membranes: pharmacological characteristics and regional distribution. *Endocrinology*, **122,** 1825–1833.

EVANS, B.K., MASON, R. & WILSON, V.G. (1992). Evidence for direct vasoconstriction activity of melatonin in 'pressurized' segments of isolated caudal artery from juvenile rats. *Naunyn-Schmiedberg's Arch. Pharmacol*., **346,** 362–365.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*, Vol. 33. *Catecholamines*. ed. Blaschko, H. & Muscholl, E. pp. 283–335. Berlin: Springer.

GEARY, G.G., DUCKLES, S.P. & KRAUSE, D.N. (1995). Melatonin is a direct vasoconstrictor in cerebral arteries. *Soc. Neurosci. Abstr*., **21,** 157.

GUARDIOLA-LEMAITRE, B. & DELAGRANGE, P. (1995). Melatonin analogues: from pharmacology to clinical application. *Eur. J. Med. Chem*., **30,** S644–S651.

HALPERN, W., OSOL, G. & COY, G.S. (1984). Mechanical behavior of pressurized in vitro prearteriolar vessels determined with a video system. *Ann. of Biochem. Engin*., **12,** 463–479.

KARSCH, F.J., BITTMAN, E.L., FOSTER, D.L., GOODMAN, R.L., LEGAN, S.J. & ROBINSON, J.E. (1984). Neuroendocrine basis of seasonal reproduction. *Recent Proc. Horm. Res*., **40,** 185–225.

KOSTER-VAN HOFFEN, G.C., MIRMIRAN, M., BOS, N.P.A., WITTING, W., DELAGRANGE, P. & GUARDIOLA-LEMAITRE, B. (1993). Effects of a novel melatonin analog of circadian rhythms of body temperature and activity in young, middle-aged and old rats. *Neurobiol. Aging*, **14,** 565–569.

KRAUSE, D.N., BARRIOS, V.E. & DUCKLES, S.P. (1995). Melatonin receptors mediate potentiation of contractile responses to adrenergic nerve stimulation in rat caudal artery. *Eur. J. Pharmacol*., **276,** 207–231.

KRAUSE, D.N. & DUBOCOVICH, M.L. (1991). Melatonin receptors. *Ann. Rev. Pharmacol. Toxicol*., **31,** 549–568.

LE GOUIC, S.L., DELAGRANGE, P., ATGIE, C., NIBBELINK, M., HANOUN, N., CASTEILLA, L., RENARD, P., LESIEUR, D., GUARDIOLA-LEMAITRE, B. & AMBID, L. (1996). Effects of both a melatonin agonist and antagonist on seasonal changes in body mass and energy intake in the garden dormouse. *Inter. J. Obesity*, **20,** 661–667.

MAHLE, C.D., GOGGINS, G.D. & YOCCA, F.D. (1995). Melatonin modulates vascular tone in rat anterior cerebral artery. *Soc. Neurosc. Abstr*., **21,** 183.

MARTINET, L., GUARDIOLA-LEMAITRE, B. & MOCAER, E. (1996). Entrainment of circadian rhythms by S20098, a melatonin agonist, is dose and plasma concentration dependent. *Pharmacol. Biochem. Behav*., **54,** 713–718.

MORGAN, P.J., BARRETT, P., HOWELL, H.E. & HELLIWELL, R. (1994). Melatonin receptors: localization, molecular pharmacology and physiological significance. *Neurochem. Intern*., **24,** 101–146.

MORGAN, P.J., LAWSON, W., DAVIDSON, G. & HOWELL, H.E. (1989). Melatonin inhibits cyclic AMP production in cultured ovine pars tuberalis cells. *J. Mol. Endocrinol*., **3,** R5.

NELSON, R.J., DEMAS, G.E., KLEIN, S.L. & KRIEGFELD, L.J. (1995). The influence of season, photoperiod and pineal melatonin on immune function. *J. Pineal Res*., **19,** 149–165.

REDMAN, J.R., GUARDIOLA-LEMAITRE, B., BROWN, M., DELAGRANGE, P. & ARMSTRONG, S.M. (1995). Dose-dependent effects of S20098, a melatonin agonist, on direction of re-entrainment of rat circadian rhythms. *Psychopharmacology*, **118,** 385–390.

SAARELA, S. & REITER, R.J. (1993). Function of melatonin in thermoregulation processes. *Life Sci*., **54,** 295–311.

SATAKE, N., OE, H. & SHIBATA, S. (1991). Vasorelaxing action of melatonin in rat isolated aorta: possible endothelium dependent relaxation. *Gen Pharmacol*., **22,** 1127–1133.

STANKOV, B., CAPSONI, S., LUCINI, V., FAUTECK, J., GATTI, S., GRIDELLI, B., BIELLA, G., COZZI, B. & FRASCHINI, F. (1992). Autoradiographic localization of putative melatonin receptors in the brain of two old world primates: *Cercopithecus aethiops* and *Papio ursinus*. *Neuroscience*, **52,** 459–468.

SUGDEN, D. (1991). Aggregation of pigment granules in single cultured *Xenopus laevis* melanophores by melatonin analogues. *Br. J. Pharmacol*., **104,** 922–927.

SUGDEN, D., DAVIES, D.J., GARRATT, P.J., JONES, R. & VONHOFF, S. (1995). Radioligand binding affinity and biological activity of the enantiomers of a chiral melatonin analogue. *Eur. J. Pharmacol*., **287,** 239–243.

TING, K.N., SCALBERT, E., DELAGRANGE, P. & WILSON, V.G. (1996). The effect of melatonin against agonist-induced and neurogenic contractions of tail arteries from juvenile Wistar rats. *Br. J. Pharmacol*., **119,** 113P.

TING, K.N., DAVIES, D.J., SCALBERT, E., DELAGRANGE, P., SUGDEN, D. & WILSON, V.G. (1997). Constant lighting does not affect the functional response of ML_1-like receptor in the tail artery of juvenile Wistar rats. *Br. J. Pharmacol*., **120,** 45P.

VISWANATHAN, M., LAITINEN, J.T. & SAAVEDRA, J.M. (1990). Expression of melatonin receptors in arteries involved in thermoregulation. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 6200–6203.

WEEKLEY, L.B. (1995). Pharmacological studies on the mechanism of melatonin-induced vasorelaxation in rat aorta. *J. Pineal Res.*, **19,** 133–138.

YING, S.W., RUSAK, B., DELAGRANGE, P., MOCAER, E., RENARD, P. & GUARDIOLA-LEMAITRE, B. (1996). Melatonin analogues as agonists and antagonists in he circadian system and other brain areas. *Eur. J. Pharmacol.*, **296,** 33–42.

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