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EFFECTS OF MELATONIN ON SLEEP AND NEUROCHEMISTRY IN THE RAT

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1 The effects of intraperitoneally administered melatonin on sleep and brain neurochemistry in the rat were studied by use of EEG recording and standard fluorescence techniques.

2 Melatonin, 10 mg/kg, reduced time to sleep onset and time spent awake but increased both slow wave and paradoxical sleep. Qualitatively similar but smaller effects were produced by a dose of 2.5 mg/kg.

3 Neither dose of melatonin altered normal EEG patterns or disrupted normal sleep behaviour.

4 Melatonin, 20 mg/kg, did not significantly alter concentrations of tryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, noradrenaline or dopamine in any part of the brain.

5 It is concluded that the sleep promoting activity of melatonin cannot be related to gross changes in brain indoleamine and catecholamine levels.

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is thought to be the primary indoleamine hormone produced by the pineal gland. Although a universal physiological rôle of melatonin in mammals has not been established, there is growing evidence that in some species it serves to transduce photoperiodic information (Reiter, 1980). Many of the effects of melatonin may occur through an action on the central nervous system (Quay, 1974; Reiter, 1977), indeed, melatonin administration has often been claimed to have psychopharmacological effects. In man there is considerable anecdotal evidence (Lerner & Nordlund, 1975) that the major effect is a mild sleepiness or sedation. However, using polygraphic techniques to define sleep stages after night-time administration of melatonin, two groups of workers have reported conflicting results: Cramer, Rudolph, Consbruch & Kendel (1974) and Cramer, Bohme, Kendel & Donnadieu (1976) have reported a specific reduction in sleep latency with no other significant changes, whereas Fernandez-Guardiola & Anton-Tay (1974) found a large increase in light sleep (slow wave stage 2) at the expense of the deeper stages (3 and 4) and a significant increase in the number of awakenings during the night.

Few studies on the effects of melatonin in animals have used polygraphic techniques or attempted to classify the EEG into the different sleep stages. In young chicks, melatonin increased slow wave sleep (S) during the first hour after injection but delayed the appearance of paradoxical or rapid eye movement sleep (PS) (Hishikawa, Cramer & Kuhlo, 1969). In contrast, Chamblin (1973) found that a large dose of melatonin did not significantly alter the awake (W), S or PS time in the cat at any time up to 30 h after administration.

This paper describes the effects of melatonin administration on the rat sleep-wake cycle, defined using chronic EEG recording techniques, and attempts to relate its sleep promoting action to changes in the concentration of brain indoles and catecholamines.

Methods

Sleep studies

Male Sprague-Dawley rats (200–250 g, Roche) were anaesthetized with pentobarbitone sodium (60 mg/kg, i.p.) and prepared for EEG recording by implanting 3 small stainless steel screw electrodes (Everbright, CHHD M 1.6 × 4) through the skull onto the surface of the cerebral cortex as described by Timo-Iaria, Negrâo, Schmidek, Hoshino, De Menezes & Da Rocha (1970). Also, a fine Trimel insulated stainless steel wire electrode (Johnson Matthey Metals, 0.4 mm diameter) was placed with its tip in the left hippocampus (stereotaxic co-ordinates: AP + 3.7, L 3.7, horizontal + 1.5, from Konig & Klippel, 1970). The electrodes were connected to a miniature 4-pin strip connector (ITT Cannon,

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MTAI 120) and the whole assembly was fixed to the skull with dental cement (Simplex Rapid). Following surgery each rat was housed singly in the environment in which EEG recordings were later made and was allowed at least one week to recover before being used in an experiment. Food (Labsure 41B) and water were available *ad libitum*, room temperature was maintained at 20 ± 2°C, lights were on from 08 h 00 min to 20 h 00 min and the light intensity at cage level varied from 100 to 140 lx.

In all experiments recordings were made on a Grass model 7B polygraph using 7P5B wide band a.c. EEG pre-amplifiers and a chart speed of 3 mm/s. Recordings were made on 3 consecutive days (pre-drug, drug and post-drug) from 12 h 00 min to 16 h 00 min. At the start of every recording session each rat was weighed, connected to the recording cable, injected intraperitoneally and returned to its home cage. All rats were adapted to these recording conditions on at least 3 days prior to experimental use. The melatonin vehicle was injected on pre-drug and post-drug days.

Cortical and hippocampal EEG's were scored visually by one investigator who did not know what treatment each rat had received. Epochs of 20 s were used and recordings were classified into 3 states: W, S and PS (Timo-Iaria *et al.*, 1970). Time to sleep onset (TSO) was defined as the time elapsed between injection and the time at which 5 min of S had accumulated. Time to paradoxical sleep onset (TPSO) was the time from injection to the occurrence of the first PS episode.

The Wilcoxon matched-pairs, signed-ranks test (Colquhoun, 1971) was used to compare sleep data from the pre-drug and drug days.

Neurochemical studies

From 13 h 30 min alternate members of each group of 10–12 male Sprague-Dawley rats (160–185 g, Roche) were injected intraperitoneally with either melatonin, 20 mg/kg, or vehicle. Sixty min later each rat was stunned, decapitated and its brain removed and dissected on a chilled glass plate into seven region designated 'cortex' comprised the forebrain Iversen (1966). Each region was frozen in less than 4 min on a stainless steel plate set in dry ice. The region designated 'cortex' comprized the forebrain remaining after removal of the striatum and hippocampus. Each region was assayed within 24 h of death for either tryptophan or 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and melatonin or noradrenaline (NA) and dopamine by use of fluorescent techniques and a Perkin Elmer model 204 fluorescence spectrophotometer and 1 × 1 cm quartz cuvettes.

Brain tryptophan was determined as described by Madras, Cohen, Messing, Munro & Wurtman (1974) and NA and dopamine by the method of Shellenberger & Gordon (1971). Brain 5-HT, 5-HIAA and melatonin were determined fluorometrically after reaction with O-pthaldialdehyde (OPT). Briefly, after weighing, each brain region was homogenized in 3.5 ml of acidified *n*-butanol (0.85 ml concentrated HCl per litre). After centrifugation, 3 ml of supernatant was added to 5 ml of cylohexane and 0.5 ml of 0.1 M HCl containing L-cysteine (1 mg/ml). Each sample was shaken by hand for 5 min. After separation of the phases, 0.3 ml of the aqueous layer was taken for 5-HT determination whilst 6.8 ml of the organic phase was added to 0.9 ml of 0.5 M sodium phosphate buffer pH 7.0 (pH titrated with 5N NaOH) and each tube was shaken for a further 10 min; 0.7 ml of the aqueous layer was then taken for 5-HIAA determination. Six ml of the remaining organic phase was warmed (≤ 40°C) and dried under a stream of nitrogen and the residue was dissolved in 1 ml of petroleum ether (30–40°C fraction). Two ml of 2 M HCl was then added and each tube was shaken by hand for 5 min; 1.75 ml of the aqueous layer was taken for melatonin determination.

5-HT and 5-HIAA fluorescence was developed by heating the samples at 80°C for 20 min after the addition of 2 ml of concentrated HCl containing OPT (0.05 mg/ml). After cooling to room temperature, the samples were read at excitation and emission wavelengths of 365 and 470 nm respectively. The tissue blank procedure described by Korf (1969) was used for both these compounds. Melatonin fluorescence was developed by adding 0.6 ml of OPT solution (0.196 mg/ml in 2 M HCl) to each sample and heating at 80°C for 20 min; after cooling the samples were read at excitation and emission wavelengths of 355 and 465 nm respectively. No tissue blanks were prepared for melatonin.

The minimum amount of melatonin which could be detected was 1.3 ng; this gave a fluorescence reading which was 2 standard deviations greater than the reagent blank. Reaction of melatonin with OPT in dilute HCl (2 M or less) gave maximal fluorescence (De Leiva & Schwarz, 1976). Under these conditions hydroxyindoles do not react; 5-HT (1.5% of melatonin), 5-hydroxytryptophan (0.4%), 5-HIAA (< 0.1%) and tryptophan (< 0.01%) gave only weak fluorescence as did the melatonin metabolites 6-hydroxymelatonin, N-acetyl 5-methoxyformyl kynurenamine (AMFK) and N-acetyl 5-methoxy kynurenamine (AMK) (< 0.001, 0.1 and 16% of melatonin respectively). Of 7 other indoles only 5-methoxytryptamine (58%), 5-methoxyindoleacetic acid (9%) and 5-methoxytryptophol (186%) gave significant fluorescence. The former two compounds, if present in tissue extracts, would be removed during extraction along with their hydroxy analogues. 5-

Methoxytryptophol is not known to be a brain constituent but has been detected in rat serum and pineal gland (Wilson, Lynch & Ozaki, 1978). In a series of recovery experiments it was found that no 5-HT or 5-HIAA were co-extracted with melatonin, although small amounts of 5-HIAA (7%) and melatonin (8%) did contaminate the 5-HT extracts.

Internal standards were included every time each assay was performed. Correcting for known sampling losses, the average ± s.e.mean recoveries of internal standards added to brain homogenates were as follows: tryptophan 80 ± 3%; 5-HT 88 ± 3%; 5-HIAA 97 ± 4%; melatonin 92 ± 3%; NA 91 ± 6%; dopamine 76 ± 5% ($n = 30-36$). Neurochemical data were compared by Student's t test.

Drugs and chemicals

Injection solutions of melatonin (Sigma) were prepared as ultrasonic dispersions (Soniprobe type 1130A, Dawe Instruments) in physiological saline containing 0.3% (v/v) Tween 80. A dose volume of 5 ml/kg was used in all studies.

L-Tryptophan, 5-hydroxytryptamine creatinine sulphate, 5-HIAA, 5-hydroxy-L-tryptophan, 5-methoxytryptamine hydrochloride, 5-methoxyindoleacetic acid, 5-methoxytryptophol, noradrenaline bitartrate, dopamine hydrochloride, L-cysteine, 6-hydroxymelatonin and Tween 80 were all obtained from Sigma; OPT was purchased from Fluka and pentobarbitone sodium (Sagatal) from May and Baker. N-acetyl 5-methoxy kynurenamine (AMK) and N-acetyl 5-methoxy formyl kynurenamine (AMFK) were synthesized in the Chemistry Department, Roche Products Ltd. All chemicals used in the neurochemical extractions were AR grade and were purchased from either BDH or Fisons and used without further purification. Solutions were made up in glass-distilled, deionized water. All glassware used in the neurochemical assays was soaked in Decon 90 (Decon Laboratories) overnight, rinsed thoroughly, washed in Chromerge cleaning solution (Manostat) overnight and then thoroughly rinsed in distilled water before use.

Results

Sleep studies

Melatonin, at a dose of 10 mg/kg (i.p.), was an effective sleep-inducing agent since it produced a significant reduction in TSO in the rat (Figure 1). A small but significant reduction in W time and increase in the proportion of time spent in S and PS was seen also on the drug day. No significant changes in sleep

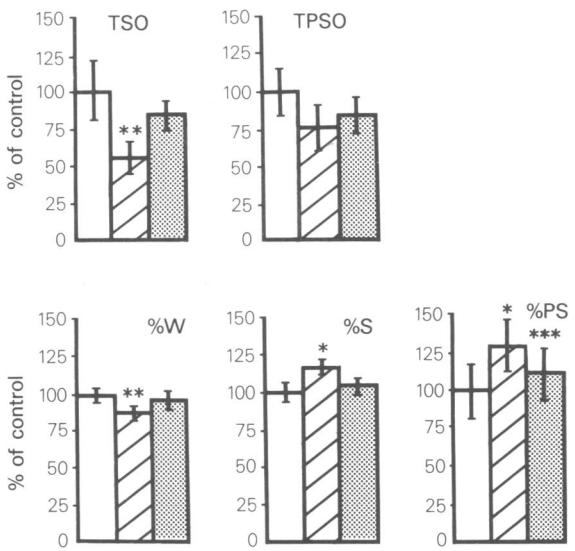

Figure 1 Effect of melatonin (10 mg/kg i.p.) on sleep in the rat. Open columns: pre-drug; hatched column: drug; stippled columns: post-drug. All data are presented as a percentage of the pre-drug day values, bars indicate s.e.mean. Pre-drug values were: time to sleep onset (TSO) 28.7 ± 5.7 min; time to paradoxical sleep onset (TPSO) 52.6 ± 8.8 min; % awake (%W) 55.8 ± 3.2; % slow wave sleep (%S) 35.1 ± 2.1; % paradoxical sleep (%PS) 9.1 ± 1.8; $n = 8$. Significant differences from pre-drug values indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.02$; $^{***}P < 0.01$.

patterns were seen on the post-drug day except in PS which was still slightly elevated. A smaller dose of melatonin, 2.5 mg/kg, had qualitatively similar effects although these reached statistical significance only in the case of PS (Figure 2). In percentage terms the increase in PS after the lower melatonin dose was greater but this probably simply reflected the lower pre-drug values (PS 5.6 ± 0.9% before 2.5 mg/kg and 9.1 ± 1.8% before 10 mg/kg).

Neither dose of melatonin had any discernible effect on normal EEG patterns and appeared not to alter sleep behaviour in the rat. Animals could be easily aroused from sleep following melatonin administration.

Neurochemical studies

Although all 7 regions of control rat brains appeared to contain small amounts of melatonin, in no case was the value found significantly greater than the detection limit. This 'apparent' melatonin in control rat brain almost certainly represents interference in the assay as more specific and sensitive techniques have failed to identify melatonin unequivocally in the rat brain (Koslow, 1974; Pang, Brown, Grota, Chambers & Rodman, 1977). Sixty min after melatonin treatment (20 mg/kg i.p.) only the striatum (control 11.6 ± 7.5, $n = 5$; treated 48.5 ± 11.3, $n = 6$, ng/g wet wt.) and cortex (control 2.1 ± 0.6, $n = 5$; treated 24.6 ± 5.0, $n = 5$, ng/g wet wt.) showed a significant ($P < 0.05$ and $P < 0.001$ respectively) increase in apparent melatonin concentration.

Melatonin treatment (20 mg/kg i.p.) had no statistically significant effect on the concentrations of either tryptophan, 5-HT or 5-HIAA in any of the 7 brain regions of the rat (Table 1). Similarly NA and dopamine concentrations were unaltered by melatonin treatment (Table 2). Dopamine levels were determined in all striatal, midbrain and cortical samples and in 8 out of 12 hippocampal samples (5 control and 3 melatonin); in all cerebellar, midbrain and hypothalamic and 4 out of 12 hippocampal samples dopamine levels were lower than the detection limit of the assay.

Discussion

The intraperitoneal injection of melatonin was found to have definite sleep promoting activity in the rat in the study described here. That this effect was produced at a dose which is less than one-twentieth of that necessary to produce signs of motor incoordination in the rotorod test (ED_{50} 260 mg/kg i.p.; Sugden, 1980), argues in favour of the suggestion that this is a specific effect of melatonin on sleep processes and not merely a general sedative action. This interpretation is also consistent with the observation that rats which had received melatonin could be easily

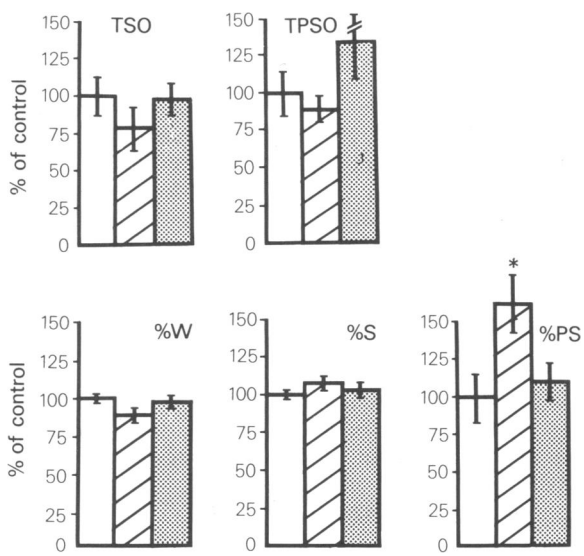

Figure 2 Effect of melatonin (2.5 mg/kg i.p.) on sleep in the rat. Data expressed as in Figure 1. Pre-drug values were: time to sleep onset (TSO) 18.5 ± 2.5 min; time to paradoxical sleep onset (TPSO) 65.0 ± 10.0 min; % awake (%W) 56.5 ± 1.5; % slow wave sleep (%S) 38.0 ± 1.0; % paradoxical sleep (%PS) 5.5 ± 0.9; $n = 9$.

Table 1 Effect of melatonin administration (20 mg/kg i.p.) 60 min before death, on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations

	Concentrations					
	Tryptophan		*5-HT*		*5-HIAA*	
	Control	Melatonin	Control	Melatonin	Control	Melatonin
Cerebellum	16.3±3.8	18.2±1.3	231± 72	123±17	139±16	130± 8
Medulla	17.2±2.2	18.0±0.8	1106± 98	888±65	654±51	627±58
Hypothalamus	17.3±3.1	21.1±1.5	1232±164	1258±79	654±67	701±93
Striatum	12.8±3.3	17.4±2.4	628± 78	636±34	565±69	526±39
Midbrain	17.0±2.4	17.2±1.2	814± 60	727±30	710±71	743±48
Hippocampus	15.5±3.4	15.7±1.9	598± 31	578±43	439±61	426±56
Cortex	15.1±2.8	16.9±1.7	208± 11	232±13	289±71	249±25

Results are expressed as mean ± s.e., $n = 6$. Tryptophan values are in nmol/g wet weight and those for 5-HT and 5-HIAA in ng/g wet weight.

Table 2 Effect of melatonin administration (20 mg/kg i.p.) 60 min before death, on brain noradrenaline (NA) and dopamine concentrations

	Concentration			
	NA		*Dopamine*	
	Control	Melatonin	Control	Melatonin
Cerebellum	138± 9	163± 9	n.d.	n.d.
Medulla	425±24	407±20	n.d.	n.d.
Hypothalamus	1268±97	1147±80	n.d.	n.d.
Striatum	157±29	197±14	2310±238	2658±329
Midbrain	342±25	363±10	142± 28	196± 28
Hippocampus	254±29	234±32	186± 46*	320± 77†
Cortex	149±16	144±11	302± 57	327± 59

Results are expressed as mean ± s.e. in ng/g wet weight, $n = 6$ unless otherwise indicated *$n = 5$; †$n = 3$. n.d. = level below assay detection limit.

aroused and showed a full expression of the normal sleep cycle.

The most marked effect of melatonin was on TSO and this is easily understood if the pharmacokinetics of melatonin are considered. Following intraperitoneal injection, peak plasma concentrations of melatonin were reached at 10 min and thereafter its concentration rapidly declined with a half-life of 15 to 20 min (Kopin, Pare, Axelrod & Weissbach, 1961; Maickel, Bosin, Harrison & Riddle, 1974). The lipophilic nature of melatonin enables it to cross the blood-brain barrier readily (Kopin *et al.*, 1961) and since in our sleep studies melatonin was administered immediately before EEG recordings were begun, it is reasonable that its hypnotic effect should have been most pronounced during the early part of the recording period as a shortened TSO. Also a decreased TSO was the only significant effect on sleep noted in two studies in man (Cramer *et al.*, 1974; 1976). In our experiments a small but significant reduction in W time and increases in both S and PS over the 4 h recording period were also apparent (Figure 1). Further analysis of the data from these experiments revealed that the changes in W and S were concentrated in the first 60 min after dosing and so were probably the consequence of decreased TSO. PS, however, appeared to be increased in each of the 4 h of recording and the observation that PS was also increased by a lower dose of melatonin (2.5 mg/kg, Figure 2) suggests that this type of sleep is particularly sensitive to the drug. The small increase in PS seen on the post-drug day was probably not a direct effect of melatonin since the drug is virtually completely eliminated from the body within 24 h (Koplin *et al.*, 1961).

Our attempt to relate the sleep promoting activity of melatonin to changes in brain neurochemistry was unsuccessful. Previously it has been reported that 500 μg of melatonin elevated brain 5-HT (Anton-Tay, Chou, Anton & Wurtman, 1968). However, these latter authors cautioned that various factors such as the time of day when melatonin is given, the interval between injection and death, the dose and its route of administration may be important in deter-

mining the effect of brain 5-HT. In the present work, care was taken to control the timing of the injection and the animals' environment but it was found that melatonin, given in sleep-inducing doses, did not alter the levels of tryptophan, 5-HT or its major metabolite, 5-HIAA, in any of the 7 brain regions studied (Table 1). Nor was there any change in NA or dopamine in these areas (Table 2). Much larger doses of melatonin have been reported to increase brain dopamine content in mice (Cotzias, Tang, Miller & Ginos, 1971). The lack of a significant change in indoleamines and catecholamines found in the study described here does not preclude the possibility that the sleep promoting action of melatonin may involve more subtle changes in these neurotransmitter systems. There is considerable evidence that 5-hydroxytryptaminergic and noradrenergic neurones are involved in the initiation and maintenance of both S and PS (Jouvet, 1972; Morgane & Stern, 1972).

Another possibility is that melatonin may induce its sleep promoting action through an interaction with the benzodiazepine receptor. In support of such a hypothesis is the recent finding that melatonin can inhibit the binding of [^{3}H]-diazepam to washed crude synaptosomal membranes prepared from rat forebrain (Marangos, Patel, Hirata, Sondhein, Paul, Skolnick & Goodwin, 1981). The same workers found AMK to be 10 times more potent than melatonin. It has been shown that cleavage of the indole nucleus of melatonin by indoleamine 2,3-dioxygenase to yield the kynurenamine metabolites AMFK and AMK is a major route of metabolism in the brain (Hirata, Hayaishi, Tokuyama & Senoh, 1974). Not only do the benzodiazepines and melatonin both promote sleep but melatonin, at higher doses, also shares the muscle relaxant and anticonvulsant properties of the benzodiazepines (Sugden, 1980), suggesting that melatonin, or a metabolite, could act as an agonist at the receptor. Furthermore there are structural similarities between AMK and the β-carbolines, one of which may be an endogenous ligand for the benzodiazepine receptor (Braestrup, Nielsen & Olsen, 1980). Although the brain concentrations of melatonin and its pyrollase metabolites are probably too low for any to be considered an endogenous ligand at the benzodiazepine receptor (Koslow, 1974; Pang, *et al.*, 1977), it is possible that the psychopharmacological effects of melatonin administration may be explained by this mechanism.

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