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Lack of Circadian Variation in the Sensitivity of Rat Terminal 5-HT_{1B} Autoreceptors

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Abstract—The sensitivity of terminal 5-HT_{1B} autoreceptors in the cerebral cortex and hippocampus to both agonist and antagonist was determined at four different time points in the light:dark cycle of the rat to evaluate whether changes in their responsiveness underlie the circadian rhythm in 5-hydroxytryptamine (5-HT) release. No significant circadian differences were evident in the apparent pIC50 values calculated for 5-HT to inhibit K^+-evoked tritium efflux or in the apparent pA_2 values calculated for methiothepin to antagonize the effect of 5-HT, at the different time points, in either brain region. These findings suggest that the sensitivity of terminal 5-HT_{1B} autoreceptors in rat cerebral cortex and hippocampus does not change in a circadian pattern, during the light:dark cycle, and argues against such a change influencing the circadian variation in 5-HT release.

Many of the factors involved in the biosynthesis of 5-hydroxytryptamine (5-HT) display a circadian variation (for review see Martin 1991). Some of these biosynthetic steps possess suitable characteristics which would enable them to act as regulators of neurotransmitter synthesis. For example, the activity of the enzyme tryptophan hydroxylase, which is the rate-limiting step in the synthesis of 5-HT, is greatest towards the end of the dark phase (Cahill & Ehret 1981; Martin & Redfern 1982; Redfern & Sinei 1985). However, this does not explain why tissue concentrations of 5-HT in several brain regions are at their highest during the light phase (Quay 1968; Hery et al 1972; Hillier & Redfern 1976). This variation in neuronal 5-HT concentration is inversely related to the release of 5-HT, which, like the rhythm in 5-HT synthesis, is greatest during the dark phase (Martin & Marsden 1985), when the firing of 5-HT neurones is highest and which coincides with the active phase of the nocturnal rat (Kalen et al 1989). Although it is possible that changes in presynaptic function are designed to replenish stores of neurotransmitter that are used at various rates during the light:dark cycle and that variations in 5-HT release contribute to or are partly responsible for the daily fluctuations in 5-HT concentrations. The situation is further complicated by the fact that the re-uptake of 5-HT is also highest when release is highest (Meyer & Quay 1976; Brunello et al 1987), suggesting that variations in 5-HT release have little effect on 5-HT concentrations.

The release of 5-HT is functionally important and measurements of extracellular 5-HT concentrations are the most relevant. The release of 5-HT is affected by many factors, including the activity of 5-HT receptors located on 5-HT neurons. These 5-HT receptors are activated by the 5-HT released from their corresponding cell bodies or nerve terminals, and are therefore termed autoreceptors. Two pharmacologically different 5-HT autoreceptors have been identified on 5-HT neurons, those located on 5-HT cell bodies have been classified as being of the 5-HT_{1A} subtype (Weissmann-Nanopoulos et al 1985; Hjorth & Magnusson 1988), while those located on 5-HT nerve terminals are of the 5-HT_{1B} subtype (Middlemiss 1984b; Engel et al 1986). These autoreceptors function by a negative feedback mechanism to regulate the release and synthesis of 5-HT in terminal regions (Hjorth et al 1982; Middlemiss 1984a,b; Sharp et al 1989a,b). In this way they could be important regulators in controlling the circadian oscillations in 5-HT synthesis and release. It was proposed recently that 5-HT_{1B} receptors exhibit a circadian variation in their sensitivity (Martin et al 1987). This variation in the sensitivity of terminal 5-HT_{1B} autoreceptors could ultimately be responsible for the circadian rhythm in 5-HT release. The aim of this study was to gauge the responsiveness of terminal 5-HT_{1B} autoreceptors, by their ability to regulate the release from brain slices of previously taken up [^{3}H]5-HT, at various time points in the light:dark cycle of the rat.

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Materials and Methods

Animals

Male Wistar rats (University of Bath strain), 100–140 g, were housed in groups of six, in polycarbonate cages, in specially constructed wooden cabinets. Inside the cabinets they were maintained on a 12-h light:12-h dark cycle (light on, 0900 h; light off, 2100 h), for at least 14 days before their use in experiments. After 14 days acclimatization, the animals weighed between 200–350 g. The majority of the experiments were performed during the normal working hours in the laboratory; however, some were performed outside normal working hours necessitating some animals to be phase shifted. The phase-shifted animals were also allowed 14 days to acclimatize to the new light:dark cycle before use (Davies et al 1974).

Housing

Rats were housed in a dark room, in specially constructed wooden cabinets, in order to regulate their light:dark cycle (Hillier et al 1973). Lighting inside each cabinet was provided by fluorescent tubes (> 200 lx) and was controlled by a time switch. The inside of each cabinet was lined with polystyrene to provide sound insulation. Plastic draught excluder foam

strips were glued to the edge of the cabinet door to ensure light proofing. Inside the cabinets, the rats had free access to both food and water so that they could be left undisturbed for several days. The cages were cleaned every two to three days, with the cleaning times being randomized to prevent them from being interpreted as a cue. During cage cleaning or when the animals were killed in the dark phase, the holding room was illuminated by a low intensity red lamp (4–6 lx), a condition known not to disrupt circadian rhythms (McGuire et al 1973). The temperature of the room varied from 18 to 22°C, while the temperature inside the cabinets varied from 20 to 23°C. Under these conditions the animals were isolated from undue environmental influences.

Superfusion studies

These studies were carried out using the cumulative dose-response technique of Frankhuijzen & Mulder (1982), in its modified version as described by Middlemiss (1984a,b). Two rats, 200–350 g, were used per experiment; they were killed by cervical dislocation in the appropriate phase of the light:dark cycle (i.e. end-dark, mid-light, end-light, mid-dark). The cerebral cortex or hippocampus was then rapidly dissected, trimmed free of white matter and adhesive blood vessels and chopped in two directions at 250 μm on a McIlwain brain-tissue chopper. The resulting slices were incubated for 15 min at 37°C in 5 mL Krebs buffer (composition (mM): NaCl 135, KCl 5, $NaHCO_3$ 25, $MgSO_4$ 1, KH_2PO_4 1.25, $CaCl_2$ 2, glucose 10, gassed with 95% O_2–5% CO_2), pH 7·4, containing 0·1 μM [^{3}H]5-HT, 200 μM ascorbic acid and 10 μM pargyline. After three washes with 5 mL Krebs buffer, about 15–30 mg tissue was transferred to each chamber, of a ten chambered superfusion apparatus and superfused against gravity with Krebs buffer at a rate of 0·4 mL min^{-1}. After a 30 min period of superfusion, eight of the chambers containing the slices were exposed to Krebs buffer containing elevated K^+ ions (25 mM, made by the iso-osmolar replacement of NaCl with KCl), for 16 min. After this period, fractions were collected every 4 min, 18 in all (i.e. t = 46 min to t = 118 min). To prevent the re-uptake of [^{3}H]5-HT or endogenous 5-HT into 5-HT-ergic neurons, paroxetine (3·2 μM) was present in the superfusion fluid from t = 0 min. At the end of each experiment the tissue slices were removed and placed in plastic scintillation vials. After the addition of 12 mL of liquid scintillation fluid (Optiphase Safe, LKB), to each vial, they were sonicated. After 3 h of stabilization in the scintillation counter, their radioactivity content was determined. Each sample was counted for 3 min and the counts were expressed as disintegrations min^{-1}.

From t = 54 min, cumulative dose-response curves to 5-HT were constructed using four concentrations of agonist (30, 100, 300 and 1000 nM), with a 16 min time interval between each successive increase in 5-HT concentration (preliminary experiments using 5-HT indicated that the maximum effect was produced after 12 min). The fractional efflux rate was calculated as the fraction of tritium content of slices at the onset of the respective 4-min collection period. In each experiment involving an agonist, the fractional efflux rate (mean of 2–4 chambers per treatment), was determined for each 4-min fraction for both basal and K^+-evoked efflux. The subtraction of basal from K^+-evoked efflux values was taken as control K^+-evoked tritium overflow. Drug effects were expressed as the percentage of the relevant control K^+-evoked tritium overflow, measured 16 min after their addition.

Dose-response curves to 5-HT were constructed at four different, equally spaced time points in the 12-h light:12-h dark cycle of the rat. Antagonist studies using the 5-HT receptor antagonist methiothepin were carried out by the addition of the drug at a concentration of 1 μM to the superflusion fluid (elevated K^+), at t = 30 min. Methiothepin was then present in the buffer, throughout the superfusion. All ten chambers received elevated K^+; four were used to assess the effect of the agonist in combination with the antagonist, while three were used to assess the effects of the agonist alone, and three others received no drug. These experiments were performed at the same time points as the agonist studies. Basal values from the agonist studies were used in the calculation of the apparent pA_2 values for methiothepin.

The highest dose of agonist and antagonist used in the superfusion studies were examined for their effects on the basal tritium efflux. 5-HT (1 μM) and methiothepin (1 μM) were added to the superfusing Krebs buffer at t = 54 min and remained present until t = 82 min. Three chambers received 5-HT, three methiothepin and the remaining four chambers served as control.

Statistics

Student's *t*-test was used to analyse the effect of temperature and that of the 5-HT uptake inhibitor paroxetine, on the uptake of [^{3}H]5-HT into slices of rat cerebral cortex and also to assess methiothepin's effect on K^+-evoked tritium efflux.

Apparent IC50 (concentration of 5-HT required to inhibit the K^+-evoked release by 50%), values were calculated for each experiment. This IC50 was converted to pIC50 ($-\log$ of IC50) for each experiment. Similarly, apparent pA_2 ($-\log$ of the molar concentration of antagonist for which the ratio of equi-effective concentrations of agonist in the presence and absence of antagonist is two) values for methiothepin were calculated for each experiment, as described by Schlicker & Gothert (1981). One-way analysis of variance was used to assess circadian differences in basal tritium efflux at t = 46 and t = 118 min, K^+-evoked tritium efflux at t = 46 and t = 118 min, enhancement of tritium efflux produced by the 5-HT-receptor antagonist methiothepin, the apparent pIC50 and pA_2 values, at the four time points, for both brain regions. Post-hoc individual comparisons were performed on values obtained at diametrically opposite time points (i.e. 12 h apart), using Dunnett's *t*-test, because these time points would be expected to correspond to the zenith and the nadir of a circadian rhythm.

Drugs

[^{3}H]5-HT, 12–20 Ci mmol^{-1} (Amersham International, Amersham, UK), 5-hydroxytryptamine creatinine sulphate (Sigma, St Louis, MO, USA), pargyline hydrochloride (Sigma), paroxetine hydrochloride (gift from Beechams, Brentford, UK) and methiothepin maleate (gift from Roche, Herts, UK) were used in the study.

Table 1. Parameters associated with tritium efflux from slices of rat cerebral cortex and hippocampus.

	End-dark	Mid-light	End-light	Mid-dark
Cerebral cortex				
Basal efflux (t=46 min)	3·15±0·21	3·14±0·19	3·28±0·18	3·16±0·08
Basal efflux (t=118 min)	2·78±0·19	2·81±0·19	2·87±0·18	2·54±0·10
K^+-evoked (t=46 min)	9·96±0·56	10·36±0·17	10·40±0·57	10·06±0·37
K^+-evoked (t=118 min)	4·04±0·29	3·59±0·32	3·72±0·42	4·20±0·11
% Inhibition 5-HT (1 μM)	72·00±4·15	76·42±3·37	78·19±6·80	72·93±2·52
Apparent pIC50	7·26±0·02	7·31±0·03	7·22±0·02	7·35±0·01
Methiothepin (1 μM)	13·2±0·20***	13·9±0·34**	13·8±0·13***	14·0±0·30**
Apparent pA_2 methiothepin	6·78±0·06	6·65±0·11	6·72±0·08	6·76±0·05
Hippocampus				
Basal efflux (t=46 min)	3·02±0·14	2·83±0·08	3·00±0·17	2·62±0·19
Basal efflux (t=118 min)	2·86±0·19	2·59±0·14	2·89±0·14	2·33±0·25
K^+-evoked (t=46 min)	9·87±0·39	9·16±0·56	9·08±0·28	9·72±0·12
K^+-evoked (t=118 min)	3·58±0·33	3·39±0·20	3·40±0·17	3·60±0·31
% Inhibition 5-HT (1 μM)	78·12±6·15	66·96±4·71	81·33±3·53	62·38±7·78
Apparent pIC50	7·13±0·06	7·16±0·02	7·02±0·04	7·17±0·05
Methiothepin (1 μM)	11·2±0·27***	12·0±0·47*	11·5±0·21***	11·9±0·22***
Apparent pA_2 methiothepin	7·00±0·05	6·91±0·06	6·85±0·07	6·87±0·11

The results are expressed as means±s.e.m. of 4–5 separate experiments for each time point. Each value is itself the mean of 2–4 measurements. Basal, K^+-evoked tritium efflux and its enhancement by methiothepin are expressed as percentage efflux of tissue stores over a 4-min period. Basal efflux represents the unstimulated tritium efflux from brain slices. K^+ (25 mM)-evoked tritium efflux represents the rate above basal obtained by subtracting the basal release rate from the K^+-evoked component, in the absence of exogenous 5-HT. The percentage inhibition and pIC50 values are calculated assuming a maximum effect at 1 μM exogenous 5-HT. Methiothepin (1 μM) was added at t=30 min and its effect on the K^+ (25 mM)-evoked tritium efflux was measured at t=46 min. Student's *t*-test was used to assess the enhancing effect of methiothepin on K^+-evoked tritium efflux. $*P<0.05$; $**P<0.01$; $***P<0.001$. One-way analysis of variance was used to examine for significant differences in basal tritium efflux, K^+-evoked tritium efflux, pIC50 or pA_2 values. Post-hoc individual comparisons were made at diametrically opposite time points using Dunnett's *t*-test.

Results

Preliminary studies revealed that the incubation of cortical slices with [^{3}H]5-HT at 0°C was associated with a significant ($P<0.001$), 80% reduction (0°C: 664±40 d min^{-1} (mg tissue)$^{-1}$, n=4; 37°C: 3279±426 d min^{-1} (mg tissue)$^{-1}$, n=9 (mean±s.e.m.)) in the accumulation of [^{3}H]5-HT as compared with those performed at 37°C. Furthermore, the inclusion of the 5-HT uptake inhibitor, paroxetine (3·2 μM) in the incubation medium was associated with a significant ($P<0.001$), 84% reduction in the uptake of [^{3}H]5-HT (control: 3279±426 d min^{-1} (mg tissue)$^{-1}$, n=9; paroxetine: 525±149 d min^{-1} (mg tissue)$^{-1}$, n=5 (mean±s.e.m.)).

Exposure of rat cortical and hippocampal slices preloaded with [^{3}H]5-HT to Krebs buffer containing elevated K^+ ions (5–50 mM), prepared by an appropriate reduction in the NaCl concentration to maintain osmolarity, was associated with a concentration-dependent increase in the fractional release rate of tritium (data not shown). From these studies, a sub-maximal K^+ ion concentration (25 mM) was chosen for assessing drug effects on tritium efflux, from both brain regions. Furthermore, the isomotic replacement of Ca^{2+} ions with Mg^{2+} ions in the Krebs buffer containing 25 mM K^+ to evoke tritium efflux, resulted in a fall in the fractional release rate of tritium towards basal levels, in both rat cortical and hippocampal slices (data not shown). In contrast, the re-introduction of Ca^{2+} ions was associated with an enhancement of tritium efflux.

Following this preliminary characterization of tritium efflux, release experiments were performed at four equally spaced time points in the 24-h light:dark cycle of the rat. Basal tritium efflux measured from rat cortical and hippocampal slices at the four time points was not significantly different at the start (t=46 min) or at the end of each experiment (t=118 min) (Table 1). Similarly, the K^+-evoked tritium efflux, calculated by subtracting basal efflux, from cortical and hippocampal slices was not significantly different at t=46 min, or at t=118 min.

The addition of exogenous 5-HT 0·03, 0·1, 0·3 and 1 μM in a cumulative manner to the superfusion medium caused a dose-related inhibition of the K^+-evoked tritium efflux, at the four time points in both brain regions (Fig. 1). The maximum effect, observed at 1 μM, represented about 70% inhibition of the K^+-evoked efflux, at the four time points tested, in both regions (Table 1). The apparent pIC50 values calculated for 5-HT in the cerebral cortex and the hippocampus were similar and not significantly different at the four time points of the light:dark cycle, in either region (Table 1).

The addition of the putative 5-HT autoreceptor antagonist methiothepin (1 μM) to the superfusion buffer caused a significant enhancement of the K^+-evoked tritium efflux (t=46 min), at all four time points in both brain regions, with respect to controls (Table 1). However, statistical analysis was unable to expose any significant differences in the magnitude of the enhancement produced by methiothepin at these time points. The presence of methiothepin in the superfusion buffer antagonized the ability of 5-HT to inhibit tritium efflux and shifted its dose-effect curve to the right. Apparent pA_2 values calculated for methiothepin in the cerebral cortex were similar to values obtained in the hippocampus, and were not significantly different with respect to the light:dark cycle (Table 1).

The highest dose of 5-HT (1 μM) and methiothepin (1 μM) used in these experiments were examined for their effects on basal tritium efflux. Basal efflux is considered to represent the release of metabolites mainly formed intracellularly

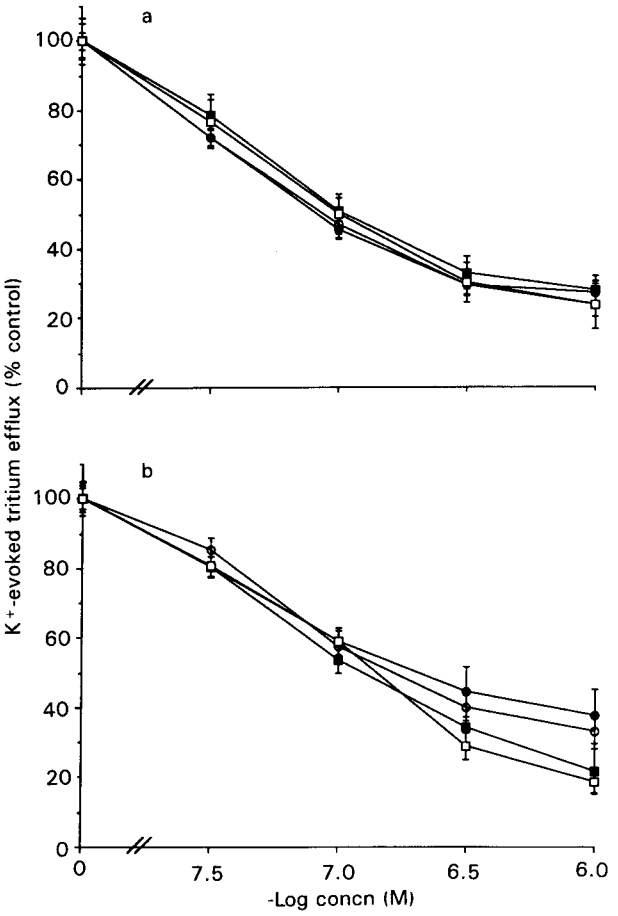

FIG. 1. Inhibition by 5-HT of the K^+-evoked tritium efflux from: rat cortical (a); and hippocampal (b) slices. Cumulative dose-response curves to 5-HT were constructed at four different time points in the light:dark cycle of the rat: mid-dark (●); mid-light (○); end-dark (■); and end-light (□). Tissue slices were exposed to increasing concentrations of 5-HT every 16 min, by its addition to the Krebs buffer starting at t = 54 min. The results, expressed as percentage of control, are the means ± s.e.m. of five separate experiments at each time point. Each value is itself the mean of 2–4 measurements per drug treatment. No significant differences were apparent between the dose-response curves at these time points.

(Moret & Briley 1988). Basal efflux, at t = 82 min, from rat cortical (control: 2·95 ± 0·09, n = 4; 5-HT = 2·76 ± 0·07, n = 3; methiothepin = 3·09 ± 0·07, n = 4) and hippocampal slices (control: 2·48 ± 0·08, n = 4; 5-HT = 2·25 ± 0·08, n = 3; methiothepin = 2·59 ± 0·08, n = 4) was not altered by drugs.

Discussion

The aim of this study was to evaluate whether 5-HT autoreceptors located on 5-HT nerve terminals display a variation in their sensitivity and thus underlie the circadian rhythm in 5-HT release. The responsiveness of these autoreceptors in the cerebral cortex and the hippocampus to both agonist and antagonist was determined at four different time points in the light:dark cycle of the rat. The ability of the natural agonist 5-HT to inhibit the K^+-evoked tritium efflux from brain slices preloaded with [^{3}H]5-HT, and of methiothepin to antagonize 5-HT's effect was not significantly different at these time points, in either brain region. Similar findings have been reported for slices prepared from the rat hypothalamus (Blier et al 1989). In conclusion, the circadian rhythm of 5-HT release in rat cerebral cortex and hippocampus is generated by factors other than any underlying variations in the sensitivity of terminal 5-HT autoreceptors. Moreover, these autoreceptors would be expected to dampen or nullify the amplitude of any circadian rhythm in 5-HT release.

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