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Circadian Studies of $5HT_2$ Receptors: Effects of Clorgyline Administration

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KOSHIKAWA, N., M. J. DURCAN, G. DUNN AND I. C. CAMPBELL. *Circadian studies of $5HT_2$ receptors: Effects of clorgyline administration.* PHARMACOL BIOCHEM BEHAV **30**(2) 347–350, 1988.—This paper demonstrates the application of an assay design that is particularly valuable for estimating receptor number (B_{max} values) and affinity (K_d values) in many small samples of tissue. It is illustrated by its application to a study of possible circadian rhythms in the numbers of $5HT_2$ receptors in the rat cerebral cortex. The assay design involves the use of only two radioligand concentrations, the lower one being close to K_d (estimated from pilot studies) and the upper one close to 4 times this concentration. The results show that chronic clorgyline (1 mg/kg/day/28 days) administration to rats results in an 18% decrease in the number of cortical $5HT_2$ receptors (as measured by specific [^{3}H]ketanserin binding). There is no significant circadian rhythm in receptor number in either the control or the MAOI-treated group. There is however, evidence of co-variation between the pairs of control animals housed in the same cage, and interestingly, that this effect is abolished by treatment with the MAOI.

Circadian studies $5HT_2$ receptors Clorgyline

THIS study presents the results of an investigation of the effects of the monoamine oxidase inhibitor (MAOI) clorgyline [1,4] on possible circadian rhythms in the concentration of $5HT_2$ receptors in the rat cerebral cortex. Clorgyline and other antidepressants have been shown to "down-regulate" α_2 and β-adrenergic and serotonergic receptors in the rat brain [2, 5, 7] and in addition, there is some evidence that clorgyline disrupts circadian rhythms of behavioural activity [11] and receptor concentration [12]. The study also demonstrates (a) an experimental design for a circadian study and (b) the use of an analytical technique for obtaining "receptor binding" data from a large number of samples.

METHOD

Adult male Wistar rats (200 g at the beginning of the study) were housed in pairs and maintained in a 12 hr:12 hr light:dark cycle throughout the 28 days of the investigation. Clorgyline was administered in drinking water to give a dose of 1 mg/kg/day. This dose is slightly higher than that used clinically (1.5-fold) [8], is relatively specific for MAO A [1,2] and has no apparent adverse effect on the animals. In total, there were 18 pairs of controls and 18 pairs of clorgyline-treated rats. At the end of the 28-day treatment period, rats were sampled every 4 hours (3, 7 and 11 hours after the beginning of the light phase, and 3, 7 and 11 hours after the begining of the dark phase) over the following 72 hours. Two control and two clorgyline-treated rats were selected at each sampling point and their brains were rapidly removed and dissected. The aim of the sampling design was to assess the reproducibility of any circadian rhythms over a period of three consecutive days.

Cerebral cortices were homogenised in a random order in Tris buffer (50 mM, pH 7.7, 10% w/v) using a Brinkman polytron (setting 7, 15 sec). They were centrifuged (48000 g × 15 min) and washed twice and the resulting pellets were stored at −80°C until used in the receptor binding assays. All cortical homogenates were dealt with in an identical way and these preparatory techniques should not be a source of bias in the experiment. We wish to thank May & Baker Pharmaceuticals (Dagenham, Essex) for a generous gift of clorgyline.

Receptor Binding Assays

In a series of preliminary receptor binding studies of the $5HT_2$ receptor subtype (a) to check the validity of the Michaelis-Menton equation for specific binding, (b) to measure the % specific binding and (c) to obtain an initial estimate of K_d, seven concentrations (0.10–9.0 nM) of the $5HT_2$ antagonist [^{3}H]ketanserin (Amersham International) were used as previously described [5,9].

The assay design chosen for studies of the receptor concentrations in the circadian rhythm experiment involved the use of only two concentrations of [^{3}H]ketanserin. The method, its validity and a comparison of it with conventional 5 and 6 point assays has already been described [3]. The lower concentration (0.45 nM) correspond to the initial estimate of K_d and the higher (1.8 nM) was four times the estimated K_d. These two concentrations of radioligand were chosen on the basis of computer simulation studies which were used to obtain the "best" experimental conditions. In fact, the choice of the two concentrations is less critical when the non-specific binding is low [3], i.e. <15% and

TABLE 1

SUMMARY OF THE OVERALL EFFECTS OF CHRONIC CLORGYLINE (1 mg/kg/DAY/28 DAYS) ADMINISTRATION ON THE NUMBER (B_{max}) OF $5HT_2$ RECEPTORS IN RAT CEREBRAL CORTEX

	Controls (n=36)	Clorgyline Treated (n=36)
Mean	7.91	6.52
% of control	100%	82.4%
Standard deviation	2.45 (31%)	2.24 (34.3%)
Standard deviation of means of pairs	2.26 (28.6%)	1.25 (19.2%)

hence in our experimental system (where non-specific binding was in this region) we could have chosen a low concentration in the range (0.25–1) $\times k_d$ (nM) and a high concentration between 4–8 $\times K_d$ (nM).

Samples of quadruplicate, containing 2.5 mg of original tissue and in a final volume of 0.5 ml, were incubated for 15 min at 37°C in Tris buffer (50 mM) and then filtered and washed through Whatman GF/B filters. The assay conditions, e.g., buffer etc., have been reported previously [5,6]. Bound radioligand was measured in a scintillation counter on the following day. Non-specific binding was measured in samples incubated as above but in the presence of methysergide (1 μM). Each binding assay therefore produced 16 data points.

The data from each binding assay were analysed using the non-linear regression procedure (NLIN) in the statistical package SAS [10]. Seventy-two separate estimates of K_d and B_{max} were obtained from the experiment. These were subsequently used as raw data in an analysis of variance to search for drug effects and possible periodicities in receptor subtype levels.

RESULTS

Examination of several 7-point saturation curves showed that the specific binding of [^{3}H]ketanserin follows a simple hyperbolic curve characteristic of a single type of binding site [3,5]. The results of these binding studies provided an estimate of the K_d of [^{3}H]ketanserin binding in the rat cerebral cortex of 0.45 nM. This estimate was used to design the 2-point assays described previously [3]. Briefly, in 250 computer simulated assays in which the non-specific binding at K_d was approximately 15% and the coefficient of variation of the binding measurements was 0.1, the standard deviations of B_{max} and K_d were as follows. Using the concentration (K_d and 4 K_d) assay, the B_{max} and its standard deviation was 100±12.53 and K_d and its standard deviation was 100±31.1. In the comparable 7-point assays (in which concentrations were 0.125, 0.25, 0.50, 1.0, 2.0, 4.0 and 8.0 times K_d), the corresponding values were B_{max} 100±9.75 and K_d 100±15.09 (Dunn *et al.*, in press). Thus, although the 2-point assay produces a greater error in the estimation of K_d, the error in the estimation of B_{max} is similar to that in the 7-point assay. Thus, under conditions of limited tissue availability or in studies in which there are large numbers of samples it is appropriate to use the 2-point assay.

The animals sampled in the investigation of possible circadian rhythms in $5HT_2$ receptor subtypes in the rat cortex each provided an estimate of B_{max} and K_d. The mean K_d value was 0.41 nM with an estimated standard error of 0.025 nM. This standard error was based on the assumption that each animal provided an independent estimate and that clorgyline had no effect on receptor affinity: there was no significant differences in the mean value of K_d between control and drug-treated animals indicating that the drug had no direct effect on the specific binding of [^{3}H] ketanserin.

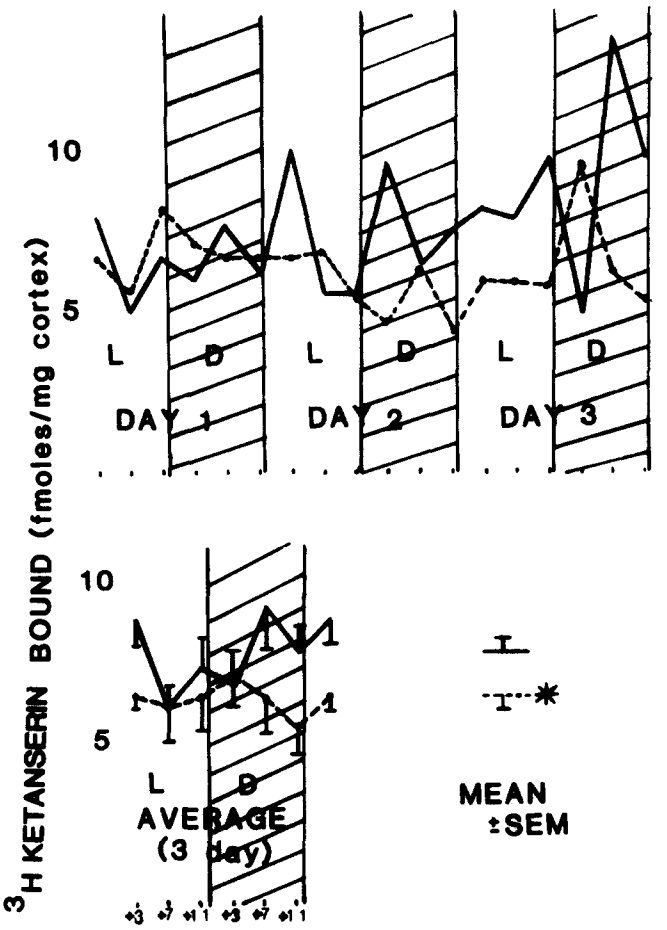

FIG. 1. B_{max} values for [^{3}H]ketanserin binding to rat cerebral cortices from control (solid lines) and clorgyline- (dashed lines) treated animals. In the top half of the figure, points are at 4-hr intervals over 72 hours and each one is the mean of 2 samples. In the lower figure, the data is "collapsed" to give a 24-hour average from the 3 days and so each point is the mean±SEM of 6 samples. B_{max} values are expressed as fmol bound/mg of original cortex (wet weight). While there is no apparent circadian rhythm in either of the groups, the mean of the clorgyline-treated group is significantly lower.

The B_{max} estimates from the control and clorgyline treated groups are summarized in Table 1. Typically each individual estimate of B_{max} had a standard error of measurement of approximately 1.03 fmol/mg tissue. In the control group the mean value for B_{max} was 7.91 (sd 2.45) fmol/mg original tissue (n=36) and in the MAOI group, the value was 6.52 (sd 2.24) fmol/mg original tissue. The drug-induced decrease (18%) is significant and it can be seen that in both the groups, the standard deviation is 30–35%. Figure 1 provides a summary of the data, (A) as individual pairs and (B) when the information is "collapsed" into one 24 hour period.

The variation due to measurement error (provided by the

standard error estimate given in the analysis of each separate binding assay) is only about 20% of the observed variation ($100 \times 1.03^2/2.34^2$) (calculated from an analysis of variance). This implies that the sampling variation due to individual differences and to variations in the efficiency of preparation is four times than that due to the assay itself. Thus, there is no evidence that the large amount of variation observed is primarily due to a lack of precision in the receptor assay.

A three-way analysis of variance on the 72 estimates of B_{max} (the three factors being drug treatment with 2 levels, day with 3 levels and time of day with 6 levels) was carried out. After incorporating the sums of squares due to all interactions into the residual squares, the F-statistic for the drug effect was 6.27 (with 1 and 63 df). This is statistically significant ($p<0.05$). There was no evidence of a day-effect, $F(2,63)=1.57$, or of a time-of-day-effect, $F(5,63)=0.87$. A test of whether there was a day by time-of-day interaction (that is, was there a different rhythm in the control animals for the drug-treated one) produced an F-statistic of 1.01 (with 5 and 58 *df*): this was not significant.

There was a possibility that the pairs of animals reared together and sampled at the same point were not actually providing independent data points. A more cautious analysis of variance was carried out using the means of these pairs of animals as separate data points. Once again the results demonstrated a significant drug effect, $F(1,27)=5.0$, but no evidence of any time effects. However, both Table 1 and Fig. 1 suggest that the two groups are differing in variability when the pair means are compared as opposed to the individual animals. If animals within a pair were independent, one would expect the standard deviation of the pair to be half of that of the individual animals. This is approximately true for clorgyline-treated animals but not for controls. Control pairs of animals are clearly showing signs of co-variation (intra class correlation coefficient=0.67) but clorgyline-treated pairs are not (intra-class correlation coefficient=0.20).

The existence of circadian rhythm implies co-variation between pairs of animals sampled together and the lack of co-variation would imply the absence of such a rhythm. However co-variation could be explained by factors other than circadian rhythms. When the analysis was restricted to the control group of rats (n=36), the following results were found. The time-of-day-effect is still not significant, $F(5,28)=1.80$. Having fitted the time-of-day-effect (and no others) we find that the intra-class correlation between the residuals is still 0.60, i.e., pairs of animals co-vary, but the source of this co-variation is not a circadian rhythm.

DISCUSSION

In this study, we have described a design for a circadian study of receptors together with the application of an analytical technique which is economical in terms of labour, animals and therefore cost. Obviously it was undesirable to pool brain preparations from different animals as one of the aims of the study was to examine individual differences in receptor concentrations. This problem was not acute with the cerebrocortical preparations but is important when considering examining samples from smaller areas of the brain such as pineal gland or the hypothalamic nuclei. Secondly, as many brain samples were studied and the work was both tedious and time consuming, an efficient assay design reduced the time involved, more importantly, reduced the chances of pipetting errors and also the number of animals used.

Most previous circadian studies suffer for the criticism that animals are only sampled over a single 24 hour period. It was considered that two rats per time per treatment group was the minimum number required to detect and periodicities in receptor levels and to obtain information about individual differences that are not under the temporal control. We considered using 3 animals per group but this would have increased the number in the experiment by one-third and would have decreased the standard error of the estimates of group means by a factor of $\sqrt{3/2}$.

In agreement with previous findings [5,7], we have observed that an antidepressant drug regimen produces a significant decrease in cortical $5HT_2$ receptors [5]. There is, however, no evidence for the existence of a circadian rhythm in the number of cortical $5HT_2$ receptors either in control animals or in those which received the MAOI regimen. The co-variation in B_{max} values between animals housed in the same cage is especially interesting as the effect is abolished by the clorgyline regimen. This suggests that the phenomenon is unrelated to some type of sampling error and that the drug may be causing some subtle type of behavioural disruption which results in a change in the 5HT receptor subtype: clorgyline, in earlier studies, has been shown to cause disruption in circadian rhythms of rest and activity in animals [11] and has been reported to be of clinical use in the treatment of rapidly cycling manic depressive patients [9]. While behavioural studies have shown some co-variance between animals housed in the same cage, we believe that this is the first receptor study in which the phenomenon has been observed. However, at this time, the significance of the effect and of its disruption by clorgyline remains unknown.

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