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THE SYNTHESIS RATE AND TURNOVER TIME OF 5-HYDROXYTRYPTAMINE IN BRAINS OF RATS TREATED CHRONICALLY WITH MORPHINE

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1 Four schedules of subcutaneous pellet implantation were used to induce tolerance to and physical dependence on morphine in Sprague-Dawley rats.

2 The schedules included implantation of four morphine pellets (each containing 75 mg of morphine free base) during a 3 day period (schedule 1); six pellets during 3 days (schedule 2); six pellets during 7 days (schedule 3) and ten pellets during a 10 day period (schedule 4).

3 A high degree of tolerance and dependence on morphine, comparable to that induced in mouse by implantation of a single morphine pellet for 3 days, was produced with schedule 4.

4 Brain 5-hydroxytryptamine (5-HT) turnover rates as measured by rate of accumulation of 5-HT after monoamine oxidase inhibition by pargyline were not different in rats rendered tolerant to and dependent on morphine according to schedules 1 to 4 when compared with corresponding placebo pellet-implanted rats.

5 The turnover rates of 5-HT in brain of morphine-and placebo pellet-implanted rats (schedule 4) from which the pellets had been removed for 24 h were also similar.

6 It is concluded that tolerance to, and physical dependence upon morphine in the rat is not associated with changes in brain 5-HT dynamics.

Introduction

Although the 5-hydroxytryptaminergic component of the central nervous system has been implicated in the pharmacological effects of morphine, its precise role in the chronic effects of morphine is still far from clear. Since the early observations of Way, Loh & Shen (1968) that tolerance to, and physical dependence on, morphine in mice is associated with increased brain 5-hydroxytryptamine (5-HT) synthesis, several reports have appeared in the literature confirming or contradicting the above hypothesis. Whole brain 5-HT turnover rates were found to be increased in morphine-tolerant mice (Shen, Loh & Way, 1970) and rats (Haubrich & Blake, 1969). However, several investigators (Algeri & Costa, 1971; Cheney, Goldstein, Algeri & Costa, 1971; Marshall & Grahame-Smith, 1971; Bhargava & Matwyshyn, 1977) have been unable to confirm the above findings.

Another approach to study the possible involvement of brain 5-HT in tolerance and physical dependence on morphine has been to alter the functional activity of the 5-hydroxytryptaminergic system by pharmacological manipulations. Such studies have also produced conflicting data. For example, depletion of whole brain 5-HT by *p*-chlorophenylalanine has been shown either to inhibit (Shen *et al.*, 1970; Ho, Lu, Stolman, Loh & Way, 1972; Maruyama, Hayashi, Smits & Takemori, 1971) or to produce no change (Algeri & Costa, 1971; Marshall & Grahame-Smith, 1971) in the development of tolerance to morphine in mice or rats.

The discrepancy in the results from various investigators could possibly be accounted for by either the species and strain differences or by the degree of tolerance and physical dependence induced. Multiple injections of morphine have been used to induce tolerance and physical dependence in the rat (Maynert & Klingman, 1962; Martin, Wikler, Eades & Prescor, 1963; Francis & Schneider, 1971). In these studies, the presence rather than the degree of tolerance and physical dependence on morphine was described. Bhargava & Matwyshyn (1977) quantitated the tolerance and dependence induced by injecting increasing doses of morphine for two weeks and observed only a low degree of tolerance and physical dependence which was not associated with changes in brain 5-HT turnover.

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Many investigators have also utilized the subcutaneous implantation of one or two pellets, each containing 75 mg of morphine base, for a period of 3 days in rats and assumed that sufficient tolerance and dependence developed without providing a quantitative measure. Under the above conditions, it has been shown (Bhargava, Afifi & Way, 1973; Bhargava, 1977; Bhargava, 1978) that an extremely low degree of tolerance and dependence develops in the rat. Furthermore, implantation of 1 or 2 morphine pellets in the rat does not alter the brain 5-HT turnover (Algeri & Costa, 1971). It has been argued that the lack of an effect on the brain 5-HT dynamics may be related to the degree of tolerance and dependence on morphine.

More recently, a high degree of both tolerance to (Bhargava, 1978) and physical dependence (Bhargava, 1977) on morphine in the rat has been developed, comparable to that produced in mice after implantation of one morphine pellet containing 75 mg of morphine base for three days (Bhargava & Way, 1976). In the present study, a series of schedules of morphine pellet implantation were used which produced different degrees of morphine tolerance and dependence, and brain 5-HT turnover rates have been measured. Brain 5-HT dynamics have also been studied in morphine-dependent rats that were withdrawn from morphine for 24 h.

Methods

Induction and quantitation of morphine tolerance and physical dependence

Male Sprague-Dawley rats weighing 150 to 200 g (Locke Erickson Laboratories, Inc., Melrose Park, Illinois) were housed 3 to a cage, given food and water *ad libitum*, and maintained on a 12 h dark-light cycle (light from 06 h 00 min to 18 h 00 min) in a room with controlled temperature (23 ± 1°C) and humidity (65 ± 2%). Rats were housed under the above conditions for at least four days before use.

Rats were rendered tolerant to, and physically dependent on, morphine by subcutaneous implantation of pellets as described previously (Bhargava, 1977; 1978). Four different schedules of pellet implantation were used. Each pellet contained 75 mg of morphine free base. The control rats received pellets containing the excipients but no drug. Briefly, in schedule 1, two pellets were implanted on the first day and two more 1.5 days later. All pellets were removed 1.5 days after the second implantation. In schedule 2, one pellet was implanted on the first day, two on the second, and three on the third day. All the pellets were removed on the fourth day. In schedule 3, one pellet was implanted on the first day, two on the third day, and three on the fifth day. All were removed on the eighth day. In schedule 4, one pellet was implanted on the first day, two on the third day, three on the fifth day, and four on the eighth day. All pellets were removed on the tenth day. After pellet removal, the tail-flick analgesic dose (AD_{50}) of morphine following its intraperitoneal administration was determined, as described by Bhargava (1978).

For assessment of physical dependence on morphine, the antagonist (naloxone) induced withdrawal jumping response, which has been shown to be a highly characteristic response of morphine-dependence, was used. A reciprocal relationship exists between the dose of naloxone required to induce the jumping response in 50% of animals (ED_{50}) and the degree of morphine-dependence in rodents (Way, Loh & Shen, 1969; Bhargava, 1977). The ED_{50} for naloxone was determined in rats from all schedules of morphine-treated rats as described earlier (Bhargava, 1977). The AD_{50} of morphine sulphate, the ED_{50} for naloxone and their 95% confidence limits were determined by the method of Litchfield & Wilcoxon (1949).

Determination of brain 5-hydroxytryptamine turnover and concentration

Steady state kinetics were used to determine the synthesis rate and turnover time of brain 5-HT as described by Tozer, Neff & Brodie (1966). Rats were implanted with morphine or placebo pellets according to schedule 1 as described above. The only difference was that the pellets were not removed; instead the rats receiving placebo or morphine were subdivided into 2 groups. Rats in 1 subgroup were given 0.9% w/v NaCl solution (saline) (1 ml/kg, i.p.) while those in the second subgroup received pargyline hydrochloride (75 mg/kg; i.p.) in 1 ml/kg volume. Thirty min after either saline or pargyline, the rats were guillotined, their brains collected and frozen at −20°C until analyzed for 5-HT. Similar experiments were carried out in rats implanted with pellets according to schedules 2, 3 and 4. An additional experiment was carried out with rats implanted with morphine or placebo pellets according to schedule 4. The pellets were removed from these rats under light ether anaesthesia and 24 h later they were treated with saline or pargyline as described above.

To test the linearity of the rate of rise of brain 5-HT following monoamine oxidase (MAO) inhibition with pargyline, groups of naive rats were killed at 0, 10, 20, 30, 45 and 60 min after pargyline and brains were analyzed for 5-HT. Since a linear increase in 5-HT was noted for 30 min this time interval was used as above. A linear relationship was also found between the logarithm of 5-HT concentration and the time after pargyline administration. In some experiments instead of measuring 5-HT concentration at

only 0 and 30 min, measurements were made at 0, 15, 30 and 60 min after pargyline treatment. Brain 5-HT concentration was determined by a modified *o*-phthaldialdehyde (OPT) method described by Curzon & Green (1970). Whole brain was homogenized in 5 ml of cold acidified *n*-butanol with a Polytron homogenizer (setting No. 4) for 60 s. The homogenate was centrifuged at 28000 *g* for 8 min in a refrigerated Lourdes Betafuge. Two 2 ml aliquots of the supernatant were mixed with 5 ml *n*-heptane and 1.2 ml of 0.1 N HCl containing 0.1% cysteine. The mixture was vortexed for 30 s followed by shaking for 5 min in a mechanical shaker (160 oscillations/min). It was centrifuged at 3000 rev/min at 4°C for 5 min; 1 ml of the aqueous phase was transferred to another tube containing 0.4 ml of 0.012% OPT in 10 N HCl. The mixture was vortexed for 15 s and heated on a boiling water bath for 15 min. After cooling, the fluorescence of the solution was read at 470 nm after being excited at 360 nm on an Aminco-Bowman Spectrophotofluorometer. The concentration of 5-HT was determined from the recovery curves obtained by the addition of known amounts of 5-HT to brain homogenates.

For each experiment, the logarithm of 5-HT concentration was plotted against time. The lines were drawn by linear regression analyses and the slopes and their standard errors were calculated. The functional rate constant, turnover rate and turnover time of 5-HT were calculated according to the method of Tozer *et al.* (1966). The 5-HT parameters were tested for statistical significance by Student's *t* test.

Results

Effect of pellet implantation on morphine tolerance and physical dependence

Implantation of morphine pellets in the rat resulted in the development of analgesic tolerance to morphine as shown by increases in the AD_{50} values of morphine over the placebo pellet-implanted rats. Morphine AD_{50}s in rats implanted with placebo pellets according to schedules 1 and 4 did not differ. Calculation of the ratios of morphine sulphate AD_{50}s in morphine pellet- to placebo pellet-implanted rats indicated 6, 8, 13 and 15 fold tolerance in morphine pellet-implanted rats according to schedules 1 to 4, respectively (Table 1).

A high degree of dependence was observed when ten morphine pellets were implanted during a 10 day period, whereas, a low degree of dependence developed in rats implanted with four morphine pellets in 3 days. As shown in Table 1, the naloxone ED_{50} in morphine-dependent rats from schedule 1 was 35 mg/kg, whereas rats from schedule 4 had a naloxone ED_{50} of 2.5 mg/kg for the withdrawal jumping response. Since naloxone ED_{50} is inversely related to the degree of morphine dependence, schedule 4-treated rats were 15 times more dependent than schedule 1-treated rats.

Effect of morphine pellet implantation and withdrawal on body weight.

Implantation of morphine pellets in the rats produced a significant reduction in the body weight gain, compared with the placebo pellet-implanted rats. As shown in Table 2, rats implanted with ten morphine pellets gained 28 g in 10 days, whereas, those receiving placebo pellets gained 40 g. More importantly, when the pellets were removed, the placebo pellet-implanted rats showed no loss in their body weight; however, the morphine pellet-implanted rats lost 34 g within 24 h.

Effect of morphine pellet implantation and morphine withdrawal on the synthesis rate and turnover time of brain 5-hydroxytryptamine

When the brain concentration of 5-HT was plotted against time after pargyline administration, a linear increase was noted for 30 min with regression coefficient $r = 0.94$ ($P < 0.00005$) and for 60 min with $r = 0.92$ ($P < 0.00005$). However, when the logarithm of brain 5-HT concentration was plotted against time an excellent linearity ($r = 0.966$; $P < 0.00005$) was obtained for 30 min values. In all the experiments the functional rate constant for the rise of 5-HT with time

Table 1 Effect of pellet implantation on the development of tolerance to and physical dependence on morphine

Schedule no.	*Pellets*	*Days*	*Morphine sulphate AD_{50}, mg/kg* (95% limits)	*Naloxone ED_{50} mg/kg* (95% limits)
Control	—	—	3.6 (1.6–8.3)	—
1	4	3	23.3 (6.6–82.0)	34.6 (20.2–59.2)
2	6	3	28.8 (13.1–63.6)	30.4 (14.7–62.6)
3	6	7	46.7 (22.9–94.9)	11.7 (5.7–23.8)
4	10	10	53.1 (29.0–97.2)	2.4 (1.4–4.3)

following pargyline administration was calculated from the slope of log [5-HT] vs. time curve.

The steady state concentration of 5-HT, the functional rate constant, turnover time and the turnover rate of brain 5-HT were unaffected in rats rendered morphine-dependent by pellet implantation according to any of the schedules used. In rats implanted with placebo pellets the steady state brain levels, rate constant, turnover time and turnover rate of 5-HT were found to be similar to those reported by Tozer *et al.* (1966). There were slight differences in these parameters in some shipments. Experiments with rats treated according to schedule 1 were repeated twice and those treated according to schedule 4 were repeated four times. As shown in Table 3, in all the experiments the brain 5-HT dynamics in placebo- and morphine pellet-implanted rats according to schedules 1 to 4 were similar. Thus, the brain 5-HT turnover rate or turnover time in morphine-dependent rats were unaffected. Rats withdrawn from mor-

Table 2 Effect of morphine pellet implantation and removal on body weights of rats

	Body weight (g) *Day*					
*Treatment**	1	3	5	8	10	11
Placebo	202.2 ± 4.7 (20)	214.2 ± 4.4 (20)	223.2 ± 4.2 (20)	229.2 ± 3.3 (20)	241.8 ± 4.1 (20)	239.8 ± 2.6 (10)
Morphine	198.0 ± 3.0 (28)	214.2 ± 3.5 (28)	219.7 ± 3.7 (28)	220.0 ± 3.8 (28)	226.7 ± 4.1 (28)	192.2 ± 4.2 (14)

Values are mean ± s.e. mean

* Rats were implanted subcutaneously with one pellet on day 1 (d1), 2 pellets on d3, 3 pellets on d5, and 4 pellets on d8. All the pellets were removed on d10. The weights on d11 represent the rats from which the pellets had been removed for 24 h. The figures in parentheses represent the number of rats used.

Table 3 Synthesis rate and turnover time of whole brain 5-hydroxytryptamine (5-HT) in rats tolerant to and physically dependent on morphine

Treatment		*Brain level of 5HT before MAO blockade* (*ng/g* ± *s.e.*)	*Rate constant of 5-HT rise after MAOI* ($k(h^{-1})$ ± *s.e.*)	*5-HT turnover time* (*min* ± *s.e.*)	*Turnover rate of 5-HT* ($ng\ g^{-1}h^{-1}$ ± *s.e.*) ($n = 5$)
Schedule 1					
A	Placebo	531.0 ± 16.0	0.90 ± 0.04	66.7 ± 3.0	478.1 ± 21.2
	Morphine	543.0 ± 31.0	0.87 ± 0.05	68.7 ± 5.2	474.1 ± 27.2
B	Placebo	377.4 ± 19.9	0.86 ± 0.10	69.8 ± 8.1	324.6 ± 37.7
	Morphine	434.2 ± 13.2	0.79 ± 0.09	76.0 ± 8.7	343.0 ± 39.1
Schedule 2					
	Placebo	434.0 ± 17.0	1.19 ± 0.07	50.4 ± 3.0	516.7 ± 30.4
	Morphine	432.0 ± 19.0	1.11 ± 0.08	54.1 ± 3.7	479.4 ± 34.6
Schedule 3					
	Placebo	489.0 ± 24.0	0.84 ± 0.11	71.4 ± 9.0	410.9 ± 53.8
	Morphine	473.0 ± 14.0	1.00 ± 0.06	60.1 ± 3.6	471.8 ± 28.4
Schedule 4					
A	Placebo	492.0 ± 7.0	0.80 ± 0.04	75.0 ± 3.4	393.6 ± 19.7
	Morphine	501.0 ± 8.0	0.85 ± 0.05	70.6 ± 3.8	425.9 ± 25.1
B	Placebo	411.0 ± 27.0	0.79 ± 0.14	76.0 ± 13.5	325.0 ± 57.5
	Morphine	423.0 ± 15.0	0.91 ± 0.08	65.9 ± 5.8	385.1 ± 33.8
C	Placebo	438.2 ± 19.7	0.71 ± 0.06	84.5 ± 7.1	311.1 ± 26.3
	Morphine	426.7 ± 9.1	0.76 ± 0.06	78.9 ± 6.2	324.3 ± 25.6
D	Placebo	390.6 ± 21.0	0.79 ± 0.16	76.4 ± 15.6	306.6 ± 62.5
	Morphine	403.3 ± 22.9	0.83 ± 0.15	72.5 ± 13.1	333.9 ± 60.5

MAO = monoamine oxidase; MAOI = monoamine oxidase inhibitor.

phine for 24 h (i.e., removal of morphine or placebo pellets from schedule 4 treatment) also did not show altered brain 5-HT dynamics. As shown in Table 4, the 5-HT turnover rate and turnover time in morphine-dependent rats were similar to those in the placebo-implanted control rats.

Discussion

The present studies indicate that chronic administration of morphine in the rat is not associated with changes in brain 5-HT turnover rates and turnover times as measured by the rate of rise of brain 5-HT following monoamine oxidase inhibitor, pargyline. These results are in agreement with those of Algeri & Costa (1971); Yarbrough, Buxbaum & Sanders-Bush (1973), Theiss, Papeschi & Herz (1975) in rats and of Marshall & Grahame-Smith (1970), Cheney *et al.* (1971) and Cheney & Goldstein (1971) in mice; however, they are in contrast to those of Haubrich & Blake (1973) in rats and of Way *et al.* (1968), Shen *et al.* (1970) and Maruyama *et al.*, (1971) in mice.

The possible reasons for the differences in the results of various investigations on the effect of chronic morphinization on brain 5-HT dynamics are (a) species and strain differences (b) methods used to determine the 5-HT turnover (c) methods used to induce tolerance to, and physical dependence on, morphine and consequently the degree of tolerance and dependence achieved. In experiments described in this paper a high degree of tolerance to morphine was produced by schedules 3 and 4 as shown by the increases in the tail-flick analgesic AD_{50} values of morphine in the tolerant rats compared with the AD_{50}s of morphine in the placebo pellet-treated rats. The degree of tolerance produced was comparable to that produced in the mouse by implantation of one morphine pellet (Bhargava & Way, 1976). Similarly, a high degree of dependence was observed in schedule 3 and 4 rats as shown by low ED_{50} values for naloxone in the jumping response.

In general, a lower degree of tolerance to, and dependence on, morphine is produced when either a multiple injection method is used (Bhargava & Matwyshyn, 1977) or when 2 to 4 morphine pellets are used to induce tolerance and dependence in the rat. Recently, it has been shown that the degree of tolerance produced by pellet implantation was directly related to the plasma and brain levels of morphine (Bhargava, 1978).

Although some assumptions are made in calculating the turnover rates (Tozer *et al.*, 1966). It is found that the turnover rates measured either from an intravenous infusion of labelled tryptophan or from the rate of rise of 5-HT following MAO inhibition by pargyline or tranylcypromine are very similar. In a number of previous investigations the increase in 5-HT during a 30 min period following MAO inhibition was multiplied by a factor of 2 to obtain the turnover rate; this could yield erroneous results. In the calculation of the turnover rates of 5-HT it is important to determine the functional rate constant and its variance and these should be multiplied by the concentration of 5-HT before MAO inhibition, instead of multiplying the functional rate constant and the concentration of 5-HT and its s.e. before MAO inhibition. The former procedure automatically allows for the variations in the steady state concentration of 5-HT.

In the present investigation, the brain 5-HT turnover rates were unaffected in rats rendered dependent on morphine by four schedules of pellet implantation. In spite of the fact that a high degree of tolerance to and physical dependence on morphine was induced, the tolerant-dependent rats failed to exhibit changes in 5-HT dynamics. It must be noted that although the morphine-dependent rats from schedules 3 and 4 consistently showed a higher absolute value for the 5-HT turnover rate compared with the corresponding placebo pellet-treated rats, a statistically significant change could not be observed. These results differ from those of Theiss *et al.* (1975) for rats. These authors used the accumulation of 5-hydroxyindole-

Table 4 Synthesis rate and turnover time of whole brain 5-hydroxytryptamine (5-HT) in morphine-tolerant-dependent rats withdrawn from morphine for 24 h

*Treatment**	n	*Brain levels of 5-HT before MAO blockade* (*ng/g* ± *s.e.*)	*Rate constant of 5-HT rise after MAOI* ($k(h^{-1})$ ± *s.e.*)	*5-HT turnover time* (*min* ± *s.e.*)	*Turnover rate of 5-HT* ($ng\ g^{-1}h^{-1}$ ± *s.e.*)
Schedule 4					
Placebo	5	336.8 ± 17.3	1.36 ± 0.115	44.1 ± 5.1	458.1 ± 38.7
Morphine	7	315.0 ± 13.3	1.60 ± 0.115	37.5 ± 4.3	504.0 ± 36.2

* Rats were implanted subcutaneously with one pellet (morphine or placebo) on day 1 (d1), 2 pellets on d3, 3 pellets on d5, and 4 pellets on d8. All the pellets were removed on d10. Brain 5-HT dynamics was studied 24 h after the pellet removal.

acetic acid (5-HIAA) following probenecid treatment, as an index of 5-HT turnover, and found that a low or medium degree of tolerance increased the 5-HT turnover, while, the highest degree of tolerance (implantation of 21 morphine pellets in 9 days) had no effect on 5-HT turnover. The explanation for such findings are not apparent. If indeed, 5-HT turnover is enhanced, then even the highest degree of tolerance should have produced some change in 5-HT dynamics, irrespective of the method used to determine it.

An attempt to relate 5-HT dynamics with the tolerance to the thermoregulatory effects of morphine in the rat was also unsuccessful (Warwick, Blake, Miya & Bousquet, 1973). Thus the results of the present study are not consistent with the view that brain 5-HT dynamics are of importance in the development of tolerance and physical dependence on morphine in the rat. It is possible that there may be some regional differences in the central 5-HT dynamics as a result of tolerance to, and dependence on, morphine in the rat. Such studies are in progress in this laboratory. The evidence so far presented does not show a causal relationship between the 5-HT dynamics and the tolerance and physical dependence on morphine in the rat.

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