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Anti-aversive role of serotonin in the dorsal periaqueductal grey matter

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Abstract. Microinjection of 5, 10, and 20 nmol serotonin (5-HT) and of 0.5, 1, and 2 nmol 5-methoxy-N, N-dimethyltryptamine (5-MeODMT) into the dorsal midbrain of rats bearing chronically implanted chemitrodes raised the electrical threshold for inducing escape behaviour following stimulation of the dorsal periaqueductal grey matter (DPAG). Linear regressions of log dose against drug-induced increase in aversive threshold were obtained for 5-HT and 5-MeODMT. The 5-MeODMT dose-effect curve was steeper and lay to the left of the 5-HT dose-effect curve. Local pre-treatment with 10 nmol metergoline or ketanserin blocked the anti-aversive effect of 10 nmol 5-HT, whereas pre-treatment with 100 nmol zimelidine potentiated this effect of 5-HT. The same dose of zimelidine raised the aversive threshold when given alone. These results suggest that 5-HT plays an inhibitory role in the DPAG controlling aversion, probably mediated by 5-HT_2 receptors.

Key words: 5-HT – 5-MeODMT – Anti-aversive effect – Antagonism by metergoline and ketanserin – Potentiation by zimelidine – Dorsal periaqueductal grey – 5-HT_2 receptors – Rat

Drugs or neuronal lesions that decrease brain serotonin (5-HT) activity release behaviour suppressed by punishment. Thus, anti-punishment effects in animal conflict tests have been described in pigeons and rats following systemic administration of several 5-HT receptor-antagonists (Graeff and Schoenfeld 1970; Stein et al. 1973; Geller et al. 1974; Graeff 1974; Schoenfeld 1976; Leone et al. 1983). Drug-induced inhibition of 5-HT synthesis also released punished behaviour in rats (Robichaud and Sledge 1969; Geller and Blum 1970; Wise et al. 1973; Schoenfeld 1976). In addition, microinjection of 5,7-dihydroxytryptamine (5,7-DHT), a neurotoxin that selectively destroys 5-HT neurones, into the ventromedial tegmentum of the rat midbrain prevented the acquisition of response suppression induced by foot-shock punishment (Tye et al. 1977).

Since the benzodiazepine anxiolytic oxazepam decreased 5-HT turnover in the rat midbrain at doses that released punished responding, it was suggested that the anti-anxiety effect of benzodiazepines is due to reduction of 5-HT in a behaviourally suppressive punishment system (Wise et al. 1972, 1973; Stein et al. 1973). Indeed, animal punishment or conflict tests are viewed as good predictors of anti-anxiety drug effects in humans (Cook and Davidson 1973; Cook and Sepinwall 1975).

However, contradictory evidence appeared when the effects of drugs that interfere with serotonergic neurotransmission were studied in rats electrically stimulated at the dorsal periaqueductal grey matter (DPAG) of the mesencephalon, a brain area belonging to the periventricular punishment system (Olds and Olds 1965) or brain aversive system (Graeff 1981, 1984). Thus, lever-pressing behaviour maintained by either reduction or termination of DPAG electrical stimulation in the rat is facilitated by the inhibitor of 5-HT synthesis para-chlorophenylalanine (Kiser and Lebovitz 1975), or by the 5-HT receptor blockers methysergide and cyproheptadine (Schenberg and Graeff 1978), and inhibited by the precursor of 5-HT synthesis 5-hydroxytryptophan (5-HTP), or by the presynaptic 5-HT uptake inhibitor chlorimipramine (Kiser et al. 1978). In addition, cyproheptadine and methysergide failed to release lever-pressing behaviour of rats maintained by water presentation, but simultaneously suppressed by response-contingent DPAG electrical stimulation (Morato de Carvalho 1981). These results suggest that 5-HT inhibits the brain aversive system rather than mediates the behavioural effects of punishment. The above results were obtained with systemic drug injections. Therefore, in order to explore the role of 5-HT in the brain aversive system more directly, chemitrodes (Leroux and Myers 1975a) aimed at the DPAG of the rat were used in the present study, allowing both microinjection of drugs and brain electrical stimulation. Dose-response relationships of the raising effect of 5-HT and of the direct 5-HT receptor agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) (Fuxe et al. 1972) on aversive threshold of DPAG electrical stimulation (Brandão et al. 1982) were determined. In addition, interactions of 5-HT with two 5-HT receptor blockers, metergoline (Fuxe et al. 1975) and ketanserin (Van Nuetten et al. 1981), as well as with the 5-HT uptake inhibitor zimelidine (Ross et al. 1976), were studied.

Materials and methods

Animals. One hundred and sixty-seven male, albino Wistar rats, bred at the animal colony of the Faculty of Medicine of Ribeirão Preto and weighing 250–300 g, were housed in individual glass-walled cages and given free access to food and water throughout the experiment.

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Surgery. Rats were anaesthetized with 40 mg/kg sodium pentobarbitone IP, and operated in a stereotaxic instrument (David Kopf, USA). A chemitrode (Leroux and Myers 1975a) made of a stainless steel guide cannula (o.d. 0.8 mm) glued to a brain electrode was implanted in the dorsal midbrain. The bipolar electrode was made of twisted stainless steel, enamel-insulated wires, 150 µm in diameter. The electrode tips were cut off square and reached 1 mm below the lower end of the cannula. With the skull horizontal between bregma and lambda, the chemitrode was introduced 2 mm lateral to the lambda at an angle of 18° with the saggital plane, until the electrode tip was 5.6 mm below the surface of the skull. The chemitrode was attached to the bone with stainless steel screws and methylmethacrylate polymer cement. A stylette was introduced inside the guide cannula to prevent its obstruction. The electrode wires were connected to male pins that could be plugged into an Amphenol socket, at the end of a flexible electrical cable, for brain stimulation (see below).

Apparatus. Brain stimuli were generated by a constant-current, sine-wave stimulator (Marseillan 1977). The stimulation current was monitored on the screen of an oscilloscope (LABO, Brazil).

A shuttle-box consisting of two compartments of 25 × 20 × 20 cm, without any barrier separating them, was placed inside a quiet, darkened room. The grid floor of the shuttle-box oscillated within a narrow angle around the midline axis whenever a rat passed from one compartment to the other. The movements of the grid floor closed a microswitch connected with standard electromechanical equipment (Grason-Stadler, USA). During the experiments, the shuttle-box was illuminated indirectly by a 15-W lamp. The rat inside the experimental chamber had its brain electrode connected to the stimulator by means of a mercury swivel and a flexible cable allowing ample movement inside the box.

Procedure. Determination of the aversive threshold was made according to the method originally described by Brandão et al. (1982) with slight modifications. Six days after surgery, the rats were placed inside the shuttle-box and allowed to habituate for 30 min. The aversive threshold was determined on the next day 10 min after a sham injection (see below). For this purpose, brain electrical stimuli (AC, 60 Hz) of 30 s maximum duration and current intensity increasing by steps of 1.4 µA (RMS) were presented at 1-min intervals until running was elicited. Brain stimulation was automatically switched off (escape) when the rat crossed the midline separating the two compartments of the shuttle-box. The aversive threshold was the lowest current inducing escape behaviour in three successive stimulations. Animals with threshold above 54 µA were discarded.

For intracerebral injections, a thin dental needle (o.d. 0.3 mm) was introduced through the guide cannula until its tip was 1 mm below the cannula end. A polyethylene sleeve occluded the upper extremity of the guide cannula, avoiding reflux of the injected solution. Fifteen seconds later, a volume of 0.5 µl was injected over 30 s using a Hamilton (USA) 10-µl microsyringe. The needle was held in place for another 15 s following injection. Whenever two injections were made, the second was given 30 min after the first one. A sham injection consisted in the introduction of a needle shorter than the guide cannula which was held in place for 1 min, no solution being injected. The aversive threshold was redetermined 10 min after the last (or the only) intracerebral injection.

The difference between the last threshold and that determined after the sham injection in the same animal (Δ threshold) was calculated to assess the effect of drug treatments.

Analysis of results. For each experiment, a single-factor analysis of variance was used. The significance levels of specific comparisons were determined by means of the multiple range test of Newman-Keuls. A log transformation of the data of the experiment on the interaction of zimelidine with 5-HT was made to homogenize the variances (Bartlett's test). In the remaining experiments no data transformation was needed. Additional comparisons among animal groups from different experiments were made using Student's *t*-test.

The dose-effect relationships of 5-HT and 5-MeODMT on aversive threshold were submitted to linear regression analysis and test of parallelism (Brownlee 1975).

Histology. Soon after the experiment, rats were sacrificed under deep anaesthesia and their heads perfused through the heart with saline solution (0.9% NaCl), followed by 10% formalin solution containing 1% potassium ferrocyanide. After decapitation, a DC current of 0.5 mA was passed through the brain electrode for 15 s to stain the tissue around its tip. The brains were removed and fixated in 10% formalin for at least 3 days. Frozen sections of 50 µm were placed on a glass slide and examined with a light microscope at low magnification. Electrode placements were localized in diagrams from König and Klippel's (1963) rat brain atlas.

Drugs. The following drugs were used: 5-hydroxytryptamine creatinine sulphate (Sigma), 5-methoxy-N,N-dimethyltryptamine (Sigma), metergoline (Farmitalia), ketanserin tartrate (Janssen Pharmaceutica), and zimelidine (Astra Läkemedel).

5-HT, 5-MeODMT, and zimelidine were dissolved in saline. A microsuspension of metergoline and of ketanserin was made in saline containing 1% Tween 80. The pH of vehicle solutions used for control injections was equalized to that of drug solutions.

Results

Localization of brain electrodes. As shown in Fig. 1, the electrode tips were localized inside the DPAG and adjoining tectum of the midbrain. Since the electrode tip and the lower end of the injection needle were both 1 mm below the guide cannula, brain injections were made close to these electrode sites.

Behavioural effects of DPAG electrical stimulation. As the intensity of the stimulating current was increased, the rats became alert and then froze. Usually urination and defaecation occurred. With higher current intensities, freezing was replaced by vigorous running, which stopped immediately after brain stimulation was switched off.

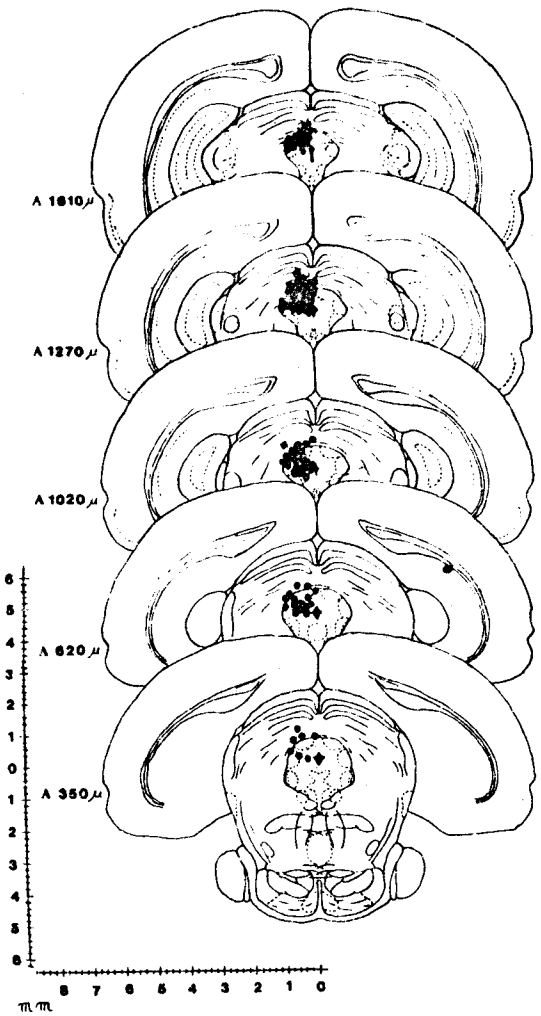

Fig. 1. Localization of electrode sites inside diagrams from König and Klippel's (1963) rat brain atlas. The number of points in the figure is less than the total number of rats used (167) because of several overlaps. Figures represent the atlas coordinates, anterior *(A)* to the interaural line

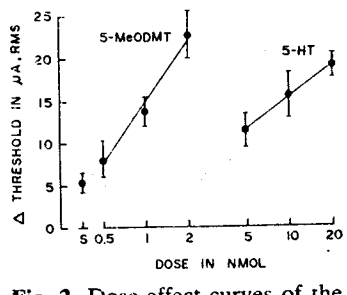

Fig. 2. Dose-effect curves of the increases in aversive threshold caused by microinjections (0.5 µl in 30 s) of 5-hydroxytryptamine *(5-HT)* and 5-methoxy-N,N-dimethyltryptamine *(5-MeODMT)* in the dorsal midbrain. The aversive threshold was the lowest current intensity inducing three midline crossing responses (escapes) in three successive trials of electrical stimulation, applied to the dorsal central grey of rats placed inside a shuttle-box. The increase in threshold was the difference between the threshold determined 10 min after intracerebral injection and the basal threshold in the same animal. Each point in the figure represents the mean and the vertical bars the SEM of 11 rats. *S* stands for saline (0.9% NaCl) injection. The straight lines are calculated linear regressions

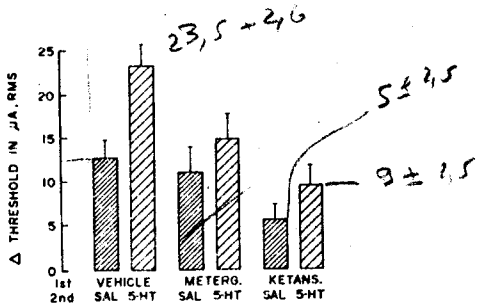

Fig. 3. Antagonism by metergoline (*METERG*, 10 nmol) and by ketanserin (*KETANS*, 10 nmol) of the anti-aversive effect of 5-HT (10 nmol). The 5-HT antagonists or vehicle (1% Tween 80) were injected 30 min before 5-HT or saline *(SAL)* administration. All injections (0.5 µl in 30 s) were inside the dorsal midbrain. Columns represent the mean increase in aversive threshold and vertical bars the SEM of nine rats (see also Fig. 2)

The aversive threshold measured after the sham injection was 20.2 ± 0.8 µA (RMS) for all 167 rats used.

Effect of 5-HT and 5-MeODMT on aversive threshold. As shown in Fig. 2, the intracerebral injection of either 5-HT or 5-MeODMT raised the aversive threshold in a dose-dependent way. Overall significance for drug effects was observed ($F = 9.14$, $df = 6$ and 70, $P < 0.001$). Comparison with control (saline), using the Newman-Keuls test, showed significant effects for the doses of 10 and 20 nmol 5-HT ($P < 0.01$), as well as of 1 and 2 nmol 5-MeODMT ($P < 0.05$ and 0.01, respectively).

By plotting log dose against drug-induced increase in aversive threshold, significant linear regressions were obtained for 5-HT ($F = 7.1$, $df = 1$ and 31, $P < 0.05$) as well as for 5-MeODMT ($F = 23.1$, $df = 1$ and 31, $P < 0.001$). The calculated equations for the regressions were: $Y = 2.61 + 12.71X$, for 5-HT and $Y = 14.85 + 24.24X$, for 5-MeODMT. The regression lines departed significantly from parallelism ($t = 3.97$, $df = 62$, $P < 0.01$).

Antagonism by metergoline and ketanserin. Figure 3 shows that intracerebral injection of 10 nmol of either metergoline or ketanserin antagonized the anti-aversive effect of the same molar dose of 5-HT, injected 30 min later into the dorsal midbrain. An analysis of variance demonstrated the overall significance of the drug effects ($F = 6.48$, $df = 5$ and 48, $P < 0.001$). A significant rise in aversive threshold, as compared to control (vehicle + saline), was caused by the intracerebral injection of 10 nmol 5-HT ($P < 0.01$) following the Tween vehicle. This effect was antagonized by pre-treatment with either metergoline ($P < 0.05$) or ketanserin ($P < 0.01$). The threshold increases caused by 5-HT following either metergoline or ketanserin were neither significantly different from control nor from the increases caused by the 5-HT antagonists, alone. The effects of metergoline or ketanserin followed by saline were not significantly different from control. Nevertheless, a non-significant tendency for the aversive threshold to be lowered was seen after ketanserin (Fig. 3).

Potentiation by zimelidine. As shown in Fig. 4, 100 nmol zimelidine (ZIM) itself caused an anti-aversive effect when injected into the dorsal midbrain of the rat, as well as

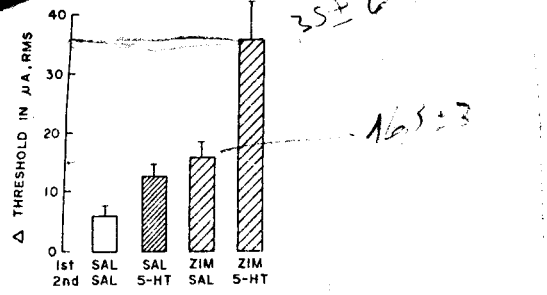

Fig. 4. Potentiation by zimelidine (*ZIM*, 100 nmol) of the anti-aversive effect of 5-HT (10 nmol). Zimelidine was injected 30 min before 5-HT or saline (*SAL*) administration. $n = 9$. Other specifications in Fig. 3

potentiating the effect of 10 nmol 5-HT when given 30 min before the amine. Variance analysis of the log transform data revealed an overall significance of drug effects ($F = 10.95$, $df = 3$ and 32, $P < 0.001$). The following meaningful differences between treatments were significant ($P < 0.05$) according to the Newman-Keuls test: saline plus 5-HT versus control, zimelidine plus saline versus control, zimelidine plus 5-HT versus saline plus 5-HT, and zimelidine plus 5-HT versus zimelidine plus saline. Subtracting the control mean from the means of all other groups in order to assess the drug effects per se, the sum of the threshold increase caused by 5-HT and by zimelidine, 16.34 μA, was considerably less than the combined effect of zimelidine plus 5-HT, 29.85 μA. Therefore, the drug interaction appears supraadditive.

Influence of repeated intracerebral injections and of Tween 80. Comparisons among different experiments showed that the threshold increase caused by two injections of saline was not significantly different from that caused by single saline injections ($t = 0.32$, $df = 18$, $P > 0.05$), but significantly smaller than the effect of the Tween vehicle followed by saline ($t = 2.63$, $df = 16$, $P < 0.05$). Similarly the raising effect of 5-HT on threshold was equivalent, whether its administration was preceded by saline or not ($t = 0.89$, $df = 18$, $P > 0.05$), though smaller than the effect of 5-HT following vehicle administration ($t = 3.68$, $df = 16$, $P < 0.01$). Therefore, in both cases a threshold-increasing effect of Tween 80 was evidenced, adding to the effects of saline or 5-HT. On the other hand, microinjection of saline apparently did not interfere with the effect of subsequent intracerebral injections.

Discussion

The present results, showing that microinjection of either 5-HT or 5-MeODMT in the dorsal midbrain of the rat raises the electrical threshold of DPAG stimulation current that induces escape behaviour, confirm and extend previously reported observations in rats with chemitrodes implanted in the hypothalamus (Leroux and Myers 1975b). In the latter study, microinjection of 5 μg 5-HT markedly increased the tolerance to aversive brain electrical stimulation. Therefore, direct application of drugs that stimulate 5-HT receptors in periventricular areas of the brain decreases the aversive effects of periventricular electrical stimulation.

Straight regression lines were presently obtained when log doses of either 5-HT or 5-MeODMT were plotted against drug-induced increases in aversive threshold. The dose-response curve of 5-MeODMT was steeper and at the left side of the 5-HT dose-effect curve, indicating its higher potency and, possibly, higher efficacy. This is probably due to the rapid inactivation of 5-HT by neuronal uptake processes (Iversen 1975). In contrast, 5-MeODMT inhibits the amine pump of serotonergic nerve endings, its effect being a combination of direct post-synaptic receptor stimulation plus potentiation of neuronally released 5-HT (Berge et al. 1983). That the latter mechanism could indeed contribute to the anti-aversive effect of locally administered 5-MeODMT is suggested by the threshold increasing effect of zimelidine, discussed below.

Metergoline and ketanserin behave as competitive 5-HT receptor blockers in vivo as well as in vitro (Fuxe et al. 1975; Peroutka et al. 1981; Van Nueten et al. 1981; Leysen et al. 1981). Therefore, the antagonism of the anti-aversive effect of intracerebrally injected 5-HT by both agents, shown by our results, strongly suggests 5-HT receptor mediation. Ketanserin has an affinity for 5-HT_2 receptors that is over 1,000 times higher than for 5-HT_1 receptors, whereas metergoline shows high affinity for both types of 5-HT receptors (Peroutka et al. 1981; Leysen et al. 1981). Since in the present results the same molar dose of either metergoline or ketanserin was equally effective in antagonizing 5-HT, it may be further suggested that 5-HT_2 receptors are preferentially involved in the anti-aversive action of 5-HT. Accordingly, a recent study using autoradiographical techniques showed that the midbrain central grey contains a moderate density of 5-HT_2 receptors (Slater and Patel 1983).

Zimelidine is a highly specific 5-HT uptake inhibitor, devoid of direct activity upon 5-HT receptors (Ross et al. 1976). In addition, immunofluorescent 5-HT fibres have been identified in the periaqueductal grey matter of the rat (Steinbush 1981). Therefore, the anti-aversive effect caused by intracerebrally injected zimelidine, shown by the present results, is probably due to an enhancement of the activity of neuronally released 5-HT. In addition, zimelidine potentiated the anti-aversive effect of exogenous 5-HT, indicating that amine uptake mechanisms are indeed operating in the DPAG. These results suggest that serotonergic nerve fibres in the DPAG inhibit its aversive function. In agreement, previously reported results using systemic drug injection revealed that either chlorimipramine, which acts like zimelidine, or the precursor of 5-HT synthesis, 5-HTP, depressed escape from DPAG electrical stimulation in the rat (Kiser et al. 1978).

Experimental evidence reviewed elsewhere (Graeff 1981, 1984) indicates that minor tranquillizers depress the functioning of the brain aversive system and that this action is likely to mediate, at least in part, their clinical anti-anxiety effect. Therefore, the present results indicating an anti-aversive role of endogenous 5-HT give little support to the suggestion that benzodiazepines relieve anxiety by decreasing 5-HT activity in brain punishment systems (Wise et al. 1972, 1973; Stein et al. 1973). The latter view is mainly based on the anti-punishment effect of drugs that either antagonize 5-HT or inhibit its biosynthesis and in the high predictive value of animal conflict tests as regards clinical anxiety (see Introduction). However, this line of evidence has been weakened by [illegible]orted results showing that in rats

repeatedly exposed to a punished drinking situation, only anxiolytics released suppressed behaviour, whereas anti-5-HT drugs were ineffective (Petersen and Lassen 1981; Kilts et al. 1982). Similar results were obtained in our laboratory with lever-pressing behaviour punished by aversive brain stimulation, in the rat (Morato de Carvalho et al. 1981). In addition, metergoline apparently enhanced subjective anxiety in healthy volunteers (Graeff et al. unpublished results), in spite of causing major increases in punished responding, in pigeons (Leone et al. 1983). Therefore, the anti-punishment effect of anti-5-HT drugs in animal conflict tests may be false-positive in terms of its predictive value for human anxiety. Two recent studies also do not support reduction in 5-HT activity as the mechanism of action of benzodiazepines (Shephard et al. 1982; Thiébot et al. 1984).

In conclusion, the present results as well as the reported evidence discussed above indicate that 5-HT plays an inhibitory role in the brain aversive system including the DPAG, probably acting through 5-HT_2 receptors. This anti-aversive function of 5-HT may be related to the clinical fear- and pain-reducing actions of 5-HT uptake inhibitors (Evans et al. 1980; Montastruc et al. 1983).

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