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Role of the Periaqueductal Gray Substance in the Antianxiety Action of Benzodiazepines[1]

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SCHENBERG, L. C. AND F. G. GRAEFF. *Role of the periaqueductal gray substance in the antianxiety action of benzodiazepines*. PHARMAC. BIOCHEM. BEHAV. 9(3) 287–295, 1978.—In order to study the interactions between serotonergic mechanism and electrical stimulation of the mesencephalic central gray substance, rats were trained to lever-press for terminating aversive electric stimuli applied at the Periaqueductal gray and adjoining tectum of the mesencephalon. Experimental sessions consisted of 40 discrete escape trials of a maximum of 30 sec duration, separated by 30 sec intervals. Dose-effect curves of two tryptamine antagonists, cyproheptadine and methysergide, as well as of the benzodiazepine minor tranquilizer, chlordiazepoxide, on average escape latencies and on frequency distribution of individual latencies were determined. Doses of 3 to 10 mg/kg of cyproheptadine decreased average latencies of escape responding in six of eight rats studied. Doses of 10 and 30 mg/kg of methysergide also facilitated escape responding in one of three rats. In contrast, doses from 1 to 10 mg/kg of chlordiazepoxide, that cause little sedation or ataxia, produced dose-dependent increases in escape latencies. Furthermore, doses of 5.6 and 10 mg/kg of chlordiazepoxide partially blocked escape responding. The facilitatory effects of the tryptamine antagonists suggest that escape behavior is inhibited by brain tryptaminergic mechanisms, whereas the specific depressant effect of chlordiazepoxide on escape from Periaqueductal gray electrical stimulation suggest that this region may be involved in the antianxiety action of benzodiazepines.

Periaqueductal gray substance Electrical stimulation Escape behavior Tryptamine antagonists
Chlordiazepoxide Behavior inhibition Anxiety

THE TRYPTAMINE antagonists, bromolysergic acid (BOL), methysergide and cyproheptadine have been shown to increase response rate of positively reinforced operant behavior, whether responding was simultaneously punished by response-contingent electric shock or not [2, 7, 8, 9, 10, 39, 45]. In the same way, inhibition of serotonin (5-HT) synthesis by para-chlorophenylalanine (PCPA), as well as selective destruction of brain serotonergic neurons by intraventricular injection of 5,6-dihydroxytryptamine have been reported to facilitate punished responding in rats [6, 30, 47]. More specifically, Tye *et al.* [41] have recently shown that injection of 5-7-dihydroxytryptamine into the ventral tegmentum of the rat mesencephalon, destroying most of the ascending serotonin system [5], desinhibits punished responding. On the other hand, directly acting tryptamine agonists, such as α-methyltryptamine and N,N-dimethyltryptamine or the 5-HT precursor, 5-hydroxytryptophan, suppress punished and non-punished responding [1, 7,10]. These results suggest the existence of serotonergic neurons in the brain causing behavior inhibition.

The serotonergic behavior-inhibitory system may be related to the antianxiety action of minor tranquilizers. In the same way as tryptamine antagonists, minor tranquilizers can enhance punished operant responding and this facilitatory action on punished behavior has good predictive value of their clinical efficacy in reducing anxiety [2, 19, 24]. Since the increases in punished responding caused by the benzodiazepine, oxazepam, on rat punished behavior correlated with the drug-induced decrease in 5-HT turnover in the midbrain-hindbrain region, Wise *et al.* [46] suggested that the antianxiety as well as the antipunishment action of the benzodiazepines is due to a decrease in functioning of the behavior-inhibitory serotonin system. More recently, Stein *et al.* [38] have also suggested that this action of the benzodiazepines is indirect, due to a facilitation of γ-aminobutyric acid-mediated presynaptic inhibition of serotonin nerve terminals. In more general terms, a relationship between behavioral inhibition and fear or anxiety has also been suggested by Gray [12].

Nevertheless, in addition to the brain serotonergic neurons inhibiting behavior, the central gray matter of the mesencephalon may also be involved in anxiety. Electrical stimulation of the periaqueductal gray has been reported to cause flight behavior or defensive aggression in cats and rats [18, 20, 31, 32, 33, 48] and these effects have been associated either with pain [33, 36, 42] or fear and anxiety [20,27]. Indeed feelings of fear, fright, and sometimes diffuse pain sensations have been reported by human patients, following the electrical stimulation of the central gray matter of the mesencephalon [26]. These results indicate that this area

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may be part of brain mechanisms mediating fear and anxiety and as a consequence, be a potential site of action of minor tranquilizers.

The effects of two potent tryptamine antagonists, cyproheptadine and methysergide, as well as of the benzodiazepine minor tranquilizer, chlordiazepoxide, on escape from electrical stimulation of the rat mesencephalon were presently studied in order to investigate the interactions between brain serotonergic systems and periaqueductal gray electrical stimulation. Since the pioneering work of Delgado, *et al.* [3], operant techniques have been used in order to accurately measure escape behavior induced by brain electrical stimulation [20, 27, 42]. Therefore, in the present study, rats were trained to press a lever in order to terminate a sequence of electrical stimuli applied to the mesencephalic central gray or adjoining tectum and dose-effect relationships for each drug on escape latencies were determined.

METHOD

Animals

Male, albino Wistar rats, weighing 250–300 g, were housed in individual, glass-walled cages and given food and water ad lib.

Surgery

Rats were anesthetized with 40 mg/kg sodium pentobarbital. Each animal was implanted with a brain electrode in a stereotaxic instrument (David-Kopf, model 900, U.S.A.). Brain electrodes were made of two twisted stainless steel wires, each coil with 160 μm dia., insulated except at the cross-section of the tip. All electrodes were aimed at the mesencephalic central gray substance following the coordinates of König and Klippel's [21] rat brain atlas (anterior −0.1 to +1.0 mm, lateral 0.0 mm and vertical: −1.4 to +1.0 mm). After insertion, the brain electrodes were anchored to the skull by means of methylmethacrylate polymer cement, held by two metal screws and a wire clamp fixed to the parietal and temporal bones, respectively.

Apparatus

A 24×28×20 cm rat chamber, provided with a lever in one of its lateral walls, placed 6 cm above the grid floor, was used. A minimum of 15 g was necessary for operation of the lever. The experimental chamber was placed inside an insulating chest provided with fan and observing screen. The animal compartment was indirectly illuminated by a 5 W light bulb, placed in the ceiling of the insulating chest. Temperature inside the chamber varied between 22 and 23°C. Standard electromechanical equipment (Grason-Stadler, Co., U.S.A.) was used for automatic programming and recording.

Brain stimuli were generated by a rectangular wave stimulator (RFM, Brazil). The stimulator pulses of 0.2 msec, were differentiated with a series, 0.01 μF capacitor (modified from Lilly, *et al.* [22]) and the stimulation current was continuously monitored by means of an oscilloscope (Heathkit, U.S.A., model 10–104). The rats, inside the experimental chamber were connected to the stimulator by means of a swivel and a flexible cable, attached to the brain electrode.

Procedure

Following a 15-day convalescence from surgery, the animals were placed in the experimental chamber and stimulated with a series of pulse-pairs at a frequency of 100/s. The first positive and the second negative components of the pulse-pair were separated by a time interval of 0.2 msec. The current was increased up to a maximum of 1.5 mA, measured at the peak of the positive wave in the oscilloscope, or until changes in the animal's behavior were observed. Next, the animals were trained to approach the lever using the removal of the brain stimulus for 15 sec as reinforcement. If shaping was successful, the stimulation effect was considered aversive. In nonlearners, the reverse procedure was attempted, that is, animals were shaped to approach the lever using the presentation of the brain stimulus for 1 sec as reinforcement. In such cases, the stimulation effect was classified as rewarding. If both positive and negative reinforcing properties were observed, the stimulation effect was named ambiguous. Finally, if no motivational effect was detected, it was classified as neutral. Only animals showing aversive brain stimulation effects were used in present experiments.

Due to the difficulty in shaping the lever-pressing response with termination of intracerebral electrical stimulation, the rats were first trained to escape continuous electric shock (0.5–1.0 mA) delivered to the rat's paws by means of a shock generator (Grason-Stadler, U.S.A., model E 106GS). A lever press turned off the electric shock for 30 sec. If no response occurred within 30 sec from the stimulus onset, the shock was turned off automatically for 30 sec. Each experimental session consisted of 40 discrete escape trials. When the animals were escaping in every trial during at least five consecutive sessions, aversive brain electrical stimulation was substituted for the electric grid shocks. The stimulus intensity was regulated for each animal in order to generate 100% escape responding at an average response latency per session varying from 4 to 6 sec. Stimulus intensity varied between 0.3 and 1.0 mA. This criterion for control latencies allowed both decreasing and increasing effects of drugs to be measured. Training to stability using brain stimulation usually required 3 to 6 weeks for completion.

The experiments were conducted daily from Monday through Friday. Drug treatments began after responding had stabilized within the above criterion for at least five days. Drug injections were made 24 hr after control sessions. Following a given drug treatment, daily sessions were run until responding returned to criterion. Drug treatments were never separated by less than 48 hr.

Analysis of Results

The response latencies of each trial were recorded in a running time meter. From these individual values frequency histograms of escape latencies were drawn. In addition, response latencies were cumulatively recorded in another running time meter along the whole experimental session. From this value the average latency in the session was calculated. The maximum latency that could be measured in each trial was 30 sec (trial without a response). This value was used for average calculations, although actual latencies should be longer.

Histology

With the rat under deep pentobarbital anesthesia, the head was perfused with 0.9% NaCl solution and 10% for-

maldehyde solution. Following decapitation, the brain was left for at least 3 days in 10% formaldehyde solution, and the electrode removed. The mesencephalon was embedded in paraffin wax and serially sectioned at a thickness of 20 μm. The sections were stained with hematoxylineosin or with Weigert-neutral red and examined with a light microscope at low magnification. Electrode placements were localized in diagrams from König and Klippel's [21] rat brain atlas.

Drugs

Cyproheptadine hydrochloride (Merck, Sharp & Dohme, Brazil), methysergide (1-methyl-d-lysergic acid butanolamide bimaleate, Sandoz, Brazil), chlordiazepoxide hydrochloride (Roche, Brazil) were used. Cyproheptadine was administered as a fine suspension in 0.9% NaCl solution. Methysergide and chlordiazepoxide were dissolved in saline solution containing 1% Tween–80. A volume of 1 ml/kg body weight of drug solution was injected IP. Cyproheptadine was administered 25 min, while methysergide and chlordiazepoxide were given 45 min before the experimental sessions. Doses of the drugs refer to salts. The different doses were given in nonsytematic order.

RESULTS

Gross-Behavioral and Motivational Effects of Dorsal Mesencephalic Stimulation

As illustrated in Fig. 1, electrical stimulation of the periaqueductal gray matter caused aversive effects in 18 of the 28 animals tested. Rewarding brain stimulation was observed in seven rats with electrodes placed inside or near the dorsal raphe nucleus. In two other animals the stimulation caused no effect and in another rat, with a ventro-lateral placement of the electrode inside the central gray, the electrical stimulation was considered ambiguous. In four additional animals showing aversive brain stimulation, and which were used in drug studies (B12, D1, H5 and H7), the electrode placement could not be anatomically determined because the animals lost the electrodes during the course of the experiment. Conversely, some animals with aversive placements shown in Fig. 1 have not been tested with drugs, having been used for the study of the motivational functions of the periaqueductal gray substance only.

The onset of aversive stimulation caused sudden immobility (freezing), immediately followed by agitated behavior. The rats wildly ran inside the experimental box or jumped against the ceiling, sometimes hurting their noses. No vocalization or motor convulsions were observed. Following training of lever-pressing escape, all signs of behavioral agitation disappeared and the animals looked calm and often groomed themselves between escape trials. In contrast, rewarding stimulation induced intense exploration and increased general motor activity.

Effects of Drugs on Escape from Brain Electrical Stimulation

Effective doses of cyproheptadine, ranging from 3 to 10 mg/kg decreased the average latencies of escape responding in six of eight rats, as shown in Fig. 2. Slight increases in latency were observed in rats H2, following 5.6 mg/kg and G2, after 10 mg/kg of cyproheptadine. In another rat (D1) doses of cyproheptadine up to 17 mg/kg (last dose not shown in the figure) did not change responding. The decrease in

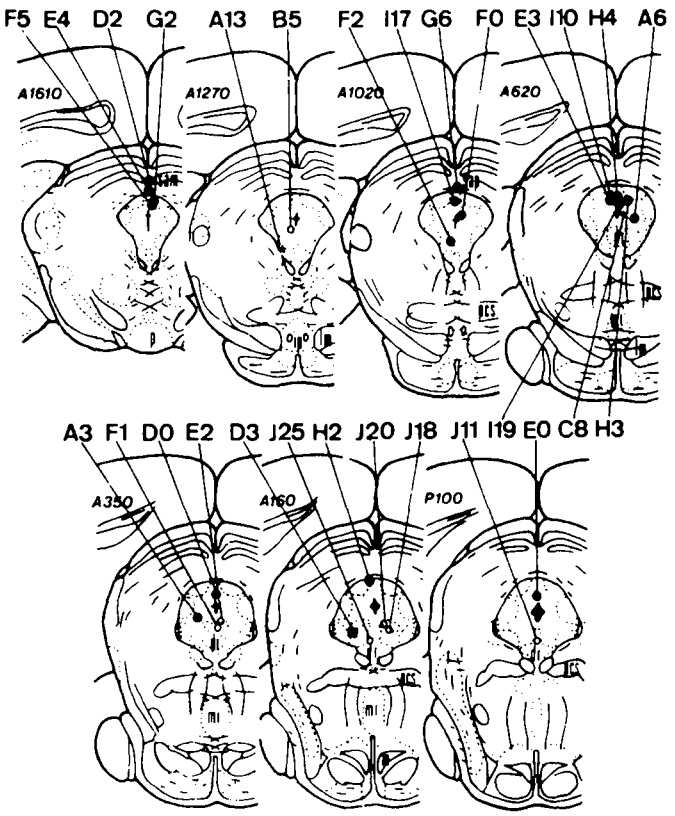

FIG. 1. Graphical representation of electrode sites and motivational effects of brain electrical stimulation. (●) aversive—(○) rewarding—(★) neutral and (■) and ambiguous. Letters and figures above graphics identify the experimental animal. Figures inside graphics represent the coordinates of König and Klippel's (1963) rat brain atlas in μm. *dr*: nucleus dorsalis raphes; *mr*: nucleus medianus raphes; *ip*: nucleus interpeduncularis; *lm*: lemniscus medialis; *pcs*: pedunculus cerebellaris superior; *sam*: stratum album mediale; *sap*: stratum album profundum. The aversive placements of Rats E3 and I10 are superimposed.

average response latencies generally caused by cyproheptadine was due to an increase in the occurrence of very short latencies (0–3 sec) at the expense of a decrement in the frequency of the longer latencies, as shown in the histograms of Fig. 3.

Doses from 1 to 30 mg/kg of methysergide were administered in three rats, F2, D1 and E3, respectively. Only the last animal showed decreases in escape latency below the control range of variation following the doses of 10 and 30 mg/kg of methysergide. In the other two animals, the drug was ineffective at the doses tested.

In contrast to the tryptamine antagonists, effective doses of chlordiazepoxide, from 1 to 10 mg/kg, caused dose-dependent increases in the average response latencies as shown in Fig. 4. This was due to the relative increase in the occurrence of long latencies (longer than 12 sec) together with a decrease in the frequency of shorter latencies, as illustrated in Fig. 5. The shaded columns in this figure also show that the highest doses of the tranquilizer progressively blocked escape responding.

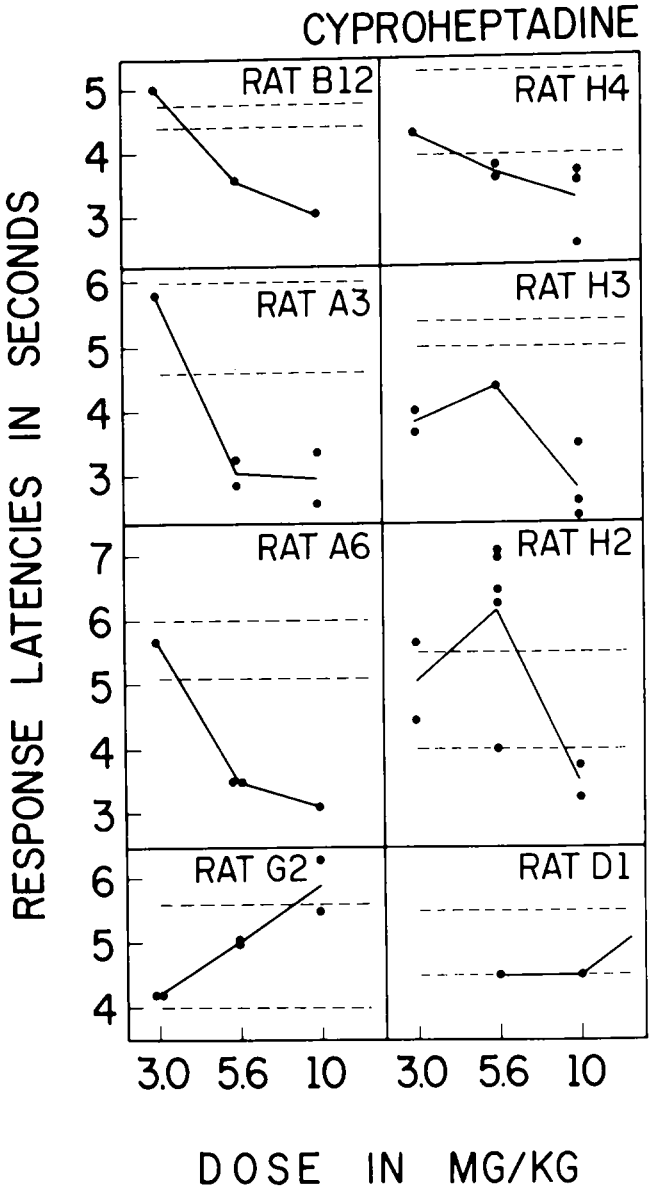

FIG. 2. Effect of cyproheptadine on escape from brain electrical stimulation in eight rats. Points in the figure represent average response latencies of 40 discrete escape trials in one experimental session. Full lines connect the session means. Dashed horizontal lines represent the range of three to nine control measurements. Cyproheptadine was injected, IP, 25 min before the experimental session.

DISCUSSION

Although a systematic study of the motivational properties of mesencephalic electrical stimulation has not been carried out in the present study, our results indicate that electrical stimulation of the dorsal portion of the mesencephalic central gray substance and tectum is aversive, whereas the stimulation of its ventral portion is rewarding. As illustrated in Fig. 1, aversive electrodes were situated above the upper limit of the dorsal raphe nucleus, with one possible exception (Rat F2), whereas rewarding electrodes were located inside or close to the dorsal raphe nucleus. Neutral or ambiguous electrodes were placed in between the two areas. These results agree with several published reports showing that the electrical stimulation of the dorsal central gray matter and tectum of the mesencephalon causes flight or escape behavior in rats [20, 27, 31, 32, 42, 48] and cats [18,33]. In addition, defensive aggression has also been reported following stimulation of the central gray, indicating that this area is part of an integral fight-flight system [11,18]. Rewarding effects of electrical stimulation in or near the dorsal raphe nucleus, as described in the present results, have also been reported by others [4, 23, 31, 32, 35]. Al-

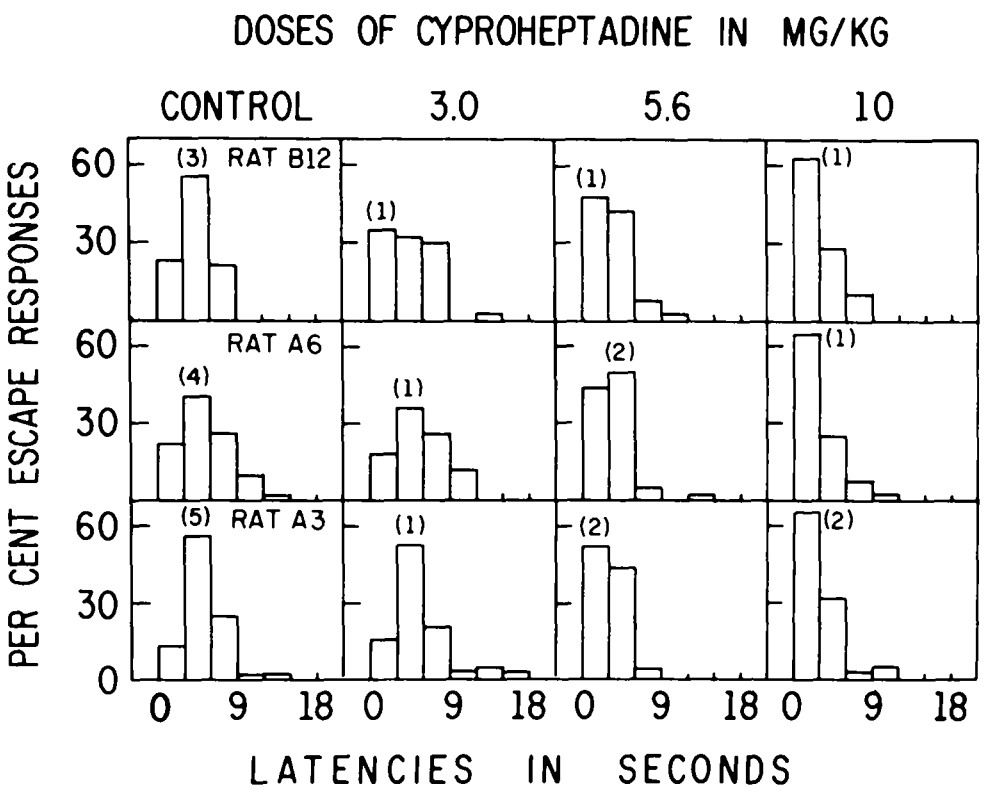

FIG. 3. Effect of cyproheptadine on escape from brain electrical stimulation in three representative rats. Frequency histograms of escape latencies in control sessions and after three increasing doses of cyproheptadine. Each escape response was classified within successive 3-sec time bins from stimulus onset. The columns show the totals within each bin expressed as percentages of total escape responses. Figures in parenthesis represent the number of experimental sessions.

though the dorsal raphe nucleus is constituted mainly of serotonergic neuron cell bodies [5], the neurochemical substrate of these rewarding effects seems to be adrenergic, rather than tryptaminergic [4, 23, 35].

The aversive character of central gray electrical stimulation has been attributed to either fear and anxiety [20, 26, 27] or pain [33, 36, 42]. Although nociceptive behavior has been reported following electrical stimulation or local morphine injection in the mesencephalic central gray and tectum [15, 36, 37], no squealing was presently observed accompanying flight behavior, even when intense electrical stimulation was used. In addition, the strong analgesic, morphine, was far less potent than chlordiazepoxide in inhibiting escape responding, as discussed below. Therefore, in our experiments, escape behavior following electrical stimulation of the mesencephalic central gray matter does not seem to be motivated by pain.

Brain tryptaminergic systems seem to inhibit escape from brain stimulation, since the two tryptamine antagonists used in the present study decreased average latencies of escape responding. Although the decrease in latency caused by cyproheptadine were small and did not occur in two of eight animals studied they are deemed important, because control average latencies were already short (between 4 and 6 sec) and therefore difficult to be even more reduced. For the same reason, the facilitation of escape behavior by methysergide, which was observed in only one out of three animals, may also be viewed as significant. Although cyproheptadine also shows powerful antihistaminic as well as antimuscarinic actions [40,43], its facilitatory effect on behavior is probably due to the antitryptaminic activity, since it has been shown that atropine is far less effective than cyproheptadine while antihistaminics are completely ineffective in enhancing punished responding, in the rat [8]. In addition, the lower potency of methysergide, in facilitating escape agrees with previous observations with punished behavior and brain electrical self-stimulation, in rats [8,34]. Although methysergide is a more specific tryptamine antagonist than cyproheptadine, in the sense that it has no important antihistaminic or antimuscarinic properties, it has also been shown to be less potent than cyproheptadine as a 5-HT antagonist in the isolated rat uterus [13]. Therefore, the presently observed facilitation of escape behavior by cyproheptadine and methysergide is likely to be due to the impairment of behavior-inhibitory serotonergic systems, as previously suggested [8, 9, 10, 34]. The hypothesis that brain serotonergic mechanisms inhibit escape from brain electrical stimulation is also supported by the results reported by Kiser and Lebovitz [20]. In this study, rats were trained to barpress in order to gradually decrease the intensity of electrical stimulation in the periaqueductal gray substance and, as presently observed with tryptamine antagonists, inhibition of serotonin synthesis by PCPA, resulted in increased escape responding.

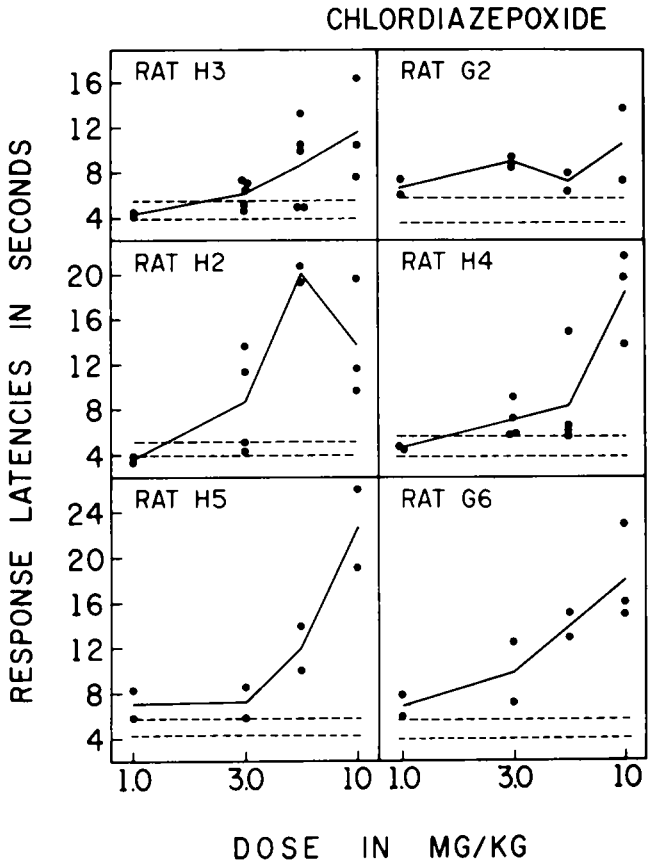

FIG. 4. Effect of chlordiazepoxide on escape from brain electrical stimulation in six rats. Dashed horizontal lines represent the range of eight to 15 control determinations. Chlordiazepoxide was injected, IP, 45 min before the experimental session. Other specifications as in Fig. 2.

Both benzodiazepine minor tranquilizers and tryptamine antagonists have been shown to increase low rates of punished and nonpunished operant behavior [8, 9, 10, 19, 24, 49]. In addition, the facilitatory effect of a benzodiazepine, oxazepam, on punished operant responding has been shown to correlate with drug-induced decrease in serotonin turnover in the rat midbrain-hindbrain region [46]. As a consequence, Wise *et al.* [46] suggested that the facilitatory action of the benzodiazepines on responding may be mediated by a reduction of serotonin in a behavioral-inhibitory system. In contrast, the present results show that chlordiazepoxide and tryptamine antagonists have opposite effects on escape from electrical brain stimulation. While cyproheptadine and methysergide tended to facilitate escape responding, chlordiazepoxide increased the latencies of escape from brain stimulation and even blocked escape responses at the highest doses used. Therefore, the inhibitory effect of chlordiazepoxide on escape from brain stimulation cannot be explained by impairment of behavior-inhibitory serotonin neurons. Kiser and Lebovitz [20] have also reported that chlordiazepoxide significantly decreased decremental escape responding. However, the doses of chlordiazepoxide used by Kiser and Lebovitz [20] were relatively high (18 and 22 mg/kg), already causing muscle relaxation. In contrast, doses of only 3 to 10 mg/kg of chlordiazepoxide were presently effective in inhibiting escape from brain stimulation. These doses are not only under the range causing major sedative or ataxic effects [17,50], but have actually been shown to increase punished and nonpunished lever-pressing maintained by water presentation, in rats [8,9]. Therefore, the presently observed inhibitory effect of chlordiazepoxide on escape from brain stimulation seems to be fairly specific.

Although the depressant effects of chlordiazepoxide on escape from brain stimulation could be due to a decrease in pain presumably produced by electrical stimulation of the mesencephalic central gray matter [36,37], this seems to be unlikely. It has been reported that high doses of chlordiazepoxide can antagonize certain responses evoked by noxious stimulation, but chlordiazepoxide is generally less potent than morphine in inhibiting pain reactions [16]. In order to study the effect of morphine on escape from periaqueductal stimulation, preliminary experiments have been conducted in this laboratory. Morphine administered to two rats under the same experimental conditions used in the present study, caused moderate escape inhibition in one animal, at the dose of 10 mg/kg, while in the other rat even 17 mg/kg of morphine did not affect escape responding. Although inconclusive in respect to the nature of morphine action on escape from periaqueductal gray stimulation, these results give little support to the view that the depressant effects of chlordiazepoxide on escape could be due to its weak analgesic actions.

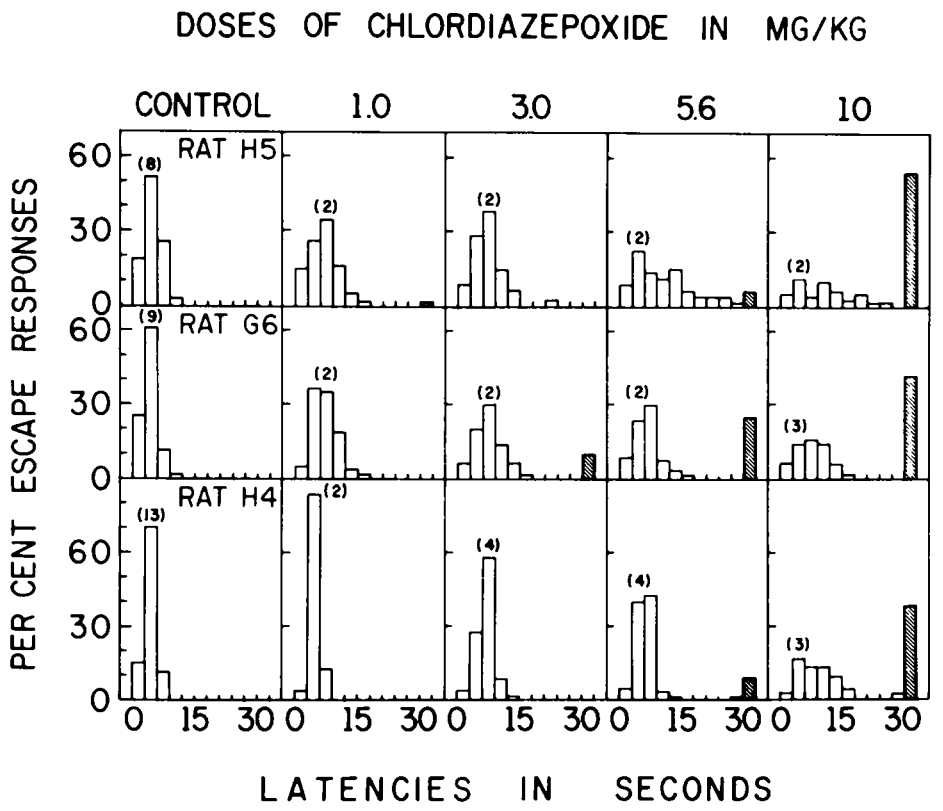

FIG. 5. Effect of chlorodiazepoxide in escape from brain electrical stimulation in three representative rats. Shaded columns represent latencies longer than 30 sec. Other specifications as in Fig. 3.

From the above discussed evidence, it may therefore be suggested that chlordiazepoxide, and possibly other benzodiazepine minor tranquilizers as well, depresses the fight-flight system by acting on the periaqueductal gray substance or on some other part, placed forward in the escape circuit. The outstanding taming effects of chlordiazepoxide originally described by Randall *et al* [29], as well as the specific inhibition of defensive aggression reported by Hoffmeister and Wuttke [17] in cats and mice, may be viewed as manifestations of drug-induced inhibition of the fight-flight system.

In addition to the decrease in functioning of behavior-inhibitory serotonin systems suggested by Wise *et al* [46], depression of the fight-flight system may be necessary for the clinical antianxiety action of the benzodiazepines, since impairment of serotonergic neurotransmission alone, by either tryptamine antagonists or PCPA, does not apparently lead to antianxiety effects, in humans. Actually, nervousness, anxiety and insomnia have been reported among their clinical side effects [14, 25, 28, 44]. Nevertheless, this suggestion does not exclude the participation of behavior-inhibitory systems in the nervous integration of fear [12]. Indeed, it has recently been observed in this laboratory (F. G. Graeff and N. G. Silveira Filho, unpublished results) that electrical stimulation of the median raphe nucleus of the rat causes suppression of ongoing positively reinforced behavior, accompanied by crouching, defecation, micturition, piloerection and sometimes teeth clattering. This fear-like reaction was attenuated by PCPA, thus suggesting its mediation by the mesolimbic serotonergic pathway, originating at the median raphe nucleus. Since the concept of fear or anxiety has a response-inhibitory connotation as well as a driving or motivating one, it is possible that the serotonergic system may subserve the inhibitory aspect, whereas the fight-flight system subserves the motivating aspect of fear. From the present results with the tryptamine antagonists and the similar report with PCPA [20], it may be further hypothesized that the serotonergic system exerts an inhibitory influence upon the fight-flight system. Both systems would be depressed by chlordiazepoxide and other minor tranquilizers in order to produce their antianxiety action.

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