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GABA Mediation of the Anti-Aversive Action of Minor Tranquilizers[1]

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BRANDÃO, M. L., J. C. DE AGUIAR AND F. G. GRAEFF. *GABA mediation of the anti-aversive action of minor tranquilizers*. PHARMAC. BIOCHEM. BEHAV. 16(3) 397–402, 1982.—Earlier observations have shown that systemically injected minor tranquilizers decrease the aversive consequences of electrical stimulation of the dorsal periaqueductal gray (DPAG) matter of the rat brain. In order to verify if these drugs can act directly on the DPAG, chlordiazepoxide (CDP) and pentobarbital (PB) were locally injected into the dorsal midbrain of rats chronically implanted with chemitrodes, allowing electrical stimulation of the same brain area. Microinjection of doses of 0.16 and 0.32 μmol of CDP and 0.16 μmol of PB significantly increased the threshold electrical current inducing flight behavior by stimulating the dorsal midbrain. Flight behavior was measured by the number of times rats crossed the dividing line while running from one compartment of a shuttle-box to the other. The same effect was caused by the intracerebral injection of 0.32 and 0.64 μmol of the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). Conversely, local injection of the GABA antagonists, bicuculline (5–20 nmol) or picrotoxin (0.3 and 0.6 nmol), into the dorsal midbrain induced flight behavior, like the electrical stimulation. On the other hand, the glycine antagonist, strychnine (40 nmol) caused convulsive behavior only, while the intracerebral injection of the cholinergic agonist, carbachol (10–40 nmol), increased locomotion, sniffing and turning behavior, but did not induce flight. Pretreatment with locally injected GABA (0.64 μmol) antagonized the aversive effect of either bicuculline (10 nmol) or picrotoxin (0.3 nmol), whereas CDP (0.32 μmol) antagonized bicuculline only and PB (0.16 μmol) was ineffective against either bicuculline or picrotoxin. These results suggest that minor tranquilizers act directly upon the DPAG by enhancing the tonic inhibitory influence of endogenous GABA. This action may underly the antiaversive affects of these drugs.

Flight behavior Dorsal periaqueductal gray Electrical stimulation Intracerebral injection
Minor tranquilizers GABA Bicuculline Picrotoxin

PREVIOUSLY reported results have shown that chlordiazepoxide (CDP) and pentobarbital (PB) decrease the aversive consequences of electrical stimulation of the dorsal periaqueductal gray (DPAG) substance of the rat brain [7, 13, 16, 21]. Also the increase in the mean arterial blood pressures, heart rate and respiration caused by DPAG electrical stimulation in urethane-anesthetized rats was shown to be attenuated by CDP [22]. On the basis of these results, the suggestion has been made that minor tranquilizers impair the functioning of the brain aversive system (BAS) comprising the DPAG, the periventricular gray substance of the diencephalon and the amygdala [5] and that this action would be responsible for at least part of their anti-anxiety effect [7, 16, 21].

In the above studies, however, the anti-anxiety drugs were injected either intraperitoneally [7, 13, 16, 21] or intravenously [22]. Therefore, it is not clear whether the drug-induced attenuation of the effects of brain stimulation was due to an action of the minor tranquilizers on the DPAG, on different areas of the BAS or on other related brain systems.

In order to verify if minor tranquilizers can directly affect the DPAG, CDP and PB were locally injected into the dorsal midbrain of the rat through a permanent guide cannula attached to a brain electrode (chemitrode), allowing electrical stimulation of the injection area. The effect of intracerebral injection of CDP and PB on the threshold electrical current eliciting flight behavior by stimulating the DPAG or its neighboring mesencephalon was measured.

The present study was also aimed at exploring the neurohumoral mechanisms of minor tranquilizers action in the DPAG. For this purpose, gamma-aminobutyric acid (GABA) and its antagonists, bicuculline and picrotoxin, were microinjected into the dorsal midbrain. The capacity of these drugs either to alter the threshold current inducing flight behavior or to cause behavior changes similar to those elicited by the electrical stimulation of the dorsal midbrain was measured. These substances were chosen on the basis of several reported results suggesting that the inhibitory neurotransmitter, GABA, is involved in the anti-anxiety as well as other pharmacological actions of benzodiazepines and barbi-

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turates [9, 10, 25]. For comparison, the glycine antagonist, strychnine was also used. Since the intraventricular or intrahypothalamic injection of the cholinergic agonist, carbachol, has been shown to induce fear-like behavior in cats [2,11] this drug was also included in the present study.

METHOD

Animals

Male, albino Wistar rats, weighing 250–300 g were housed in individual glass-walled cages and given free access to food and water throughout the experiment.

Surgery

Rats were anesthetized with 40 mg/kg of sodium pentobarbital, IP, and operated in a stereotaxic instrument (David Kopf, U.S.A.). A chemitrode made of a stainless steel guide cannula (o.d. 0.8 mm) glued to a brain electrode was implanted in the dorsal midbrain. The electrode was made with stainless steel wire, 250 μm in diameter, Diamel-insulated except at the cross-section of the tip, reaching 1 mm below the lower end of the cannula. With the skull horizontal between bregma and lambda, the chemitrode was vertically introduced at the lambda, until the electrode tip was 5.0 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 obstruction. The electrode wire was connected to a male pin, parallel to the outer end of the cannula. Together, they could be plugged into an Amphenol socket, at the end of a flexible electrical cable (see below), for brain stimulation.

Apparatus

Brain stimuli were generated by a constant current, sine-wave stimulator [15]. The stimulation current was monitored by means of an oscilloscope (Heathkit, U.S.A.).

A shuttle-box consisting of two compartments of 25×20×20 cm without any barrier between them was placed inside an insulating chest provided with fan and a wide angle lens allowing one-way vision of both compartments. The grid-floor of the shuttle-box oscillated within a narrow angle around a midline axis whenever a rat passed from one compartment of the box to the other. The movements of the grid floor closed one of two microswitches connected with standard electromechanical equipment (Grason-Stadler, U.S.A.), automatically recording the crossing responses. During the experiments, the shuttle-box was illuminated by a 2 W lamp. The rat inside the experimental chamber had its brain electrode connected with the stimulator by means of a mercury swivel and a flexbile, bite-proof cable allowing ample movement inside the box. Environmental temperature was kept between 20° and 22°C.

Procedure

Five to seven days after the surgery, the animals were placed inside the shuttle-box and allowed to habituate for 30 min. In the next day, the threshold stimulation for eliciting flight behavior was determined. For this purpose, brain electrical stimuli (AC, 60 Hz) of 15 sec duration and current intensity increasing by steps of 1.4 μA (RMS) were presented at 1-min intervals, until the rat began to run from one compartment to the other during the stimulation period. The current intensity eliciting running behavior during three consecutive periods of brain stimulation was considered as threshold. Animals with threshold current above 88.4 μA (RMS) were discarded at any time during the experiments.

The intensity of the running behavior elicited by brain stimulation was measured by recording in digital counters the cumulative number of midline crossings made by the rat during 10 successive periods of brain stimulation (total 150 sec) with constant current intensity.

For intracerebral injections, the stylette of the guide cannula was removed and a thin dental needle (o.d. 0.3 mm) was introduced through the guide cannula until its lower end was 1 mm below the guide cannula, reaching the same depth as the electrode tip. A polyethylene sleeve shut the upper end of the guide cannula, to minimize reflux of injected solutions. A volume of 1 μl was injected during 30 sec using a hand-driven Hamilton 10 μl microsyringe. The needle was held inside the cannula for another 30 sec after the injection and the animals were placed into the shuttle-box immediately thereafter. No more than six injections were given to the same rat.

In order to measure the effect of local drug injections on brain stimulation, the flight threshold was determined before as well as after the intracerebral injections. The time interval between each drug injection and threshold measurement was chosen on the basis of preliminary experiments with the same drugs. If the injected drug, per se, induced locomotion or running, the total number of midline crossings was recorded during a continuous period of 150 sec at the peak of the drug effect. The animals were unsystematically observed during brain stimulation as well as during 30 min after intracerebral drug injections.

Analysis of Results

Statistical comparisons between groups of paired or unpaired observations were made using Wilcoxon's signed rank or rank sums test [4], respectively.

Histology

Rats were sacrificed under deep pentobarbital anesthesia and their heads removed after perfusion through the heart with saline, followed by 50 ml of 10% Formalin solution and by 50 ml of 10% Formalin containing 1% potassium ferrocyanide. After decapitation, a DC current was passed through the brain electrode for 15 sec. The heads were maintained in 10% Formalin for at least three days. The brains were then removed and frozen sections of 50 μm were placed on a glass slide. Enlarged photographs were taken with an amplifying projector. The placements of the electrode tips were localized in diagrams from König and Klippel's [14] rat brain atlas.

Drugs

The following drugs were used: chlordiazepoxide hydrochloride (Psicosedin®, Farmasa), sodium pentobarbital (Nembutal®, Abbott), gamma-aminobutyric acid (Sigma), bicuculline (Sigma), picrotoxin (Sigma), strychnine sulphate (Mann Research Laboratories) and carbamylcholine hydrochloride (Carbachol, Sigma).

Bicuculline was dissolved according to the method described by Stevens *et al.* [27]: 5 mg of the drug were dissolved in 0.1 ml of 1.0 N HCl and 0.1 ml of 0.1 N $NaHPO_4$ was added to the solution. The pH was raised to 4.8 by

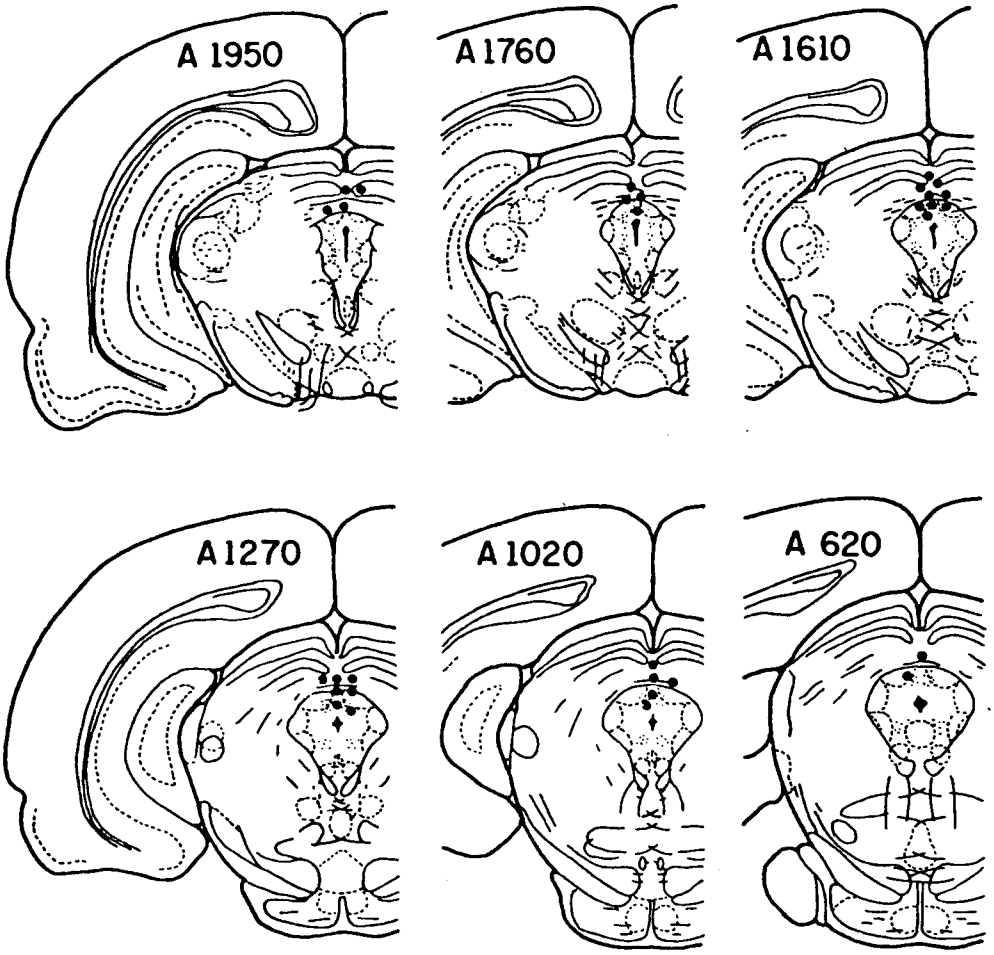

FIG. 1. Localization of electrode sites (●) inside diagrams from König and Klippel's rat brain atlas [14]. Figures represent the atlas coordinates in μm, anterior (A) to the inter-aural line. The number of points in the figure is less than the total number of rats used (80) because of several overlaps.

slowly dropping 0.1 N NaOH. The control solution was similarly prepared. Picrotoxin was dissolved in warmed saline solution, but injected at room temperature. Other drugs were dissolved in saline solution at room temperature.

RESULTS

Localization of the Brain Electrodes

As shown in Fig. 1, the electrode tips were localized in the mesencephalic tectum and inside the DPAG.

Behavioral Effects of Brain Electrical Stimulation

When the intensity of the stimulating current was increased stepwisely, the first change observed was behavioral arrest or freezing. The animals suddenly stopped, became tense and immobile and often urinated and defecated. With higher current intensities, freezing behavior was followed by vigorous running from one compartment of the shuttle-box to the other. Alternatively, the animals reared at the corners of the box or poked their noses vigorously between the bars of the grid floor, as if attempting to escape from the shuttle-box. Higher stimulus intensities induced vigorous jumps against the ceiling of the box. Whenever this happened, the brain stimulation was immediately turned off. The animals tended to freeze most of the time in between stimulations.

At threshold stimulus intensity (see Procedure), the brain stimulation induced coordinated running. Under these circumstances, the number of midline crossings made by the animal during the stimulation period was a convenient measure of the magnitude of the flight response.

Effect of Drugs on the Aversive Threshold of Brain Stimulation

As shown in Fig. 2, intracerebral injection of 0.16 and 0.32 μmol of CDP, 0.32 and 0.64 μmol of GABA as well as 0.16 μmol of PB into the dorsal midbrain significantly increased the threshold of electrical current inducing flight behavior by stimulating the dorsal midbrain. The magnitude of the drug effect was proportional to the injected dose.

The remaining drugs studied caused behavioral activation

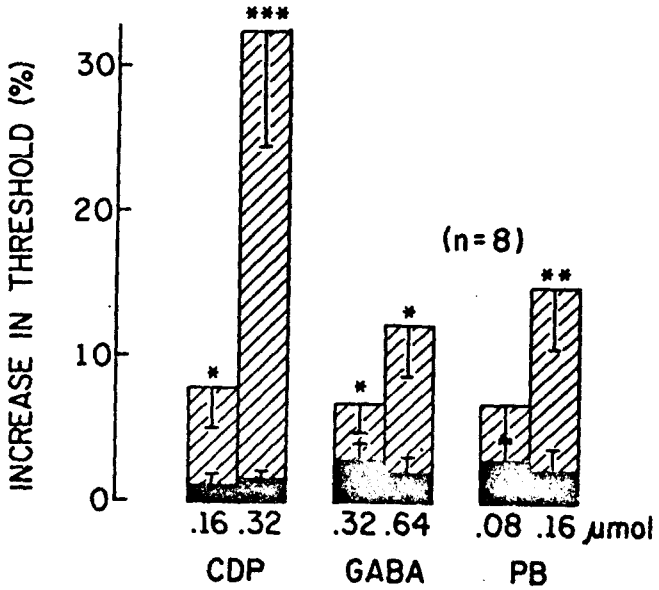

FIG. 2. Effect of local administration (1 μl injected during 30 sec) of chlordiazepoxide (CDP), gamma-aminobutyric acid (GABA) and pentobarbital (PB) on the aversive threshold of electrical stimulation (AC, 60Hz, 15 sec) of the dorsal midbrain of the rat. Increases in threshold are expressed as percent of pre-drug individual values of the threshold current intensity inducing running behavior (flight) inside a shuttle-box. Hatched columns represent mean and bars the SEM. Filled areas at the bottom of the hatched columns represent the control mean following intracerebral injection of vehicle in the same animals. Post-injection thresholds were determined 30 min after CDP or PB and 5 min after GABA administration. The symbol * indicates significant difference from control at $p<0.05$, $**p<0.01$ and $***p<0.005$, using Wilcoxon's signed rank test [4] while n is the number of animals in each group.

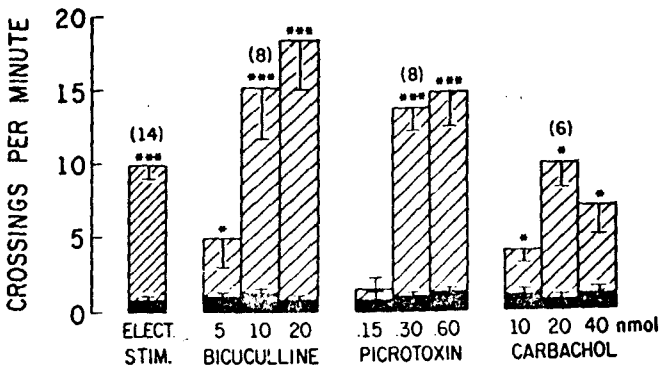

FIG. 3. Running behavior induced by electrical stimulation of the dorsal midbrain of the rat with threshold current intensity or by intracerebral injection into the same brain area of two GABA antagonists, bicuculline and picrotoxin, as well as carbachol. The intensity of flight behavior was measured by the number of times the animal crossed the midline between the two compartments of the shuttle-box during 10 successive periods of 15 sec of electrical stimulation, given at 1 min interval, or during a continuous 150 sec period beginning immediately after bicuculline or 3 min after picrotoxin administration. Hatched columns represent mean and bars the SEM. Filled areas at the bottom of hatched columns represent control means obtained after either electrical stimulation with half the threshold current intensity or vehicle injection in the same animals. The symbol * indicates significant difference from control at $p<0.05$ and $***p<0.005$, using Wilcoxon's signed rank test [4]. Figures in parentheses indicate number of animals in each group.

(see below) that hindered the determination of the aversive threshold following their intracerebral injection.

Behavioral Effects of Intracerebral Drug Injections

The dose of 10 nmol of bicuculline caused marked behavioral changes in the eight rats studied, beginning immediately after its injection into the dorsal midbrain and lasting for approximately 5 min. The animals usually ran continuously inside the shuttle-box, but sometimes reared at its corners or poked their noses between the bars of the grid floor. This behavior was indistinguishable from that observed during the electrical stimulation of the dorsal midbrain at threshold current intensity. As the drug effect disappeared, the rats became quiet, usually lying for several minutes at one corner of the experimental chamber. The dose of 5 nmol of bicuculline caused similar behavior changes in only two out of eight rats studied. On the other hand, the dose of 20 nmol of bicuculline induced intense running as well as violent jumps against the ceiling of the experimental box. Occasionally, some jerking movements of head and limbs occurred at the peak of the drug effect. Nevertheless, generalized convulsions were not observed. The running behavior caused by bicuculline resulted in dose-dependent increases of the number of crossing responses made by the rats inside the shuttle-box, as shown in Fig. 3.

The dose of 0.3 nmol of picrotoxin caused behavioral effects similar to 10 nmol of bicuculline, except for their long latency of nearly 3 min and duration of approximately 10 min. A lower dose of 0.15 nmol of picrotoxin affected only one out of eight animals studied while the dose of 0.6 nmol of the alkaloid caused intense running and jumping as well as some jerking movements. As also shown in Fig. 3, the doses of 0.3 and 0.6 nmol of picrotoxin caused the animals to cross the dividing line between the two compartments of the shuttle-box significantly more than after control injections with nearly the same intensity.

The injection of 10, 20 and 40 nmol of carbachol inside the dorsal midbrain also significantly increased the number of midline crossings made by the animals, measured 10 min after the drug administration. The intermediate dose caused the greatest effect (Fig. 3). However, the behavior of the animals injected with carbachol was markedly different from that caused by the GABA antagonists or the electrical stimulation of the dorsal midbrain. Soon after the intracerebral injection of 20 nmol of carbachol, rats showed mild body tremors followed by continuous locomotion of moderate intensity, accompanied by sniffing behavior directed at several places. The rats returned to normal after approximately 15 min from the injection. The dose of 10 nmol of carbachol caused lesser effects, whereas the dose of 40 nmol induced locomotion as well as turning behavior towards either the right or the left side. In no instance running or other flight-like behavior were observed following carbachol.

The dose of 40 nmol of strychnine caused generalized

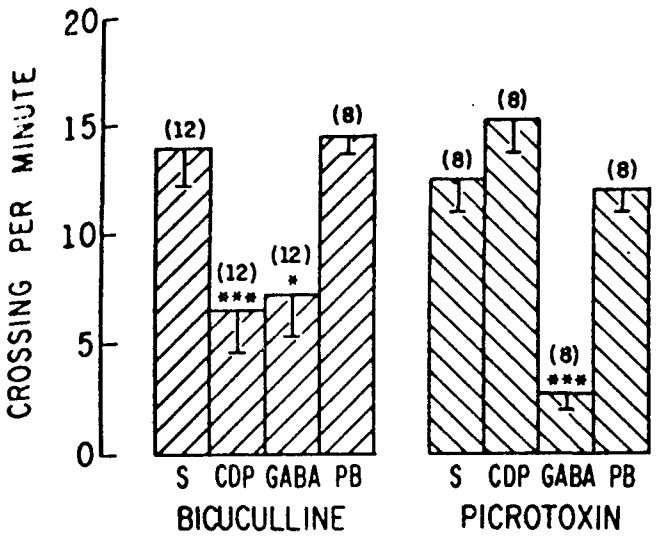

FIG. 4. Effect of local pretreatment with 0.32 μmol of CDP, 0.16 of PB or 0.64 μmol of GABA on running behavior induced by 10 nmol of bicuculline or 0.3 nmol of picrotoxin, injected into the dorsal midbrain of the rat. The GABA antagonists were given 30 min after CDP or PB and 5 min after GABA administration. The symbol * indicates significant difference from the saline-pretreated group (S) at $p<0.05$ and $^{***}p<0.005$, using Wilcoxon's rank sums test [4]. Columns represent mean and bars the SEM. Figures in parentheses indicate the number of animals in each group.

convulsions in four out of six animals, 5 min after its intracerebral injection. The two other rats showed no effect. A lower dose of 20 nmol of strychnine was ineffective in all six rats studied.

The doses of the drugs that significantly increased the threshold electrical current inducing flight behavior, namely CDP, GABA and PB (see above) did not cause appreciable behavior changes following their injection into the dorsal midbrain.

Effect of CDP, PB and GABA Pretreatment on Bicuculline or Picrotoxin-Induced Flight

As shown in Fig. 4, doses of CDP (0.32 μmol) and GABA (0.64 μmol), that increased the threshold of electrical stimulation inducing fight behavior (Fig. 2), also significantly decreased the flight behavior induced by 10 nmol of bicuculline, when previously injected into the dorsal midbrain. However, PB (0.16 μmol) was ineffective. Only GABA pretreatment significantly attenuated the behavior induced by picrotoxin (0.3 nmol), both CDP and PB being ineffective.

DISCUSSION

Several reported results suggest that the DPAG and adjoining tectum of the mesencephalon play a major role in the integration of aversive behavior, in the rat [19, 21, 23, 28]. Accordingly, the present results show that the electrical stimulation of the dorsal midbrain elicits vigorous running and jumping as well as other behavior changes characteristic of flight responses, in this species.

Systemically administered minor tranquilizers decrease the aversive consequences of DPAG electrical stimulation. Thus, previous studies have shown that doses of 3–22 mg/kg of CDP, given IP, decrease skilled escape from DPAG electrical stimulation [13,21] as well as release operant responding punished by brief electrical stimuli applied to the same area of the rat brain [7,16]. The last effect was also caused by doses of 5.6–17 mg/kg of PB, given IP [16]. These results led to the suggestion that minor tranquilizers depress the BAS comprising the periaqueductal-periventricular gray matter and the amygdala [5] and that this action mediates at least part of their anti-anxiety effect [7, 16, 21]. The present results showing that local injection of either CDP or PB increased the threshold electrical current eliciting flight behavior by stimulating the dorsal midbrain of the rat strongly suggest that minor tranquilizers act directly upon the DPAG and/or adjoining tectum of the mesencephalon, in order to depress the functioning of the BAS.

The above suggestion, however, does not exclude the possibility that other areas of the BAS may also be affected by the minor tranquilizers. In this regard, recently reported results have shown that the microinjection of CDP inside the amygdala releases food-rewarded lever-pressing punished by foot-shock, in the rat [17]. Therefore, systemically injected minor tranquilizers may impair the BAS by acting simultaneously on several components of this system.

Concerning the neurohumoral mechanisms of minor tranquilizer's action, several reported results support the view that benzodiazepines as well as barbiturates enhance the action of GABA on its receptors and therefore facilitate GABA neurotransmission in the central nervous system [1, 8, 12]. Both benzodiazepine receptors and GABA have been found in the DPAG as well as other areas of the BAS, such as the medial hypothalamus and amygdala [3, 6, 18, 24, 26]. In addition, the present results show that microinjection of GABA into the dorsal midbrain of the rat increased the flight threshold in the same way as CDP and PB. Therefore the anti-aversive action of minor tranquilizers, evidenced by the present as well as by previously reported results [7, 13, 16, 21], may be due to the facilitation of GABA-mediated neurotransmission in the DPAG. The fact that two GABA antagonists, bicuculline and picrotoxin, induced flight behavior when injected into the dorsal midbrain strongly supports this suggestion. The last results further indicate that endogenous GABA exerts a tonic inhibitory influence on the aversive DPAG. The specificity of the behavioral effects of bicuculline and picrotoxin is demonstrated by the fact that the glycine antagonist, strychnine, caused only convulsive behavior when injected into the dorsal midbrain. A participation of GABA in the aversive function of the central gray has recently been suggested by G. Sandner, D. Dessort, R. Lappuke, P. Schmitt and P. Karli (personal communication).

On the basis of many reported evidence, it has been hypothesized that bicuculline competes with GABA for its specific recognition site in the GABA-receptor complex, whereas picrotoxin antagonizes GABA actions by interfering with the opening of chloride ion channels associated with the GABA receptor [9, 20]. The present results showing that GABA pretreatment antagonized the flight behavior induced by either bicuculline or picrotoxin is consistent with this model. In the same way, the presently observed antagonistic action of CDP on the effect of bicuculline could be due to an increased affinity of GABA receptors for the neurotransmitter caused by the benzodiazepine, as demonstrated in experiments in vitro using binding techniques [8]. In this regard,

however, the lack of antagonism of CDP on picrotoxin-induced flight does not conform to the expectations generated by the above model. Also the ineffectiveness of PB on both bicuculline and picrotoxin-induced behavior is puzzling, since barbiturates have also been shown to enhance GABA receptor actions [12] and are supposed to compete for the picrotoxin recognition site in the GABA-receptor complex [20]. It is possible that insufficient doses of the minor tranquilizers were used in the present study. However, higher concentrations of solutions already precipitated at room temperature and thus could not be appropriately injected.

Previously reported results have shown that intraventricular or intrahypothalamic injection of carbachol induced seemingly aversive behaviors, such as retreat and defensive displays in cats, suggesting that brain cholinergic neurons are involved in fear integration [2,11]. The present results, however, do not support this hypothesis since no flight behavior was observed following the microinjection of carbachol into the rat midbrain. Instead, only locomotor, sniffing and turning behaviors were seen following carbachol administration. Nevertheless, the participation of cholinergic mechanisms in fear cannot be entirely dismissed because of the differences in animal species and site of injection between the present and previously reported results [2,11].

In summary, the above discussed evidence suggests that minor tranquilizers act directly upon the DPAG and possibly other areas of the BAS by enhancing the tonic inhibitory influence of GABA-mediated neurotransmission. The resulting impairment of the BAS may, at least in part, bring about the anti-aversive, anti-punishment as well as the anti-anxiety effects of these drugs, as previously suggested [7, 16, 21].

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