Pharmacology Biochemistry and Behavior, Vol. 65, No. 2, pp. 267–273, 2000

0091-3057/00/$–see front matter

PII S0091-3057(99)00200-2

# Pontine Tegmentum Lesions Increase Anxiety-Like Behavior in Rats: A Comparison With Anxiety Produced by β-CCE

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Received 30 October 1998; Revised 18 June 1999; Accepted 6 August 1999

PODHORNA, J. AND K. B. J. FRANKLIN. *Pontine tegmentum lesions increase anxiety-like behavior in rats: A comparison with anxiety produced by β-CCE*. PHARMACOL BIOCHEM BEHAV **65**(2) 267–273, 2000.—Electrolytic lesions of the pedunculopontine tegmental nucleus (PPTg) have been previously reported to increase anxiety-like behavior in rats. The aim of the present study was to compare these behavioral changes with those produced by an anxiogenic compound, the partial inverse agonist at benzodiazepine receptors, β-CCE. Three groups of rats, sham-lesioned treated with vehicle, sham-lesioned treated with 10 mg/kg of β-CCE, and PPTg-lesioned rats treated with vehicle, were tested in the elevated plus-maze, the social-interaction test, and for spontaneous locomotion. Histology showed that lesions were concentrated on the caudal half of the PPTg. Measures of both the PPTg-lesioned and β-CCE–treated rats indicated increased anxiety-like behavior in the elevated plus-maze and in the social-interaction test. Spontaneous locomotion, measured in the open- field arena, did not differ between sham controls and PPTg-lesioned rats, but was decreased in rats treated with β-CCE. Our results confirmed that electrolytic lesions of the caudal PPTg produce increased anxiety-like behavior. This behavior is quantitatively and qualitatively similar to that produced by 10 mg/kg of β-CCE. © 2000 Elsevier Science Inc.

Anxiety-like behavior PPTg Lesion Rat Elevated plus-maze Social-interaction test β-CCE

THE pedunculopontine tegmental nucleus (PPTg) has been implicated in the control of a variety of behavioral functions including the sleep–wake cycle and arousal, attention and cognitive processing, locomotor activity, and reinforcement (1,6,7,17,23,28,35–37,39,44,46,47). Recently, it has also been implicated in emotionality and defensive behavior (8,30,34,42,43,51). Bilateral NMDA-induced lesions of the pontine tegmentum, which included the PPTg, were found to increase behaviors indicative of anxiety in the elevated plus maze test (34). This effect lasted for several day, and was reversible by 1 and 2 mg/kg of diazepam (34). Electrolytic lesions of the caudal PPTg also produced increased anxiety-like behavior detectable in the elevated plus-maze and in the social interaction test in rats (42,43). These behavioral changes were found to persist over several weeks of the experiment (43). Moreover, lesions of the PPTg attenuated the habituation to a novel environment, which occurs when animals are repeatedly exposed to the novel environment, i.e., the plus-maze apparatus (43). Both neurotoxin-induced and electrolytic lesion to the PPTg diminish the prepulse inhibition of the acoustic startle response (30,31,51), which is believed to be an important element of defensive flight response (15,16). In addition, bilateral lesions of the midbrain reticular formation, which includes the PPTg, decreased habituation of the acoustic startle response (9,27).

Anxiety is a subjective human experience, and there is no physiological measure that reflects changes in anxiety alone (19). Rather, anxiety is assayed by comparing the behavioral effects of putative and known anxiogenic or anxiolytic stimuli. Although we have found that PPTg lesions induce behavioral changes indicative of increased anxiety in two well-established animal models, and that these are reversed by diazepam (34,42,43), no comparison with known anxiogenic stimuli has been made. The

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present study, therefore, compared the magnitude and profile of the behavioral effects of PPTg lesions with the behaviors induced by a known anxiogenic drug, ethyl β-carboline-3-carboxylate (20,52).

Compounds acting at benzodiazepine receptors have both antianxiety (agonists) and proanxiety (inverse agonists) effects (25). Ethyl β-carboline-3-carboxylate (β-CCE) is a partial inverse agonist at benzodiazepine receptors (5) that is anxiogenic in several animal models of anxiety (for review: 20,52). Beta-CCE is rapidly metabolized in rodents (32), but behavioral effects have been reported for doses equivalent to between 5 and 16 mg/kg, IP. Thus, β-CCE dose dependently (0.5–5 mg/kg, IV) increased plasma corticosteroid levels (18) and 8 mg/kg β-CCE SC elevated 3,4-dihydroxyphenylacetic acid levels in the frontal cortex and nucleus accumbens, similarly to the effect of the footshock stress (11). Beta-CCE dose dependently decreased the time rats spent in social interaction over the range 4–16 mg/kg, IP (10), and 1–2 mg/kg, IV (20). In the social conflict test in mice, 8 mg/kg of β-CCE SC increased defenses and escapes in timid mice, i.e., male mice exhibiting defenses and escapes in a control interaction with a strange mouse (50).

On the basis of the previous data we selected 10 mg/kg β-CCE SC as a dose that would be expected to produce strong behavioral and physiological anxiety-like effects. The effects of β-CCE and pedunculopontine lesions were compared in two well-established models of anxiety—the elevated plus-maze and the social interaction test—as well as in the spontaneous locomotion test.

## METHOD

### *Subjects*

Male Long–Evans rats (Charles River Laboratories), weighing 250–275 g at the beginning of experiment, were housed individually and maintained on a light–dark cycle with light on from 0600 to 1800 h. Food and water were available ad lib.

### *Electrolytic Lesions of the PPTg*

Previously we have found an increase in anxiety-like behaviors after both excitotoxin or electrolytic lesions of the PPTg (34,42). Excitotoxin lesions are demyelinating (4,17), and are associated with a high postoperative mortality that may indicate damage remote from the injection sites. For these reasons the present study used electrolytic lesions.

Rats were randomly assigned to two groups, either sham-lesioned ($n = 17$) or PPTg-lesioned ($n = 12$) rats. They were anesthetized with a combination of pentobarbital sodium (45 mg/kg IP) and xylazine (5 mg/kg IM), and placed in a stereotaxic instrument (Kopf) in the skull-flat position. Coordinates for the PPTg were: AP = −7.8, ML = ±2.0, DV = +2.9 (41). An insulated stainless steel electrode was lowered into the PPTg, and an anodal current was passed (1 mA) for 10 s. The electrode remained in place for 1 min after current was terminated. Sham lesions were made by an electrode lowered to a point 0.5 mm dorsal to the target structure and left in place for 70 s without current application. Rats were handled daily for 1 week before behavioral testing started.

### *Behavioral Testing*

Behavioral testing started 1 week after surgery. Testing was performed in the following order with 2- to 3-day recovery periods between tests: the elevated plus-maze, the social interaction test, and the spontaneous locomotion test. All animals were used for each test, and all tests were carried out in the same room.

*The plus-maze apparatus.* This was made from wood, and consisted of two open arms (50 × 10 cm), with 1 cm-high edges, and two closed arms (50 × 10), with 40 cm-high walls, and a central platform (10 × 10 cm). The floor of the apparatus was raised 50 cm above the floor. Open-arm activity was facilitated by testing under red dim light (45). In random order, rats were placed onto the central platform facing an open arm and allowed to explore apparatus for 5 min. To reduce any lingering olfactory cues, the apparatus was cleaned with a 70% ethanol solution after each animal. Behavior was scored directly by a trained observer using a computer program for registration of behavior. The scored behavior included both traditional measures (frequency of rearing, the number of open- and closed-arm entries, and time spent on various parts of the maze) and novel "risk assessment" activities (13,45). Novel measures included open- and closed-arm entry latencies and "risk assessment" activities, such as closed-arm returns (exiting a closed arm with the forepaws only, and then returning into the same arm), freezing, self-grooming, head dipping (scanning over the sides of the maze towards the floor), and stretch-attend postures (forward elongation of head and shoulders, followed by retraction to original position) (13). The later two parameters were differentiated as "protected" (occurring on or from the closed arms or central platform) or "unprotected" (occurring on the open arms).

Increased anxiety-like behavior was indicated by decreased open arm activities and increased closed arm activities, by prolonged latency to the first open-arm entry, by increased numbers of closed-arm returns and freezings, and by decreased numbers of unprotected head dips and unprotected stretch-attend postures (13,33). To compare habituation in sham- and PPTg-lesioned subjects, rats were exposed to the apparatus for 5 consecutive days.

*The social interaction test.* The test arena was a Plexiglas box (41 × 41 × 30 cm). To increase the amount of social interaction in controls, testing was carried out under low light intensity (approximately 60 lx), and subjects were familiarized to the boxes for 10 min on 3 days prior to testing day. Rats to be used as partners did not undergo the familiarization. On the test day, each subject was placed into the Plexiglas box before its partner was introduced. The partner (which served only as a stimulus) was then placed in the box as far away from the subject as possible. Social behavior was then videotaped for 10 min. The number, duration, and latency of the following behavioral activities were scored from the video record by a trained observer: sniffing (sniffing the partners's body, head, genitals or tail), partner following (following the partner while trying to sniff its tail or genitals) and mounting the partner, partner and self-grooming, defensive postures (including defensive sideways, upright, and full submissive postures), stretched-attend posture (elongation of head and shoulders toward the partner, with eyes and ears being directed towards the partner), aggressive boxing, threat (including offensive sideways, upright, and full aggressive postures), and locomotion (walking and rearing). Total time spent in social interaction was calculated as the sum of the durations of social sniffing and following. Increased anxiety-like behavior was defined as a decrease in social investigation (the numbers and total durations of social sniffing and following the partner) and an increase of freezing or defensive behavior without a concomitant decrease in motor activity (19,20).

*Spontaneous locomotor activity.* The apparatus was an arena

made from wood with floor 100 × 100 cm divided into 25 squares of 20 × 20 cm. Walls were 50-cm high. Illumination from overhead white fluorescent lights was approximately 100 lx. Animals were placed into the center of the arena, and numbers of line crossings and rearings were recorded for 5 min by an experienced observer. Line crossings were recorded separately for the center of the arena and for the periphery (up to 20 cm from walls). Total line crossings were calculated as a sum of crossings in the center and in the periphery. Overall locomotor activity was calculated as a sum of line crosses and rears.

*Drugs and Procedure*

Ethyl-β-carboline-3-carboxylate (RBI, Natick, MA) was dissolved in propylene glycol (90%) with a 10% of absolute alcohol.

Rats were randomly assigned to three groups: 1) sham-lesioned rats treated with vehicle = controls; 2) sham-lesioned rats treated with 10 mg/kg of β-CCE = β-CCE treated group; 3) PPTg-lesioned rats treated with vehicle = PPTg-lesioned group. Beta-CCE (10 mg/kg SC) or vehicle were administered in the volume 2 ml/kg 15 min before behavioral observations began.

*Histology*

After behavioral testing was completed, animals were killed by an overdose of chloral hydrate (1 ml IP) and perfused with saline. Brains were removed, parallel 20-μm sections were cut on a cryostat and stained either with NADPH stain or cresyl violet to verify the lesions.

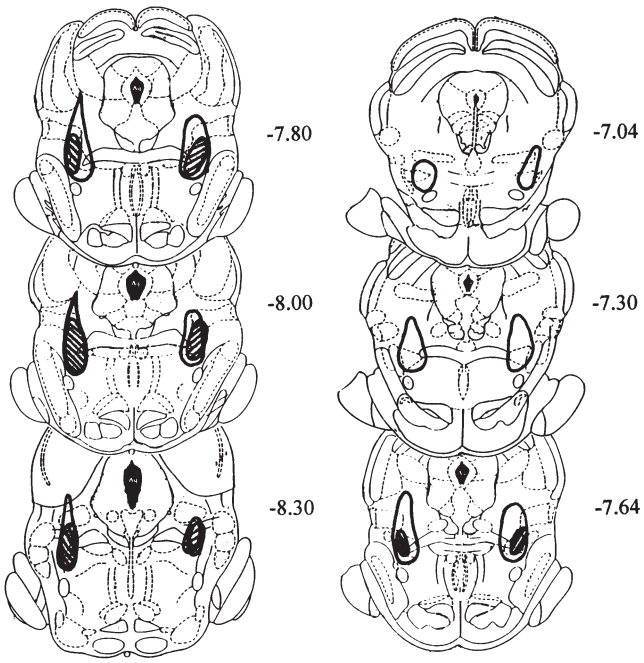


FIG. 1. Outlines of the smallest (hatched) and the largest electrolytic lesions (1 mA for 10 s) traced onto coronal sections of the atlas of Paximos and Watson (41). Numbers adjacent to each section represent the anterior–posterior level relative to bregma.

*Statistical Analysis*

Statistical analysis was carried out with SigmaStat (SPSS Science, Chicago, IL) statistical software. The data showed severe nonhomogeneity of variance. The nonparametric Kruskal–Wallis test and the Friedman Repeated-Measures test were used in place of ANOVA. Multiple comparisons were carried out using nonparametric versions of Dunn's test or the Student–Newman–Keuls method (24). For all tests $\alpha = 0.05$.

## RESULTS

*Histology*

Lesions in eight animals were symmetrical, and destroyed the caudal PPTg with the maximum destruction 7.60 and 8.40 mm posterior to bregma. The smaller lesions destroyed only the caudal PPTg and a narrow surrounding area (Fig. 1). The damage of larger lesions extended to the cuneiform nuclei, the microcellular tegmental nuclei, lateral parts of the superior cerebellar peduncle, the nuclei of the lateral lemniscus, the paralemniscal nuclei, the central tegmental tract, the external cortex of the inferior colliculus, the retrorubral nuclei, and the retrorubral field.

Four animals were removed from the experiment because the lesions missed the PPTg either uni-or bilaterally. In two of the rejected subjects the PPTg was damaged unilaterally; the others had minimal damage to the PPTg. In the rejected subjects, lesions damaged variously: the cuneiform nuclei, the external cortex of the inferior colliculus, the microcellular tegmental, the lateral lemniscus and its nuclei, the paralemniscal nuclei, the rubrospinal tract, the superior cerebellar peduncle, and the central tegmental tract.

*Elevated Plus-Maze*

Kruskal–Wallis analysis indicated significant differences between groups for the following measures over all 5 testing days: percent frequency of open-arm entries ($p < 0.001$; Fig. 2), percent time spent on open arms ($p < 0.001$; Fig. 2), percent frequency of closed-arm entries ($p < 0.001$), percent time spent on closed arms ($p < 0.01$), latency to open-arm entry ($p < 0.01$), total arm entries frequency ($p < 0.05$; Fig. 3)

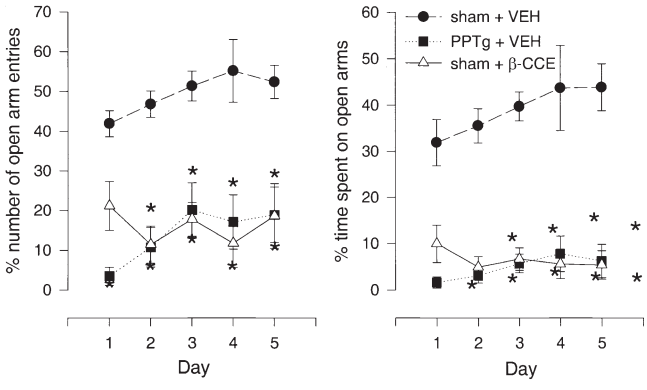


FIG. 2. The percent number of open-arm entries (left) and percent time spent on open arms (right) over 5-day exposure to the plus-maze apparatus. * < 0.05 vs. sham controls (Mann–Whitney). No significant differences were found between PPTg-lesioned and sham-lesioned rats treated with β-CCE.

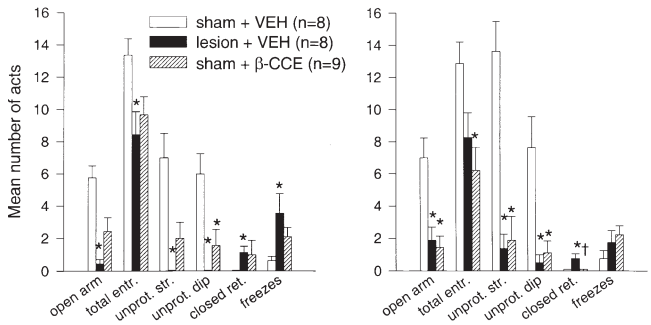


FIG. 3. Mean number (± SEM) of behavioral scores on the 1st day (left) and fifth day (right) of testing on the plus-maze. Op = open-arm entries, To = total entries, S.u = unprotected stretch postures, D.u = unprotected head dips, Re = closed-arm returns, Fr = freezes. * < 0.05 vs. sham controls (Mann–Whitney).

and number of rears ($p < 0.05$), unprotected stretch postures, and unprotected head dips ($p < 0.001$; Fig. 3), and closed arm returns and freezes ($p < 0.05$; Fig. 3). Post hoc multiple comparisons (Dunn's test) revealed that most scores indicative of increased anxiety remained significantly different in PPTg-lesioned rats from control scores over the 5 testing days (see Figs. 2 and 3). Beta-CCE treated rats also had scores indicating increased anxiety on each testing day except for day 1. On day 1, behavioral scores in β-CCE treated rats were biased toward anxiety but, except for number of unprotected head dips, were not significantly different from control scores. The PPTg-lesioned and the β-CCE–treated groups did not differ on any measure for any of the 5 days of testing.

The groups of animals with asymmetric lesions was too small for statistical analysis but the behavioral scores were intermediate between PPTg-lesioned rats and controls. For instance, on day 1 the mean "percent frequency of open arm entries": for controls was 42% (standard deviation 9.3), for rats treated with β-CCE 21.2% (19.8), for PPTg-lesioned rats 3.1% (6.1), and for incompletely lesioned rats 12.9% (12.7).

*Spontaneous Locomotor Activity*

Data are summarized in Table 1. Numbers of line crossings, rears, and total locomotor activity did not significantly differ between the PPTg-lesioned rats and sham-controls. Unlesioned rats treated with β-CCE showed fewer rears ($p < 0.05$) when compared to sham controls. Line crossings and total locomotor activity were lower in unlesioned β-CCE treated rats ($p < 0.01$) when compared to PPTg-lesioned rats treated with vehicle.

*The Social-Interaction Test*

Data are summarized in Fig. 4. There were significant differences between groups (Kruskal–Wallis) for the following behavioral scores: time spent in active social interaction ($p < 0.001$), durations of social sniffing ($p < 0.001$), duration of freezing ($p$ , 0.001), number of social sniffs ($p$ 0.001), number of partner follows ($p < 0.001$), and number of freezings ($p < 0.001$). The post hoc multiple-comparison test (Dunn's method) revealed significant differences between controls (sham-lesioned rats treated with vehicle) and PPTg-lesioned rats in all above-mentioned measures. Controls and sham-lesioned rats treated with β-CCE also differed in all the above measures except number of freezings, which was not significantly different from controls in rats treated with β-CCE. Thus, PPTg-lesioned rats and sham-lesioned rats treated with β-CCE showed significantly prolonged freezing episodes, significantly fewer social sniffs and partner follows, reduced duration of social sniffing and of time spent in active social interaction. In addition, PPTg-lesioned rats had significantly higher number of freezings than controls. There were no significant differences among groups in locomotor behavior (walking and rearing; Fig. 4). PPTg-lesioned rats and sham-lesioned rats treated with β-CCE did not differ on any measure.

In the rats with lesions that did not damage the PPTg bilaterally, behavioral measures lay within the range of sham-lesioned subjects. For instance, the mean time spent in social interaction for controls was 134.7 (standard deviation 26.05), for rats treated with β-CCE 65.6 (28.05), for PPTg lesioned rats 69.4 (13.24), and for incompletely lesioned rats 100.2 (42.87).

## DISCUSSION

The results of the present study confirmed our previous findings that electrolytic lesions of the caudal PPTg produce behavioral changes indicative of increased anxiety in rats (42,43). Moreover, the data showed that these behavioral changes produced by electrolytic lesions of the caudal PPTg are, in severity, comparable to those produced by 10 mg/kg of the partial inverse agonist at benzodiazepine receptors, β-CCE.

Anxiety-like behavior in the elevated plus-maze is indicated by fewer or shorter episodes of behaviors indicative of confidence (open-arm activities, unprotected head dips, and unprotected stretch-attend postures), and by increased episodes of behaviors indicating fearfulness (number of freezings, closed-arm returns, prolonged latency to the first open-

TABLE 1

COMPARISON OF THE SPONTANEOUS LOCOMOTOR BEHAVIOR OF EXPERIMENTAL GROUPS IN THE OPEN-FIELD TEST

| | Sham Lesioned + Vehicle | PPTg Lesioned + Vehicle | Sham Lesioned + β-CCE |
|---|---|---|---|
| Lines crossed in the periphery | 89.63 ± 7.14 | 112.75 ± 12.72 | 77.89 ± 4.70 |
| Lines crossed in the center | 24.50 ± 5.93 | 33.63 ± 4.63 | 13.89 ± 2.06 |
| Total lines crossed | 114.13 ± 10.08 | 146.38 ± 14.53 | 91.78 ± 5.53† |
| Rearing | 22.00 ± 2.19 | 21.25 ± 2.37 | 13.78 ± 1.43* |
| Total activity | 136.13 ± 11.02 | 167.63 ± 15.08 | 105.56 ± 6.53† |

Scores are mean numbers (±SEM).
*$p < 0.05$ vs. sham controls.
†$p < 0.01$ vs. PPTg-lesioned animals.

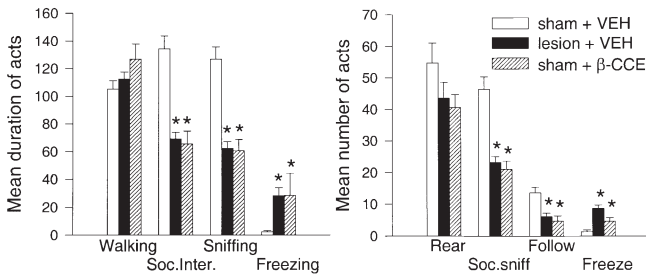


FIG. 4. Behavioral scores in the social interaction test. The left figure shows mean duration (± SEM), and the right figure shows mean number (± SEM) of selected scores. * < 0.05 vs. sham controls (Mann–Whitney). No significant differences were found between PPTg-lesioned and sham-lesioned rats treated with β-CCE.

arm entry). Both classes of indicators of anxiety were increased in the PPTg-lesioned and β-CCE–treated rats when compared to sham-lesioned controls. Except for the number of unprotected head dips, behavioral indicators of anxiety did not reach significance in β-CCE–treated rats on the first testing day, but they were increased over the following 4 days. In all cases, the trend for β-CCE–treated animals was in the same direction as for PPTg-lesioned rats, and there were no significant differences between the two groups on any measure. It remains unclear why behavioral scores in β-CCE–treated rats did not reach significance on the first day. Cumulation of doses after repeated daily administration of β-CCE can be excluded, because β-carbolines have short half-life (32). As we have previously suggested (43) the ability of the plus-maze test to detect proanxiety effects might increase with repeated exposures. As control subjects become habituated to the apparatus, they exhibit fewer behaviors indicative of anxiety, and the difference between anxious and nonanxious subjects becomes larger (e.g., Fig. 1). Our hypothesis is supported by study of Cole et al. (12), who found no effect of β-CCE in the elevated plus-maze in naive rats.

In the social interaction test in rats, a decrease in social interaction without a concomitant decrease in motor activity is interpreted as increase in anxiety-like behavior (19,20). Our results showed that most behavioral scores of both PPTg-lesioned rats and sham-lesioned rats treated with β-CCE indicated increased "anxiety." In the social interaction test, β-CCE was previously found to be anxiogenic at a dose of 1–2 mg/kg, when administered intravenously (20), and at 2–16 mg/kg, when administered intraperitonally (10). The bioavailability of lipid-soluble drugs, such as β-CCE, after subcutaneous administration is comparable to that after intraperitoneal administration. The 10 mg/kg dose of β-CCE administered subcutaneously is, therefore, a sufficient dose to produce behavioral changes of increased anxiety. Because PPTg-lesioned animals showed anxiety-indicating scores as high or higher than sham-lesioned rats treated with 10 mg/kg of β-CCE, "anxiety" produced by PPTg lesions appears to be quite severe.

Total arm entries and number of rears, as measured by performance on the elevated plus-maze, were lower in both the PPTg-lesioned and sham-lesioned rats treated with β-CCE vs. sham controls. The number of line crossings and total spontaneous locomotor activity measured in the open-field arena did not differ between sham-lesioned controls and PPTg-lesioned or sham-lesioned β-CCE–treated animals. The finding that PPTg lesions produce no changes in spontaneous locomotor activity agrees with previous studies (1,27,29,38, 42). Therefore, the decrease in plus-maze locomotion and exploration found in PPTg-lesioned rats is consistent with increased "anxiety" rather than with motor effects of the lesion. In addition, PPTg-lesioned rats have been subjected to neurological testing (38), and no gross motor deficits were found. Beta-CCE–treated rats reared less than controls in both the spontaneous locomotion and plus-maze tests. Although some beta-carbolines have been reported to exert minimal sedative (14,21,22) and ataxic properties (40,49,53), β-CCE decreased rearing in our tests as well as in the light/dark box (26), which might indicate its antiexploratory effect.

We found no correlation between the size of the lesion and anxiety-indicating behavioral scores, provided that the PPTg was damaged bilaterally. Moreover, comparison of the lesions in different subjects suggested that lesions to the caudal half of the PPTg were as effective as more extensive lesions. This is consistent with our previous studies (42,43), as well as with a study of Kodski and Swerdlow (31).

In summary, the present study demonstrated that PPTg lesions produce behavioral changes indicative of increased anxiety comparable to those produced by a relatively high dose of β-CCE, an anxiogenic drug (20,26,52) that is a partial inverse agonist at the benzodiazepine receptors (5). As in previous studies (34,43), the increase in anxiety-like behavior in PPTg-lesioned rats had long duration. It was not reduced by repeated exposure to the plus-maze apparatus, suggesting that PPTg-lesioned rats do not habituate to a novel environment. Lesions of the caudal PPTg were as effective as larger lesions, indicating the critical site may be in the caudal PPTg. Because removal of this region leads to increased anxiety, we hypothesize that the region of the caudal PPTg is inhibitory to anxiety. Because neither electrolytic and excitoxin lesions are selective, it is not possible to determine whether the effects are due to loss of PPTg cells or fibers of passage. The hypothesis that PPTg cells are involved is consistent with a recent study of brain c-*fos* induction during fear, which found that the PPTg was activated only when fear reactions were suppressed by a conditioned inhibitor (8). There is also evidence of strong connections between the PPTg and other midbrain areas known to be involved in anxiety and defensive behavior such as the periaqueductal gray and the dorsal raphe nucleus (2,3,48). We suggest that PPTg could be a part of a neural circuit of anxiety in the midbrain. However, the exact origin of "anxiety" produced by lesions of the caudal PPTg remains to be determined.

ACKNOWLEDGEMENTS

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada, and the program Formation de Chercheurs at L'Aide à la Recherche du Quebec. J. P. was a PDF supported by the W. Stairs Memorial Fund of McGill University.

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