Pharmacology Biochemistry & Behavior, Vol. 19, pp. 225–229, 1983. © Ankho International Inc. Printed in the U.S.A.

The Involvement of Nigral Serotonin Innervation in the Control of Punishment-Induced Behavioral Inhibition in Rats

MARIE-HÉLÈNE THIÉBOT,* MICHEL HAMON† AND PHILIPPE SOUBRIÉ†

**Département de Pharmacologie, Faculté de Médecine Pitié-Salpêtrière 91, boulevard de l'Hôpital, 75634 Paris Cedex 13, France*
†INSERM—U 114—Collège de France, 11 place Marcelin-Berthelot, 75231 Paris Cedex 05, France

Received 27 December 1982

THIÉBOT, M. H., M. HAMON AND P. SOUBRIÉ. *The involvement of nigral serotonin innervation in the control of punishment-induced behavioral inhibition in rats.* PHARMACOL BIOCHEM BEHAV **19**(2) 225–229, 1983.—In rats, serotonergic innervation of the substantia nigra plays a role in the control of experimentally-elicited anxiety: punishment-induced inhibition is lessened following bilateral intra-nigral infusion of 5,7-dihydroxytryptamine (2 μg; 0.5 μl). A significant correlation (0.62) is found between the loss of nigral, but not hippocampal, tryptophan hydroxylase activity and the release of behavior in two situations of shock-induced suppression of responding. Likewise, infusion of this neurotoxin (1 μg; 0.4 μl) into the nucleus raphé dorsalis causes an attenuation of punishment-induced suppression. These findings suggest an involvement of serotonergic raphé-nigral neurons in experimentally-elicited anxiety.

Substantia nigra Serotonin Raphé nuclei Punishment-induced inhibition Rat

SEROTONIN containing neurons located within both the dorsal and the median raphé nuclei and which provide most of the serotonergic innervation of the forebrain [2, 12, 16] have been implicated in the control of punishment-induced inhibition in animals, and in the anti-punishment activity of benzodiazepines [14, 15, 17, 19, 20].

However, the nucleus of origin (dorsal or median raphé nucleus), as well as the areas of projection of those serotonergic neurons chiefly involved in the control of punishment remain to be elucidated. There is evidence to suggest the involvement of hippocampal serotonin [8], and consequently of the median raphé [2,16], the major source of serotonergic innervation of the hippocampus.

Evidence also exists implicating the dorsal raphé in punishment. Application of benzodiazepines to this nucleus elicits a typical anti-punishment effect that is cancelled by prior destruction of the serotonergic neurons of the dorsal raphé [17]. Since this nucleus sends a massive projection to the substantia nigra [2, 12, 16], it was of interest to investigate the influence of nigral serotonergic innervation in the control of punishment-induced suppression. The present work was undertaken in order to determine the effect of 5,7-dihydroxytryptamine (5,7-DHT) lesions of the raphé-nigral serotonergic neurons using microinjections of the neurotoxin. Lesions of the substantia nigra (Experiment 1) and of the dorsal raphé (Experiment 2) were analyzed in two paradigms in which inhibition of appetitive behavior is caused by electric foot-shock.

GENERAL METHOD

The experiments were carried out on male Wistar A. F. rats housed 10 per cage, under standard conditions (12-hr light-dark cycle; room temperature = 21±1°C). All the experiments were performed blindly.

Behavioral Testing

Punishment-induced behavioral inhibition was studied in two situations:

The first is modified from the punishment procedure used by Vogel *et al.* [21]. During the 2 days preceding the test-session (15 days after the surgery), free access to water in the home cage was restricted to 2 hours a day (between 3 and 5 p.m.) and the rats were trained (one 15 min session a day, between 10 a.m. and 1 p.m.) to drink in the test-apparatus (30×30×32 cm Plexiglas box with one transparent wall and electrified grid-floor). Each box was equipped with one bottle located in a corner, the end of the drinking tube being set 5 cm above the floor. During the test-session, the animals were allowed to drink water during an initial 15 sec period and were subsequently given one electric foot-shock (0.40 mA, 45 msec) every time a 3 sec period of licking was completed. The number of shocks received during 3 min following the first shock was recorded.

In the second situation, rats maintained at 80–85% of their normal body weight were trained (12 daily 15 min sessions) in a Skinner box to press a lever for food-reward on a con-

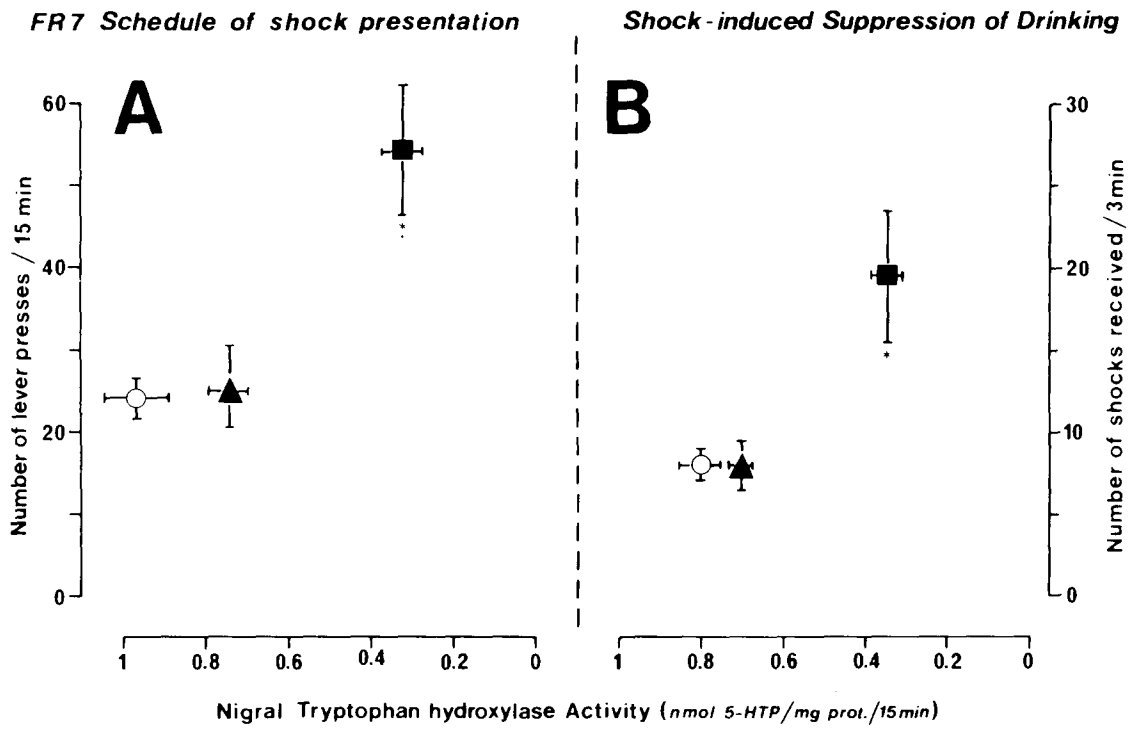

FIG. 1. Effects of the severity of nigral serotonergic lesions on punished behavior. Punished behavior was assessed in: Control rats (○); 5,7-DHT-lesioned rats for which the loss of nigral tryptophan hydroxylase activity was less than 40% (▲) or more than 55% (■) when compared to control values. (A) Suppression of lever pressing for food in a CRF/FR7 schedule of shock presentation expressed as the mean number of lever presses ± SEM (vertical bars) effected during a 15 min period. ○, n=12; ▲, n=5; ■, n=10. (B) Shock-induced suppression of drinking expressed as the mean number of shocks ± SEM (vertical bars) received during a 3 min period. ○, n=15; ▲, n=8; ■, n=8. Nigral tryptophan hydroxylase activity is presented as the mean n mol of 5-hydroxytryptophan synthesized by mg of protein in 15 min ± SEM (horizontal bars). $*p<0.05$; $**p<0.01$ as compared to respective control values (Student's t test).

tinuous reinforcement schedule (CRF). The test-apparatus was an operant chamber (housed in a ventilated, sound-insulating cubicle) with an automatic magazine delivering 45 mg Noyes pellets. Each box was equipped with a lever which the rat had to press to obtain reward, and with an electrified grid-floor. After 6 days of post-surgery recovery, the rats (280–300 g) were food restricted and were given 5 additional CRF trials (20 min). Fifteen days after surgery, the animals were subjected to an experimental session divided into two components: a 5 min CRF period followed by a 15 min period of CRF with a Fixed Ratio 7 (FR 7) schedule of shock presentation (each 7th press was paired with the delivery of one electric foot-shock, 0.50 mA, 45 msec, through the grid-floor of the Skinner box). The number of lever presses was recorded at 5 min intervals.

In order to assess the reliability of the experimental conditions, a group of non-lesioned rats received a standard dose of diazepam (2 mg/kg IP), 30 min before behavioral testing.

5,7-Dihydroxytryptamine Lesion

The rats, pretreated with desipramine (25 mg/kg IP), were anaesthetized with chloral hydrate (400 mg/kg IP) 60 min later and 5,7-DHT (dissolved in saline containing 0.02% ascorbic acid) was infused into the chosen structure over a 4 min period, using stereotaxic procedures. Under these conditions, 5,7-DHT is known to markedly destroy serotonergic neurons with minimal damage to other monoaminergic cells [7]. Control rats were sham-operated.

Tryptophan Hydroxylase Assay

The day following the behavioral test-session, the rats were killed by decapitation, their brains quickly removed and dissected on ice. Tryptophan hydroxylase activity was measured according to Hamon *et al.* [9], in the 35,000 × g supernatant of tissue homogenates, with 0.15 mM tryptophan, 0.16 mM 6-MPH4 and 0.01% sodium dodecyl sulfate.

Statistical analyses were performed using either Student's t test or analysis of variance. Correlations were estimated using the Bravais-Pearson test or partial coefficient of correlation [13].

EXPERIMENT 1

This experiment was carried out in order to explore the role of nigral serotonergic innervation in punishment paradigms.

Method

Fifteen days before being subjected to one of the two punishment procedures (described in the General Method Section), the rats were given bilateral injections of 5,7-DHT (2 μg of free base, in 0.5 μl) into the substantia nigra (stereotaxic coordinates according to König and Klippel

TABLE 1

SHOCK-INDUCED SUPPRESSION OF DRINKING IN RATS PRESENTING A MINIMAL LESION OF THE NIGRAL SEROTONERGIC INNERVATION ASSOCIATED WITH A SIGNIFICANT LESION OF HIPPOCAMPAL SEROTONERGIC INNERVATION

	N	Tryptophan hydroxylase activity (nmol 5-HTP/mg prot/15 min*) Substantia Nigra	Hippocampus	Punished drinking (number of shocks received/3 min*)
Control rats	15	0.796 ± 0.052	0.301 ± 0.020	8.00 ± 1.13
5,7-DHT lesioned rats	4	0.647 ± 0.051	0.144 ± 0.018	8.50 ± 3.52

Rats were given 5,7-DHT into both substantia nigra 15 days before behavioral testing.
*Mean ± SEM.

[10]: A=2.2; L=1.8; H=−2.8, the incisor bar being set 2.4 mm under the inter-aural plane).

The extent of the lesion was estimated by assessing tryptophan hydroxylase activity in each substantia nigra. Since serotonergic fibers projecting to limbic structures pass in the vicinity of the substantia nigra, tryptophan hydroxylase activity within each hippocampus was also measured.

Results and Discussion

As compared to control values, bilateral nigral infusion of 5,7-DHT induced a significant decrease in tryptophan hydroxylase activity in the substantia nigra (0.879±0.065 vs. 0.452±0.035, $t=6.28$, $p<0.001$) and in the hippocampus (0.301±0.020 vs. 0.187±0.014, $t=4.12$, $p<0.001$). Significant correlations were found between the reduction of tryptophan hydroxylase activity in the substantia nigra and the hippocampus ($r=0.47$, $p<0.02$). The correlation was greater between the decrease in nigral tryptophan hydroxylase activity and the attenuation of punishment-induced behavioral suppression ($r=0.62$, $p<0.01$). This coefficient only decreased to 0.55 ($p<0.01$) when the reduction of hippocampal tryptophan hydroxylase activity was kept constant by calculating the partial coefficient of correlation. A more detailed a posteriori analysis revealed that 5,7-DHT-lesioned rats can be divided into two subgroups according to the loss of nigral tryptophan hydroxylase activity. Severely-lesioned animals (more than 55% loss of tryptophan hydroxylase activity) exhibit a significant release of punished behavior. Indeed, rats of this subgroup effected a significantly greater number of lever presses for food during the 15 min punished period of the CRF/FR7 schedule ($t=3.40$, $p<0.01$) (Fig. 1A). Lever presses were not altered during the initial 5 min non-punished period (42±5 and 39±12 for lesioned and control rats, respectively, data not shown). Similarly, the rats of the severely-lesioned group received significantly more shocks than control animals ($t=2.47$, $p<0.05$) in the drinking test (Fig. 1B). It is worth noting that rats with severe damage of nigral serotonergic innervation displayed attenuated punished behavior similar to that observed in non-lesioned animals treated with diazepam (2 mg/kg IP): 49±7 lever presses for food in the CRF/FR7 schedule of shock presentation (n=14) or 25±5 shocks during the drinking test (n=8) (data not shown).

In rats whose nigral serotonergic innervation was less severely damaged (less than 40% loss of tryptophan hydroxylase activity) punished behavior did not differ statistically from controls (Fig. 1A and 1B).

The coefficient of correlation between the reduction of hippocampal tryptophan hydroxylase activity and the attenuation of punishment-induced behavioral suppression failed to reach a statistically significant level ($r=0.36$, $0.05<p<0.10$). The coefficient decreased to 0.10 (NS) when the reduction of nigral tryptophan hydroxylase activity was kept constant by calculating the partial coefficient of correlation. As an illustration, 4 rats showing a marked decrease in hippocampal (−52±6%) but not in nigral (−19±6%) tryptophan hydroxylase activity received a number of shocks identical to that received by controls in the drinking test (Table 1).

These data do not exclude a possible role of serotonergic projections to the hippocampus in punishment-induced inhibition. However, they suggest a minimal involvement of this structure in the behavioral changes reported in this study.

Experiment 1 clearly indicates the important contribution of serotonergic innervation of the substantia nigra in the control or expression of shock-induced suppression of ongoing behavior. This anti-punishment effect seems rather specific since nigral lesion neither altered pressing for food during the additional post-surgery training sessions nor non-punished pressing in the CRF/FR7 schedule of shock presentation was altered by nigral lesion (controls; 37.3±3.2; 5,7-DHT: 37.2±5.5 presses during the initial 5 min non-punished period).

EXPERIMENT 2

The substantia nigra receives a mixed serotonergic innervation from the dorsal and the median raphé nuclei. Previous experiments support the role of the dorsal raphé in benzodiazepine-induced release of punished behavior [17]. Therefore experiment 2 was planned in order to investigate whether the projection arising from the dorsal raphé is involved in the control of suppression of lever pressing for food in the CRF/FR7 schedule of shock presentation.

Method

Fifteen days before being subjected to the CRF/FR7 schedule of shock presentation (described in the General Method Section), the rats pretreated with desipramine (25 mg/kg IP) were given an infusion of 5,7-DHT (1 μg of free

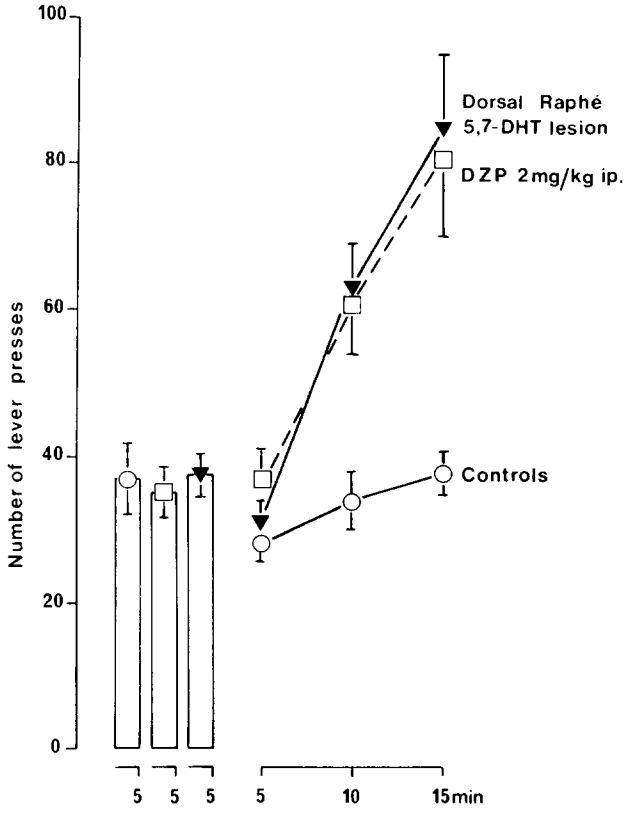

FIG. 2. Effects of intra-raphé dorsalis application of 5,7-DHT on responding for food during a CRF/FR7 schedule of shock presentation. The values are the mean number of lever presses±SEM effected during the initial 5 min of CRF non-punished period and the mean cumulated number of presses effected per 5 min during the subsequent 15 min of FR7 schedule of shock presentation. ○, controls (n=7); ▼, 5,7-DHT-lesioned rats (n=6); □, non-operated rats treated with diazepam (2 mg/kg IP) 30 min before the test (n=7).

base in 0.4 μl) into the nucleus raphé dorsalis (sterotaxic coordinates: A=0.16; L=0; H=−0.8, the incisor bar being set 5 mm above the inter-aural plane and the injection cannula being lowered at a 12° angle to the sagittal plane).

Tryptophan hydroxylase activity was assessed 24 hours after testing, as described in the general method.

Results and Discussion

As compared to controls, 5,7-DHT-lesioned rats effected a significantly greater number of lever presses for food during the 15 min punished period, $F(1,55)=19.76$, $p<0.001$. During the initial non-punished period, lever pressing was not modified (Fig. 2). A similar pattern was obtained in non-lesioned animals given a standard dose of diazepam (2 mg/kg IP).

Intra-raphé dorsalis infusion of 5,7-DHT induced a significant decrease (−60 to −70%) in tryptophan hydroxylase activity in the dorsal raphé as well as in various forebrain structures innervated by this nucleus. No division into relevant subgroups can be made in relation to behavior since raphé dorsalis serotonergic neurons in all the rats given 5,7-DHT were severely damaged.

This finding confirms and extends previous work concerning the involvement, although probably not exclusive, of serotonergic neurons of dorsal raphé in the release of punished behavior [17]. This is not a consequence of a general behavioral facilitation as non-punished behavior was not altered following 5,7-DHT infusion in the dorsal raphé (Fig. 2) nor was pressing for food during the post-surgery training sessions.

GENERAL DISCUSSION

These results confirm that serotonergic neurons contribute significantly to the control of punished behavior [14, 15, 17, 19, 20]. They indicate on one hand, the involvement of serotonergic innervation of the substantia nigra and, on the other hand, the role of serotonergic neurons of the dorsal raphé. These findings may suggest additional functional roles for the substantia nigra since this structure has never been claimed to be crucially implicated in the control of emotional responses. This might be expected in the light of anatomical data showing the substantia nigra to be in a position to influence, through its projection to the dorsal raphé, serotonergic cells of this nucleus [3].

Taken together, Experiments 1 and 2 strongly suggest that serotonergic neurons of the dorsal raphé projecting to the substantia nigra are chiefly involved in the control of behavioral suppression. To be fully established, this assumption requires specific experiments aimed at investigating the role of serotonergic median raphé cells ascending to the substantia nigra. Furthermore, a contribution of forebrain structures other than the substantia nigra receiving serotonergic innervation from the dorsal raphé also has to be assessed. However, dorsal raphé neurons projecting to the amygdaloid complex or to the nucleus accumbens do not seem to be involved in behavioral suppression since marked destruction of serotonergic innervation of these forebrain structures fails to release punished behavior [18].

The question arises as to whether an attenuation of punished behavior following damage of nigral serotonergic terminals is really associated with lessened fear or anxiety. In fact, 5,7-DHT in the substantia nigra has been reported to cause a limited hyperactivity, to enhance amphetamine-induced excitation and to reduce the potency of neuroleptics to elicit catalepsy [4,5]. This may indicate that nigral serotonergic innervation is essential for the control of motor components of behavior and, in particular, for the expression of fear or anxiety through the inhibition of ongoing responding.

A large amount of data support the contention that serotonergic innervation in the substantia nigra inhibit dopaminergic neurons [3, 4, 7]. One can therefore assume that this is essential for response suppression. However, two points deserve specific comments. First, since dopamine metabolism within the substantia nigra can be altered by stressful situations [1,6], the results of the 5,7-DHT lesion may be not specific to punished behavior, but rather reflect an effect on behavioral inhibition to any aversive event. Second, other dopaminergic neurons, such as those located within the ventral tegmental area, can be influenced by intra nigral 5,7-DHT and participate in the observed behavioral changes. Indeed, A10 neurons project to and control the frontal cortex, the ablation of which has been reported to attenuate conflict behavior [11]. Nevertheless, current preliminary experiments suggest that 5,7-DHT infusion into the ventral tegmental area fails to modify punishment-induced suppression.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from INSERM (ATP 80 79 112).

REFERENCES

1. Anisman, H. and R. M. Zacharko. Depression: The predisposing influence of stress. *Behav Brain Sci* **5:** 89–99, 1982.
2. Azmitia, E. and M. Segal. Autoradiographic analysis of differential ascending projections of the dorsal and median raphé nuclei in the rat. *J Comp Neurol* **179:** 641–668, 1978.
3. Beckstead, E. M., V. B. Domesick and W. J. H. Nauta. Efferent connections of substantia nigra and ventral tegmental area in the rat. *Brain Res* **175:** 191–217, 1979.
4. Carter, C. J. and C. J. Pycock. A study of the sites of interaction between dopamine and 5-hydroxytryptamine for the production of fluphenazine-induced catalepsy. *Naunyn Schmiedebergs Arch Pharmacol* **304:** 135–139, 1978.
5. Carter, C. J. and C. J. Pycock. The effects of 5,7-dihydroxytryptamine lesions of extrapyramidal and mesolimbic sites on spontaneous motor behaviour and amphetamine-induced stereotypy. *Naunyn Schmiedebergs Arch Pharmacol* **308:** 51–54, 1979.
6. Fadda, F., A. Argiolas, M. R. Melis, A. H. Tissari, P. L. Onali and G. L. Gessa. Stress-induced increase in 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the cerebral cortex and in the nucleus accumbens: its reversal by diazepam. *Life Sci* **23:** 2219–2224, 1979.
7. Giambalvo, C. T. and S. R. Snodgrass. Biochemical and behavioral effects of serotonin neurotoxins on the nigrostriatal dopamine system: comparison of injection sites. *Brain Res* **152:** 555–566, 1978.
8. Graeff, F. G., S. Quintero and J. A. Gray. Median raphé stimulation, hippocampal theta rhythm and threat-induced behavioral inhibition. *Physiol Behav* **25:** 253–261, 1980.
9. Hamon, M., S. Bourgoin, F. Hery and G. Simonnet. Phospholipid-induced activation of tryptophan hydroxylase from the rat brainstem. *Biochem Pharmacol* **27:** 915–922, 1978.
10. König, J. F. R. and R. A. Klippel. *The Rat Brain*. Baltimore: Williams and Wilkins, 1963.
11. Lippa, A. S., D. Critchett, M. C. Sano, C. A. Klepner, E. N. Greenblatt, J. Coupet and B. Beer. Benzodiazepine receptors: cellular and behavioral characteristics. *Pharmacol Biochem Behav* **10:** 831–843, 1979.
12. Parent, A., L. Descarries and A. Beaudet. Organization of ascending serotonin systems in the adult rat brain. A radioautographic study after intraventricular administration of ^{3}H-5-hydroxytryptamine. *Neuroscience* **6:** 115–138, 1981.
13. Schwartz, D. *Méthodes Statistiques à l'Usage des Médecins et des Biologistes*. Paris: Flammarion, 1963, pp. 239–241.
14. Stein, L., C. D. Wise and J. D. Belluzi. Effects of benzodiazepines on cerebral serotoninergic mechanisms. In: *Mechanism of Action of Benzodiazepines*, edited by E. Costa and P. Greengard. New York: Raven Press, 1975, pp. 29–44.
15. Stein, L., J. D. Belluzi and C. D. Wise. Benzodiazepines: behavioral and neurochemical mechanisms. *Am J Psychiat* **134:** 665–669, 1977.
16. Steinbusch, H. W. M. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience* **6:** 557–618, 1981.
17. Thiébot, M. H., M. Hamon and P. Soubrié. Attenuation of induced-anxiety in rats by chlordiazepoxide: role of raphé dorsalis benzodiazepine binding sites and serotonergic neurons. *Neuroscience* **7:** 2287–2294, 1982.
18. Thiébot, M. H., M. Hamon and P. Soubrié. Serotonergic neurons and anxiety-related behavior in rats. In: *Psychopharmacology of the Limbic System*, edited by E. Zarifian and M. Trimble. London: Wiley, in press.
19. Tye, N. C., B. J. Everitt and S. D. Iversen. 5-Hydroxytryptamine and punishment. *Nature* **268:** 741–743, 1977.
20. Tye, N. C., S. D. Iversen and A. R. Green. The effects of benzodiazepines and serotoninergic manipulations on punished responding. *Neuropharmacology* **18:** 689–695, 1979.
21. Vogel, J. R., B. Beer and D. E. Clody. A simple and reliable conflict procedure for testing antianxiety agents. *Psychopharmacologia* **21:** 1–7, 1971.