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BENZODIAZEPINE RECEPTOR LIGAND INFLUENCES ON LEARNING: AN ENDOGENOUS MODULATORY MECHANISM MEDIATED BY BENZODIAZEPINES POSSIBLY OF ALIMENTARY ORIGIN

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In rats, pre- but not post-training ip administration of either flumazenil, a central benzodiazepine (BZD) receptor antagonist, or of n-butyl-B-carboline-carboxylate (BCCB), an inverse agonist, enhanced retention of inhibitory avoidance learning. Flumazenil blocked the enhancing effect of BCCB, and the inhibitory effect of the BZD agonists clonazepam and diazepam also given pre-training. Post-training administration of these drugs had no effect. The peripheral BZD receptor agonist/chloride channel blocker Ro5-4864 had no effect on the inhibitory avoidance task when given ip prior to training, but it caused enhancement when given immediately post-training either ip or icv. This effect was blocked by PK11195, a competitive antagonist of Ro5-4864. These results suggest that there is an endogenous mechanism mediated by BZD agonists, which is sensitive to inverse agonists and that normally down-regulates the formation of memories through a mechanism involving GABA-A receptors and the corresponding chloride channels. The most likely agonists for the endogenous mechanism suggested are the diazepam-like BZDs found in brain whose origin is possibly alimentary. Levels of these BZDs in the cortex were found to sharply decrease after inhibitory avoidance training or mere exposure to the training apparatus.

Key words: learning – memory formation – endogenous benzodiazepines – flumazenil – benzodiazepine receptor ligands

Pre-training administration of central BZD agonists impair learning of a variety of behaviors in many species (Thiebot, 1985; Pereira et al., 1989). BCCB and other B-carboline esters are BZD inverse agonists: they bind to BZD receptors but cause opposite behavioral actions: anxiety, seizures, etc. (Novas et al., 1988). Recently, diazepam and other BZDs (De Blas et al., 1987; De Robertis et al., 1988; Medina et al., 1988) and BCCB (De Robertis et al., 1988) have been detected in the brain. Possibly the BZDs present in brain are of alimentary origin, since they are also found in cow milk (Medina et al., 1988) and in a variety of plants that serve as food (De Robertis et al., 1988).

This study investigates the effect of flumazenil (Ro-15-1788), a central BZD receptor antagonist, of BCCB, of clonazepam and diazepam, and of the peripheral BZD receptor ligands and GABA-A receptor chloride channel blockers Ro5-4864 (agonist) and PK11195 (antagonist) (Basile et al., 1988), on inhibitory avoidance learning in rats.

MATERIALS AND METHODS

Female Wistar rats from our own breeding stock (age, 3 months; median weight, 150 g) were trained in a step-down inhibitory avoidance task (Pereira et al., 1989) and tested 24 h later. The apparatus was a 50 x 25 x 25 cm acrylic box with a floor of 1 mm caliber bronze bars spaced 8 mm apart. A 5 cm high, 7 cm wide formica platform was on the left end of the box. On the training session, animals were placed on the platform and their latency to step down, that is to place their four paws on the grid, was measured with an automatic device. On stepping down, they received a 0.3 mA, 60 Hz, 2 sec footshock. The test session was similar, but the footshock was omitted. Test minus training step-down latency (ceiling, 300 sec) measured retention test performance (Pereira et al., 1989).

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Drug injections were given 30 min prior to training (Tables I, II,), and/or immediately after training (Table II). The icv experiments shown in Table II were performed using chronically implanted cannulae (for refs., see Izquierdo, 1989).

TABLE I

Effect of the vehicle, flumazenil, BCCB, BCCB plus flumazenil, clonazepam, clonazepam plus flumazenil, diazepam, diazepam plus flumazenil, and Ro5-4864, given ip. 30 min prior to training, on retention test performance in a step down inhibitory avoidance task. Training-test interval was 24 h. Data are expressed as median (interquartile range) test minus training step-down latency. N = 11 for all groups

Treatment (mg/kg)	Retention score (sec)
Vehicle	20 (10 / 31)
Flumazenil (2.0)	40 (17 / 180)
Flumazenil (5.0)	124 (46 / 300)[c]
BCCB (0.2)	64 (24 / 300)[b]
BCCB (0.5)	187 (31 / 300)[c]
BCCB (0.5) + Flumazenil (2.0)	12 (4 / 27)[d]
Clonazepam (1.0)	−1 (−9 / 8)[c]
Clonazepam (1.0) + Flumazenil (2.0)	13 (3 / 29)[d]
Diazepam (1.0)	0 (−5 / 3)[c]
Diazepam (1.0 + Flumazenil (2.0)	18 (10 / 49)[d]
Ro5-4864 (2.5)	15.7 (11.0/26.5)[a]
Ro5-4864 (6.25)	14.8 (5.5/16.5)

a: significant difference from vehicle, $p < 0.05$ in a Mann-Whitney test, two-tailed; *b:* same, $p < 0.02$; *c:* same, $p < 0.002$; *d:* significant difference from clonazepam or diazepam without flumazenil, $p < 0.02$.

TABLE II

Effect of the vehicle, BCCB, clonazepam and diazepam, given ip 30 min prior to training, and of either the vehicle of flumazenil given immediately post-training, on retention test performance in a step down inhibitory avoidance task. Training-test interval, 24 h. Data expresses as in Table I. N = 13 per group.

Treatment (mg/kg) Pre-training	Post-training	Retention score (sec)
Vehicle	Vehicle	26 (13/ 41)
Vehicle	Flumazenil (2.0)	23 (9/ 24)
BCCB (0.5)	Vehicle	115 (66/300)[b]
BCCB (0.5)	Flumazenil (2.0)	165 (13/300)[b]
Clonazepam (1.0)	Vehicle	2 (−5/ 5)[b]
Clonazepam (1.0)	Flumazenil (2.0)	−1 (−4/ 4)[b]
Diazepam (1.0)	Vehicle	3 (−2/ 5)[a]
Diazepam (1.0)	Flumazenil (2.0)	5 (1/ 11)[a]

a: significant difference from vehicle-vehicle group, $p < 0.02$ in a Mann-Whitney U test, two-tailed; *b:* same, $p < 0.002$.

BZD-like immunoreactivity was measured with a monoclonal antibody in cortical tissue from intact control, trained rats, and rats placed for 30 sec in the training box. The brain was removed within 1 min after the behavioral procedure(s) and extracted and processed as recommended by De Robertis et al. (1988) and Medina et al. (1988).

RESULTS AND DISCUSSION

Flumazenil and BCCB enhanced, clonazepam and diazepam depressed, and Ro5-4864 had no effect on, retention, when given prior to training. Flumazenil antagonized the effect of BCCB, clonazepam and diazepam (Table I). Post-training flumazenil had no effect and was unable to antagonize the effects of BCCB and the BZDs (Table II). This suggests either that the drugs act on acquisition, or that they do act on post-training consolidation but flumazenil reaches the brain too slowly to be effective upon post-training administration. The post-training effect of the peripheral receptor/chloride channel ligands, Ro-5-4864 and PK11195, particularly that observed upon icv administration (Table III), favors the latter possibility.

TABLE III

Effect of the immediate post-training ip administration of vehicle, flumazenil, BCCB, clonazepam, diazepam, and of the ip or icv administration of Ro5-4864, alone or with PK11195, on retention test performance in a step down inhibitory avoidance task. Training-test interval, 24 h. Data expressed as in Table I

Treatment (mg/kg)	N	Retention score (sec)
Vehicle, ip	11	24 (12/ 36)
Flumazenil (5.0, ip)	10	19 (7/116)
BCCB (0.5, ip)	10	17 (8/ 39)
Clonazepam (1.0, ip)	10	18 (5/ 23)
Diazepam (1.0, ip)	10	19 (15/ 27)
Vehicle, ip	11	19 (10/ 23)
Ro 5-4864 (1.0, ip)	11	41 (5/300)[a]
Ro 5-4864 (2.0, ip)	11	51 (41/ 79)[b]
Ro 5-4864 (5.0, ip)	11	300.0 (71/300)[b]
Vehicle, icv	9	36 (29/ 73)
Ro 5-4864, 2.5 μg/rat, icv	9	300 (300/300)[b]
PK11195, 2.5 μg/rat, icv	9	24 (15/ 34)
Ro 5 + PK (2.5 + 2.5 μg/rat, icv)	9	56 (35/ 64)[c]

a: significant difference from its own vehicle control group at $p < 0.05$ level in a Mann Whitney U test, two-tailed; *b:* same, $p < 0.01$; *c:* significant difference from Ro 5 alone, $p < 0.02$.

TABLE IV

Benzodiazepine-like immunoreactivity in the cerebral cortex of control rats, of rats sacrificed less than 1 min after inhibitory avoidance training, and of rats sacrificed less than 1 min after mere exposure to the training box without footshock

Group	N	Benzodiazepine immunoreactivity (Mean ± SE ng/g diazepam)
Control	8	31.8 ± 5.4
Footshock-trained	8	13.8 ± 4.5[a]
Exposed to apparatus	8	14.8 ± 3.9[a]

a: significant difference from control group at $p < 0.01$ level in Duncan multiple range test.

Anyway, the effect of flumazenil on its own, and the high sensitivity of this task to BCCB, suggest that memory formation of inhibitory avoidance learning is normally down-regulated by endogenous BZD agonists, possibly diazepam or the diazepam-like molecules found in brain (De Blas et al., 1987; Medina et al., 1988). The experiment of Table IV indeed suggests that there is a release of such substances during and/or after training in the cortex. Experiments in progress show a similar release in septum, hippocampus and amygdala which, like the cortex, are structures involved in memory processing, particularly in the early stages of memory formation (Isquierdo, 1989).

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REFERENCES

BASILE, A. S.; BOLGER, G. T.; LUEDDENS, H. W. M. & SKOLNICK, P., 1989. Electrophysiological actions of Ro5-4864 on cerebellar Purkinje neurons: Evidence for "peripheral" benzodiazepine-mediated depression. *J. Pharmacol. Exptl. Therap., 248:* 463-469.

DE BLAS, A. L.; PARK, D. & FRIEDRICH, P., 1987. Endogenous benzodiazepine-like molecules in the human, rat and bovine brain studied with a monoclonal antibody to benzodiazepines. *Brain Res., 413:* 275-284.

DE ROBERTIS, E.; PENA, C.; PALADINI, A. C. & MEDINA, J. H., 1988. New developments on the search for the endogenous ligand(s) of central benzodiazepine receptors. *Neurochem. Int., 13:* 1-11.

FILE, S. E. & PELLOW, S., 1986. Intrinsic actions of the benzodiazepine receptor antagonist Ro 15-1788. *Psychopharmacol., 88:* 1-11.

IZQUIERDO, I., 1989. Different forms of post-training memory processing. *Behav. Neural Biol., 51:* 171-202.

MEDINA, J. H.; PENA, C.; PIVA, C.; PALADINI, A. C. & DE ROBERTIS, E., 1988. Presence of benzodiazepine-like molecules in mammalian brain and milk. *Biochem. Biophys. Res. Comm., 152:* 534-539.

NOVAS, M. L.; WOLFMAN, C.; MEDINA, J. H. & DE ROBERTIS, E., 1988. Proconvulsant and 'anxiogenic' effects of n-butyl B carboline-3-carboxylate, an endogenous BZD binding inhibitor from brain. *Pharmacol. Biochem. Behav., 30:* 331-336.

PEREIRA, M. E.; ROSAT, R.; HUANG, C. H.; GODOY, M. C. G. & IZQUIERDO, I., 1989. Effect of additional training on the retention of shuttle avoidance behavior in rats, and the influence of diazepam thereupon. *Behav. Neurosci., 103:* 105-107.

THIEBOT, M. H., 1985. Some evidence for amnesic-like effects of benzodiazepines in animals. *Neurosci. Biobehav. Revs., 9:* 95-100.