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Evaluation of Perinatal Flumazenil Effects on the Behavior of Female RLA/Verh Rats in Anxiety Tests and Shuttle Box Avoidance

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FERRÉ, P., R. M. ESCORIHUELA, A. TOBEÑA AND A. FERNÁNDEZ-TERUEL. *Evaluation of perinatal flumazenil effects on the behavior of female RLA/Verh rats in anxiety tests and shuttle box avoidance.* PHARMACOL. BIOCHEM. BEHAV. **55**(4) 475–480, 1996— The present study investigated the effects of perinatal flumazenil (Ro 15-1788, a benzodiazepine receptor antagonist), given from gestational day 15 to the 14th day after giving birth, at two doses (3.5 and 6.3 mg/kg/day) on the behavior of female RLA/Verh rats. This rat line has been selectively bred for non-acquisition of two-way active avoidance. Offspring of treated/non-treated dams were tested when adults for two-way active (shuttle box) avoidance acquisition. For comparison reasons additional offspring coming from the same treatments were used in an hyponeophagia test and in both an open field and a plus maze tests. The results show that perinatal flumazenil, specially the lower dose (and also the highest dose, although only in the final phases of the shuttle box training) was able to improve two-way active avoidance acquisition, as it has been previously found with males, whereas it failed to show any effect in the hyponeophagia task, the open field and the plus-maze tests. As two-way active avoidance acquisition can be enhanced both by reducing emotionality/anxiety or by improving memory, it is suggested that flumazenil treatment could have affected either one or both processes.

RLA/Verh rats Flumazenil Hyponeophagia Two-way active avoidance acquisition Learning Anxiety

THE SWISS sublines of Roman High- and Low-avoidance rats (RHA/Verh and RLA/Verh) have been selected and bred for their rapid acquisition vs. failure to acquire two-way active (shuttle box) avoidance (7). Performance in this task is improved by anxiolytic (environmental and pharmacological) manipulations and impaired by anxiogenic treatments (11, 12,23,27). Accordingly, RLA/Verh rats reactions are more pronounced, hormonally and behaviorally, to most environmental stressors, than those of RHA/Verh rats (1,4,7,14,18,24).

Previous studies have also reported a lower GABA-stimulated $^{36}Cl^-$ uptake in cerebral cortex in RLA/Verh rats than in RHA/Verh animals (19), thus suggesting a role for the $GABA_A$-Benzodiazepine receptor-Chloride channel ($GABA_A$-BZR/Cl^-) complex in the emotional differences seen between both rat lines.

Both the ontogeny of the $GABA_A$-BZR/Cl^- complex and some behavioral processes related to its function can be modified by prenatal, perinatal or early postnatal administration of BZR-related compounds (2,9,10,15,16,17,20,22). Particularly meaningful for the present study was the report that perinatal treatment with low doses of flumazenil (a BZR antagonist) in Sprague-Dawley rats promoted enduring increases in hippocampal BZR binding, as well as long-lasting decreases in emotional reactivity and improvements in learning ability in a radial maze (22). However, the effects of developmental treatments with BZR-related compounds can be different depending upon the developmental stage in which they are given or tested (15–17), the dose used (8,15,16,22) and the sex of the animals (8,20).

Therefore, the present study was aimed at evaluating shuttle box avoidance acquisition, as well as performance in several other anxiety-related tasks (an hyponeophagia test, an open field and a plus-maze test), in adult female RLA/Verh rats receiving perinatal flumazenil (FZ) at two different doses.

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Perinatal flumazenil has been shown to improve two-way avoidance acquisition in male RLA/Verh rats (10), and it was our purpose both to determine whether sex differences might surface with this drug as had been the case with diazepam (8) and to test the effect of an additional dose of FZ. The hyponeophagia (14,25,26), open field and plus-maze tests (21) were chosen as typical situations used to test emotional reactivity.

MATERIALS AND METHODS

Subjects

Pregnant RLA/Verh female rats were used. They were kindly provided by Dr. Peter Driscoll (Animal Science Institute, ETH-Zentrum, Zürich, Switzerland) and mated in our Laboratory. The rats were kept with food and water freely available, with a 12h light-dark cycle schedule (light on 8:30–20:30 h), and controlled temperature (22 ± 2°C) and relative humidity (50 ± 10%).

Perinatal Flumazenil Treatment

Flumazenil was kindly supplied by Hoffman-La Roche, Basel (Dr. J.R. Martin).

From gestation day 15 to the 14th day after giving birth some dams (randomly assigned to FZ treatment; RLA/FZ1 and RLA/FZ2 groups) were given drinking water containing FZ (prepared fresh daily, dissolved in ethylene glycol plus water; final concentration 0.5%), whereas Control (RLA/C) and vehicle-treated (RLA/V) mothers received tap water or water plus vehicle, respectively. The daily solution intake by each litter was measured every 24 h (in the morning) in order to adjuste the actual FZ dose consumed with that expected at the end of the treatment. There were no between-group differences in the average consumed volumes of the corresponding solutions. The average daily dose of FZ was 3.5 mg/kg/day (relative to the weight of the dams plus the litter) in the RLA/FZ1 group and 6.3 mg/kg/day in RLA/FZ2 rats.

Behavioral Testing

In experiment 1, thirty-two naive 7-month-old female offspring (n = 8 per group, obtained from at least 6 different litters) were tested for two-way active avoidance. Testing was performed in two identical shuttle boxes (Letica Inst. LI 916, Barcelona-Spain) housed within two independent, sound-attenuating boxes constructed of plywood. Dim and diffuse illumination inside the latter boxes was provided by a fluorescent bulb which emitted a level of illumination <50 lx. The experimental room was kept dark. The shuttle boxes were each divided into two equally sized compartments (25 × 25 cm, 28 cm high) connected by an opening 8 cm wide and 10 cm high. Training consisted of five 50-trial sessions (spaced 24 h). Immediately before starting the first session animals were allowed to familiarize to the box for 5 min, and crossings during this period (habituation crossings) were considered as a measure of locomotor activity. Each trial was composed of a 10s CS (a 2400 Hz, 63 dB tone plus a light from a 7 W small lamp, simultaneously delivered), followed by a 20s US (scrambled electric footshock of 0.7 mA, delivered through the grid floor) and an intertrial interval of 60 s. Crossings to the other compartment in the presence of the CS alone were considered as avoidance responses. The number of avoidances for each 10-trial block, the number of trials required to achieve the criterion of 10 consecutive avoidances (criterion 10) and the mean response latency/session were scored. A between-session index was also calculated as the number of avoidances in each session minus the number of avoidances in the previous one.

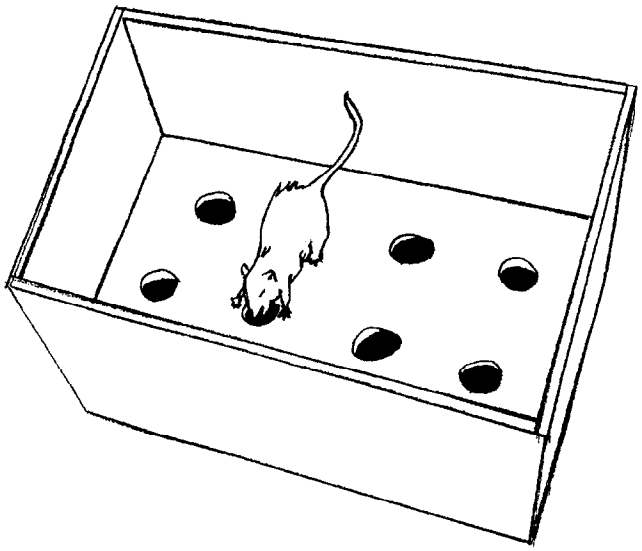

FIG. 1.

In experiment 2, twenty-nine naive 9-month-old female offspring (n = 7–9/group, obtained from at least 6 different litters) from the same experimental groups performed an hyponeophagia test. They were food-deprived following a progressive schedule resulting in a daily hour of food freely available (from 17:00 to 18:00), during 10 days. Testing was carried out from 16:00 to 19:00 and it was performed (Figure 1) in a brown wooden box (57 × 28 × 32 cm) with eight holes in the bottom (diameter 5 cm) each containing a plastic recipient with a pellet of the animals' normal food. Testing (10 min) started by placing each rat in the box facing a wall corner. Eating latency (EL) and the total time spent eating (TE) were scored.

In experiment 3, thirty-five naive 15-month-old female offspring from the same experimental groups were used. They performed an open field test, consisting of a beige arena (diameter: 83 cm) with white 34 cm high walls, and divided into 19 equal sectors. During 5 min, the number of ambulations (AMB) and defecations (DEF) were scored. The open field test was performed under normal room illumination (a fluorescent bulb). Twenty-four hours later, the same rats were tested in an elevated plus-maze, consisting of two open arms, 50 × 10 cm, and two enclosed arms 50 × 10 × 40 cm, with an open square 10 × 10 cm in the center of the + sign, and elevated to a height of 50 cm. During 5 min, the number of entries into the open arms (EOA) and the total number of arm entries (TAE) were recorded. The plus-maze test was performed under dim red light.

The overall experimental protocols of the present experiments have been approved by an Ethical Committee of the Autonomous University of Barcelona.

Statistical Analysis

In Experiment 1, multivariate analyses of variance (MANOVA), adjusting for habituation crossings (i.e. including this variable as a covariate in order to control for possible differ-

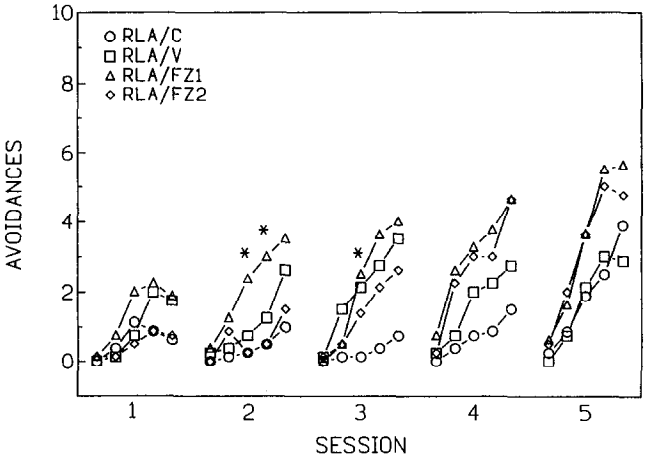

FIG. 2. Means of avoidances in each 10-trial block are represented. *$p < 0.05$ vs. RLA/C group (Duncan's test).

ences between groups in spontaneous activity), were applied to avoidances across 10-trial blocks in each session. They were followed by Duncan's tests for comparisons between groups. The number of animals/group achieving Criterion 10 and mean response latencies/session lower than 10s (i.e. lower than the duration of the CS) were analyzed by non-parametric tests. Data from the hyponeophagia test (Exp. 2), open field and plus-maze tests (Exp.3) were analyzed by oneway ANOVAs.

RESULTS

In experiment 1, the MANOVAs with repeated measures (for avoidances across 10-trial blocks in each session) showed significant influences of the covariate (all $F > 20.0, p < 0.001$). The values of habituation crossings were: RLA/C, 6.0 ± 1.1; RLA/V, 7.0 ± 0.9; RLA/FZ1, 4.6 ± 0.8; RLA/FZ2, 6.7 ± 0.7. Nevertheless, after adjusting for the influence of the covariate MANOVAs showed treatment effects on avoidances across 10-trial blocks [all $F(3,27) > 9.6\, p < 0.001$], as well as treatment X 10-trial blocks interactions [all $F(3,24) = 8.4$ $p < 0.002$]. Duncan tests showed that in the third and fourth 10-trial block, in session 2, and in the third block, in session 3, RLA/FZ1 performed significantly more avoidances than RLA/C rats ($p < 0.05$, Duncan's test; Fig. 2). Moreover Fig. 2 shows that RLA/FZ2 subjects improved their performance in the last three sessions, thus achieving a performance level similar to RLA/FZ1 rats (although in the initial 2 training sessions they had a poor performance level). Furthermore the number of subjects performing a mean response latency lower than 10 seconds was significantly greater in RLA/FZ2 group ($p < 0.025$ vs RLA/C group, Fisher's exact test) in session 4 (Latency 4 in Table 1). No differences appeared in reponse latency values along the other sessions. With regard to the between-session index, significant differences appeared only in the progression between sessions 3 and 4 (AV4-AV3 in Table 1). Thus, the AV4-AV3 variable showed that the increase of avoidances (between sessions 3 and 4) was higher in the RLA/FZ2 group than in RLA/V rats ($p < 0.05$, Duncan's test). Finally the number of subjects performing 10 consecutive avoidances (Criterion 10) was greater in both FZ-treated groups than in RLA/C and RLA/V groups ($p < 0.05$, Chi-square test applied to treated vs. nontreated animals; Figure 3), and the number of trials required to achieve that criterion was lower in RLA/FZ1 than in RLA/C group ($p < 0.05$, Mann Whitney's U test; Figure 3).

Although the scores of RLA/Verh rats in the remaining tasks are concordant with those usually seen with those animals, no FZ effects appeared in the hyponeophagia test (Exp. 2; Table 1) nor in the open field and plus-maze tests (Exp. 3; Table 2).

DISCUSSION

The results show that, when using appropriate training schedules, RLA/Verh female rats are able to acquire two-way active avoidance behavior at least to a moderate degree. Moreover, the present is, to our knowledge, the first report demonstrating a long-lasting improvement of two-way active avoidance performance in RLA/Verh female rats as a long-lasting consequence of a drug treatment. In line with previous results obtained in our laboratory using males (10), perinatal FZ given at a moderate dose (3.5 mg/kg) was able to improve shuttle box performance of RLA/Verh female rats (which achieved 34% avoidances by the 5th session, vs. 18% for the RLA/C group). However, the performance level achieved by female RLA/Verh rats in the present study is somewhat lower than that previously seen in males (10). These partial discrepancies could be explained by sex or age differences. On the other hand, the results from experiment 1 also confirm (10) that the warm up effect is very marked in RLA/Verh female rats during the initial trials of each session (Fig. 2). That phenomenon could explain the failure to observe shuttle box

TABLE 1

EFFECTS OF PERINATAL FLUMAZENIL ON SEVERAL MEASURES OF SHUTTLE BOX AVOIDANCE ACQUISITION AND HYPONEOPHAGIA

	Shuttlebox Avoidance		Hyponeophagia	
	Latency 4 (rats with mean latency <10 s)	AV4-AV3	EL	TE
RLA/C	0/8	2.1 (1.1)	335.6 (61.9)	121.0 (36.0)
RLA/V	2/8	−1.4 (0.6)	381.3 (51.1)	148.0 (30.6)
RLA/FZ1	4/8	4.5 (2.3)	424.3 (73.8)	121.3 (49.9)
RLA/FZ2	5/8*	6.4† (3.5)	304.5 (23.9)	169.7 (9.3)

Means and SEM (in parentheses) are shown. *, $p < 0.025$ vs RLA/C, Fisher's Exact Test; †, $p < 0.05$ vs RLA/V, Duncan's multiple range test. See text for variable symbols.

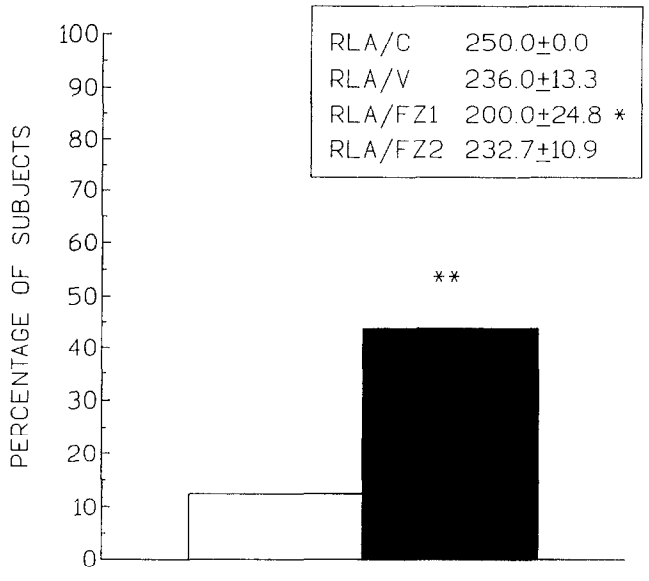

FIG. 3. The percentage of subjects in RLA/C and RLA/V groups (unfilled bar) and RLA/FZ1 and RLA/FZ2 groups (filled bar) that achieved the criterion of 10 consecutive avoidances along training are represented. The mean ± S.E.M. of the number of trials required to achieve the criterion in each group are shown in the top part of the Figure (*$p < 0.05$ vs. RLA/C group; Mann-Whitney's U test; **$p < 0.05$ vs. untreated animals, Chi-Square Test).

avoidance acquisition in such rat line when using shorter training procedures (4,7,8,10,11).

A further finding was that FZ 6.3 mg/kg was also capable of inducing a slight performance improvement, but only in the final training phases (in sessions 4-5 both FZ-treated groups had identical avoidance performance). This was also supported by measuring the number of subjects in each group that were able to perform 10 consecutive avoidances. In this difficult criterion both drug-treated groups showed better performance (Fig. 3).

The FZ-induced enhancement of shuttle box avoidance, which could be consistent with a reduction of anxiety in those animals (11,12,27,28), appears to be in line with several findings obtained with pre- or perinatal FZ treatments in Sprague-Dawley rats and using other emotionality measures (2,22).

An interpretation of the previous results exclusively on the basis of FZ effects on anxiety is precluded, however, by the fact that FZ treatment did not affect behavior in the hyponeophagia test, nor in the open field and plus-maze tests. Importantly, these results are consistent with the lack of (perinatal) FZ effects found in previous studies using RLA/Verh males of different ages (aproximately from 1 to 8 months) which were evaluated in similar tests (10,13; and unpublished results). Thus, the described results (in females as well as in males) in tests of spontaneous fearfulness lead to the suggestion that if the observed improvement of shuttle box avoidance is related to a decrease in anxiety, it would probably be the conditioned fear involved in avoidance acquisition. However, this possibility remains to be further explored.

Alternatively, it is also worth mentioning that shuttle box avoidance (especially when it is carried out along several sessions) is sensitive to the effects of several behavioral or pharmacological treatments (5,6,9,23) which similarly affect performance in other learning/memory tasks. In this regard, although shuttle box acquisition (as well as the deficit of RLA/Verh rats in that task) has been consistently related to emotionality/anxiety (10–12,27,28), the lack of FZ effects in the other forementioned tests does not allow us to exclude a possible memory-enhancing effect of FZ treatment which could explain the improvement observed in shuttle box avoidance acquisition. However, the lack of flumazenil action in the warming up precludes an unspecific effect on task retention; maybe the process affected could be related to memory of conditioned stimulus (as indicated by the fact that flumazenil effect is especially seen after the second 10-trial block, in sessions 2 and 3). The possibility of a memory effect would also be consistent with Marczynski's et al. findings (22) showing a better memory performance of FZ-treated rats (given a perinatal dose of 3 mg/kg/day) in a radial maze test.

TABLE 2

BEHAVIOR OF RLA/Verh FEMALE RATS IN THE OPEN FIELD AND PLUS MAZE TESTS

	Open Field		Plus Maze	
	AMB	DEF	TAE	EOA
RLA/C	20.8 (3.1)	4.7 (0.9)	5.6 (0.7)	1.5 (0.4)
RLA/V	17.3 (3.5)	5.2 (1.5)	6.0 (1.5)	2.6 (0.7)
RLA/FZ1	15.4 (1.7)	4.7 (0.8)	3.6 (1.3)	1.4 (0.7)
RLA/FZ2	19.8 (3.1)	4.2 (0.9)	5.2 (1.2)	2.0 (0.7)

Mean and SEM (in parentheses) are shown. See text for variable symbols.

The vehicle used in the present study could have contributed to some of the observed flumazenil effects in the shuttle box, although vehicle-treated animals did not differ from control rats in any parameter. In fact, ethylene glycol is an alcohol that produces CNS depression, and at very high doses induces toxic and deleterious effects on cholinergic neurons (3). It cannot even be excluded that some contribution of ethylene glycol to flumazenil effects could be due to both substances sharing, at least partially, the same site of action. Further experiments would be needed to elucidate the contribution of each factor in the main treatment effects obtained.

Cross-fostering, which allows testing for possible changes in maternal behavior, was precluded with the present experimental design, i.e. a perinatal treatment during which the pups receive the drug, after birth, through the mother's milk. Nevertheless, in a study concerning the effects of prenatal diazepam (Driscoll, personal communication), no effects of that treatment were seen in the RLA/Verh mothers maternal behavior, even with a dose that significantly improved shuttle box performance in their offspring (8). Still related to that, fostering RLA/Verh pups to RHA/Verh dams (whose maternal behavior show clearcut differences with that of RLA/Verh dams; (7)) doesn't influence adult shuttle box performance of the fostered rats (7), thus indicating that behavior of RLA/Verh rats is very resistant to maternal influences and, therefore, the present shuttle box results are probably not a consequence of changes in maternal behavior.

On the other hand, although it could be argued that the oestrus cycle had to be controlled, the similarity of the present results with those obtained in males (10) and the fact that the rats were tested in a counterbalanced manner along the 10 days of experiment duration, make the possibility of an influence of the oestrus rather unlikely.

In summary, the present set of experiments has produced several new findings. First, an environmental manipulation (i.e. extensive training) allows female RLA/Verh rats to acquire, to some extent, two-way active avoidance behavior. This also provides the oportunity (as seen in males; (10)) to observe that the warm-up phase (i.e. the initial 10–20 trials) of each session appears to be less influenced by training, thus suggesting that psychogenetic selection of RLA/Verh rats has especially influenced the so-called warm-up effect. Secondly, the long-lasting improvement of shuttle box avoidance observed after perinatal FZ, as well as similar results obtained in males and also after prenatal diazepam (8,10), tend to suggest that the $GABA_A/BZR/Cl^-$ complex could be (at least partially) related to the genetic avoidance deficit of RLA/Verh rats (7,12,19). Finally, and taking into account the lack of FZ effects in tests of unconditioned emotionality/anxiety, it remains to be established to what extent perinatal FZ may be affecting fear conditioning (known to be involved in shuttle box acquisition) and/or processes related to memory of the specific warning stimulus in the RLA/Verh rat line.

ACKNOWLEDGEMENTS

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