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Benzodiazepine Receptors Increase Induced by Stress and Maze-Learning Performance in Chick Forebrain

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MARÍN, R. H. AND A. ARCE. *Benzodiazepine receptors increase induced by stress and maze-learning performance in chick forebrain.* PHARMACOL BIOCHEM BEHAV **53**(3) 581–584, 1996.—Two-day-old chicks were selected on their second escape performance in a one-trial, maze-learning task, and termed high-performance (H-P), moderate-performance (M-P), and low-performance (L-P) chicks. The learning degree was expressed by the escape time improvement being respectively the 64, 46, and 24%. Then, the three selected groups were maintained to reach 15 days of age and then submitted to acute swimming stress, and [^{3}H]flunitrazepam and [^{3}H]Ro 5-4864 receptor bindings were performed on synaptosomal/mitochondrial membranes from forebrain. The receptor number for both radioligands in stressed high-performance chicks was significantly higher than in stressed low-performance chicks. The results suggest that higher performance chicks were more susceptible than lower performance chicks to acute stress associated to increase of both central and peripheral type benzodiazepine receptors, probably due to differences in the degree of endogenous emotionality.

Stress susceptibility Maze-learning selected chicks Benzodiazepine receptors

PERIPHERAL-TYPE benzodiazepine receptors (PBR) have been identified in peripheral organs and in nonneuronal cells of the central nervous system (8). These receptors bind selectively and with high affinity the ligands, Ro 5-4864 (4′-chlorodiazepam) and PK 11195 (an isoquinoline carboxamide derivative) (4) and are primarily localized on mitochondria, although a plasma membrane fraction has been also identified (5). Acute stress causes immediate increases in PBR binding in several tissues, whereas prolonged or chronic stress tends to cause decreases (4,6). Rat exposure to swimming acute stress produces an increase in the PBR in mitochondrial membranes from olfatory bulb, without changes in affinity (11).

The central-type benzodiazepine receptor (CBR) is an allosteric modulatory site localized in the $GABA_A$ receptor-chloride channel complex, in neuronal cells (3). These receptors bind selectively and with high affinity ligands clonazepam and flunitrazepam (4). The CBR have also been implicated in the organism's response to physical (4,10) and psychological stress (4,9). Chick exposure to swimming acute stress produces an increase in the [^{3}H]flunitrazepam receptors in synaptosomal membranes from forebrain, without changes in affinity (10). Furthermore, young chicks selected on their performance in imprinting behavior and then submitted to swimming stress showed that the degree of CBR increase was inversely related to the degree of imprinting performance (13).

In the present investigation, we studied whether three chick subpopulations, selected on the basis of escape speed performance in a one-trial maze-learning task, display different increments of both the [^{3}H]flunitrazepam ([^{3}H]FNZ) and [^{3}H]Ro 5-4864 receptor binding on forebrain membranes, after acute swimming stress.

METHOD

Animals

Chicks (Cobb Harding, of both sexes) were obtained from a commercial hatchery, INDACOR (Argentina). A total of 280 birds in groups of 20 were housed in brooders, in a room with constant temperature (32°C) and humidity at a 12 L : 12 D cycle (lights on at 0700 h) with food and water freely avail-

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able and maintained in these conditions until they reached 2 days old.

Apparatus

The apparatus, as described by Gilbert et al. (7), consisted on T maze with isolation chamber, placed inside a communal brooder, but separated from the brood area by chicken wire. A small 10 × 10 cm mirror was located at the T junction of the maze just above the floor of the T maze to facilitate the arrival of the chick to the T junction. Then, the chick could choose to enter any of the maze arms. The brood area was illuminated with a bright lamp (250 W) suspended immediately above it, and food and tapwater were freely available. The apparatus and brooder were contained in a 95 × 60 cm box that was kept in a small room (2 × 3 m) held at constant temperature and humidity during testing.

Procedure

The procedure was essentially as described by Gilbert et al. (7). Before training, all chicks were individually marked with spray dye and placed in the communal brooder area for at least 1 h to interact with each other freely. Training always commenced at 1000 h. Each chick was placed individually in the center of the isolation box facing away from the T corridor. After the chick arrived at the exit of the apparatus the first escape time was recorded and the chick was immediately replaced with its brood mates. After 3 h the chick was again placed in the apparatus. The time taken for the chick to arrive at the exit of the apparatus was recorded as second escape time and then the chick was immediately replaced with its brood mates. It was reasoned that chicks who remembered the way out from the first trial would show an escape improvement, leaving the isolation apparatus more quickly on the second escape. Three chick groups were selected on their second escape time. Chicks that escaped in less than 10 s were termed high-performance (H-P), the ones that escaped in 10–40 s were termed moderate-performance (M-P), and the ones that escaped in 40–120 s were termed low-performance (L-P) chicks. Then, the escape time improvement was calculated for each selected chick group, according to the following ecuation:

$$\text{Escape time improv. (\%)} = \Sigma \frac{(t\ \text{1st escape} - t\ \text{2nd escape})}{t\ \text{1st escape}} \times \frac{100}{n}$$

where n = number of chicks in each group, and t = time (s). The percent of escape time improvement expresses the relationship between the second escape and the first escape, and this gives us information about the learning degree for each group of chicks in this task.

Sexing of Chicks

After maze-escape selection of 120 chicks, they were sacrificed by decapitation, the abdomen dissected and the gender determined.

Acute Swimming Stress

After maze-escape selection, the marked chicks were grouped at random and maintained in batches of 12, on regular daily light-dark cycles at 32°C. Food and water were freely available. When the chicks were 15 days old, they were stressed as follows (13). Four chicks of each H-P, M-P, and L-P groups were simultaneously removed. Two birds of each group were immediately sacrificed by decapitation and the other two were individually placed in a basin (22 × 22 × 22 cm) previously filled with 38°C clean water to a depth of 15 cm, during 15 min. The birds did not show symptoms of exhaustion during this time period. They were sacrificed by decapitation immediately after the end of the swimming period. Then the brains were removed and forebrain hemispheres quickly dissected on ice.

Preparation of Crude Mitochondrial/Synaptosomal Fraction

All the procedures for preparing the membranes were carried out at 4°C, essentially as described by Awad and Gavish (2). Tissue material was homogenized in 50 vol of 50 mM Tris-HCl buffer, pH 7.4/g original tissue, using a Potter-S glass Teflon homogenizer and centrifuged at 35,000 × g for 15 min. Each pellet was suspended in 150 vol of this buffer and used as PBR and CBR preparation.

Binding Assay

Binding assays were conducted in 50 mM Tris-HCl buffer, pH 7.4, at 4°C (2). The binding assay mixture contained 400 μl of membrane suspension at a final concentration of 300 μg of protein/ml and 50 μl of [^{3}H]Ro 5-4864 (85.4 Ci/mmol; New England Nuclear) or [^{3}H]flunitrazepam (86.0 Ci/mmol; New England Nuclear) solution in the absence (total binding) or presence (non specific binding) of 10 μM diazepam (Hoffmann-La Roche). [^{3}H]Ro 5-4864 was at concentrations ranging from 1 to 25 nM, and [^{3}H]flunitrazepam was at concentrations ranging from 0.5 to 10 nM. After incubation for 60 min at 4°C, samples were filtered under vacuum over Whatman GF/B filters using a Brandel M-24R filtering manifold. Samples were washed three times with 4 ml of ice-cold Tris buffer and the radioactivity was counted in a LKB-1219-Rack-Beta Counter at 48% efficiency. B_{max} and K_d values for PBR and CBR were determined by computer aided nonlinear regression analysis of the experimental data.

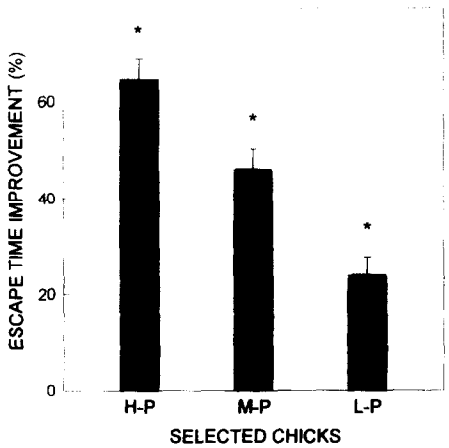

FIG. 1. Percentage of escape time improvement in selected chicks. Each percentage is the mean ± SEM of values obtained from eight experiments (20 animals were used in each experiment). *Significant differences between groups ($p < 0.014$).

TABLE 1

PERCENTAGES OF MALE AND FEMALE IN SELECTED CHICKS ON THE BASIS OF THEIR MAZE-ESCAPE PERFORMANCE

Groups	Male (%)	Female (%)
(A) H-P	51.75 ± 9.6	48.25 ± 8.9
(B) M-P	51.20 ± 8.7	48.80 ± 8.3
(C) L-P	48.75 ± 8.2	51.25 ± 8.6

Each percentage of male and female chicks is the mean ± SEM of values obtained from four experiments (30 animals were used in each experiment). Two-way ANOVA on the percentages of male and female chicks in each selected group do not show a significant effect either for maze-escape selection or for gender.

Statistics

Results are expressed as the mean ± SEM. Experimental data were analyzed by two-way ANOVA followed by post hoc comparisons (Newman-Keuls test), or one-way ANOVA followed by Tukey test comparison of means. A p-value ≤ 0.05 was considered to represent a significant difference between groups.

RESULTS

Maze-Learning Performance

The percentages of selected L-P, M-P, and H-P chicks after the test were, respectively, 24%, 59%, and 17% on a total of 280 birds. The learning degree is expressed by the escape time improvement and was, respectively, 64%, 46% and 24% (Fig. 1). One-way ANOVA shows a significant main effect of escape time improvement, $F(2, 28) = 25.23$, $p < 0.001$. Tukey test (HSD) pairwise comparisons of means shows significant differences between groups ($p < 0.014$).

Percentages of Male and Female Chicks in Selected Birds on the Basis of Maze-Escape Performance

Two-way ANOVA on the percentages of male and female chicks in each selected group (Table 1) do not show a significant effect either for maze-escape selection, $F(2, 18) = 0.000$, or for gender, $F(1, 18) = 0.077$.

Effect of Swimming Stress on Specific [^{3}H]FNZ-Binding in Forebrain of Previously Selected Chicks

Two-way ANOVA on the B_{max} shows (Table 2) a significant interaction between swimming stress effects and maze-learning selection factors, $F(2, 26) = 3.390$, $p < 0.05$. That is, the three chicks subpopulations significantly differ with relation to the influence of swimming stress. Newman-Keuls test shows that the H-P B_{max} is 41% higher ($p < 0.01$) in stressed chicks (928 fmol/mg proteins), than in controls chicks (657 fmol/mg proteins). Furthermore, the M-P B_{max} is 28% higher ($p < 0.01$) in stressed chicks (851 mol/mg proteins) than in control chicks (666 fmol/mg proteins), while the L-P B_{max} is 17% higher ($p < 0.05$) in stressed chicks (772 fmol/mg proteins) than in control chicks (657 fmol/mg proteins). In addition, the B_{max} in stressed H-P chicks is higher than in stressed L-P chicks ($p < 0.01$). On the other hand, significant differences in B_{max} are not observed between nonstressed groups, H-P, M-P, and L-P. At all cases, the receptor affinity remains unchanged.

Effect of Swimming Stress on Specific [^{3}H]Ro 5-4864 Binding in Forebrain of Previously Selected Chicks

Two-way ANOVA on the B_{max} shows (Table 3) a significant interaction between swimming stress effects and maze-learning selection factors, $F(2, 36) = 3.353$, $p < 0.05$. That is, the three chick subpopulations significantly differ with relation to the influence of swimming stress. Newman-Keuls test shows that the H-P B_{max} is 40% higher ($p < 0.01$) in stressed chicks (806 fmol/mg proteins), than in controls chicks (575 fmol/mg proteins). Furthermore, the M-P B_{max} is 25% higher ($p < 0.05$) in stressed chicks (735 fmol/mg proteins) than in control chicks (590 fmol/mg proteins), while the L-P B_{max} is only the 9% higher (nonsignificant) in stressed chicks (629 fmol/mg proteins) than in control chicks (578 fmol/mg proteins). In addition, the B_{max} in stressed H-P chicks is higher than in stressed L-P chicks ($p < 0.01$). On the other hand, significant differences in B_{max} are not observed between nonstressed groups, H-P, M-P, and L-P. At all cases, the receptor affinity remains unchanged.

DISCUSSION

Two-day-old chicks were selected on their second-escape performance in a one-trial, maze-learning task, and termed H-P, M-P, and L-P chicks. The learning degree was expressed by the escape time improvement and was respectively the 64%, 46%, and 24% (Fig. 1). The selected chick groups were of both sexes, and because it is known that male and female chicks exhibit differences in spatial and attentional processes (1), it is possible that there was some segregation of sexes during the maze training. However, the percentages of male and female chicks did not show differences between the selected groups (Table 1). So, the sex variable is equally distrib-

TABLE 2

EFFECT OF SWIMMING STRESS ON SPECIFIC [^{3}H]FNZ-BINDING IN FOREBRAIN OF CHICKS SELECTED ON THE BASIS OF THEIR MAZE-ESCAPE PERFORMANCE

Groups	B_{max} (fmol/mg protein)	%	K_d (nM)	%
(A) H-P				
Control	657 ± 49 (4)	100	2.67 ± 0.45	100
Stressed	928 ± 23 (6)*†	141	2.70 ± 0.20	101
(B) M-P				
Control	666 ± 22 (5)	100	2.49 ± 0.26	100
Stressed	851 ± 30 (7)*	128	2.34 ± 0.27	94
(C) L-P				
Control	657 ± 32 (4)	100	2.67 ± 0.28	100
Stressed	772 ± 22 (6)‡	117	2.37 ± 0.16	89

Each value of B_{max} or K_d is the mean ± SEM of values obtained by computer aided nonlinear regression analysis in 15-day-old chicks. The number of binding assays is indicated in parentheses (two animals were used in each assay). Two-way ANOVA of B_{max} reveals a significant interaction between stress effects and maze-learning selection factors, $p < 0.05$. *$p < 0.01$ as compared to B_{max} in control H-P and M-P chicks; ‡$p < 0.05$ as compared to B_{max} in control L-P chicks; †$p < 0.01$ as compared to B_{max} in stressed L-P chicks (Newman-Keuls test).

TABLE 3

EFFECT OF SWIMMING STRESS ON SPECIFIC [^{3}H]Ro 54864 BINDING IN FOREBRAIN OF CHICKS SELECTED ON THE BASIS OF THEIR MAZE-ESCAPE PERFORMANCE

Groups	β_{max} (fmol/mg protein)	%	k_d (nM)	%
(A) H-P				
Control	575 + 27 (7)	100	7.80 ± 1.54	100
Stressed	806 ± 37 (7)*†	140	8.47 ± 1.04	109
(B) M-P				
Control	590 ± 29 (7)	100	8.20 ± 1.46	100
Stressed	735 ± 31 (7)‡	125	7.52 ± 1.05	92
(C) L-P				
Control	578 ± 43 (7)	100	7.12 ± 1.27	100
Stressed	629 ± 47 (7)	109	7.62 ± 1.44	107

Each value of β_{max} or K_d is the mean ± SEM of values obtained by computer aided nonlinear regression analysis in 15-day-old chicks. Number of binding assays is indicated in parentheses (two animals were used in each assay). Two-way ANOVA of B_{max} reveals a significant interaction between stress effects and maze-learning selection factors, $p < 0.05$. *$p <$ 0.01 as compared to B_{max} in control H-P chicks; ‡$p < 0.05$ as compared to B_{max} in control M-P chicks; †$p < 0.01$ as compared to B_{max} in stressed L-P chicks (Newman-Keuls test).

uted throughout the three experimental groups and could not contribute to any particular group.

Then, the three selected groups were maintained in standard conditions to reach 15 days of age and then submitted to acute swimming stress and [^{3}H]FNZ and [^{3}H]Ro 5-4864 bindings, respectively, for CBR and PBR, were performed on forebrain membranes of chicks. B_{max} increase for both radioligands was significantly higher in H-P as compared to L-P chicks after swimming (Tables 2 and 3).

In the maze-learning task, it is posible that the social reward motivated the birds to find their way out of the box. It is possible also that individual differences in reactivity to stress affect the learning performance of chicks. The results suggest that higher performance chicks are more susceptible than lower performance chicks to acute stress associated to increase of both CBR (Table 2) and PBR (Table 3), probably due to differences in the degree of endogenous emotionality. In addition, their escape time improvement is directly correlated with the second-escape performance (Fig. 1). These results appear opposite to the results obtained with imprinting performance (13), in which less-imprinted chicks are more susceptible than more-imprinted chicks to swimming stress.

On the other hand, it has been reported that the [^{3}H]FNZ receptor number in the cerebral cortex was higher in nonanxious rats that exhibited high exploratory activity in the elevated plus maze than in anxious rats that exhibited low exploratory activity (12). Nevertheless, our results showed that the [^{3}H]FNZ receptor B_{max} in control chicks did not differ in H-P, M-P, and L-P chicks (Table 2). In addition, the [^{3}H]Ro 5-4864 receptor B_{max} in control chicks did not differ in H-P, M-P, and L-P chicks (Table 3). These results indicated that escape speed performance did not select chick subpopulations with differences in the basal RCB and RPB density.

In conclusion, our results indicating that the degree of escape speed and escape time improvement are directly related to the degree of CBR and PBR increase associated to acute stress suggest that chicks with a higher grade of learning are more susceptible to acute stress than chicks with a lower grade of learning in this maze-learning test.

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