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Role of Polyamines in Experience-Dependent Brain Plasticity

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Received 21 August 1986

FERCHMIN, P. A. AND V. A. ETEROVIĆ. *Role of polyamines in experience-dependent brain plasticity*. PHARMACOL BIOCHEM BEHAV **26**(2) 341–349, 1987.—Enriched experience increases brain growth, neuronal differentiation and learning abilities. Polyamines are modulators of growth and differentiation. We studied the effect of difluoromethylornithine (DFMO, an inhibitor of putrescine synthesis) on brain growth of rats exposed either to a complex or an impoverished environment. In both environmental conditions, DFMO decreased cortical putrescine by 50% and increased spermine by 13%; spermidine remained constant. Cortical RNA was not affected significantly by DFMO but DNA was decreased exclusively in rats exposed to the impoverished environment. Environmental complexity increased cortical weight, RNA and spermidine content. These differences were larger in DFMO-injected rats than in saline controls. Since stimulants such as amphetamines also enhance the environmental effects it was conceivable that DFMO might act as a stimulant. We have measured the effect of DFMO on rats' exploratory activity and found it decreased by the drug. Therefore the enhancing effect of DFMO cannot be explained by its behavioral activity. We propose that DFMO enhances the experience-dependent brain plasticity by facilitating differentiation of neurons.

Brain polyamines Experience Brain plasticity Difluoromethylornithine Rat brain Brain RNA and DNA

BRAIN has two modes of growth: the ontogenic development and a second component which depends on the subject's experience caused by interaction with the environment [11,29]. This last process is mainly differentiation, in the sense that the number of neurons remains constant but their dendritic trees become larger and the synaptic contacts more differentiated [14, 35, 37]. The experience-dependent growth takes place at any age although it is faster in younger animals [10]. Experience-dependent brain growth is accompanied by improvement in learning abilities [30].

In the present work we address the role of polyamines in experience-dependent brain growth. Polyamines are small molecular weight aliphatic amines, ubiquitous in all cells. They are crucial modulators of growth and development [26,27]. A great advance in the study of polyamines was made possible when specific inhibitors of polyamine synthesis became available. Alpha-difluoromethylornithine (DFMO) is the most frequently used inhibitor of polyamine synthesis. It inhibits specifically ornithine decarboxylase (ODC), the first and rate limiting enzyme of polyamine biosynthesis [22]. ODC synthesizes putrescine, the simplest polyamine, which is the precursor of the other two: spermidine and spermine.

Despite great advances in the field the mechanism of action of polyamines is not completely understood yet [26,27]. However, there is a steadily increasing body of data which suggests that, among other effects, polyamines might either act as or modulate the action of second messengers. Cytosolic Ca^{++} concentration appears to be regulated by spermine in liver [24] and heart contractility might be regulated by polyamines due to their role in the regulation of Ca^{++} fluxes [2]. Polyamines could mediate the action of insulin [20] and stimulate the phosphorylation of phosphatidylinositol [36]; both processes probably involve regulation of Ca^{++} fluxes. Koenig and collaborators have reported that the effect of testosterone requires a transient increase in ODC activity; the effect is blocked by DFMO and restored by exogenous putrescine [18]. The release of GABA and norepinephrine from cortical synaptosomes was also reported to require a transient increase in ODC activity [15]. These results, however, were only partially replicated by others [6]. The possibility that transient ODC stimulations play a role in brain seems plausible because in brain there is high content of antizyme-ODC complex which could represent a pool of rapidly activable ODC [19].

The role of polyamines in the ontogenic brain development was extensively studied using DFMO and other inhibitors by the group of Slotkin [1, 33, 34]. There are no studies on the effect of inhibitors of polyamine biosynthesis on the experience-dependent brain growth. As mentioned before this type of growth is better described as differentiation rather than proliferation. Proliferation requires active synthesis of polyamines and is usually accompanied by high levels of putrescine [21]. On the other hand, a decreased putrescine level produced by administration by DFMO induces differentiation in embryonal teratogenic carcinomas

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[31] and melanomas [17]. Although this finding has not been generalized yet to normal tissue, an attractive hypothesis presents itself: that in certain cells the inhibition of putrescine synthesis leads to an enhanced degree of differentiation. In agreement with this hypothesis, in the present work DFMO did not impair the experience induced brain changes.

EXPERIMENT I

The effect of inhibition of polyamine synthesis by DFMO was studied in rats exposed to either environmental complexity (EC) or impoverished condition (IC). EC and IC rats injected with saline were run simultaneously to determine the effect of DFMO and to account for the previously described effect of injections [8]. The effect of experience provided by the interaction with EC was measured as the difference between littermates exposed to EC and those kept in IC. The following brain measures were studied: brain weights and the content of RNA and DNA in cortex.

METHOD

Subjects

The animals were seventy-six male Tryon S_1 gray rats, originally from the Department of Psychology at Berkeley, bred in our colony.

The selection of subjects and standardization of preweaning conditions is critical for this experiemnt in which the duration of exposure to EC is only seven hours a day for four days and the animals are stressed by two daily injections. Litters were selected within five days after birth. The size of the litters was reduced to six pups of which at least four were males. To habituate the animals to injections all pups received two daily subcutaneous injections of saline during the third and fourth week of life. By the end of the fourth week the rats were ear-marked. At the age of 30±3 days the animals were weaned and assigned at random to one of the four experimental conditions described later. For the purpose of statistical analysis these four experimental subjects from one litter constituted one block. Usually four such blocks were run simultaneously, that is eight EC rats (four "saline" and four "DFMO") and eight IC rats (four saline and four DFMO).

Behavioral and Pharmacologic Treatment

All animals were housed in metal cages 12×20×13 cm with solid walls. The rats assigned to IC remained in these cages for the length of the environmental treatment, being removed only for weighing and injecting as described below. The littermates assigned to EC were removed from the individual cages and exposed together to large cages with objects for seven hours a day. During the first day the EC rats were housed in a medium size cage (45 W×45 D×30 H cm) furnished with objects but without any other rats with previous experience in EC. The remaining days during the morning, EC rats were housed in a wide EC cage (110 W×41 D×75 H cm) and at noon were moved to a tall EC cage (50 W×50 D×120 H cm). Both cages had shelves, wooden ladders, open cans and similar objects that stimulated interaction with the environment. Additionally, in those cages there were eight rats of similar age but with previous experience in EC, called here "teachers." The teachers facilitated the interaction of experimental rats with EC.

There were four experimental conditions:

(1) D-EC: rats kept in EC cages for four days, seven hours a day, from 9 a.m. to 4 p.m. They were injected with 200 mg/kg of DFMO before and after each daily exposure to EC. The remaining 17 hours of the day they were housed in individual cages.

(2) D-IC: rats kept in IC during four days, injected with DFMO in the same manner and approximately at the same time as the former group.

(3) S-EC: same treatment as D-EC but injected with saline instead of DFMO.

(4) S-IC: same as D-IC but injected with saline.

All injections were subcutaneous in the back of the rat. The isotonic DFMO solution was prepared by neutralizing 1 g of DFMO·HCl with NaOH and the osmolarity was adjusted with saline solution; the final volume was 50 ml.

The animals were sacrificed in the morning of the fifth day, approximately 17 hours after the last injection.

Quantitative Brain Dissections

Brains were dissected into occipital cortex, remaining cortex, subcortex and cerebellum plus medulla, following the procedure described in [4] as modified in [10]. Immediately after dissection brain sections were wrapped in aluminum foil, weighed, frozen in liquid nitrogen or over dry ice and stored at −80°C. The person doing the dissections did not know the experimental condition to which each animal belonged.

RNA and DNA Determination

The method of Morimoto *et al.* [23] was used because it overcomes interferences due to substances particularly abundant in brain.

Statistical Analysis

Those data that were normally distributed and homoscedastic (with homogeneous variances) were analyzed by a two-way analysis of variance for blocked data; the main factors were environment and drug. Where the interacting factor was significant, ANOVA was followed by the Neuman-Keuls multiple comparison test. Values for nucleic acid concentrations and ratios were not normally distributed. These data were analyzed by Friedman's non parametric ANOVA for blocked data, followed by Friedman's multiple comparison test. All tests mentioned here are described in [38].

RESULTS

The results are shown in Table 1. Brain weight was increased significantly by EC, $F(1,55)=8.453$, $p<0.005$, but not by DFMO. The EC vs. IC brain weight difference was more than double in the DFMO EC vs. IC pairs than in the saline injected pairs, but the environment by drug interaction was not significant. The weight of remaining cortex did not show a significant effect of environment or drug but did show a significant environment by drug interaction, $F(1,55)=5.988$, $p<0.02$. This significant interaction was the result of a relatively large environmental effect in DFMO injected EC vs. IC pairs. Cortical RNA content showed a

TABLE 1

DFMO AND ENVIRONMENT AFFECT THE METABOLISM OF NUCLEIC ACIDS IN CEREBRAL CORTEX

	Mean ± s.e.m.			
	DFMO	Saline	% Dif. DFMO vs. Saline	p—Drug
Total Brain				
Weight (mg)				
EC	1300.2 ± 13.0	1283.3 ± 11.4	1.3	n.s.
IC	1270.1 ± 13.1	1270.4 ± 9.0	0.0	
% Dif. EC vs. IC	2.4	1.0		
p—Environment	0.005			
Remaining Cortex				
Weight (mg)				
EC	610.6 ± 6.1	596.7 ± 7.6	2.4	n.s.
IC	591.7 ± 7.4	601.8 ± 7.1	−1.8	
% Dif. EC vs. IC	3.4	−0.8		
p—Environment	n.s.			
p—Environ. × Drug 0.02				
RNA Content (μg)				
EC	1138 ± 29	1117 ± 22	1.9	n.s.
IC	1084 ± 21	1076 ± 23	0.7	
% Dif. EC vs. IC	5.0	3.8		
p—Environment	0.0005			
DNA Content (μg)				
EC	539 ± 13	529 ± 13	1.9	n.s.
IC	511 ± 14	543 ± 15	−5.9	
% Dif. EC vs. IC	5.5	−2.6		
p—Environment	n.s.			
p—Environ. × Drug 0.005				
RNA Concentration (μg/g)				
EC	1852 ± 45	1877 ± 31	−1.3	
IC	1840 ± 32	1794 ± 36	2.6	
% Dif. EC vs. IC	0.7	4.6		
p—Friedman 0.0001				
DNA Concentration (μg/g)				
EC	876 ± 20	887 ± 19	−0.9	
IC	866 ± 19	902 ± 20	−3.9	
% Dif. EC vs. IC	1.2	−1.6		
p—Friedman 0.011				
RNA/DNA Ratio				
EC	2.128 ± 0.058	2.127 ± 0.046	0.1	
IC	2.142 ± 0.060	2.007 ± 0.061	6.7	
% Dif. EC vs. IC	−0.7	6.0		
p—Friedman 0.0005				
Body				
Final Weight (g)				
EC	70.1 ± 2.0	74.6 ± 1.5	−6.0	n.s.
IC	69.6 ± 2.2	69.1 ± 2.3	1.3	
% Dif. EC vs. IC	0.1	8.0		
p—Environment	0.01			
p—Environ. × Drug 0.025				

The table shows the levels of nucleic acids in the "remaining cortex," that is total brain cortex minus occipital cortex. There were 19 rats per experimental conditions for weights and 18 for nucleic acids (one block was lost during analysis). Unless indicated otherwise, significance levels were obtained by a two-way analysis of variance for blocked data. In those cases where a significant interaction was observed, ANOVA was followed by a Newman-Keuls multiple comparison test. If two groups were significantly different ($p<0.05$) by the Newman-Keuls test, the percent difference between these groups was underlined.

The values for the RNA and DNA concentration and the RNA to DNA ratios were not normally distributed, and for this reason they were analyzed by the non-parametric Friedman's analysis of variance, followed by Friedman's multiple comparison test.

significant effect of environment, $F(1,55)=14.500, p<0.0005$. Administration of DFMO did not impair the EC vs. IC difference in brain and cortical weights or RNA content. DNA content showed a significant environment by drug interaction, $F(1,55)=8.653$, $p<0.005$. D-IC and S-IC groups were significantly different by Newman-Keuls test, as well as the D-EC and D-IC groups. The results suggest that DFMO reduces cell number in IC, but not EC rats. Like for DNA content, DNA concentration was also decreased by DFMO in IC rats (Friedman's statistics=11.38; $p<0.011$). This measure was not affected significantly by the environment.

The cortical RNA to DNA ratio, a measure of average RNA per cell, was affected both by environment and DFMO (Friedman's statistics=19.2; $p=0.0005$). DFMO has increased the RNA to DNA ratio in IC rats because it decreased the DNA but not the RNA content (compare D-IC vs. S-IC). In other words, in DFMO-injected rats the exposure to EC has increased both RNA and DNA content (compare D-EC vs. D-IC). Therefore, the RNA to DNA ratio remained relatively constant in both DFMO injected groups. The saline group instead followed the usual [29] pattern of RNA increase and slight DNA decrease by exposure to EC (compare S-EC vs. S-IC). Consequently, in saline injected EC rats RNA to DNA ratio was significantly higher than in saline injected IC littermates.

Final body weight was slightly but significantly higher in S-EC than in other groups. However, D-EC vs. D-IC difference in brain and cortical weight were larger than the S-EC vs. S-IC differences, indicating no obvious correlation between final body weight and brain weights in this group of animals.

Occipital cortex weight and RNA content were not affected significantly by the environment or DFMO. DNA content and concentration were reduced by DFMO in IC rats (data not shown).

DISCUSSION

It is clear from this experiment that DFMO does not impair changes induced by experience. Cortical weight and RNA content were both increased by EC in spite of the inhibitor of putrescine synthesis. RNA concentration seems to behave differently, since this measure displayed only a very small increase by EC in the DFMO group (0.7%). However, we must remember that the increase in RNA concentration by EC is transient: it disappears after about four days of environmental treatment [12]. It is possible that this process is accelerated in the presence of DFMO, and that this is the reason for the small difference in RNA concentration between DFMO EC and IC rats.

The combined effect of impoverished environment and DFMO decreased cortical DNA. DFMO has either arrested glial multiplication or decreased brain cell survival, but only in cortices of young rats deprived of experience. It has been reported previously that DFMO can decrease cell survival [25] while the administration of polyamines seems to have the opposite effect [13]. In these studies the environment was not particularly enriched, thus the rats were more similar to our IC than EC condition.

Stress caused by injections usually interferes with the effect of short exposures to EC [8]. In the present experiment we exposed the subjects to EC for only 7 hours per day for four days coupled with two daily injections. To obtain significant EC brain effect we habituated our subjects to injections during two weeks previous to the beginning of the actual experiment. Even with the habituation, the EC vs. IC differences in saline groups were smaller and more variable than in previous studies where noninjected rats were used.

EXPERIMENT 2

In this experiment we determined the effectiveness of DFMO in lowering the levels of polyamines in cortices of EC and IC rats. Only a limited decrease in putrescine content could be expected due to the low putrescine content in brains of 30-day-old rats and the difficulty to completely deplete putrescine in intact animals. The duration of the treatment was three instead of four days, because we assumed that changes in the concentrations of polyamines should precede those of brain weight, or RNA and DNA content.

METHOD

A total of 32 male Tryon S_1 rats were used. Subject selection, experimental design and the behavioral and pharmacologic treatment were as in experiment 1, except that the treatment lasted for three instead of four days.

Polyamine Determination

The method is based on the procedure of Redmond and Tseng [28] as adapted for biological samples by Tyms (personal communication). The brain cortices, about 0.6 g each, were homogenized in 7 ml of ice cold 5% trichloroacetic acid (TCA) and left in ice for 15 min. The homogenates were centrifuged at 45000×g, at 4°C for 20 min. Four 1 ml aliquots of the clear supernatants were transferred to glass screw cap tubes. The excess of TCA was extracted with 2 ml of ethyl ether. One ml of 2 N NaOH was added to the aqueous phase, followed by 5 μl of benzoyl chloride added with shaking until the oily drops vanished. After 30 min at room temperature the derivatized polyamines were extracted twice with 1 ml of ethyl ether. The pooled ether extractions were dried with nitrogen in vials suitable for the automatic injector of the Waters QA-1 analyzer. Air cannot be used to dry the samples because it produces a bulky white precipitate which interferes with subsequent steps. The dry samples were dissolved in 2.5 ml of 40% acetonitrile in water. The separation and quantification of the benzoylated polyamines was done using the above mentioned Waters QA-1 chromatographic analyzer with a 254 μm detector, Waters 740 integrator and Altex Ultrasphere C_{18} 4.5×150 mm column. The conditions of the run were: 40% acetonitrile, flow rate 1 ml/min, total volume 15 ml and pressure of 800 psi. Typically the retention times for putrescine, spermidine and spermine were respectively, 3.9, 6.8 and 11.2 min. All determinations were done in quadruplicates and appropriate standards were run simultaneously.

Statistical Analysis

All data were normally distributed and homoscedastic. They were analyzed by a two-way analysis of variance for blocked data, one factor being the environment and the other, drug.

RESULTS

Table 2 shows the results. As in experiment 1, total brain

TABLE 2

CORTICAL POLYAMINES CHANGE IN RESPONSE TO ENVIRONMENT AND DFMO

	Mean ± s.e.m. DFMO	Saline	% Dif. DFMO vs. Saline	p—Drug
Total Brain				
Weight (mg)				
EC	1365.3 ± 11.2	1332.2 ± 15.2	2.5	n.s.
IC	1321.8 ± 17.0	1320.8 ± 11.4	0.1	
% Dif. EC vs. IC	3.3	0.9		
p—Environment	0.017			
Remaining Cortex				
Weight (mg)				
EC	653.1 ± 8.1	644.1 ± 8.8	1.4	0.013
IC	623.7 ± 11.0	602.7 ± 9.1	3.5	
% Dif. EC vs. IC	4.7	6.9		
p—Environment	0.0001			
Putrescine Content (nmoles)				
EC	3.5 ± 0.4	6.5 ± 0.5	−46.4	0.0001
IC	3.3 ± 0.3	6.8 ± 0.4	−50.8	
% Dif. EC vs. IC	4.7	−4.0		
p—Environment	n.s.			
Spermidine Content (nmoles)				
EC	176.7 ± 12.6	160.2 ± 13.0	10.3	n.s.
IC	156.9 ± 14.9	153.8 ± 16.5	2.1	
% Dif. EC vs. IC	12.6	4.2		
p—Environment	0.05			
Spermine Content (nmoles)				
EC	182.8 ± 25.3	159.3 ± 23.7	14.7	0.019
IC	171.3 ± 26.4	153.1 ± 24.3	11.9	
% Dif. EC vs. IC	6.7	4.1		
p—Environment	n.s.			
Body				
Final Weight (g)				
EC	81.3 ± 2.2	82.0 ± 3.0	−0.9	n.s.
IC	77.9 ± 3.3	82.1 ± 3.5	−5.2	
% Dif. EC vs. IC	4.4	−0.2		
p—Environment	n.s.			

The polyamines levels shown are those for the remaining cortex. There were seven values in each experimental condition. One block was lost during chemical analysis. Significance levels were obtained by a two-way analysis of variance for blocked data. There were no significant environment × drug interactions.

weight was increased significantly by EC, $F(1,18)=6.967$, $p<0.017$, and the EC vs. IC difference was larger in DFMO rats than in saline groups although not significantly so, $F(1,18)=2.38$, $p<0.14$. The weight of remaining cortex increased by the EC treatment, $F(1,18)=42.01$, $p<0.0001$, and by DFMO, $F(1,18)=7.516$, $p<0.02$. Although differing in some aspects these results are similar to those from experiment 1 in the sense that DFMO injected EC rats had the largest cortical weight of all four experimental groups. Clearly DFMO did not inhibit the EC mediated cortical weight increase.

At the dose used here DFMO halved the putrescine content, $F(1,18)=162.8$, $p<0.0001$. Both EC and IC groups showed a similar effect of DFMO on putrescine content. Spermidine content was significantly increased by EC treatment, $F(1,18)=6.680$, $p<0.05$, but was not affected by DFMO injections. Spermine content was significantly increased by DFMO, $F(1,18)=6.680$, $p<0.019$. This paradoxical spermine increase after DFMO administration has been observed before [33,34]. Data for polyamine concentrations (nmoles/g weight) are not shown in Table 2 to avoid redundancy. DFMO lowered putrescine concentration by 50%, $F(1,18)=170.3$, $p<0.0001$, and increased spermine concentration by 11%, $F(1,18)=4.674$, $p<0.044$. Environment did not affect significantly the concentration of any polyamine.

The four experimental groups did not differ significantly in final body weights.

DISCUSSION

This experiment shows that DFMO was effective in de-

TABLE 3

LEVEL-CROSSING AS A MEASURE OF ACTIVITY IN ENRICHED ENVIRONMENT

Days of Exposure to EC	Level-Crossing Scores d,l-Amphetamine	Saline	% Difference
17	38	10	
20	52	10	
22	65	2	
24	65	13	
27	98	4	
29	85	25	
	Mean ± S.D.		
	67 ± 22	11 ± 8	509%

Sprague-Dawley male rats were exposed to EC for one hour a day, from 30 to 60 days of age. They received a daily subcutaneous injection of 2 mg/kg d,l-amphetamine or saline solution. Level-crossing was measured as described in the Method section of experiment 3.

creasing the levels of putrescine to about 50% of saline controls. In thirty-day-old rats it is probably not possible to achieve a deeper depletion of brain putrescine by administration of DFMO. Putrescine levels are very low at this age and any further decrease slows down its catabolism and increases the synthesis of ODC [16, 33, 34]. Spermidine content was not significantly affected by the drug because the levels of polyamines are well regulated by compensatory mechanisms which maintain their levels despite inhibition of putrescine synthesis [27, 32, 33]. Spermine content was significantly increased in DFMO injected animals due probably to increased activity of the biosynthetic enzymes and decreased catabolism [32,33].

Cortical spermidine content was significantly increased by EC treatment probably reflecting increased RNA content and enhanced protein synthesis. The increase in spermidine content produced by exposure to EC suggests that spermidine, but not putrescine, is necessary for the experience-dependent brain plasticity.

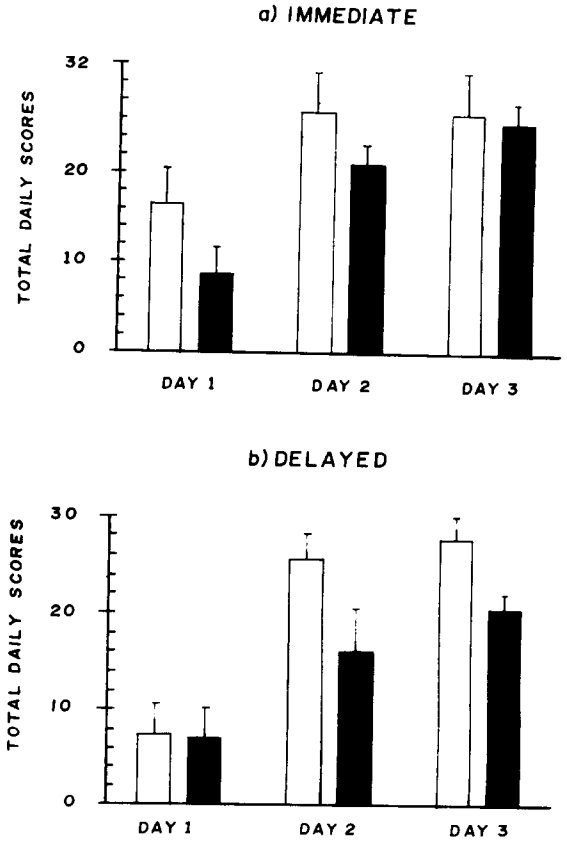

FIG. 1. Effect of DFMO on exploratory activity. Solid bars correspond to drug injected animals, open bars to saline controls. "Scores" represent level-crossings as described in the Method section (Experiment 3). a: DFMO (200 mg/kg each injection) was injected immediately before and immediately after the behavioral session. b: DFMO, at the same dose, was injected three hours before the session and immediately after. Significant differences are discussed in the Results section—Experiment 3.

EXPERIMENT 3

Since several brain measures showed greater responsiveness in the presence of DFMO, this experiment was done to determine whether DFMO was acting as a stimulant. It is known that stimulants such as amphetamines increase the interaction with the environment and by doing so increase the effect of exposure to EC [5]. A stimulatory activity of DFMO could compensate for a possible impairment of growth due to inhibition of polamine synthesis.

In this experiment we measured the effect of DFMO on rats' interaction with the environment. The conditions of the test used were chosen to be similar to the actual EC conditions used in experiments 1 and 2. That is, four rats were exposed to the tall EC cage together, they received injections of either saline or DFMO, and we studied the cumulative effect of daily experience and injections for four days. As measure of interaction the crossing from one level of the tall EC cage to another was chosen. The crossing from level to level is an unequivocal event easy to observe in the intricate setting of the EC cage.

METHOD

There were two variants of this experiment, in each of them DFMO injected rats were compared with saline injected rats. In the first variant (Experiment 3a), the injections of either DFMO (200 mg/kg) or saline were given immediately before and after the testing. In the other experiment (Experiment 3b), the first injection was given four hours before the testing and the second immediately after the test. Injections were given during the four days of treatment.

Subjects and Experimental Design

The preweaning treatment of the subjects did not include habituation to injections, otherwise it was like in experiments 1 and 2. At 30±3 days of age four male rats from each of two litters were assigned, by splitting the litters, to either DFMO or saline injected groups. Each experiment was replicated four times. A total of 32 rats was used for each of Experiment 3a and b, 16 per experimental condition.

Behavioral Procedure

The first day in the morning the animals were housed in the wide EC cage. At noon each group of four rats, assigned to the same injection mode, were housed in plastic cages 30×45×25 cm. During the afternoon each group of four rats was exposed to the tall EC cage for 30 min. After the 30 min period each group was returned to the plastic cage until all groups were treated so. After that all the animals were returned to the wide EC cage. At 4:30 p.m. all rats were moved to the tall EC cage where they spent the night. No observations were done the first day during which the rats were simply allowed to habituate to injections and to the EC cages.

This routine was repeated for four days. During the last three days the rats were observed and scored by two independent observers. These observations were done during the first 15 min of exposure to the tall EC cage. This cage has four levels communicated by wooden ladders that allow animals to cross from level to level through openings in the floors of the levels. A score was recorded whenever any of the four rats stuck its head and both front paws into one of the openings. The score was recorded even if the rat withdrew afterwards.

This test was first applied to two groups of rats injected subcutaneously either with 2 mg/kg of d,l-amphetamine or with saline solution. Both groups were exposed to EC for 30 days, one hour a day. Behavioral observations were performed three times a week, for the last two weeks of environmental treatment.

Statistical Analysis

Total daily scores for each day and for each drug condition were normally distributed and homoscedastic. These data were analyzed by two-way ANOVA for blocked data, one factor being the drug and the other, days of testing.

RESULTS

We have developed a behavioral test for activity in the context of a group of rats living in the EC cage. When applied to rats injected with d,l-amphetamine, this test detected a clearcut increase in activity over saline controls (Table 3). Therefore, the test seems appropriate to measure the level of activity in the enriched environment.

Figures 1a and b show the cumulative scores for each of the three testing days for every experimental group. DFMO decreased significantly the level-crossing scores when the injections were given four hours before testing, Experiment 3b: $F(1,15)=10.76$, $p<0.005$. DFMO given immediately before the test did not significantly decrease the scores, but the trend of the data suggests a decrease in activity. Experiment 3a: $F(1,15)=3.344$, $p<0.09$.

In both experiments, 3a and b, scores were increasing with days of testing, Experiment 3a: $F(2,15)=10.26$, $p<0.002$, and Experiment 3b: $F(2,15)=33.79$, $p<0.0001$. There was no significant interaction between drug treatment and days of testing. That is, the activity of both, the saline and the DFMO groups, increased similarly with days of testing. Analysis of scores per five-minute period for each day indicated the following (data not shown): during the first day of observation the activity was sustained throughout the 15 min period, while on the third day of observation the activity decayed abruptly with time. There was no significant interaction between drug and time.

DISCUSSION

DFMO did not stimulate the interaction with EC either immediately or 4 hours after injection. On the contrary it seems to be a mild depressant. Therefore from the behavioral perspective DFMO could only decrease the EC vs. IC differences, and the observed increases must result from a different mechanism of DFMO action.

We have been observing for many years that rats increase the quality and quantity of interaction with the objects and the spatial arrangements in the EC cages from one day of exposure to another. The level crossing behavior follows this general pattern: Fig. 1 shows that the scores increased from the first day of observation to the third. So, we conclude that level crossing is an appropriate measure of interaction with EC because it follows the observed pattern of behavior.

The fact that level crossing increased from day to day does not mean that there was no habituation to EC. After 2 or 3 exposures to the same EC cage there was habituation within each session, but during the next session the activity is renewed and increased, perhaps because of the experience acquired in previous sessions. We have observed a very similar pattern of activity when studying squirrel monkeys interaction with small objects [9].

GENERAL DISCUSSION

Inhibition of putrescine biosynthesis by DFMO did not abolish the experience-induced brain changes. This rather surprising result illustrates the difference between ontogenic development and experience induced growth. The inhibition of ODC by DFMO impairs ontogenic brain development [1,33]. Slotkin and his group found that the profound disturbances in development produced by DFMO administration take place during a critical period of development specific for each area. For cortex this period is 15–17 gestational days and for cerebellum 1 to 19 days after birth [3]. We minimize and control for any ontogeny related effect by working with slower growing 30-day-old rats and using saline injected controls of same age. Therefore there is no contradiction between this work and the findings of Slotkin and colleagues.

Contrary to its effect on cell proliferation, DFMO increases differentiation of certain cells in culture [17, 21, 31]. If we consider that experience increases the degree of differentiation of neurons rather than proliferation [14, 35, 37], then our results seem to point in the same direction as the work done in cell culture.

The effect of DFMO on polyamine levels agrees with previous studies provided the age of the animals is considered [16, 33, 34]. The dose of DFMO used by us was effective enough to render approximately a 50% reduction in putrescine content 17 hours after the last injection. The inhibition of putrescine synthesis was probably larger in the first few hours after the rats were removed from EC. Putrescine was the only polyamine to be significantly decreased by DFMO. Spermidine content was not significantly affected by the inhibitor but spermine content was increased. This can be attributed to the exquisite regulation of polyamine biosynthesis [26, 27, 32]. The decreased putrescine content causes

a compensatory increase in the half life of spermidine and spermine and in the activity of the enzymes involved in their biosynthesis. By this mechanism the depletion of spermidine is prevented and the content of spermine is even increased.

The environmental treatment did not affect significantly the content of putrescine. The significant increase in spermidine content in cortices of EC rats probably reflects increased RNA content and enhanced protein biosynthesis. In brain, but not in liver, protein biosynthesis and RNA content are directly proportional [7].

Stimulants increase the effect of exposure to EC by increasing the interaction of the animals with the EC; sedatives have the opposite effect [5]. We have shown in experiment 3 that DFMO decreased the interaction of the rats with the EC. Therefore, we cannot explain the lack of inhibition of the EC effect by DFMO through behavioral compensation of a putative biochemical inhibition. These two effects, the behavioral and the biochemical, are probably mediated by two different mechanisms and the slight behavioral inhibition is overcome by the stimulation of growth.

The role of polamines in experience induced brain plasticity is far from being elucidated by this work. As discussed above, we propose that DFMO facilitates neuronal differentiation induced by experience, but more experiments will have to address this issue.

ACKNOWLEDGEMENTS

This work was funded by MBRS-NIH grant RRO 8159-07A1. We thank Mr. Jose Cotto Aponte for technical assistance and Dr. Anne D. Frame for her able direction of the MBRS program. The following students from Interamerican University participated in this work: Carlos Cintron, Ana Cordoba and Jose R. Fernandez. This work would not be possible without the generous gift of DFMO from Dr. P. P. McCann of Merell Dow Research Institute. Data were analyzed using the Prophet computer system funded by DRR-NIH.

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