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Effect of Proline Administration on Rat Behavior in Aversive and Nonaversive Tasks

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MOREIRA, J. C. F., C. M. D. WANNMACHER, S. M. COSTA AND M. WAJNER. *Effect of proline administration on rat behavior in aversive and nonaversive tasks.* PHARMACOL BIOCHEM BEHAV **32**(4) 885–890, 1989.—Sustained levels of proline comparable to those of human type II hyperprolinemia were achieved in blood and brain of rats by subcutaneous proline administration twice a day from the 6th till the 28th day of life. Control rats were treated with saline in the same volumes. Behavioral studies using aversive and nonaversive tasks were performed one week or one month after treatment. Proline treatment did not affect rats' performance in the inhibitory avoidance task, but reduced significantly habituation in the open field. Our results seem to indicate that early postnatal administration of proline to rats affects habituation to a novel environment. If this happens to be so the present tendency to consider hyperprolinemia as a benign condition should be revised.

Proline Behavior Hyperprolinemia Aversive and nonaversive tasks

HYPERPROLINEMIA results from inherited deficiences of either proline oxidase (type I) or Δ^1-pyrroline-5-carboxilic acid dehydrogenase (type II) activities (15,39). Iminoglycinuria occurs in both disorders, whereas high serum and urinary excretion of Δ^1-pyrroline-5-carboxilic acid (PC) is characteristic of type II hyperprolinemia (18, 22, 34). Although an apparent association of hyperprolinemia with certain clinical features (nephropathy, deafness and photogenic epilepsy in type I, and seizures, mental retardation and EEG anomalies in type II) had been previously described in the first families affected by the disorders (2, 19, 33, 36), it has been increasingly demonstrated that various hyperprolinemic individuals are asymptomatic (30,31). In addition, no clinical improvement has been detected in symptomatic patients submitted to low proline diet (33).

On the other hand, experimental studies demonstrated that proline administration affects memory (9, 10, 20). The retrograde amnesia effect has been shown in both chick and mice (11). EEG tracing records showed that chicks injected with amnestic doses of proline do not exhibit seizure spiking or abnormal electrical activity (12).

In 1972 Blake described a strain of mice, Pro/re, deficient in proline oxidase (7). By using these mice as a model for hyperprolinemia, investigators searched for causes of renal failures and other metabolic defects associated with the disorder. However, this model cannot be applied to the study of type II hyperprolinemia, since plasma proline levels are much lower than those encountered in patients affected by this type of hyperprolinemia. The present study aimed to investigate the effects of high concentrations of proline on rat behavior. We have produced sustained levels of proline in blood and brain of young rats, comparable to those of patients affected by type II hyperprolinemia, by injecting subcutaneously the drug twice a day. We have also investigated the effect of early chronic proline administration on the behavior of rats in aversive and nonaversive tasks, one week and one month after treatment, in the hope that this might contribute to a certain extent to clarify the controversy about the association between hyperprolinemia and neurological dysfunction.

METHOD

Wistar rats from our breeding stock were used. Pregnant rats were housed in individual cages and left undisturbed throughout gestation. Twenty-four hours after delivery the litters were culled to eight pups. Half of them were assigned to the experimental condition and the other half served as controls. The rats were weaned at 21 days. All animals had free access to commercial chow and water and were kept on a 12-hour light-dark cycle.

Proline was administered subcutaneously from the sixth till the twenty-eighth day of life. Doses were determined by measuring pharmacokinetic parameters, such as apparent volume of distribution (V_d), plasmatic half-time ($t\frac{1}{2}$) and plasmatic clearance (CL_p)

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TABLE 1

PHARMACOKINETIC PARAMETERS OF PROLINE AND DOSES ADMINISTERED ACCORDING TO AGE OF RATS*

Age (days)	t½ (min)	V_d ($ml \cdot g^{-1}$)	CL_p ($\mu l \cdot min^{-1} \cdot g^{-1}$)	Proline Doses ($\mu mol \cdot g^{-1}$)
6–13	102	0.64	4.4	12.8
14–17	87	0.73	5.7	14.6
18–21	78	0.82	7.4	16.4
22–28	66	0.91	9.0	18.2

*Data expressed as mean of 4 rats.

t½ = plasmatic half-time; V_d = apparent volume of distribution; CL_p = plasmatic clearance.

after drug administration (5). Using these parameters, doses were calculated in order to achieve plasma levels of 1.2–1.4 $mmol \cdot dl^{-1}$, similar to those described in children affected by type II hyperprolinemia (Table 1). Control rats received saline solution in the same volumes. Because of its high plasmatic clearance, proline was administered twice a day with an interval of 10 hours between injections.

Rats were sacrificed either at 7, 14, 21 or 28 days of life by decapitation. Blood was collected on heparin for plasma separation. Brain was rapidly removed. Medulla, pons and olfactory lobes were discarded. The rest of the brain (cerebrum) and cerebellum were weighed. Cerebrum was homogenized in saline. Proline was determined in cerebrum homogenate and in plasma by adaptation of the Summer and Roszel colorimetric method (38).

When saline and proline administration was completed, rats were left undisturbed in their cages for a week. Half of them were submitted to behavioral tasks, and the other half were separated by sex and left undisturbed in their cages up to 60 days of age. Rats from both sexes were used because we observed no sex influence on the studied parameters. The animals were submitted to one aversive task (inhibitory avoidance) and one nonaversive task (open field habituation), all consisting of two sessions (training and test) 24 hours apart.

The inhibitory avoidance task was carried out in a $45 \times 30 \times 30$ cm metal box with a frontal glass wall. The right side of the floor consisted of a grid of bronze bars spaced 1 cm apart and the left side was a $25 \times 6 \times 4$ cm wood platform. The rats were gently held by their body and placed on the platform facing the rear left corner. During training, when rats stepped down on the grid, a shock of 0.2 mA intensity was delivered until they climbed back to the platform. The test session was similar to the training except that animals did not received foot-shock when they stepped down. Training-test step-down latency difference was considered as a measure of retention (memory) and was limited to 270 seconds (21,25).

Rats were submitted to an open field task in a wooden box measuring $60 \times 40 \times 50$ cm with a frontal glass wall. The floor was divided by white lines drawing 12 equal squares. Each session lasted two minutes. The latency on leaving the first square (timidity) and the defecation (number of stools) were considered as measures of rats' emotionality (3,16). The number of crossings from one square to another was indicative of motor activity and the number of orientation responses (rearings) considered as a measure of habituation (41,42). Each parameter was counted in both sessions, training and test. The training-test difference in the number of rearings was considered as a measure of retention (memory).

TABLE 2

PROLINE PLASMA LEVELS OF RATS TREATED CHRONICALLY WITH PROLINE FROM THE SIXTH UNTIL THE TWENTY-EIGHTH DAY OF LIFE*

Age (days)	Time (min) 0	60	180	360
	Saline-Treated Rats			
7	91.52† ±6.13	103.19 ±4.12	101.88 ±11.31	86.95 ±1.68
14	66.17 ±2.71	67.12 ±2.71	78.92 ±8.12	76.22 ±10.48
21	81.23 ±5.23	81.72 5.70	79.86 ±8.12	91.06 ±6.46
28	75.18 ±2.78	82.28 ±4.53	70.46 ±5.13	81.45 ±4.79
	Proline-Treated Rats			
7	109.46 ±9.21	1423.82 ±45.97	693.49 ±47.02	259.97 ±34.64
14	127.94 ±28.98	1438.93 ±50.21	384.07 ±34.40	195.56 ±22.90
21	97.08 ±12.77	1272.06 ±47.12	382.46 ±50.32	192.04 ±21.77
28	88.20 ±7.61	1223.13 ±19.02	358.81 ±60.16	131.10 ±9.36

*Levels expressed as $\mu mol \cdot dl^{-1}$.

†Data expressed as mean ± SEM for 4 rats per group.

Data from behavioral tasks were analysed by a two-way ANOVA with repeated measures (training and test session) and the other factor was drug treatment. For post hoc comparisons, F-test for simple effect was used. To meet the assumptions for an analysis of variance, data of inhibitory avoidance and defecation were subjected to log(x) transformation before analysis (8). Body, cerebrum and cerebellum weight between groups were compared by Student's *t*-test for unpaired samples.

RESULTS

Sixty minutes after injection, plasma levels of proline in rats were similar to those of patients with type II hyperprolinemia, i.e., about ten times higher than normal values (Table 2). Cerebral proline levels in the same rats had a different pattern. In 7-day-old rats, the highest level was observed at 180 minutes after injection. In 14- and 21-day-old rats, the highest levels were observed at 60 minutes after injections and in 28-day-old rats no peak was identified (Table 3).

Sixty minutes after injection, brain/plasma ratios of injected proline were 0.11 in 7-day-old rats, 0.06 in 14- and 21-day-old rats and 0.01 in 28-day-old rats, indicating a low permeability of proline to the blood-brain barrier, more evident in older animals.

Body, cerebrum and cerebellum weight of rats submitted to chronic proline administration did not differ from those of control rats (Table 4).

Table 5 shows the results of the inhibitory avoidance task. The analysis of variance for 35-day-old rats showed a significant main effect of repeated measures, $F(1,42) = 260.55$, $p < 0.001$. Post hoc

TABLE 3

PROLINE BRAIN LEVELS OF RATS TREATED CHRONICALLY WITH PROLINE FROM THE SIXTH UNTIL THE TWENTY-EIGHTH DAY OF LIFE*

Age (Days)	Time (min) 0	60	180	360
	Saline-Treated Rats			
7	67.47† ±14.91	64.04 ±8.94	68.56 ±11.95	74.17 ±10.46
14	42.00 ±1.73	40.78 ±3.15	36.32 ±3.52	44.90 ±4.94
21	55.28 ±5.24	56.53 ±5.71	52.53 ±8.12	42.65 ±6.46
28	43.60 ±4.94	43.24 ±3.18	44.12 ±4.93	44.69 ±4.23
	Proline-Treated Rats			
7	210.92 ±22.72	360.79 ±20.85	477.34 ±40.56	270.79 ±20.75
14	73.18 ±12.77	152.24 ±32.87	114.19 ±14.97	75.91 ±8.64
21	55.28 ±5.24	127.72 ±9.59	87.90 ±11.90	57.09 ±5.87
28	41.43 ±3.75	55.93 ±3.49	49.35 ±2.21	43.69 ±3.77

*Levels expressed as $\mu mol \cdot 100\ g^{-1}$.
†Data expressed as mean ± SEM for 4 rats per group.

comparisons by F-test for simple effect demonstrated that both groups saline, $F(1,42) = 122.16$, $p<0.001$, and proline, $F(1,42) = 139.22$, $p<0.001$, performed well, as was shown by the significant increase in latencies detected in the test session. The analysis of variance of 60-day-old rats indicated a significant main effect of repeated measures, $F(1,54) = 167.45$, $p<0.001$. Post hoc comparisons by F-test for simple effect demonstrated that both groups saline, $F(1,54) = 81.25$, $p<0.001$, and proline, $F(1,54) = 71.84$, $p<0.001$, performed well, as was shown by the significant

TABLE 4

EFFECT OF CHRONIC PROLINE ADMINISTRATION ON BODY, CEREBRUM AND CEREBELLUM WEIGHT OF RATS*

	Treatments Saline	Proline
35-Day-Old		
Body Weight (g)	58.69 ± 1.37	59.64 ± 1.03
Cerebrum Weight (g)	1.02 ± 0.011	1.01 ± 0.014
Cerebellum Weight (g)	0.23 ± 0.004	0.24 ± 0.005
60-Day-Old		
Body Weight (g)	176.30 ± 5.89	170.30 ± 5.25
Cerebrum Weight (g)	1.11 ± 0.009	1.06 ± 0.035
Cerebellum Weight (g)	0.26 ± 0.004	0.26 ± 0.005

Data expressed as mean ± SEM for 24 rats per group.

TABLE 5

EFFECT OF CHRONIC PROLINE ADMINISTRATION ON STEP-DOWN LATENCY OF ADULT RATS SUBMITTED TO INHIBITORY AVOIDANCE SESSIONS USING 0.2 mA FOOTSHOCK INTENSITY*

	35-Day-Old Training	Test	60-Day-Old Training	Test
Saline	2.33 ± 0.14†	4.48 ± 0.28	1.75 ± 0.13†	4.23 ± 0.35
Proline	1.91 ± 0.14†	5.16 ± 0.25	1.49 ± 0.10†	4.44 ± 0.25

*Log (x) transformed data expressed as mean ± SEM, N = 22 per group.
†Statistically different from test values, $p<0.001$.

increase in latencies detected in the test sessions. In addition, no group-by-task interaction was observed between both groups, indicating that proline probably does not interfere with this task.

Table 6 displays the number of rearings in the open field task. The analysis of the number of rearings of 35-day-old rats demonstrated a significant main effect of repeated measures, $F(1,28) = 11.32$, $p<0.005$. Post hoc comparisons by F-test for simple effect showed a significant decrease only in saline-injected rats, $F(1,28) = 7.38$, $p<0.025$, suggesting that proline affects this task.

The analysis of the number of rearings of 60-day-old rats indicated a significant main effect of repeated measures, $F(1,40) = 4.92$, $p<0.005$. Post hoc comparisons by F-test for simple effect showed that the number of rearings decrease significantly only in saline rats, $F(1,40) = 9.86$, $p<0.005$. In addition, group-by-task interaction was significant, $F(1,40) = 4.92$, $p<0.05$, reinforcing the idea that proline affects this task.

The analysis of the motor activity (crossing) of the 35-day-old rats submitted to open field task (Table 7) indicated a significant main effect of repeated measures, $F(1,28) = 5.14$, $p<0.05$, but there was no significant difference between groups (saline and proline). In 60-day-old rats the same analysis showed no significant effect of repeated measures and also no difference between groups (Table 7).

Table 8 shows timidity of rats in the open field task. In 35-day-old rats no differences between groups were encountered, but a significant main effect of repeated measures, $F(1,28) = 23.15$, $p<0.001$, was found. Sixty-day-old rats showed no difference between groups and a significant main effect of repeated measures, $F(1,40) = 176.43$, $p<0.001$.

Data on defecation of rats submitted to the open field task is shown in Table 9. Thirty-five-day-old rats had no significant difference between groups, but presented a significant main effect

TABLE 6

EFFECT OF CHRONIC PROLINE ADMINISTRATION ON RATS' HABITUATION IN THE OPEN FIELD TASK*

	35-Day-Old Training	Test	60-Day-Old Training	Test
Saline	13.94 ± 1.18†	9.14 ± 1.28	22.05 ± 0.79‡	17.90 ± 0.35
Proline	12.13 ± 1.96	8.47 ± 1.79	18.52 ± 1.19	18.52 ± 1.64

*Data representing number of rearings expressed as mean ± SEM, N = 15 per group (35-day-old) and N = 21 per group (60-day-old).
†Statistically different from test values, $p<0.025$.
‡Statistically different from test values, $p<0.005$.

TABLE 7

EFFECT OF CHRONIC PROLINE ADMINISTRATION TO RATS ON MOTOR ACTIVITY IN THE OPEN FIELD TASK*

	35-Day-Old		60-Day-Old	
	Training	Test	Training	Test
Saline	43.67 ± 3.69	35.41 ± 4.29	51.95 ± 1.89	47.05 ± 2.86
Proline	36.93 ± 4.24	30.33 ± 4.46	49.09 ± 2.49	44.52 ± 3.68

*Data representing the number of crossing as mean ± SEM, N = 15 (35-day-old) and N = 21 (60-day-old).

of repeated measures, $F(1,28) = 10.20$, $p<0.005$. Sixty-day-old rats also showed a significant main effect of repeated measures, $F(1,40) = 176.43$, $p<0.001$, and no difference between groups.

DISCUSSION

In this study we produced high sustained levels of proline in blood and brain (cerebrum) of rats similar to those described in type II hyperprolinemia (35). The drug was administered during a period characterized by intense synaptogenesis and where various cerebral structures involved with learning/memory have a rapid development in rats (14, 24, 29). In a previously described model of hyperprolinemia in mice, proline levels were much lower and like those encountered in type I hyperprolinemia (6,13).

It was shown that animals chronically exposed to proline treatment have no differences in physical growth and brain weight when compared with control rats (saline-injected rats). These findings are in accordance with the inherited enzyme defect in man, and enabled us to study behavior without interference of malnutrition, present in various experimental models of inborn errors of metabolism.

Our results on proline pharmacokinetic parameters and on the permeability of blood-brain barrier to proline revealed that they change with age. While the pharmacokinetic parameters examined indicated an increase in proline metabolization and excretion as age advances (28), proline permeability through the blood-brain barrier decreased with age. It was seen that blood-brain barrier of young adult rats (28-day-old rats) is practically impermeable to exogenous proline, which emphasizes the importance of studying the effect of peripherically administered proline on central nervous system (CNS) in early postnatal life.

The present investigation was undertaken to assess the effect of chronic postnatal administration of proline on rats' behavior. The behavioral studies were performed one week and one month after treatment. By doing so, we aimed to investigate a possible effect of proline on certain behavioral tasks and also to test whether this effect is reversible with time. As behavioral tasks performed during or soon after a chronic treatment may be difficult to interpret (1), the behavioral tests were done after at least one week of treatment cessation.

In the open field task, we observed no significant differences in training-test rearing responses of proline-treated animals, in contrast to the control rats where these differences were apparent. Furthermore, the training-test differences themselves differed between groups, demonstrating a group-by-task interaction. These findings were observed even 30 days after treatment, indicating that they are probably permanent. As habituation to a novel environment such as the open field box is usually measured by the decrease in the number of rearing responses in the test session, we presume that proline-injected rats did not present habituation to the open field task. Besides, since a diminution of number of rearings along sessions in the open field task can be interpreted as indicative that the animal learned about the environment (41,42), allied to the fact that in the present experiment proline-treated rats did not habituate, it is assumed that this lack of habituation may reflect a nonessential learning impairment.

We also found that motor activity and emotionality were not affected by proline treatment in the open field task. This facilitated interpretation of habituation results, since an alteration in locomotor activity and in the level of stress caused by proline could otherwise have affected the performance of the rats in orientation responses (rearings). Chronic postnatal proline administration did not affect the overall performance of adult animals in the inhibitory avoidance sessions. The experimental group has performed similarly as the control group in this task. Thus, it is possible that this aversive task is less sensitive to detect fine learning disabilities than nonaversive tasks. Our findings match with the view of other investigators who, working with phenylketonuric rats, concluded that nonessential tasks are more appropriate to identify small learning deficits than essential learning tasks where animals must learn in order to escape or reach immediate biological needs, such as to obtain food or to escape aversive stimulation (37). In this context, it should be mentioned that mentally retarded humans perform equally as nonretardates in tests of classical conditioning, but, by having poor ability to spontaneously acquire information, present much difficulty in tackling a new situation which requires past experience (17,43). It is therefore possible that behavioral studies in experimental models, particularly those using tasks that do not use aversive stimuli, may serve as a screening to detect tenuous learning disabilities in certain pathologies having in common mental retardation.

Our findings are also in agreement with those of other investigators, who demonstrated that cerebral, intraperitoneal and intravenous proline administration causes retrograde amnesia in

TABLE 8

EFFECT OF CHRONIC PROLINE ADMINISTRATION ON RATS' LATENCY IN LEAVING THE FIRST SQUARE ON THE OPEN FIELD TASK*

	35-Day-Old		60-Day-Old	
	Training	Test	Training	Test
Saline	3.43 ± 0.48†	2.16 ± 0.77	3.54 ± 0.39‡	1.53 ± 0.63
Proline	3.66 ± 0.54‡	1.52 ± 0.17	3.53 ± 0.34‡	1.82 ± 0.20

*Data expressed as mean ± SEM, N = 15 (35-day-old) and N = 21 (60-day-old).
†Statistically different from test; $p<0.025$.
‡Statistically different from test; $p<0.001$.

TABLE 9

EFFECT OF CHRONIC PROLINE ADMINISTRATION ON RATS' DEFECATION IN THE OPEN FIELD TASK*

	35-Day-Old		60-Day-Old	
	Training	Test	Training	Test
Saline	0.80 ± 0.37†	0.26 ± 0.12	1.38 ± 0.46	1.24 ± 0.45
Proline	0.86 ± 0.32‡	0.00	1.71 ± 0.15§	0.76 ± 0.36

*Data expressed as mean ± SEM, N = 15 (35-day-old) and N = 21 (60-day-old).
†Statistically different from test, $p<0.05$.
‡Statistically different from test, $p<0.025$.
§Statistically different from test, $p<0.01$.

chicks and mice (4, 9–12). Although so far there is no convincing explanation for this deficit in memory, one alternative could be that high levels of proline in CNS interfere with normal release of glutamate from neuron, affecting secondarily the memory pathway (23, 26, 32, 40). It has been already demonstrated that glutamate release during neuronal activity is involved in the mechanism of memory (27). This amino acid increases sodium permeability of the plasma membrane of dendritic elements, provoking an accelerated uptake of sodium, chloride and water. It is proposed that the resulting diminished length resistance of dendritic spines could enhance the likelihood of a discharge of neurons when synapses situated on such spines are activated, and this would be an important mechanism in memory formation. In this context, L-proline would suppress this effect of glutamate.

In summary, our data seem to indicate that early postnatal administration of proline to rats prejudices habituation to a novel environment. The significance of these findings and their possible association to the human inherited type II hyperprolinemia is far from clear. However, we are tempted to speculate that brain proline levels achieved in this condition may be high enough to cause metabolic alterations in pathways involved with learning/memory and with other neurological functions, which may otherwise not happen in previously used models of hyperprolinemia where tissue proline levels are lower.

If this happens to be so the present tendency to consider both hyperprolinemia disorders as benign conditions should be revised, at least in so far as type II hyperprolinemia is concerned. Indeed, the presence of variants of hyperprolinemia accompanied by neurological impairment has been already postulated (6) and identification of other metabolites with altered concentration in brain of hyperprolinemic rats, including lower levels of glycine, a neurotransmitter, has been demonstrated (4).

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