Pharmacology Biochemistry & Behavior, Vol. 22, pp. 1053–1059, 1985. © Ankho International Inc. Printed in the U.S.A. 0091-3057/85 $3.00 + .00

High Proline Levels in the Brains of Mice as Related to Specific Learning Deficits

CLAUDE F. BAXTER,*§ ROGER A. BALDWIN,* JOEL L. DAVIS‡§ AND JAMES F. FLOOD†§

Neurochemistry, Psychobiology† and Aging and Behavioral Biology Laboratories,‡ V.A. Medical Center Sepulveda, CA 91343*

and Department of Psychiatry and Biobehavioral Sciences,§ UCLA School of Medicine University of California Los Angeles, CA 90024

Received 4 September 1984

BAXTER, C. F., R. A. BALDWIN, J. L. DAVIS AND J. F. FLOOD. *High proline levels in the brains of mice as related to specific learning deficits.* PHARMACOL BIOCHEM BEHAV 22(6) 1053–1059, 1985.—Hyperprolinemic PRO/Re mice have been studied as potential models for hyperprolinemia in man. In addition to high proline levels, some heretofore unreported amino acid abnormalities in the brains of PRO/Re mice are described. The T-maze and shuttlebox learning abilities of PRO/Re mice were compared with those of CD-1 mice having normal proline levels. PRO/Re mice had a significant deficit for T-maze learning, but a significantly greater aptitude for shuttlebox learning when compared to CD-1 mice. By studying the F3 progency of the PRO/Re × CD-1 cross, these strain-specific differences in learning ability for different tasks were shown to be unrelated to the differences in brain proline levels. F3 mice could be subdivided into two distinct groups: those with high proline (HP+) and low proline (HP−) titers. Other amino acids in brain tissues were essentially identical in both groups. A comparison of learning abilities of these HP+ mice with their HP− littermates showed no meaningful differences. However, the slightly slower *rate* at which HP+ mice acquired shuttlebox learning was sufficiently consistent over the 8 day training period so that it became significant. These results do not support the hypothesis that high levels of proline in brain tissues and blood are necessarily accompanied by impaired learning and memory, but are in agreement with those studies of hyperprolinemia in man that suggest no consistent learning deficits in hyperprolinemic subjects. The results seem to validate the suitability of the PRO/Re mouse as a model for hyperprolinemia in man. The data suggest also that the altered amino acid pattern in brains of PRO/Re mice has multiple etiologies. The possibility remains that in PRO/Re mice, the differences in ability to learn the T-maze and shuttlebox are the result of the interaction of high proline levels with some of the other biochemical abnormalities in the brain tissues of this mouse strain.

Proline Hyperprolinemia Aminoaciduria Brain amino acids PRO/Re Mice Learning deficits

HYPERPROLINEMIA and prolinuria can occur in man under a variety of pathological conditions [7, 15, 17] and as an expression of a genetic defect. In the latter category, two types of hyperprolinemia have been identified [1,9]. In type I, it has been shown that the defect resides in the mitochondrial proline oxidase activity [22]. In type II, the defect involves both proline and hydroxyproline in that the dehydrogenase catalyzed conversion of Δ-pyrroline-5-carboxylic acid and Δ-pyrroline-3-hydroxy-5-carboxylic acid is inhibited [1,11]. In addition, hyperprolinemia as an adjunct to other inborn errors of amino acid transport and metabolism has been described [27].

In recent years, the study of hyperprolinemia has been aided by the discovery of a hyperprolinemic strain of mouse, PRO/Re [4], with a metabolic deficiency of proline oxidase activity [5,13] that resembles closely the deficit in the human type I hyperprolinemia. Studies with the mouse model have to date emphasized changes in liver metabolism and renal excretion [5, 6, 13, 16] with little attention given to the consequences of high proline levels upon the central nervous system (CNS).

In the CNS, there are metabolic and physiological interrelationships between proline and other important neuroactive substances (for review, see [2, 14, 21, 31]). Proline has also been related to the loss of learned behavior [8,29]. In man, high proline levels in blood and urine have been correlated with mental retardation by some investigators, particularly in cases of type I hyperprolinemia [9,12]. Other investigators have failed to correlate mental retardation with hyperprolinemia [18]. This inconsistency may reflect genetic heterogeneity, the expression of occasionally linked genes for mental retardation and hyperprolinemia, or it may be due to the purely coincidental coexistence of hyperprolinemia and mental retardation in the same individual. Furthermore, in human studies, the *in vivo* level of proline in CNS tissues is never measured, and all conclusions are based upon blood plasma proline levels. Since the blood-brain barrier towards proline develops early in maturation [20], it is questionable whether unusual proline levels in the blood plasma can accurately reflect proline levels in the CNS tissues.

In order to resolve some of these uncertainties, we have

carried out studies using the mouse strains PRO/Re (hyperprolinemic), CD-1 (non-hyperprolinemic) and the F3 progeny of a PRO/Re × CD-1 cross. Whereas these studies were not designed to reveal any mechanisms by which learning abilites and biochemical anomalies were inherited, they permitted a clear examination of the possible correlation between selected learning abilities and levels of proline in brain tissues of these mice.

METHOD

Subjects

The two strains of mice used (CD-1 and PRO/Re) were obtained from Charles River Breeding Laboratories, Wilmington, MA and Jackson Laboratory, Bar Harbor, ME respectively. One control for the high levels of brain proline in the PRO/Re (pro-1^b/pro-1^b) strain could have been its congenic strain PRO/Re-pro-1^a which has low levels of brain proline. However, the viability of this strain is poor and only 4 male mice became available from Jackson Laboratories over a 6 month period. Thus hybridization of CD-1 and PRO/Re mice offered a more practical means for obtaining a sufficient number of male mice in the same litter to study the effects of high cerebral proline levels upon the ability to learn, while at the same time eliminating some possible nurturing variables. A hybrid was developed by mating a CD-1 female with a PRO/Re male. The F1 generation of this cross was inbred and the F2 progeny was used as the breeding stock for the F3 stock used in the experiments. F1 and F2 breeders were randomly selected (i.e., selection was not based on either proline levels or learning ability). All breeders were experimentally naive.

The F3 mice used for the biochemical and behavioral studies, reported here, were littermate males, 60 to 90 days of age. They were housed in plastic cages with a bedding of pine shavings and were fed Purina 5001 diet and water ad lib. At the conclusion of the behavioral experiments, proline levels in the brains of all mice were measured. The results of the behavioral tests were not made available to those doing the biochemical assays until the proline measurements were completed. Thus, those training the mice did not know which littermates were hyperprolinemic and those performing the biochemical assays did not have information about the learning ability of the mice.

Behavioral Studies

Training procedures. Learning ability was determined by training mice to avoid footshock in two situations: when the T-maze was used, training trials were performed consecutively in one session. When the shuttlebox was used, training consisted of twenty trials per day.

T-Maze footshock avoidance acquisition. The T-maze training procedure has been described in detail previously [10]. The mice were trained to avoid footshock (0.35 ma) in a T-maze constructed of black plastic. The maze consisted of an alley with a start box and guillotine door at one end and two opposed goal boxes at the other end. A shock grid floor ran throughout the maze. At the beginning of any trial, the mouse was placed in the start box. Then, simultaneously, the guillotine door was raised and a buzzer sounded, followed 5 sec later by a continuous scrambled footshock. The goal box which the mouse entered on the first trial was designated "incorrect" and the footshock continued until the mouse entered the opposite goal box. The mouse was removed from this goal box using a plastic liner to avoid unnecessary handling. In all subsequent training trials with this mouse, the latter goal box was designated "correct." The mouse was placed in the start box, the buzzer was sounded as the guillotine door was raised, and a 5-sec non-shock interval was allowed for the mouse to reach its correct goal box and thereby avoid footshock. If the mouse did not reach the correct goal box in 5 sec, it received footshock until it did so. The training continued with a variable 30–60 sec interval between trials until a mouse made 5 avoidances in 6 training trials. Two measures of acquisition were recorded: (a) the number of trials required to achieve a first avoidance response and (b) the number of trials required to reach criterion.

Shuttlebox acquisition. The shuttlebox consisted of a black plastic box with a 10 × 22 cm floor constructed of brass rods through which 0.4 ma of scrambled footshock was administered. The floor space was divided into two equal compartments by a 2.2 cm roller that the mouse crossed to avoid or escape footshock. A miniature lamp was in the ceiling of each compartment. Light onset indicated a "safe" compartment in which the mouse would receive no footshock. At the beginning of each trial, the light was turned on in the compartment that the mouse was not occupying at the time. Simultaneously, a loud, door-bell type buzzer was activated. To avoid footshock and terminate both light and buzzer signals, the mouse had to cross the roller into the "safe" compartment within 5 seconds. Failing to do so resulted in footshock. Light and buzzer were terminated only if the response occurred in less than 5 sec or light, buzzer and footshock were terminated upon successful escape from the footshock. The intertrial intervals were randomized at 30, 45 and 60 seconds. For each mouse the daily training session consisted of 20 trials. Such training sessions were conducted

TABLE 1

A COMPARISON OF SOME FREE AMINO ACID LEVELS IN THE BLOOD PLASMA OF CD-1 AND PRO/Re MALE MICE

Amino Acid	μmol/ml CD-1	PRO/Re
proline	0.35 ± 0.12	3.77 ± 0.47
alanine	0.34 ± 0.06	0.45 ± 0.08
glycine	0.30 ± 0.05	0.41 ± 0.06
valine	0.22 ± 0.01	0.34 ± 0.08
leucine	0.16 ± 0.01	0.26 ± 0.07
threonine	0.15 ± 0.03	0.25 ± 0.07
isoleucine	0.09 ± 0.01	0.15 ± 0.04
phenylalanine	0.07 ± 0.005	0.10 ± 0.02
tyrosine	0.07 ± 0.010	0.11 ± 0.05
tryptophan	0.06 ± 0.006	0.10 ± 0.02
asparagine	0.06 ± 0.004	0.08 ± 0.02
glutamate	0.031 ± 0.007	0.045 ± 0.019
aspartate	0.008 ± 0.001	0.012 ± 0.003
l-methyl-histidine	0.005 ± 0.001	0.010 ± 0.001

Each value is the mean ± S.E. of 5 aminals. Only those amino acids are listed where the difference between CD-1 and PRO/Re appears to be 30% or more.

TABLE 2

SIGNIFICANT DIFFERENCES IN AMINO ACID COMPOSITION OF BRAIN TISSUE FROM YOUNG ADULT CD-1 AND PRO/Re MALE MICE

Amino Acid	μmol/g tissue wet wt. CD-1	PRO/Re	p	% diff.
proline	0.095 ± 0.012	0.67 ± 0.11	<0.001	+701
β-alanine	0.042 ± 0.003	0.058 ± 0.008	<0.001	+38
GABA	2.43 ± 0.11	3.02 ± 0.26	<0.001	+24
phospho-ethanolamine	1.55 ± 0.15	1.81 ± 0.16	<0.025	+17
carnosine	0.16 ± 0.040	0.10 ± 0.014	<0.005	−38
glycine	1.11 ± 0.19	0.89 ± 0.008	<0.001	−20
lysine	0.19 ± 0.007	0.15 ± 0.029	<0.01	−19

The following amino acids were not significantly different in the brains of the two strains of mice: taurine, aspartic acid, threonine, serine, asparagine, glutamic acid, glutamine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ethanolamine, histidine, arginine and homocarnosine. Each value is the mean ± S.E. of 5–7 animals.

TABLE 3

A COMPARISON OF SOME FREE AMINO ACID LEVELS IN BRAINS OF HP+ AND HP− MALE MICE OF THE F3 CROSS

Amino Acid	F3 hybrid μmol/g tissue wet wt. (HP−)	(HP+)	p*
proline	0.11	0.62	<0.001
β-alanine	0.05	0.05	ns
GABA	3.1	3.0	ns
phosphoethanol-amine	1.7	1.8	ns
carnosine	0.15	0.14	ns
glycine	1.4	1.4	ns
lysine	0.21	0.21	ns
taurine	8.2	8.3	ns
aspartic acid	3.7	3.5	ns
threonine	0.39	0.37	ns
serine	0.82	0.75	ns
asparagine	0.11	0.10	ns
glutamic acid	11.3	11.0	ns
glutamine	6.4	5.6	ns
alanine	0.66	0.62	ns
valine	0.10	0.10	ns
methionine	0.05	0.05	ns
isoleucine	0.02	0.02	ns
leucine	0.05	0.05	ns
tyrosine	0.08	0.07	ns
phenylalanine	0.04	0.04	ns
ethanolamine	0.06	0.08	ns
histidine	0.08	0.08	ns
arginine	0.12	0.13	ns
homocarnosine	0.10	0.10	ns

Each value is the mean of 19 to 23 animals.
*Any difference with a random probability greater than 2% is considered insignificant (ns) for these comparisons.

for 6 days in Experimental Series 1 and for 8 days in Experimental Series 2. The measure of acquisition was the number of avoidance responses made per training session and the rate at which such responses increased with each successive day of training.

Biochemical Studies

The source of chemicals used in the biochemical analyses of amino acids are described elsewhere [2,26]. All other reagents used were of analytical reagent grade.

Tissue preparation. Each mouse was anesthetized with methoxyflurane, and an incision made to open the thoracic cavity. The brain was perfused *in situ* with 20 ml of a 0.9% solution of physiological saline containing 4 units /ml of heparin. The solution, at room temperature, was infused through a hypodermic needle placed in the left heart ventricle, extending into the ascending aorta. The blood was permitted to efflux through the incision made in the right atrium of the heart. The operation and perfusion took from 2 to 3 min, after which the mouse was decapitated, the exsanguinated brain excised, hemisected, quick frozen and weighed. One-half of the brain (including pons and medulla) was stored at −70°C for future reference. The other half was homogenized in four volumes (w/v) of 10% trichloracetic acid (TCA). Deproteinized particulate matter was precipitated by centrifugation (12,000 g for 15 min) and the supernatant removed and stored at −70°C until it was analyzed for proline and other amino acids.

Proline analysis. Ten μl of the TCA tissue extract, representing approximately 2 mg of brain tissue, was mixed with 10 μl of distilled water and 10 μl of a 10 μM solution of L-[U^{14}C] proline with a specific activity of 30 mCi/mmol. The TCA was removed from this mixture by extracting it three times with 100 μl of diethyl ether. After each extraction, the sample was centrifuged to obtain well-defined aqueous and organic phases. The organic phase was removed and the remaining aqueous phase used for the analysis of proline. The double-label isotope ratio method is described elsewhere [2].

Amino acid analysis. Fifty μl of the TCA tissue extract (representing approximately 10 mg of brain tissue) plus 5.0 μl of a solution containing 0.53 mM norleucine (as an internal standard) were mixed and adjusted to pH 2.0 with 1.0 N lithium hydroxide. The mixture was centrifuged to precipitate extraneous particulates. Of the clear supernatant, 15 μl were used for the separation of 25 amino acids and amines, using our automated, home-assembled, amino acid analyzer [26]. Briefly, the sample was applied to a Dionex DC4A ion exchange microcolumn, and amino acids were eluted from this column using a programmed five-buffer system (derived from three Pico buffers and two Hi-Phi buffers). During the 5-1/2-hour run, buffers were pumped through the column at a rate of 7 ml/hr. The column pressure started at 550 psi and, as the column temperature was gradually increased from 30°C to 61°C, the pressure dropped to 350 psi. Effluent amino acids were complexed with 0-phthaladehyde at 37°C for 5 min and measured quantitatively with an Aminco

Fluoro Monitor (Model 10-222). Fluorometric readings were automatically integrated using a Spectra Physics minigrator. In addition, fluorometric readings were also recorded on a Sargent-Welch recorder (Model DSRLG) so that any dubious results from the minigrator (due, for example, to a partial overlap of peaks) could be resolved and measured manually. Corrections were applied based upon the recovery of the internal standard.

RESULTS

Chemical Analyses

A tenfold elevation of proline levels in the blood plasma of PRO/Re as compared to CD-1 mice was noted (Table 1). The absolute proline values reported are in close agreement with those published by another research group [4]. Other amino acids also appeared to be higher in the blood of PRO/Re mice. Those for which the average difference between the two strains of mice exceeded 30% are listed in Table 1. However, our sample size and biological variability precludes a clear-cut differentiation except for the elevated levels of tryptophan ($p<0.01$) and 1-methyl-histidine ($p<0.001$) in the blood of PRO/Re mice. Amino acids unlisted in Table 1 were close to equal or were elevated to a lesser extent. A few basic amino acids and amines, notably lysine, 3-methyl-histidine and ethanolamine, appeared to be lower in the blood of PRO/Re mice as compared to CD-1 mice.

Because of the relatively high proline levels in the blood of all mice, the exact measurement of proline in brain required exsanguination of this tissue before analysis. It was found that the proline level of *non*-perfused brains from CD-1 mice was routinely 30 to 50% higher than the level found in perfused brains. The need for perfusion, with the unavoidable time lapse of 2 to 3 minutes before freezing the tissue, was undoubtedly responsible for some post-mortem artifact such as the unusually high GABA levels (Table 2). The proline levels in the brains of PRO/Re male mice were six to seven times greater than in CD-1 mice. In addition, differences were found consistently also in six other amino acids: three were higher and three were lower in the PRO/Re as compared to the CD-1 mouse brains (Table 2). Although the differences for β-alanine, GABA and glycine are very small in comparison to proline, they are consistent and significant ($p<0.001$, t-test).

The mice of F3 stock were of two distinct types: one with abnormally high proline levels (HP+) in brain tissues and the other with normal proline levels (HP−) in brain tissues. No mice with intermediate proline levels in their brains were encountered in this study.

The complete amino acid analyses for brain tissues from F3 stock HP+ and HP− mice are shown in Table 3. Surprisingly, the *only* amino acid that differed significantly was proline. Those amino acids that differed in concentration in the brains of their progenitors, were generally intermediate in the F3 stock. The one notable exception was glycine which was significantly higher in the brains of mice of both HP+ *and* HP− of the F3 stock as compared to the levels found in CD-1 and PRO/Re brains (compare Tables 2 and 3).

Behavioral Analyses

Experimental Series 1: testing learning ability of CD-1 and PRO/Re mice. Since, in humans, hyperprolinemia has been associated by some investigators with impaired learning and memory [8, 9, 29], the purpose of the first set of experiments with T-maze and shuttlebox training was to determine whether PRO/Re mice, with elevated proline in brain tissues, exhibited difficulty in acquiring footshock avoidance response compared to CD-1's, with normal levels of proline in brain tissue.

TABLE 4

CORRELATION OF PROLINE LEVELS IN BRAIN TISSUES OF CD-1 MICE, PRO/Re MICE AND THEIR F3 CROSS WITH ABILITY TO LEARN FOOTSHOCK AVOIDANCE IN A T-MAZE

	CD-1	PRO/Re	F3 Cross (HP−)	F3 Cross (HP+)
Proline				
mol/g tissue	0.10	0.67	0.11	0.65
(±SD)	(0.01)	(0.17)	(0.02)	(0.15)
n/group	9	9	23	15
	$p<0.001$		$p<0.001$	
T-Maze				
acquisition score*	5.31	13.25	6.29	6.75
(±SEM)	(0.02)	(1.03)	(0.48)	(0.72)
n/group	16	16	14	8
	$p<0.001$		$p=$ns	

*The T-maze acquisition score reflects the average number of trials required for mice in each group to make their first avoidance responses. When results were analyzed on the basis of the number of trials required for mice to avoid footshock in five out of six successive trials, a similar result and statistical difference between CD-1 and PRO/Re mice ($p<0.001$) was obtained.

PRO/Re mice took significantly longer to make a first avoidance response as compared to the CD-1 mice for T-maze training (mean trials to first avoidance response 13.2 and 5.3, respectively; $p<0.001$; Table 4). The PRO/Re mice also required more trials to reach the 5 out of 6 avoidance response criterion. On the other hand PRO/Re mice acquired the shuttlebox avoidance habit significantly faster than the CD-1 mice (Fig. 1; 2-way ANOVA with repeated measures and a log transform to correct for covariance of mean and variance, strain × days, strain effect, $F(1,96)=14.13$, $p<0.0005$).

Experimental Series 2: test of learning ability of HP+ and HP− mice of the PRO/Re × CD-1, F3 cross. In Experimental Series 1, the CD-1 mice were controls only as far as the high proline levels in the PRO/Re mice were concerned. They did not control for the other genetic differences between the two strains that might affect learning. To minimize such differences, experiments were carried out using male littermates of the F3 cross. Although litters contained both HP+ and HP− animals, the level of all other amino acids measured in the brain tissue of the F3 stock appeared to be alike (Table 3).

Two separate groups of F3 mice were used, one to determine T-maze acquisition and the other to test shuttlebox acquisition. At the conclusion of each experiment the levels of proline in brain tissues were determined. Only animals with normal levels of proline (HP−) ranging from 0.10 to 0.12 μmol/g brain tissue, or animals with high proline levels (HP+) ranging from 0.40 to 1.00 μmol/g brain tissue were found. The hyperprolinemic mice (HP+) of the F3 stock did

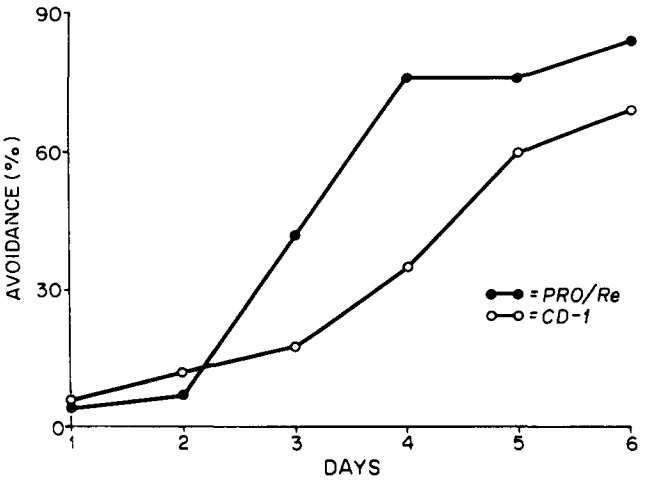

FIG. 1. Strain differences in footshock avoidance acquisition: comparison of PRO/Re (n=9) and CD-1 (n=9) male mice in shuttlebox learning trials. PRO/Re (n=9) and CD-1 (n=9) male mice were given 20 training trials on each of six successive days. The avoidance % score reflects the number of correct footshock avoidances made on each day. The PRO/Re, hyperprolinemic mice were more rapid learners of footshock avoidance in the shuttlebox ($p<0.0005$).

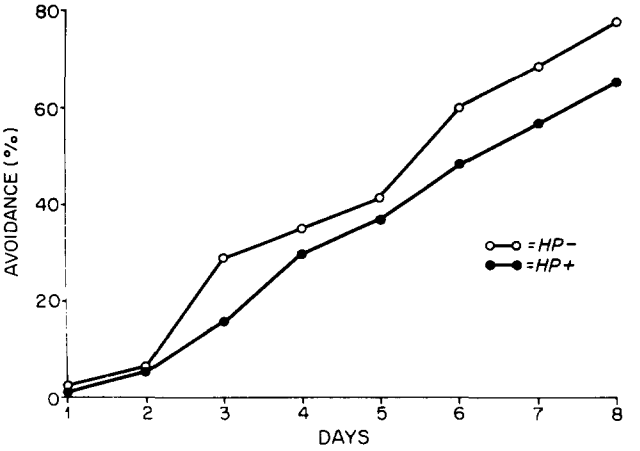

FIG. 2. Littermate differences in shuttlebox footshock avoidance acquisition. The male mice of the F3 generation of a PRO/Re × CD-1 hybrid were given 20 training trials/day for 8 days. After training was completed, mice were assigned to hyperprolinemic (HP+) or non-hyperprolinemic (HP−) groups based on brain proline levels. Whereas HP− mice made, on average, more avoidances on any given day of testing ($p<0.005$ for consistency), the actual numerical difference in the avoidance score between the HP+ and HP− groups was too small to have any statistical significance.

not differ significantly from the non-hyperprolinemic (HP−) littermates in the number of training trials required to make their first avoidance response in the T-maze (Table 4; 6.75 and 6.29 mean trials to first avoidance.)

During shuttlebox training, it became apparent that very few mice of the F3 stock would achieve 70% or more avoidances by the sixth day of training. For this reason, training was extended to eight days. As illustrated in Fig. 2, the HP− mice acquired the shuttlebox avoidance habit a little faster than their HP+ littermates (2-way ANOVA with repeated measures and a log transform to correct for covariance of mean and variance, strain × days; strain, $F(1,176)=5.48$, $p<0.02$). Furthermore, a sign test indicated that on each of the 8 days of training the HP− mice made slightly but consistently more avoidance responses than did HP+ littermates ($p<0.005$ for consistency; Fig. 2). The daily differences in the mean number of avoidances made in 20 trials ranged from 0.14 to 2.6 or a difference of 0.7 to 12.9%. Thus taking into account the variability within each of the two groups, the difference in the shuttlebox performance of the HP− and HP+ mice, on any one day, did not differ statistically.

DISCUSSION

Differences in the Biochemical Composition of Brain Tissue from Different Strains of Mice

The pioneering work of Blake, his coworkers and others [3, 4, 5, 13] characterized the PRO/Re strain of mice as suffering from hyperprolinemia and prolinuria as the result of a single gene autosomal recessive defect [5,13]. The defect has been shown to affect specifically the oxidative metabolism of proline in liver mitochondria [5,13]. Our present data shows that this condition is accompanied by a tenfold increase of proline in the blood and a sevenfold elevation of proline in tissues of the central nervous system. In addition, we have found a generalized elevation of many acidic and neutral amino acids in blood and more selective differences in the level of some nitrogenous compounds in the brains of PRO/Re mice (Tables 1 and 2). Since in our experimental procedures the residual blood in brain tissues was removed by perfusion with physiological saline solution (see the Method section), the elevated levels of proline and other amino acids in brain tissue are not the reflection of residual blood in these tissues. Whereas the blood brain barrier prevents transiently high levels of plasma amino acids from influencing cerebral levels of such amino acids [19], persistently high levels of amino acids in blood plasma can eventually affect the levels of these amino acids in the brain [28].

In PRO/Re mice, hyperprolinemia and hyperaminoacidemia are presumably present at birth and persist thereafter. It is likely, therefore, that the high levels of proline and altered levels of other amino acids in the nervous system are, to some extent, a reflection of the high levels of proline and other amino acids in the blood plasma.

Several lines of evidence suggest that most of the differences in amino acid composition that distinguish brains of PRO/Re mice from those of CD-1 mice (Table 2) *could* be the metabolic consequence of only the high proline levels in the PRO/Re mice: the elevated levels of GABA could be related to an increased metabolism of proline to polyamines [16]. Glutamate-γ-semialdehyde, a compound at a branch point of this pathway, can also be converted to glutamate and thence to GABA in neuronal tissues [31,32]. Similarly, such glutamate could give rise to larger amounts of α-ketoglutarate which, in turn, could react with lysine to form α-aminoadipic acid and thereby lower lysine levels. The metabolism of α-ketoglutarate could favor an increased production of phosphoethanolamine through an increased availability of ATP. The high level of β-alanine in the brains of PRO/Re mice is balanced exactly by an equivalent lower level of carnosine (Table 2). Since these two compounds are interre-

lated through a single enzymatic reaction sequence [30], the altered levels in the brains of PRO/Re mice can be explained by assuming a very slight shift in the equilibrium of this reaction. Finally, it has been suggested on the basis of studies with mice and men that glycinuria [4, 24, 25] and taurinuria [6,23], which often accompany hyperprolinemia and prolinuria, are the results of an overloading of common reabsorptive mechanisms for proline, glycine, taurine and β-alanine in kidney tubules [6].

Yet none of these logical and theoretically satisfying considerations can be invoked to account for the altered amino acid levels in the brains of PRO/Re mice. An overloaded reabsorptive mechanism does not explain why levels of taurine in the brains of these animals are normal, or why the levels of glycine and β-alanine differ from the normal (CD-1) brain in *opposite* directions. Neither can a metabolic hypothesis, based exclusively upon the effects of high proline levels, explain the lack of any change in amino acids (other than proline) in the brain of mice of the HP+ F3 stock (Table 3). The lack of any change other than proline in the brains of these mice suggests that the factors responsible for the changes in amino acid patterns in the brains of PRO/Re mice are independent of the high proline levels. Thus any apparent relationship of proline levels in brain to the behavior of the PRO/Re mouse are complicated by a variety of other biochemical differences that heretofore were unknown or were assumed to be related directly to the altered proline metabolism.

Analysis of Behavioral Differences in CD-1, PRO/Re and the F3 Cross

Neither the results of Experimental Series 1 or 2 support the hypothesis that high levels of brain proline are *necessarily* associated with an impairment of learning and memory. In Experimental Series 1, genetic differences and/or nurturing variables could account for all the differences in T-maze and shuttlebox acquisition. In Experimental Series 2, hyperprolinemic and non-hyperprolinemic littermates did not differ significantly in the acquisition of T-maze footshock avoidance training. Although, on average, the HP+ mice consistently made more avoidances on any given day than the HP− littermates, the difference between the two groups was not statistically significant on a day to day basis.

Our data with hyperprolinemic and non-hyperprolinemic mice appears to resemble that obtained from clinical studies. In hyperprolinemic humans, high proline levels were thought to be responsible for cognitive impairment. However, siblings of those showing cognitive dysfunction have been tested and were found to have hyperprolinemia without apparent learning disabilities [18]. Thus our observations with mice and those reported by some investigators in clinical practice suggest that high proline levels in brain tissues are by themselves insufficient to account for learning or memory deficits. Hyperprolinemia in man is frequently associated with other metabolic disorders. A parallel phenomenon may be reflected by some unusual amino levels (in addition to proline) in brain tissues of PRO/Re mice. It remains possible, therefore, that high proline levels, in conjunction with abnormal levels of other amino acids and amines in the nervous system, might account for learning and memory impairment in some individuals with hyperprolinemia.

ACKNOWLEDGEMENT

We wish to acknowledge the assistance of Sandra Saniga for assistance in training the mice and Sharon Pruess for assistance in running the biochemical assays. The research was supported by the Medical Research Service of the Veterans Administration and by the Sepulveda Geriatric Research, Education and Clinical Center.

REFERENCES

1. Applegarth, D. A., P. Ingram, J. Hingston and D. F. Hardwick. Hyperprolinemia Type II. *Clin Biochem* **7**: 14–28, 1974.
2. Baxter, C. F., R. A. Baldwin, N. Pomara and S. D. Brinkman. Proline in the cerebrospinal fluid of normal subjects and Alzheimer's disease patients as determined with a new double-labeling technique. *Biochem Med* **32**: 189–198, 1984.
3. Blake, R. L. Animal model for hyperprolinemia: Deficiency of mouse proline oxidase activity. *Biochem J* **129**: 987–989, 1972.
4. Blake, R. L. and E. S. Russell. Hyperprolinemia and prolinuria in a new inbred strain of mice PRO/Re. *Science* **176**: 809–811, 1972.
5. Blake, R. L., J. G. Hall and E. S. Russell. Mitochondrial proline dehydrogenase deficiency in hyperprolinemic PRO/Re mice: Genetic and enzymatic analysis. *Biochem Genet* **14**: 739–75, 1976.
6. Blake, R. L., R. V. Grillo and E. S. Russell. Increased taurine excretion in hereditary hyperprolinemia of the mouse. *Life Sci* **14**: 1285–1290, 1974.
7. Cerra, F. B., J. Caprioli, J. H. Siegel, R. R. McMenamy and J. R. Border. Proline metabolism in sepsis, cirrhosis and general surgery. *Ann Surg* **190**: 577–586, 1979.
8. Cherkin, A., M. J. Eckhardt and L. K. Gerbrandt. Memory; Proline induces retrograde amnesia in chicks. *Science* **193**: 242–244, 1976.
9. Efron, M. L. Familial hyperprolinemia. *New Engl J Med* **272**: 1243–1254, 1965.
10. Flood, J. F., G. E. Smith and A. Cherkin. Memory retention: Potentiation of cholinergic drug combinations in mice. *Neurobiol Aging* **4**: 37–43, 1983.
11. Goodman, S. I., J. W. Mace, B. S. Miles, C. C. Teng and S. B. Brown. Defective hydroxyproline metabolism in type II hyperprolinemia. *Biochem Med* **10**: 329–336, 1974.
12. Harries, J. T., A. T. A. T. Piesowicz, S. W. T. Seakins, D. E. M. Francis and S. H. Wolff. Low proline diet in type I hyperprolinaemia. *Arch Dis Child* **46**: 72–81, 1971.
13. Kanwar, Y. S., C. A. Krakower, J. R. Manaligod, P. Justice and P. W. K. Wong. Biochemical, morphological and hybrid studies in hyperprolinemic mice. *Biomedicine* **22**: 209–216, 1975.
14. Keller, E., J. L. Davis, K. H. Tachiki, J. T. Cummins and C. F. Baxter. L-proline inhibition of glutamate release. *J Neurochem* **37**: 1335–1337, 1981.
15. Kershenobich, D., G. Garcia-Tsao, S. A. Saldana and M. Rojkind. Relationship between blood lactic acid and serum proline in alcoholic liver cirrhosis. *Gastroenterology* **80**: 1012–1015, 1981.
16. Manen, C., R. L. Blake and D. H. Russell. Altered polyamine metabolism in the PRO/Re strain of inbred mice. *Biochem J* **158**: 529–533, 1976.
17. Mezey, E. and J. J. Potter. Effect of ethanol on hepatic proline-metabolizing enzymes in the rat. *J Lab Clin Med* **98**: 776–783, 1981.
18. Mollica, F. and L. Pavone. Hyperprolinemia: A disease which does not need treatment? *Acta Paediatr Scand* **65**: 206–208, 1976.
19. Oldendorf, W. H. Brain uptake of radiolabeled amino acids, amines and hexoses after arterial injection. *Am J Physiol* **221**: 1625–1639, 1971.

20. Purdy, J. L. and S. C. Bondy. Blood-brain barrier: selective changes during maturation. *Neuroscience* **1**: 125–129, 1976.
21. Rhoads, D. E., N. A. Peterson and E. Raghupathy. Biochemical evidence of a neuroactive role for proline in mammalian brain. *Fed Proc* **42**: 2009, 1983.
22. Scriver, C. R. and M. L. Efron. Disorders of proline and hydroxyproline metabolism. In: *The Metabolic Basis of Inherited Disease*, edited by J. B. Stanbury, J. B. Wyngaarden and D. S. Frederickson. New York: McGraw-Hill, 1972, p. 351.
23. Scriver, C. R., M. L. Efron and I. A. Schafer. Renal tubular transport of proline, hydroxyproline and glycine in health and in familial hyperprolinemia. *J Clin Invest* **43**: 374–385, 1964.
24. Scriver, C. R., I. A. Schafer and M. L. Efron. New renal tubular aminoacid transport system and new hereditary disorder of amino acid metabolism. *Nature* **192**: 672–674, 1961.
25. Statter, M., A. Ben-Zui, A. Shina, R. Schein and A. Russell. Familial iminoglycinuria with normal intestinal absorption of glycine and amino acids in association with profound mental retardation, a possible "cerebral phenotype." *Helv Paediatr Acta* **31**: 173–182, 1976.
26. Tachiki, K. H. and C. F. Baxter. Role of carbon dioxide fixation, blood aspartate and glutamate in the adaptation of amphibian brain tissues to a hyperosmotic internal environment. *Neurochem Res* **5**: 993–1010, 1980.
27. Teijema, H. L., H. H. van Gelderen, M. A. H. Giesberts and M. S. L. Laurent de Angulo. Dicarboxylic aminoacidurias: An inborn error of glutamate and aspartate transport with metabolic implications, in combination with a hyperprolinemia. *Metabolism* **23**: 115–123, 1974.
28. Toth, E. and A. Lajtha. Elevation of cerebral levels of nonessential amino acids *in vivo* by administration of large doses. *Neurochem Res* **6**: 1309–1317, 1981.
29. Van Harreveld, A. and E. Fifkova. Involvement of glutamate in memory formation. *Brain Res* **81**: 455–467, 1974.
30. White, A., P. Handler, E. L. Smith R. L. Hill and I. R. Lehman. *Principles of Biochemistry*, 6th ed. New York: McGraw Hill Book Co., 1978, p. 707.
31. Yoneda, Y. and E. Roberts. A new synaptosomal biosynthetic pathway of proline from ornithine and its negative feedback inhibition by proline. *Brain Res* **239**: 479–488, 1982.
32. Yoneda, Y., E. Roberts and G. W. Dietz, Jr. A new synaptosomal biosynthetic pathway of glutamate and GABA from ornithine and its negative feedback inhibition by GABA. *J Neurochem* **38**: 1686–1694, 1982.