Reduced Myelinogenesis and Recovery in Hyperphenylalaninemic Rats

Correlation Between Brain Phenylalanine Levels, Characteristic Brain Enzymes for Myelination, and Brain Development

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ABSTRACT

In a previous paper (Burri et al., 1990), we have shown that experimental hyperphenylalaninemia (hyper-Phe) in 3–17 d-old rats leads to reduced myelinogenesis. Such treated rats recover during a 6 w low phenylalanine (Phe) period between days 17 and 59. In order to get more detailed information about the disturbed myelinogenesis and recovery, we measured in hyper-Phe rats the developmental pattern of two brain enzymes typical for myelination, cerebroside sulfotransferase (CST), and 2′, 3′-cyclic nucleotide 3′-phosphohydrolase (CNP), and other developmental parameters. Further, we correlated brain Phe levels with the brain damage in hyper-Phe rats, and we measured brain acetylcholinesterase (AChE) as a neuronal marker.

Experimental hyper-Phe rats, injected between postnatal days 3 and 17 with α-methylphenylalanine and phenylalanine, showed a delayed age-dependent increase of CST activity, compared to that of

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controls. In hyper-Phe rats, CST peak activity was reached 2–4 d later, and was lower than in controls. The age-dependent decrease of the CST activity, however, started in test and control rats at the same time, at day 21. Betwen days 24 and 59, hyper-Phe rats had normal CST activity. CNP activity in hyper-Phe rats was lower than in controls from day 10 to 35, and recovered to normal values between days 35 and 59.

Our results indicate that recovery from reduced myelinogenesis is possible after the period of fast myelination without compensatory increased CST activity. Further, the brain damage in test rats with Phe levels higher than average is more severe than in test rats with Phe levels lower than average; and there is no effect of hyperphenylalaninemia on brain neurons containing AChE.

Index Entries: Hyperphenylalaninemia; phenylalanine; development; reduced myelinogenesis; recovery; cerebroside sulfotransferase; 2′, 3′-cyclic nucleotide 3′-phosphohydrolase; acetylcholinesterase; rat.

INTRODUCTION

To reach pathological brain phenylalanine (Phe) levels in an experimental animal model for the human disease phenylketonuria (PKU), Phe has to be administered in combination with α-methylphenylalanine (α-MP), an inhibitor of the phenylalanine hydroxylase (Greengard et al., 1976; Chester and Johnson, 1978; DelValle et al., 1978; Lane et al., 1980; Huether and Neuhoff, 1981). Plasma Phe concentrations in such rats are comparable to Phe plasma concentrations in untreated PKU patients (Greengard et al., 1976). In a previous paper on the α-MP/Phe model (Burri et al., 1990), we showed that hyper-Phe in rats between postnatal days 3 and 17, during the period of intensive myelinogenesis (Lane et al., 1980; Huether and Neuhoff, 1981; Huether et al., 1983), caused significantly reduced brain myelin content. If the animals are allowed to recover from days 17 to 59, there is a complete recovery of brain myelin, as demonstrated biochemically and immunohistochemically. Age-dependent myelinogenesis correlates directly with the activity of the enzyme cerebroside sulfotransferase (CST), a key enzyme for the synthesis of galactosulfatides, that are typical brain lipids (Farrel and McKhann, 1971; Tennekoon and McKhann, 1978). An age-dependent CST pattern is characterized by a strong increase at the beginning (turning on point), CST peak activity, and a decrease (turning off point) to adult levels. Another important brain enzyme, 2′, 3′-cyclic nucleotide 3′-phosphohydrolase (CNP), that is thought to be a compound of myelin-forming oligodendrocytes, can be correlated with brain myelinogenesis (Figlewicz and Druse, 1980; Trapp et al., 1988; Vogel and Thompson, 1988; Burri et al., 1990; *review:* Sprinkle, 1989). In this study, we measured at 10 different time points the *developmental pattern* of brain CST and CNP activities in hyper-Phe rats, and in rats that had been allowed to recover.

Hyper-Phe disturbs brain myelinogenesis, but how are neurons themselves affected? As an example of a certain population of neurons, acetylcholinesterase (AChE) containing neurons can be studied. During development of the rat brain, the AChE level increases, and a change in the pattern of multiple molecular forms occurs that is characterized by an increase in the ratio G_4/G_1 (Rieger and Vigny, 1976; Wade and Timiras, 1980). These changes, observed during maturation of the brain in vivo also occur during differentiation of neuroblastoma cells in vitro (Vimard et al., 1976; Lazar and Vigny, 1980). Here, we analyzed AChE in hyper-Phe and control rats as a measure for the maturation of AChE containing neurons.

The following questions were investigated:

1. What is the time course of the brain damage induced by hyper-Phe, and especially of the brain recovery after the damage, measured by following the pattern of brain CST and CNP activities and other developmental parameters between postnatal days 3 and 59? Which enzyme activities are necessary to achieve the previously observed recovery of brain myelinogenesis?
2. Is there a correlation between brain Phe levels in hyper-Phe rats and their brain damage?
3. How does hyper-Phe affect the development of AChE containing neurons?

METHODS

Sprague-Dawley rats were housed in a temperature and light controlled room (22°C, 12-h light/dark cycle). Food (NAFAG, Gossau, Switzerland), and water were supplied *ad libitum*. Newborn rats were grouped in litters of eight animals. Two females and two males were chosen as test animals, two females and two males as control animals. Test animals were injected s.c. every day, once between 9 and 10 AM, and once between 3 and 4 PM, with 4.8 μmol/g body wt α-methylphenylalanine and 10.4 μmol/g body wt phenylalanine. Controls received 0.9% saline. The injections were given from postnatal day 3 to 17. For this experiment, we used nine litters of eight rats. At the following time points, all rats of one litter were sacrificed by decapitation between 1 and 2 PM: postnatal day 8, 10, 14, 17, 21, 24, 28, 35, 59. Brains were excised, immediately frozen on dry ice, and stored at −80°C prior to biochemical determinations.

For biochemical determinations, brains were homogenized 1:10 in a buffer containing 50 m*M* NaH_2PO_4, 1 m*M* EDTA, and 1% Triton X-100; pH was adjusted to 7.0. Total proteins (Prot.) were determined by the method of Lowry et al. (1951). For DNA determination, a fluorometric assay was used (Hinegardner, 1971). Aliquots of brain homogenates

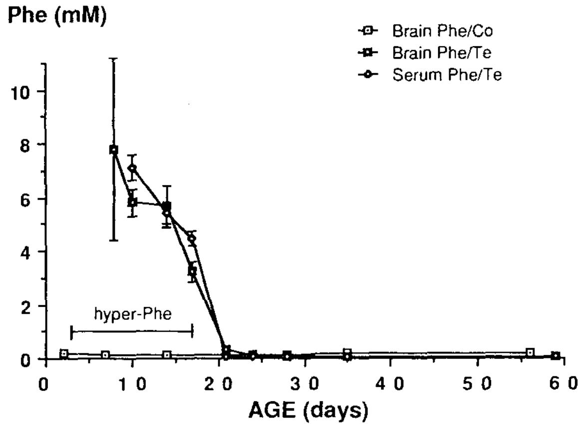

Fig. 1. Brain and serum Phe levels in hyper-Phe rats. Means ± standard deviations. Co = control rats, Te = Phe rats.

were incubated with 3′, 5′-diamino-benzoic acid-dihydrochloride (DABA). The resulting fluorescence was measured at 510 nm, and compared with standards. CNP activity was measured according to a modified method of Sogin (1976), using 2′, 3′-cyclic nicotinamide adenine dinucleotide monophosphate (cNADP) as a substrate. CST activity was determined by using [^{35}S]phosphoadenosine-5-phosphosulfate as a sulfur donor (Siegrist et al., 1976, 1981). Soluble and detergent soluble AChE were extracted by a sequential extraction procedure according to Sorensen et al. (1982). AChE activity was measured according to Ellman et al. (1961). The distribution of the molecular forms of AChE was analyzed by density gradient centrifugation, and the amount of monometric G_1-form and tetrameric G_4-form was calculated from the area under the peaks (1989).

For amino acid analysis, 30–60 mg brain tissue was homogenized in 0.5 *M* perchloric acid containing norvaline (0.08 m*M*) as internal standard. An aliquot was analyzed using automated ion exchange chromatography (Biotronic LC 7000, Munich, FRG.)

Statistical analysis was performed using the two-tailed Student's *t*-test for independent samples and one-way analysis of variances (ANOVA).

RESULTS

Time Course of the Brain Damage and Recovery in Hyper-Phe Rats

Brain Phe concentrations (Fig. 1) in control animals were below 0.2 m*M* during the whole period from days 3 to 59. In the experimental animals, the highest Phe levels were found at day 8 with 7.82 ± 3.41

Table 1
Developmental Parameters of Control and Hyper-Phe Rats

AGE	Body w. (g)	Brain w. (mg)	Prot. (mg/g brain)	DNA (μg/g brain)
8 d Co 8 d Te	20.0 ± 0.9 11.0 ± 2.1 p<0.001	562.4 ± 14.7 410.4 ± 61.9 p<0.001	78.3 ± 8.1 80.3 ± 5.7 n.s.	2.73 ± 0.20 2.26 ± 0.23 p<0.05
10 d Co 10 d Te	26.2 ± 2.4 13.7 ± 1.5 p<0.001	677.1 ± 14.1 443.1 ± 16.3 p<0.001	92.2 ± 7.9 86.0 ± 3.0 n.s.	2.54 ± 0.17 2.28 ± 0.23 n.s.
14 d Co 14 d Te	40.4 ± 1.7 25.0 ± 2.7 p<0.001	875.5 ± 37.2 617.7 ± 42.5 p<0.001	115.0 ± 3.3 111.8 ± 4.3 n.s.	2.21 ± 0.11 2.47 ± 0.12 p<0.05
17 d Co 17 d Te	55.0 ± 1.5 35.6 ± 1.5 p<0.001	983.0 ± 19.3 671.3 ± 18.6 p<0.001	126.3 ± 5.5 117.2 ± 6.7 n.s.	2.05 ± 0.15 2.19 ± 0.18 n.s.
21 d Co 21 d Te	65.5 ± 3.1 36.5 ± 2.4 p<0.001	1015.0 ± 34.0 704.7 ± 28.3 p<0.001	145.3 ± 4.6 150.0 ± 3.7 n.s.	2.08 ± 0.18 2.15 ± 0.11 n.s.
24 d Co 24 d Te	84.0 ± 5.1 44.2 ± 5.4 p<0.001	1046.3 ± 30.0 698.8 ± 54.2 p<0.001	149.4 ± 4.6 146.9 ± 7.1 n.s.	2.55 ± 0.11 2.37 ± 0.15 n.s.
28 d Co 28 d Te	121.0 ± 6.3 86.2 ± 4.1 p<0.001	1120.3 ± 59.8 830.3 ± 10.7 p<0.001	144.4 ± 7.8 152.3 ± 6.9 n.s.	2.25 ± 0.06 2.26 ± 0.13 n.s.
35 d Co 35 d Te	167.7 ± 6.4 126.3 ± 18.0 p<0.05	1153.7 ± 11.2 854.3 ± 61.8 p<0.001	164.4 ± 2.6 164.5 ± 3.2 n.s.	2.33 ± 0.02 2.30 ± 0.11 n.s.
59 d Co 59 d Te	347.0 ± 102.2 270.3 ± 46.9 n.s.	1268.3 ± 89.3 951.8 ± 23.8 p<0.001	157.7 ± 7.9 159.9 ± 11.6 n.s.	2.17 ± 0.11 2.29 ± 0.29 n.s.

Mean ± standard deviation; $n = 3$ or 4; Co: control rats, Te: hyper-Phe rats

m*M*. Phe concentrations dropped to 3.24 ± 0.38 m*M* at day 17, at the end of the phenylalanine hydroxylase inhibition. Seven days later, Phe levels reached control values. The age-dependent decrease of brain Phe levels between days 8 and 17 was significant ($p < 0.05$, one-way ANOVA). Serum Phe concentration in hyper-Phe rats was 7.14 ± 0.46 m*M* on day 10, and 4.50 ± 0.29 m*M* on day 17 at the end of the hyper-Phe period. This age-dependence was also significant ($p < 0.001$). The correlation

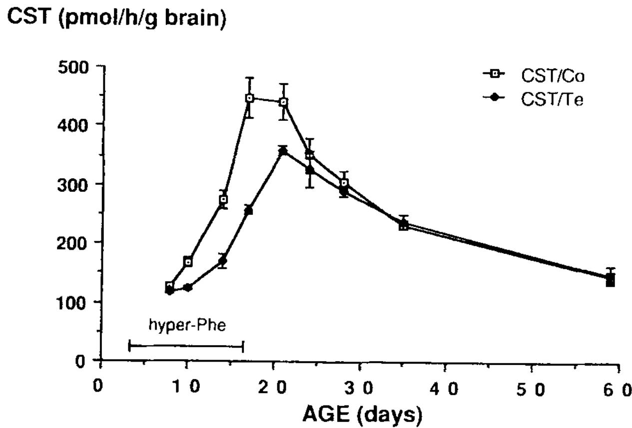

Fig. 2. Brain CST activity in control and hyper-Phe rats. Means ± standard deviations. Co = control rats, Te = hyper-Phe rats.

coefficient between brain and serum Phe levels for days 10, 14, and 17 was 0.82. If day 21 was included, the correlation factor was 0.95.

During hyper-Phe and up to day 35, the body wt (Table 1) of test animals were significantly lower than those of control rats. At the end of the recovery period, at day 59, this difference between test and control rats was not significant. Brain weights of test animals were already reduced at postnatal d 8 (d 5 of hyper-Phe). In control rats, brain growth was the fastest between days 8 and 17, whereas it was much slower during this period in treated animals. After day 17, brain growth in test rats was the same as in normal rats. At day 59, after 6 wk of recovery, brain weights of the test animals were still significantly lower than those of control animals. Brain protein contents were the same for hyper-Phe and control animals at all time points. Protein concentration steadily increased during development, and reached adult levels already at postnatal day 21. At postnatal day 8, DNA levels in test rats were lower, at postnatal day 14, higher than in controls. No other differences were found. DNA content per g brain did not change significantly during development.

Brain CST (Fig. 2) in test and control rats were not different after 5 d of hyper-Phe (postnatal day 8). After day 8, CST activity in control rats increased very rapidly (turning on point: 8 d). CST peak activity was reached at day 17, and lasted till day 21. In test rats, the turning on point CST was at day 10, and CST activity increased at a much slower rate than in control rats. In hyper-Phe animals, CST peak activity was reached at postnatal day 21, 4 d later than in control rats. From day 10 up to day 21, CST activity in test rats was always significantly lower than in controls ($p < 0.001$, day 21: $p < 0.01$). After day 21, during the period of CST activity decrease, the difference between control and test rats got smaller,

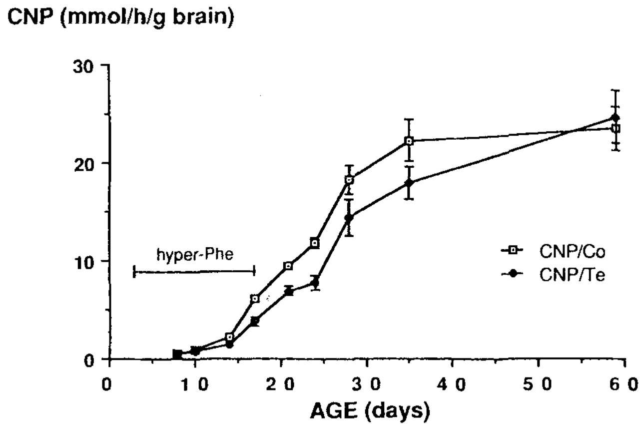

Fig. 3. Brain CNP activity in control and hyper-Phe rats. Means ± standard deviations. Co = control rats, Te = hyper-Phe rats.

and was insignificant in 24-d-old and older rats. For test and control animals, the turning off point of CST activity seemed to be the same, at postnatal day 21.

Brain CNP activity (Fig. 3) of test and control animals was the same at day 8. Between days 10 and 35, test rats had significantly lower CNP values. The confidence levels of significance were: day 10: $p < 0.05$, day 14: $p < 0.01$, day 17–24: $p < 0.001$, days 28 and 35: $p < 0.05$. No difference in CNP activity between test and control rats was found at day 59. The normal developmental curve of CNP showed a steep increase starting at day 8. A plateau was reached at day 35. In test animals, the CNP curve was less steep, and there was still an increase between days 35 and 59. The catchup of CNP activity in treated rats occurred between days 35 and 59.

Correlation Between Brain Phe Levels and Degree of Brain Damage in Hyper-Phe Rats

For the evaluation of the strength of the effect of hyper-Phe, we selected the test groups day 10, 14, 17, 21, and 24. Group day 8 was excluded because these animals had normal CST activity and CNP activity. Group day 24 was included because in this group, although Phe injections were stopped 7 d earlier, there was still a certain variation in brain Phe content measurable. Brain Phe concentration of one animal of this group was still elevated. From day 28 on, all test animals had normal brain Phe levels.

The 18 test animals (Fig. 4) of the five groups selected were divided into animals with brain Phe levels higher than average (each animal compared to the mean of its group, that is 100%), and animals with brain Phe level lower than average. This led to a group Phe > 100% with seven

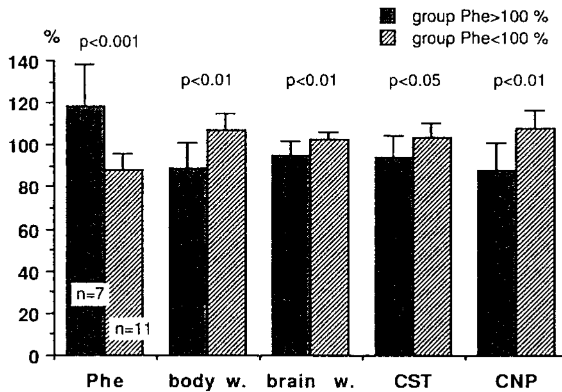

Fig. 4. Hyper-Phe rats: Brain Phe levels and its influence on body weights (body w.), brain weights (brain w.), CST, and CNP.

animals, and a group Phe < 100% with 11 animals. The animals with high Phe levels (Phe > 100%) had significantly lower body wt, brain wt, CST activity, and CNP activity, compared to the animals with low Phe levels (Phe < 100%). The confidence level of significance for body wt, brain wt, and CNP was $p < 0.01$, for CST, $p < 0.05$.

Effect of Hyper-Phe on Brain Neurons Containing AChE

As an indicator for the maturation of neurons containing AChE, the level of soluble and detergent-soluble AChE, and the distribution of molecular forms were analyzed in the anterior part of the right brain half (mainly cortex and striatum) at days 10, 17, 28, and 59. The specific activity of soluble AChE remained nearly constant between days 10 and 59 at about 40 mIU/mg protein, whereas detergent-soluble AChE greatly increased during this time period in hyper-Phe rats from 92 to 192 mIU/mg protein, as well as in control animals from 96 to 202 mIU/mg protein. Furthermore, between days 10 and 59, a four to five fold increase in the ratio of the tetameric G_4-form and monomeric G_1-form of detergent-soluble AChE was observed in both the test animals (from 4.0 to 15.6), and the control animals (from 2.8 to 15.6), whereas a much less pronounced increase in this ratio was seen for soluble AChE (hyper-Phe: from 1.0 to 3.4, controls: from 0.9 to 3.0).

DISCUSSION

In our experiment, all hyper-Phe animals had elevated brain Phe levels. Although the dosage of Phe/α-MP per gram body wt was the same for all test animals, younger rats had higher brain Phe levels than the

older rats. This age-dependent decrease between days 8 and 17 is significant, and there is a correlation with serum Phe levels. Therefore, the uptake of the subcutaneously injected material into the blood must be age-dependent, high in young rats, and low in older rats. Because brain Phe levels depend on serum Phe levels, this may mean that there is no difference in Phe uptake through the blood–brain barrier (BBB) between younger (8, 10-d-old) and older rats (14, 17-d-old). However, Greengard and Brass (1984) and McChesney et al. (1988) have seen a decrease of BBB permeability between 4-d-old and 16-d-old rats. Taking this result into account, together with our results, we can say that changes in serum Phe levels and BBB permeability cause the age-dependent decrease of brain Phe concentration in young hyper-Phe rats.

Body wt and brain wt were severely affected by hyper-Phe. In a previous paper (Burri et al., 1990), we could exclude the possibility that the brain damage measured in our hyper-Phe animal model is just a result of undernutrition. In this former paper, we measured the effects of hyper-Phe at day 17, and the recovery at day 59. At day 17, we found reduced CNP and CST activities, and reduced MBP concentration in hyper-Phe rats. Brain cerebrosides and sulfatides, brain lipids that were enriched in myelin, were also reduced. In recovered, 59-d old rats, all these compounds were normal. We sustained our data with immunohistochemical MBP stainings. In this new paper, the main goal was to get information about the *time course* of the brain damage and recovery in hyper-Phe rats, using exactly the same experimental setup as in Burri et al. (1990).

Brain CST and CNP are clearly affected by hyper-Phe. At day 8, after 5 d of hyper-Phe, these two enzymes are still normal. Between days 8 and 10, CST activity, as a measure of myelinogenesis, stays at about the same level in test rats (turning on point: day 10), whereas it increases greatly in control rats (turning on point: day 8). In hyper-Phe rats, the turning on point of CST is delayed by two days, and CST peak activity is reached about 4 d later than in control rats. The turning off point of CST at day 21, however, is not affected by hyper-Phe. Test rats 24 d-old and older have CST values indistinguishable from those of normal rats. CNP activity in test animals, another indicator for myelinogenesis, is significantly lower from days 10 to 35 compared to controls, but normal at day 59, indicating myelin recovery. During the recovery period from days 17 to 59, we expected to see in test animals compensatory higher CST activities than normal. This is not the case, that means that in test animals, the CST activities after day 17 (lower than normal or normal) are sufficient for a complete biochemical and histochemical recovery of brain myelin. It is of special interest that recovery is possible after CST peak activity, after its age-dependent maximal metabolic activity. However, as seen earlier (Burri et al., 1990), brain wt of formerly hyper-Phe rats were still reduced at the end of the recovery period at day 59. Although we could not detect any biochemical or morphological differences, we can-

not exclude the possibility that the brain function and the behavior of 59-d-old rats allowed to recover were affected.

CGalT (UDP galactose:ceramide galactosyltransferase), another key enzyme for myelination that is involved in the formation of cerebrosides, shows the same developmental pattern as CST (Siegrist et al., 1980; Monge et al., 1988). During the time period of highest CST activity (days 17–21), also forebrain MBP mRNA peaks (Kristensson et al., 1986), corresponding with the marked increase in MBP immunoreactivity in the rat forebrain between days 14 and 21 (Bjelke and Seiger, 1989). [^{3}H]lysine incorporation into myelin proteins is highest between days 17 and 30 (Berger et al., 1980). These points, besides our results (Burri et al., 1990), show the relevance of CST activity measurements to follow brain development. We do not have an explanation for the significant differences in DNA content at days 8 and 14, and cannot exclude that this is caused by experimental variations.

To exclude the possibility that our Phe measurements were affected by diurnal Phe variations in the rats, injection times and time of sacrifice were the same for all animals. Thus, the measured individual variation of brain Phe levels in the test groups at the time of sacrifice probably reflects individual differences during the whole experimental time period. To correlate these brain Phe levels with the degree of the brain damage in hyper-Phe rats, we selected the animals with elevated brain Phe levels and measurable brain damage (groups day 10, 14, 17, 21, 24). To combine the results for these animals of different ages, all data had to be calculated as percentage of the mean of each group for all parameters. Then, it was possible to divide the test animals into a group with Phe $> 100\%$ and a group with Phe $< 100\%$. The comparison of these two groups shows a positive correlation between brain Phe levels and the degree of brain damage. In untreated PKU patients, Partington (1978) could not find a direct correlation between plasma Phe level and brain damage. Therefore, to evaluate a PKU patient's situation, one should be able to measure brain Phe levels in vivo. This should be possible soon with in vivo ^{1}H magnetic resonance spectroscopy.

The facts that brain Phe level determines the degree of brain damage, and that a newborn rat has a more permeable blood brain barrier in regard to Phe than an adult rat (Geengard and Brass, 1980; McChesney et al., 1988), have consequences for pregnant PKU mothers. It seems clear that a very strict Phe-poor diet should be kept during pregnancy, or even before. An uncarefully controlled Phe-restricted diet, that may lead to hyperphenylalaninemia in the unborn infant, may be one cause of the very frequently observed mental retardation of children of PKU mothers (Lewis et al., 1985; Okano et al., 1986; Vorhees and Berry, 1989).

Hyper-Phe affects myelination. The question remains whether the development of the neurons themselves is also influenced. We found an increase in the AChE level, and in the ratio G_4/G_1 in both the hyper-Phe animals and in the control group. Our results thus indicate that there is

no difference between hyper-Phe and control rats with respect to maturation of neurons containing AChE. To study other neurons, e.g., catecholaminergic neurons, would be of interest.

CONCLUSIONS

1. Recovery from reduced brain myelinogenesis in hyper-Phe rats is possible with brain CST activities never exceeding control values;
2. The brain damage in test rats with Phe levels higher than average is more severe than in test rats with Phe levels lower than average; and
3. There is no effect of hyperphenylalaninemia on brain neurons containing AChE.

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