Neurochemical Research, Vol. 9, No. 6, 1984

DEVELOPMENTAL CHANGES OF CEREBRAL PHENYLALANINE UPTAKE FROM SEVERELY ELEVATED BLOOD LEVELS*

OLGA GREENGARD AND CLIFFORD A. BRASS
Department of Pediatrics and Pharmacology
Mount Sinai School of Medicine
New York, New York 10028

Accepted January 5, 1984

Brain phenylalanine concentrations at plasma levels raised to that in phenylketonuric subjects were studied in rats from fetal through postnatal life. Suppression of the hepatic phenylalanine hydroxylase with α methylphenylalanine, and injections of age-adjusted doses of phenylalanine on the next day, assured the persistence of the same elevation of plasma levels for at least four hours prior to assay. The net phenylalanine uptake determined under these conditions underwent several-fold decreases between the fourth day and the end of the suckling period, and by about the age of 30 days it was as low as in adulthood. The development of transport properties studied here could contribute to the change with age in the vulnerability of the brain to the same degree of hyperphenylalaninemia and, since the cerebral phenylalanine uptake may decrease to non-damaging levels during childhood, it is pertinent to defining the age at which the rigorous diet of phenylketonurics might be safely relaxed.

INTRODUCTION

Phenylketonuria has been referred to as a "development disease" because exposure to high plasma phenylalanine levels in the first few years of life alone has been found to lead to mental retardation, and dietary treatment through these years has obviated this effect despite lack of subsequent plasma phenylalanine control (1). The classical explanation of these findings invoked the "critical growth period" of the brain, the concept that

* Dedicated to Henry McIlwain.

0364-3190/84/0600-0837$03.50/0

insults during the time when the brain is growing at its maximal rate and is establishing new connections lead to more severe or qualitatively different damage than that incurred during other stages of life. This theory, consistent with observations on experimental animals exposed to various chemical insults (2), provides a general explanation of the hazard that environmental or genetic aberrations represent to the immature human brain. However, there is another possible explanation, of specific relevance to conditions where the genetic lesion is restricted to the liver and the blood is the primary location of the chemical principle that is potentially harmful to the brain: if in untreated phenylketonurics the brain contained a greater excess of phenylalanine during the period of early infancy than at any other time, then this would be *a* reason for the particular severity of damage that it incurs during that period.

Although there is evidence for maturational changes in the activity of transport systems (see Discussion), little is known about the manifestations of these changes under pathologically high plasma amino acid concentrations. It is difficult to maintain such concentrations in the normal experimental animals for any length of time. In the case of phenylalanine, this difficulty was partly overcome by imitating in animals the genetic lesion of phenylketonurics. There have been extensive studies in recent years (3, 4, 5, 7) on rats rendered hyperphenylalaninemic with the aid of the non-toxic suppressor of phenylalanine hydroxylase, α methylphenylalanine (6); however, age-dependent alterations in cerebral phenylalanine excess had not been investigated. The present study is the first one showing that under conditions of equally severe hyperphenylalaninemia, the younger rat brain is exposed to much higher levels of phenylalanine than that of the more mature animals. This developmental change occurs primarily during the first few weeks of life and thus coincides with the time period during which exposure to experimental phenylketonuria was found to lead to permanent behavioral deficits (7, 8).

EXPERIMENTAL PROCEDURE

The animals used in these studies were CDF Fisher rats purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts. They received food (Purina Rodent Chow) and water ad libitum. Suckling rats were weaned on the 20–21st day of age. In experiments involving the dietary induction of hyperphenylalaninemia in pregnant or weanling rats, the solid diet contained 0.5% α methyl-DL-phenylalanine (Sigma, St. Louis, Missouri) plus the indicated amounts of L-phenylalanine. All other experimental rats received a subcutaneous injection of α methylphenylalanine (2.4/μmol/g body weight) which was followed on the next day by 2 hourly injections of phenylalanine. The doses per g body weight of phenylalanine given in experiments of Figures 1–4 (and doubled for those of Figure

TABLE I

PLASMA AND BRAIN PHENYLALANINE LEVELS OF RATS ON DIETS CONTAINING PHENYLALANINE PLUS α-METHYLPHENYLALANINE

Age (days)	Phenylalanine in Diet	Plasma Phenylalanine nmol/ml	Cerebral Phenylalanine nmol/g	Plasma/Brain Phenylalanine Ratio
Fetal	0	243 ± 62 (6)	292 ± 52 (6)	0.83
Fetal	3	2922 ± 801 (8)	1893 ± 520 (10)	1.54
Fetal	7	3300 ± 383 (3)	3315 ± 1301 (4)	1.15
23	1.5	667 ± 408 (5)	271 ± 106 (5)	2.46
23	3	3332 ± 803 (7)	848 ± 365 (7)	3.93
23	7	2112, 5654	814, 1542	2.59, 3.67
25	3	1400 ± 131 (5)	750 ± 54 (5)	1.81
25	7	4779, 4904	1416, 1257	3.38, 3.90
27	7	4835 ± 427 (3)	615 ± 49 (3)	7.86
29	7	8903, 5223	2163, 1818	4.11, 2.88
30	3	1175 ± 702 (8)	364 ± 182 (10)	3.23
32	7	2268, 4718	1370, 1393	1.65, 3.89
120+	0	96 ± 31 (7)	80 ± 17 (5)	1.20
120+	3	1080 ± 345 (10)	296 ± 87 (7)	3.65
120+	7	1743 ± 1485 (6)	314 ± 66 (3)	5.55

The experimental diets contained 0.5% α methylphenylalanine plus the indicated amounts of phenylalanine. Adult (120+), pregnant rats were placed on these diets on the 12th gestational day; analysis of their tissues and those of their fetuses were carried out between the 17th and last (22nd) days of gestation. The dietary treatment of all other rats began after weaning, on the 20th postnatal day. Values represent means ± SD (with the number of animals in parentheses) or refer to individual animals.

5) were 5.2 μmol for day old rats and were progressively increased with age to a maximum of 15.6 μmol.

All chemical analyses were done on freshly obtained tissues. The whole brain was homogenized in 4 volumes of trichloroacetic acid (0.3 M final concentration) and centrifuged for 20 minutes at 105,000 *g*. Blood, collected in heparinized tubes, was centrifuged and deproteinized in the same manner. Phenylalanine was assayed fluorimetrically by the method of McCaman and Robins (9) as modified by Faulkner (10).

RESULTS

Studies on the recently developed rat model for gestational phenylketonuria (11) provided the first indication of a striking maturational change of cerebral phenylalanine uptake in hyperphenylalaninemic animals. The results in Table I, representing an extension of those studies, show that the hyperphenylalaninemic dams were able to concentrate phenylalanine

into the brain at only 314 nmol/g, whereas their fetuses with only 2–3 times higher levels of plasma phenylalanine accumulated 6–10 times higher brain concentrations (1893–3315 nmol/g brain), resulting in plasma/brain phenylalanine ratios 2–5 times lower than in the adult animals. The development of this difference in the ability to transport and accumulate phenylalanine into the brain was further studied by placing young weanling rats on the experimental diets. The 23–32 day old rats were intermediate between the fetal and adult animals in their ability to accumulate brain phenylalanine. For example, 23 day old rats on a 3% phenylalanine diet had brain phenylalanine levels of only 848 nmol/g brain compared to levels of 3315 nmol/g brain in fetal animals with similar plasma phenylalanine levels. Twenty-five day old rats on the 3% phenylalanine diet had brain phenylalanine concentrations (750 nmol/g) much higher than adult rats on the 7% phenylalanine diet (314 nmol/g) who had even higher plasma phenylalanine levels (Table I).

This dietary approach to the study of the development of phenylalanine transport into the brain is limited in that it could not be applied during the important suckling period of life, nor could it produce equally high levels of plasma phenylalanine in all age groups. With the alternative, injection approach, fluctuations of plasma phenylalanine content presented some difficulty. From the V_{max} of phenylalanine entry into the brain, 30 nmol/min/g (12), it can be estimated that the accumulation of 3000 nmol/g brain would take almost 2 hours. (In actuality this time was even longer, see Figure 1). Thus, determination of maximal cerebral phenylalanine levels attained must be preceded by a prolonged period of constant, high plasma level, which is precluded by the normal organism's effective homeostatic mechanisms. An injection of α methylphenylalanine circumvents this problem to a large extent. Also, the suppression of the hepatic phenylalanine hydroxylase it causes persists for at least 48 hours (6) so that, by studying cerebral phenylalanine uptake on the second day, possible interference of α methylphenylalanine with cerebral transport can be avoided or minimized. However, study of maturational changes in transport still presented a problem. The dosages of phenylalanine which maintained the desired elevation of plasma phenylalanine in four day old rats for 3 or 4 hours, did not do so in older animals. During the normal early postantal increase in activity of the hepatic phenylalanine hydroxylase (13) there is also a small rise in its activity remaining after the injection of α-methylphenylalanine (3), and at puberty additional physiological changes may modify the capacity for phenylalanine clearance. Therefore, at each different age to be studied we had to empirically determine the phenylalanine doses required to maintain approximately the same elevations of plasma phenylalanine.

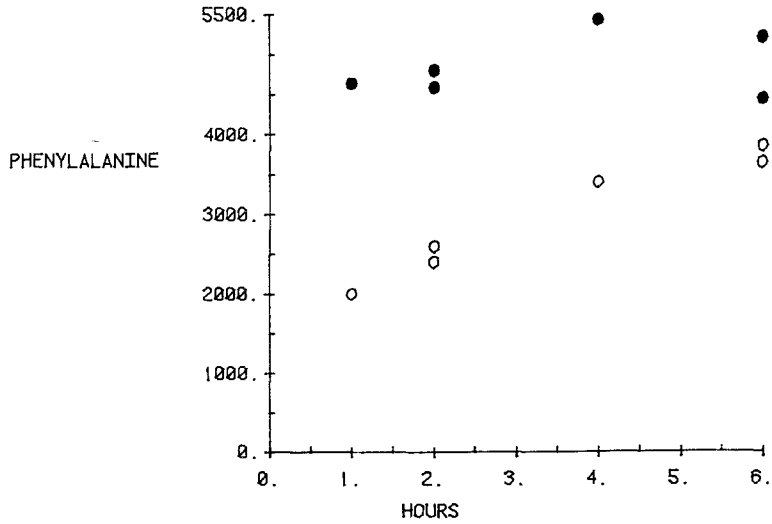

FIG. 1. The time course of elevation of cerebral phenylalanine content in 4 day old rats. Three day old rats were given one injection of α methylphenylalanine and multiple doses of phenylalanine on the next day as described under Method of Procedure. The nmol of phenylalanine (see ordinate) per ml plasma (full circles) and per g brain (open circles) in each 4 day old rat were determined at the indicated hours after the first phenylalanine injection. Each point refers to a single animal; for variability see SD in Figures 4–5.

Figures 1, 2 and 3 display some representative results at three important ages, 4, 16, and 70 days. As seen in Figure 1, the 4 day old experimental rats attained brain phenylalanine levels greater than 3000 nmol/g. In contrast, 16 day old animals with comparable plasma phenylalanine levels had brain phenylalanine concentrations below 3000 nmol/g (Figure 2), and in 70 day old rats these concentrations were even somewhat lower (Figure 3). Thus, a progressive decrease in net phenylalanine transport into the brain was noted with increasing age in animals with plasma phenylalanine levels in the phenylketonuric range.

The developmental increase in the ability to restrict phenylalanine transport into the brain can be seen in more detail in Figure 4. Here, the mean brain phenylalanine levels (plotted as % of the 4 day value) achieved after three hours or more of plasma phenylalanine elevation to 5000–7000 nmol/ml is displayed for the different age groups studied. We see that the most rapid decrease in brain phenylalanine content occurs between days 4 and 16, with a more gradual decrease to adult levels occurring after that.

To further define the changes in the early suckling period (between ages 4 and 16) a similar study was done which includes more age groups and

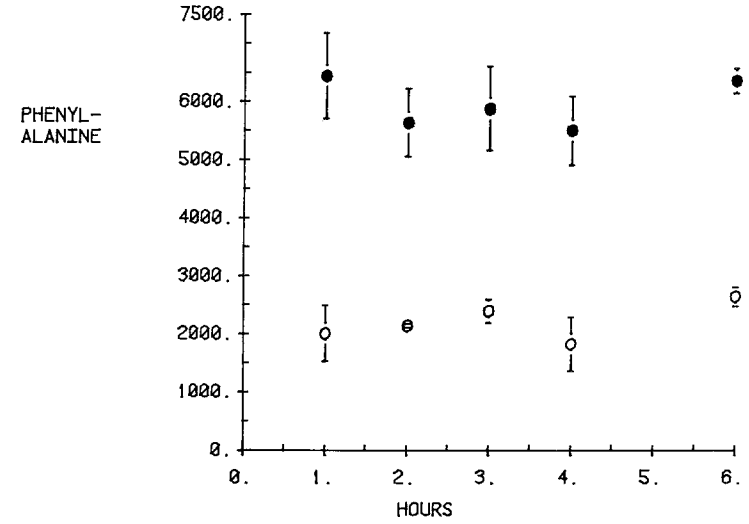

FIG. 2. The cerebral phenylalanine content in 16 day old, hyperphenylalaninemic rats. For experimental details see Figure 1 and Experimental Procedure. The values are means of results on 3–5 rats; bars indicate Standard Deviations. In untreated rats the phenylalanine levels are 95 nmol per ml plasma and 90 nmol per g brain.

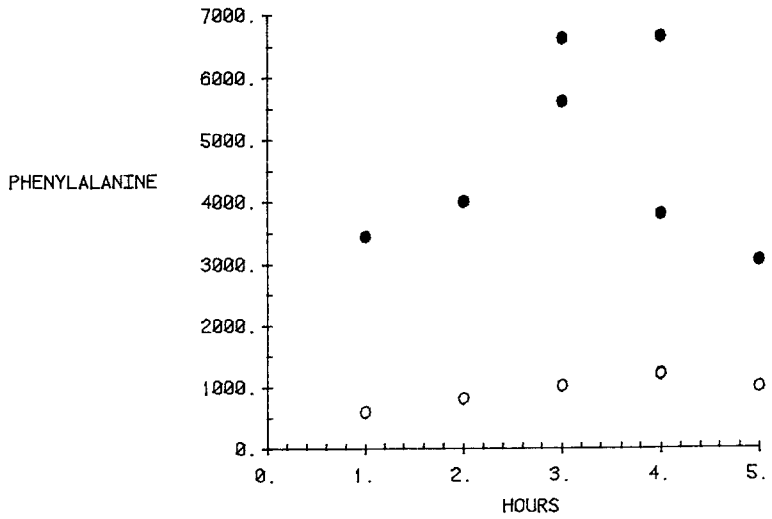

FIG. 3. Cerebral phenylalanine uptake in 70 day old rats. For details see Figure 1.

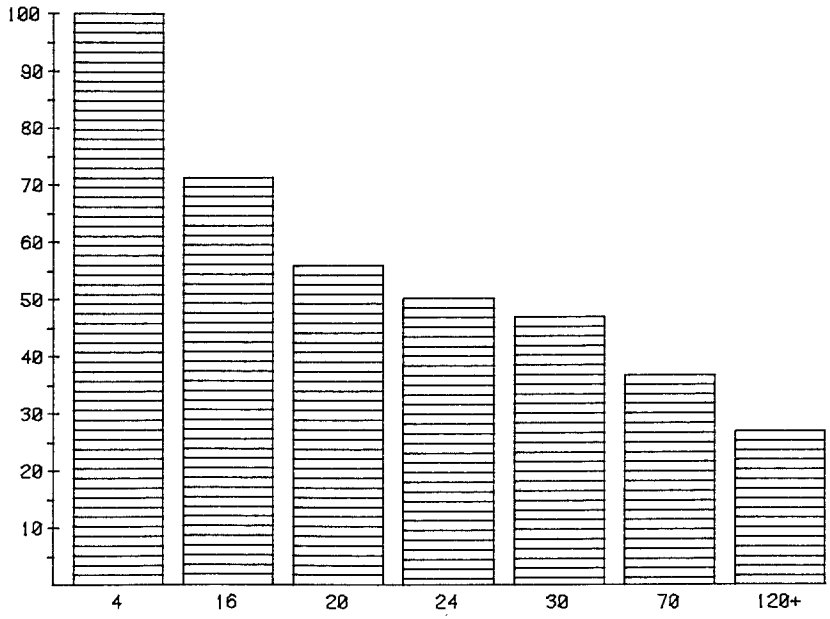

FIG. 4. Developmental decrease in net phenylalanine uptake at plasma levels of 5–7000 nmol per ml plasma. Net phenylalamine uptake (ordinate), i.e., the cerebral concentration after 3 or more hours plasma phenylalanine elevations of 5,000–7000 nmol per ml, was determined in rats on different days of age (abscissa). The results averaged for 5–11 animals are expressed as percent of that in 4 day old rats. The Standard Deviations (in the order of increasing age) were 5.8, 19.4, 4.4, 3.2, 8.3, 4.0 and 2.1, respectively.

where the plasma phenylalanine levels were raised to greater than 10,000 nmol/ml for more than three hours (Figure 5). It was thought that the use of such high levels (in excess of that observed in most phenylketonurics) would accentuate and more accurately define the changes in cerebral uptake capacity occurring during early development. Indeed, while the results in Figure 5 generally confirm those in the previous illustrations, they demonstrate more clearly that within the first 3 postnatal weeks, the net phenylalanine transport to the brain undergoes its most rapid decline.

DISCUSSION

It is well established that all substances that enter the brain, except for small uncharged molecules, must be primarily transported by cerebral capillaries through what is referred to as the "blood brain barrier". The

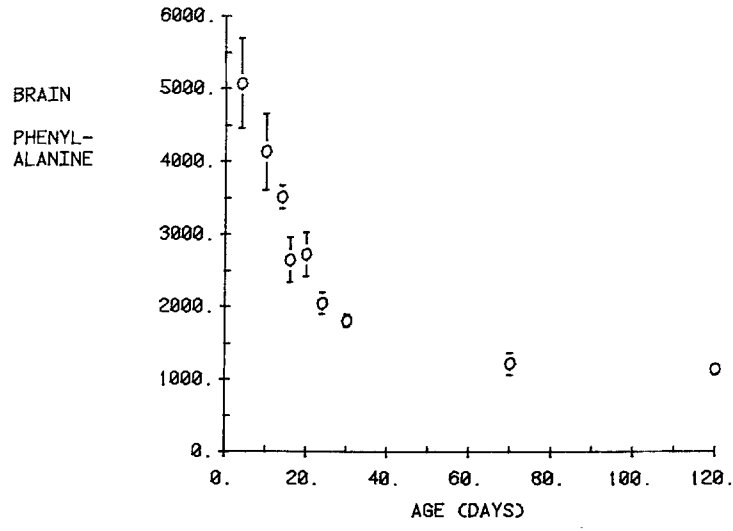

FIG. 5. Developmental decrease in net cerebral phenylalanine uptake at plasma levels of 10–20,000 nmol per ml. The experimental procedure was the same as in Figures 1–4 except that higher amounts of phenylalanine were injected (see Experimental Procedure) so that phenylalanine levels of 10–12,000 nmol per ml prevailed for at least 3 hours prior to brain assay. The nmol of phenylalanine per g brain (ordinate) are means of results on 4–8 animals of the indicated age; bars represent Standard Deviations.

systems responsible for the transport of specific groups of amino acids, including the L-system whereby phenylalanine is transported (12, 14), have been shown to be fully operative shortly after birth (e.g., 15). Therefore, a greater activity of these systems, rather than an incomplete blood brain barrier permitting passive diffusion, is thought to contribute to the higher concentrations of several amino acids in the immature than in the adult brain. Daniel, Banos and Pratt (15) have demonstrated that the increase of cerebral radioactivity upon the intravenous administration of [C^{14}]leucine, valine or tryptophan was faster in suckling than in adult rats. While they did not detect such a difference for two other amino acids transported by the L-system, phenylalanine and tyrosine (15), comparison of neonatal (rather than suckling) and adult animals by Sershen and Lajtha (16) indicated that the cerebral entry of phenylalanine was also diminished in the latter. Such a change, followed for the first time throughout postnatal development, was recently reported for the uptake of another L-system amino acid, tryptophan, into rabbit brain (17). However, these age differences were established under physiological amino acid concentrations. Developmental changes in net amino acid transport at non-phys-

iological levels over a long period of time had not been studied in detail previously.

The present studies demonstrate that the blood brain barrier also limits phenylalanine uptake from pathological plasma levels. The net cerebral transport of phenylalanine decreased with age so that the young rat's brain is exposed to much higher levels of phenylalanine than the mature one's, despite equivalent plasma phenylalanine elevations. The results show a difference, as well as a resemblance, to those by Cornfeld and Oldenburg (17), who followed the cerebral radioactivity during the initial seconds after intracarotid injection of labelled tryptophan to rabbits. In this and other investigations (16, 18) using the same technique, the rate of increase of radioactivity, referred to as the "brain uptake index" (BUI), might reflect not only the influx but also the rate of utilization of the amino acid. Decreased utilization was probably the reason for the rise in BUI which occurred after the 30th day and which had no equivalent in the present observations made at abnormally high phenylalanine levels (Figures 4 and 5). On the other hand, the early postnatal decline in the BUI for tryptophan (17) must have been due to a decrease of the influx rate itself. This decrease was probably also a major factor in the decline we now saw of net phenylalanine uptake at an equivalent age in rats. However, at least two other factors must be considered.

In studies involving short term infusion and physiological amino acid concentrations, transport across cerebral capillaries plays the primary role, whereas uptake over long time periods and at pathologically high concentrations may also involve the less restrictive transport from extracellular spaces into brain cells (19). Thus, age differences in the efficacy of the later system might contribute to the now observed maturational changes in net phenylalanine uptake. Secondly, the steady state concentration of an amino acid under these conditions is determined by both influx and efflux rate. Lajtha and Toth (20) have shown that adult rats will transport leucine out of the brain against a concentration gradient, whereas newborns will not do so. Although phenylalanine is not transported against concentration gradients, the adult brain does have an effective system for its efflux (21). This, coupled with the known pial vessel transport from the cerebrospinal fluid into the blood (22), may thus be another factor contributing to the control of cerebral phenylalanine in older rats. The mechanisms underlying the development of this control remain to be elucidated. The significance of the present results lies in defining the time course and magnitude of change in cerebral phenylalanine concentrations prevailing in the face of persistently high circulatory levels, and in pointing to early infancy as the period when the mammalian brain becomes mature with respect to its ability to restrict the entry of

phenylalanine. By about the 30th day this ability was the same as in adult rats. The cerebral phenylalanine uptake was much higher in the first 3 postnatal weeks (Figures 4 and 5) and this also happened to be the period when exposure of rats to chronic hyperphenylalaninemia was shown to cause irreversible behavior alterations (8).

Critical roles in the pathogenesis of mental retardation and small brain size of phenylketonurics is attributed to the imbalanced amino acid precursor pool for protein synthesis and subnormal cerebral content of the neurotransmitters dopamine, norepinephrine and serotonin, evidenced in untreated subjects of this disease (23, 24). The mechanisms whereby hyperphenylalaninemia can evoke the same chemical alterations in the brain of genetically normal animals has been extensively investigated (4, 5, 7, 25). The interference by excess phenylalanine itself with cerebral protein synthesis and with the activity of cerebral enzymes (n.b. tyrosine hydroxylase, mandatory for catecholamine synthesis) are among the observations illustrating that the *brain* concentration of phenylalanine is what ultimately determines the magnitude of the chemical alterations and thus the magnitude of the ensuing functional defects in the brain. Among the several variables that might underly the age-dependence of cerebral vulnerability, the amounts of the insulting chemical principle entering the brain is the most obvious one. It is reasonable to hypothesize that the higher cerebral concentrations of phenylalanine prevailing in the period of early infancy is causally related to the severe damage that phenylketonurics incur during that period. (This may or may not be significantly aggravated by the somewhat higher plasma phenylalanine levels in phenylketonurics during the first 10 months than in later life (26)).

Changes in cerebral transport not only have important implications to the age-dependence of vulnerability to hyperaminoacidemias but may also provide a basis for explaining individual variations in this vulnerability. An extreme example of such a variation is that some untreated phenylketonurics show normal mental development (27). Extending the above hypothesis to these individuals, one may postulate that, owing to an aberrant or precociously mature transport system, severe hyperphenylalaninemia did not raise the phenylalanine concentration of the brain sufficiently to have interfered with its functional development.

Because of the hardship of adhering to the strictly phenylalanine restricted diet, the question of how long this treatment of phenylketonurics must continue is an important and controversial one. According to some investigators treatment up to the age of 4–5 years is adequate, while others recommend that normal plasma levels of phenylalanine should be maintained for as long as possible (24, 28, 29). The decision is made difficult by the knowledge that the growth and functional differentiation of brain

areas continue up to puberty. The present discovery of a maturation change in cerebral transport which (beginning long before puberty) enables the animal to restrict large amounts of phenylalanine from entering the brain, may lead to the development of rational criteria for this decision. Extension of the rodent findings to man could define the stage in infancy when the brain no longer concentrates enough phenylalanine to damage its still-developing functions, and when the dietary treatments of phenylketonurics can be safely discontinued or at least made less rigorous.

ACKNOWLEDGMENT

We are grateful to Miss Michele Gambetta for her skilled assistance in these investigations.

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