

# Branched Chain Amino Acids Improve Complex Maze Learning in Rat Offspring Prenatally Exposed to Hyperphenylalaninemia: Implications for Maternal Phenylketonuria

CHARLES V. VORHEES AND HELEN K. BERRY

*Institute for Developmental Research, Children's Hospital Research Foundation; and Department of Pediatrics, University of Cincinnati, Cincinnati, OH*

**ABSTRACT.** Maternal phenylketonuria results in a high incidence of children born who are mentally retarded. It has been suggested that blood-brain-barrier transport of phenylalanine may be reduced by competitive inhibition of transporter uptake by supplemental administration of other large neutral amino acids. We hypothesized that large neutral amino acids might also be effective at improving the outcome of fetuses exposed to hyperphenylalaninemia *in utero*. If correct, sparing of embryonic CNS development might be possible. Pregnant rats were given a hyperphenylalaninemic diet alone or the same diet supplemented with a combination of valine, isoleucine, and leucine. As adults, the progeny exposed *in utero* to hyperphenylalaninemia showed characteristic learning deficits in a complex maze, while those exposed *in utero* to hyperphenylalaninemia combined with valine, isoleucine, and leucine showed no deficits in maze acquisition. The valine, isoleucine, and leucine supplement may show promise as a treatment for intrauterinely acquired mental deficiency associated with maternal phenylketonuria. (*Pediatr Res* 25:568-572, 1989)

## Abbreviations

PKU, phenylketonuria  
LNAA, large neutral amino acid  
PCPA, D,L-*p*-chlorophenylalanine  
PFC, pair-fed control  
VIL, valine, isoleucine, and leucine

PKU, an inherited disorder of phenylalanine metabolism, has been treated during the last 30 y through a combination of phenylalanine-free formulas and dietary restriction of phenylalanine-containing foods (1). The result is an increasing number of nonmentally retarded, noninstitutionalized PKU adults starting families. Unfortunately, maternal PKU causes a high incidence of mental retardation and congenital defects in infants due to transplacental hyperphenylalaninemia (2-4). Relying upon evidence developed concerning blood brain barrier transport of amino acids, we sought to reduce the embryotoxic effects of hyperphenylalaninemia using the branched-chain amino acids in a murine model of PKU.

A review of worldwide cases (2) found that 95% of pregnant PKU women with serum phenylalanine levels  $\geq 20$  mg/dL delivered at least one child who was mentally deficient. Other findings were microcephaly (90%), growth retardation (56%), and congenital heart defects (40%). Reinstatement of a phenylalanine-restricted diet before or after recognition of pregnancy has produced mixed, but generally unsatisfactory, results (2-7).

This has led to the search for new approaches. Phenylalanine and other LNAA share a common transport carrier for selective CNS uptake (8-12). It has been established that increasing the concentration of one or more LNAA competitively inhibits the transport into brain of other amino acids in this group. This approach has been suggested as a means of reducing the influx of phenylalanine in PKU (13-18). Early experiments to assess this have shown promise (13-15, 17-22), but in no case was evidence provided that such approaches could produce lasting cognitive improvement. Given that mental retardation is the most prevalent untoward effect of maternal PKU, we sought to test the efficacy of the branched-chain LNAA to improve embryo-fetal development in pregnant hyperphenylalaninemic rats to determine if sparing of CNS development in the progeny was possible.

## MATERIALS AND METHODS

Nulliparous female Sprague-Dawley CD (VAF/Plus) rats (Charles River Breeding Laboratories, Portage, MI) were bred and on d 4 (sperm plug = d 0) were randomly assigned to one of three treatment groups on a wt matched basis. Experimental diets were provided on gestation d 5-15. The PKU group received diet containing 2.5% added L-phenylalanine (phe) and 0.12% PCPA by wt (23-25) *ad libitum*. PCPA is a phenylalanine hydroxylase inhibitor (26-27). The PKU-VIL group received the phe+PCPA diet *ad libitum* with VIL added at concentrations of 1.0% L-valine, 1.0% L-isoleucine, and 1.33% L-leucine by wt (18-21). The PFC group received the base diet in the amount consumed by its matched pair in the PKU group. Neither phe alone nor PCPA alone induces long-term effects on water maze performance or on any other measure of behavioral performance characteristic of PKU animal models using similar treatment regimens (23, 24, 28-30). On d 11 and 15 of gestation, every other dam in each group was anesthetized with methoxyflurane and a blood sample drawn from the tail for determination of serum amino acids. Number of litters/group were: PKU = 10; PKU-VIL = 12; PFC = 12.

At birth, litters were adjusted to eight (four males and four females) using a random selection method. Offspring were weaned and housed in same-sexed pairs on postnatal d 28. Offspring were assessed on four measures of neurobehavioral

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Correspondence and reprints to Charles V. Vorhees, Ph.D., Institute for Developmental Research, Children's Hospital Research Foundation, Cincinnati, OH 45229-2899.

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and cognitive functioning by personnel blind to treatment group assignment.

On d 50–52 all offspring were assessed for 15 min/d in an automated open-field as a test of locomotor activity under low-level illumination during the diurnal phase after a 10 min dark adaptation period. Each rat was tested individually and at the same time each day. The fields were circular and had diameters of 45.7 cm. Males and females were tested in separate fields, which were cleaned between subjects. There were four LED-phototransistor pairs aligned in the *x*-axis and 4 in the *y*-axis of each field (San Diego Instruments, San Diego, CA).

On d 55, half of the male and female offspring from each litter received four trials in a 150-cm straight water channel to acclimate them to a swimming task, as a measure of performance capacity, and as a measure of their motivation to escape from water. Time to swim the channel and reach an escape ladder were analyzed by ANOVA for sum of trials, last trial, and fastest trial. Data for rats within each litter were averaged together and litter, stratified for sex, was the unit of statistical analysis (31). Water temperature was  $22 \pm 1^\circ\text{C}$ .

Rats tested in the straight channel were next evaluated for learning in a complex water maze. The maze chosen was an expanded version of the Biel maze (32). Path A consisted of the sum of errors committed on six trials (two trials/d) given on d 56–58 on the initial maze problem (acquisition). Path B consisted of the sum of errors committed on six additional trials (two trials/d) administered on d 62–64 with the start and goal areas reversed (reversal). Two types of errors were recorded, *cul-de-sac* errors, which were whole body entries into any blind alley, and excursion errors, which were whole body entries into any channel of the maze other than the path leading to the goal. Elapsed maze times were also recorded (s). Four offspring (two males and two females) were tested/litter. Rats failing to reach the goal within 6 min were assisted to the goal by placing barriers in the maze behind them thereby preventing re-entry into previously chosen channels. Errors and maze times were analyzed by fixed-effect factorial ANOVA with litter, stratified for sex, as the unit of analysis (31).

On d 65 two males and two females/litter were tested for modified startle responses. Rats were tested in a San Diego Instruments SR apparatus. Rats were placed in a sound-attenuated chamber and acclimated for 5 min before their first trial. Each rat was given 72 trials. The first 36 trials used a white noise auditory prepulse stimulus of 90 dB(A) and an auditory startle-eliciting stimulus of 115 dB(A) presented against a continuous 70 dB(A) background noise. All signals were of mixed frequency with a predominant band at 4 kHz. The second 36 trials used the same auditory prepulse followed by a tactile startle-eliciting stimulus (12 psi air-puff). The interstimulus interval (gap) ranged from 0–4000 ms and were presented in a Latin square design with an intertrial interval of 30 s. The first trial was always a no-prepulse trial. Data for all trials with the same interstimulus interval were averaged together. Interstimulus gaps were measured from prepulse signal onset to startle stimulus onset. Signal duration was 20 ms and the response window was 100 ms from startle signal onset. Dependent measures were  $V_{\max}$  = maximum response (voltage change) occurring during the response window,  $V_{\text{mean}}$  = mean voltage change during the recording window taken each ms, and  $T_{\max}$  = response latency (ms) from signal onset to  $V_{\max}$ .

Serum amino acids were analyzed on a Beckman model 119B amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA) (33–34). Offspring data were analyzed using the litter mean as the unit of statistical analysis (31). Statistical inference was based on general linear model ANOVA (factorial and mixed models, as appropriate), with follow-up analyses using simple-effect ANOVA and Duncan *a posteriori* multiple-group comparisons. Anesthesia/blood sampling history was a factor in a series of initial analyses. These uniformly demonstrated that there were no effects of this procedure on any dependent measure.

## RESULTS

Serum amino acid patterns of dams in the PKU and PKU-VIL groups exhibited several differences (Table 1). The serum concentrations of phenylalanine were in the phenylketonuric range at both time points and were greater on d 15 than on d 11 in both groups. No differences in serum phenylalanine between the PKU and PKU-VIL groups were obtained confirming that VIL did not alter maternal phenylalanine or PCPA absorption or metabolism. As expected, the PKU-VIL group showed substantial increases in serum VIL. Minor differences were also noted between groups in serum threonine, serine, and glycine.

No differences were found in maternal food consumption throughout gestation. No differences in maternal body wt were observed from d 0–14 of gestation. A minor effect on maternal wt appeared on d 15 and 20 (PKU and PKU-VIL groups compared to PFC,  $p < 0.10$ ). On the d of birth, but not thereafter, there was a group effect on maternal wt ( $p < 0.01$ ), such that the

Table 1. Serum amino acids in hyperphenylalaninemic pregnant rat dams with and without branched chain dietary supplementation\*

Amino Acid	Gestation d 11 ( $\mu\text{mol}/\text{liter}$ )		Gestation d 15 ( $\mu\text{mol}/\text{liter}$ )	
	PKU	PKU-VIL	PKU	PKU-VIL
Tau	399 $\pm$ 53	297 $\pm$ 22	399 $\pm$ 51	313 $\pm$ 55
Asp	21 $\pm$ 6	13 $\pm$ 2	19 $\pm$ 3	12 $\pm$ 3
Thr	319 $\pm$ 25	249 $\pm$ 20†	293 $\pm$ 11	259 $\pm$ 10†
Ser	318 $\pm$ 26	241 $\pm$ 15†	263 $\pm$ 24	231 $\pm$ 12†
Asn	65 $\pm$ 9	54 $\pm$ 5	62 $\pm$ 6	54 $\pm$ 5
Glu	74 $\pm$ 15	51 $\pm$ 7	84 $\pm$ 12	66 $\pm$ 10
Gln	556 $\pm$ 29	461 $\pm$ 38	473 $\pm$ 26	441 $\pm$ 30
Pro	184 $\pm$ 21	166 $\pm$ 16	199 $\pm$ 12	206 $\pm$ 16
Gly	254 $\pm$ 21	209 $\pm$ 9†	236 $\pm$ 26	190 $\pm$ 15†
Ala	562 $\pm$ 61	497 $\pm$ 36	488 $\pm$ 28	525 $\pm$ 14
Cit	79 $\pm$ 3	73 $\pm$ 9	43 $\pm$ 6	81 $\pm$ 10
Val	192 $\pm$ 14	321 $\pm$ 57‡	204 $\pm$ 10	358 $\pm$ 36‡
Cys	14 $\pm$ 4	27 $\pm$ 5	20 $\pm$ 5	22 $\pm$ 9
Met	64 $\pm$ 3	55 $\pm$ 3	58 $\pm$ 2	52 $\pm$ 8
Ile	87 $\pm$ 5	130 $\pm$ 26‡	86 $\pm$ 3	151 $\pm$ 13‡
Leu	143 $\pm$ 10	205 $\pm$ 38‡	141 $\pm$ 7	239 $\pm$ 20‡
Tyr	148 $\pm$ 7	128 $\pm$ 9	148 $\pm$ 15	152 $\pm$ 12
Phe	1693 $\pm$ 447	2025 $\pm$ 429	2635 $\pm$ 429§	2920 $\pm$ 426§
PCPA	365 $\pm$ 32	400 $\pm$ 29†	466 $\pm$ 11	555 $\pm$ 29†
Phe mg/dL	28 $\pm$ 7	33 $\pm$ 7	44 $\pm$ 7	48 $\pm$ 7
Dams bled in each group (n)	7	7	7	7

\* Pregnant rats were given diets containing either 2.5% L-phenylalanine + 0.12% D,L-p-chlorophenylalanine (PKU group) or the same amounts of phenylalanine + PCPA plus VIL (1.0% L-valine, 1.0% isoleucine, and 1.33% L-leucine (PKU-VIL group) by wt) on d 5–15 of gestation. One-half of the dams in each group (including PFC) were anesthetized with methoxyflurane on d 11 and 15, and a blood sample was drawn from the tail. Samples were centrifuged, and serum was removed and frozen for later amino acid analysis. Amino acids were analyzed on a Beckman model 119B amino acid analyzer, including separation of PCPA. Amino acid concentrations were analyzed using treatment group  $\times$  gestational age ANOVA. All maternal and offspring data were analyzed with anesthesia/blood sampling history as a factor in ANOVA for all dependent variables. These analyses showed no main effects of sampling history and no interactions of sampling history with treatment group on any dependent variable.

†  $p < 0.05$  compared to PKU group.

‡  $p < 0.01$  compared to PKU group.

§  $p < 0.01$  increase compared to d 11 levels.

PKU dams weighed less than the PKU-VIL (7.6%;  $p < 0.05$ ) or PFC dams (12.4%;  $p < 0.05$ ). There were no differences in the number of delivering dams, litter size, or offspring sex ratio at birth among groups. There were no significant group differences in offspring body wt at any age. Offspring mortality was increased before weaning in the PKU group (8.8%) and PKU-VIL group (8.5%) compared to PFC (2.1%, both comparisons  $p < 0.05$ ).

No differences in basic swimming performance were obtained in straight channel trials (Fig. 1). This indicated that the groups did not differ in swimming ability or motivation. In contrast, large differences occurred in maze performance (Fig. 2). Using two methods of scoring errors, the PKU offspring committed more *cul-de-sac* and excursion errors than PFC offspring on both phases of maze testing and required more time in the maze. Rats in the PKU-VIL group committed fewer errors than the PKU group and did not differ from PFCs on path A. On path B, the PKU-VIL group committed fewer errors than the PKU group and more errors than the PFC group; however, they did not commit significantly more errors than PFC or significantly fewer than PKU.

In the open-field test, there were group  $\times$  d and group  $\times$  test-interval interactions for peripheral activity ( $p < 0.05$  and  $p < 0.01$ , respectively), but no effects on central section activity. Further analyses showed only one clear effect, which occurred on d 2 of testing during the second 5-min test interval ( $p < 0.01$ ). During this interval, the PKU group was less active than either of the other groups, which did not differ from one another ( $\bar{x} \pm$  SEM, PKU =  $118.5 \pm 6.3$ ; PKU-VIL =  $139.8 \pm 5.0$ ; PFC =  $134.3 \pm 4.5$ ; PKU < PKU-VIL and PKU < PFC by Duncan comparisons, both  $p < 0.05$ ).

No differences were found on the startle reflex using either auditory or tactile stimuli, with or without a prepulse response inhibition signal (Fig. 3).

#### DISCUSSION

The data revealed that the VIL treatment had a selective beneficial effect on CNS development *in utero*. The effect was noteworthy for its specificity and magnitude on learning ability. VIL had a large effect on the learning of a complex water maze, only a modest effect on locomotor activity, and no effect on

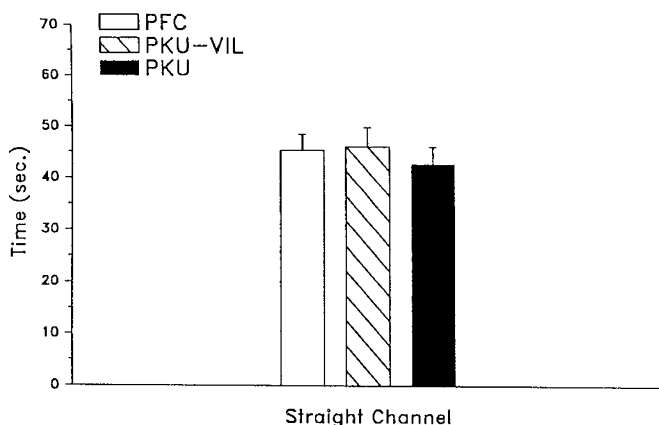


Fig. 1. Mean  $\pm$  SEM straight channel swimming times (s) summed across trials. There were no group differences found by ANOVA. Similarly, there were no differences when the data were analyzed separately by last trial or fastest trial. All litters were coded and tested by personnel blind to treatment group on this and all other tests. PKU = offspring of 10 dams given 2.5% L-phe + 0.12% PCPA in the diet on d 5–15 of gestation; PKU-VIL = offspring of 12 dams given the PKU group's diet supplemented with a mixture of valine, isoleucine, and leucine (L-isomers) on the same days of gestation; PFC = offspring of 12 control dams given standard diet in the amount consumed by the dams in the PKU group (pair-fed).

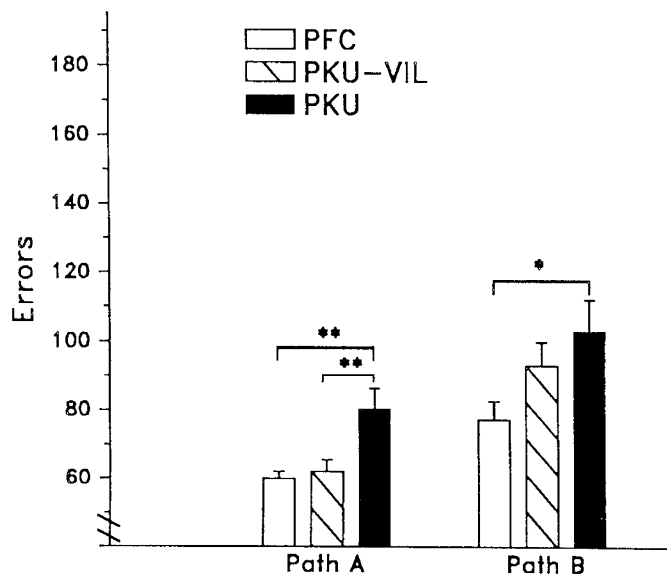


Fig. 2. Mean  $\pm$  SEM of the sum of *cul-de-sac* errors committed in the water maze during each phase of testing (6 trials/phase). Group differences were found on path A ( $p < 0.001$ ) and path B ( $p < 0.05$ ) by fixed-effect factorial ANOVA. Individual group comparisons were made using Duncan's multiple range test. Duncan comparisons are shown as \* $p < 0.05$  and \*\* $p < 0.01$ . An essentially identical pattern was found for excursion errors, *i.e.* group differences were found for path A ( $p < 0.05$ ) and path B ( $p < 0.05$ ). For excursion errors, group differences for path A were ( $\bar{X} \pm$  SEM): PKU =  $43.5 \pm 3.2$ , PKU-VIL =  $35.7 \pm 2.0$ , PFC =  $35.4 \pm 1.2$  (PKU > PKU-VIL or PFC, both  $p < 0.05$ ); for path B: PKU =  $70.7 \pm 5.5$ , PKU-VIL =  $62.8 \pm 3.7$ , PFC =  $55.1 \pm 3.4$  (PKU > PFC  $p < 0.05$ , PKU-VIL < PKU, NS, and PKU-VIL > PFC, NS, all by Duncan comparisons). The same pattern was also seen for maze times (not presented), but differences were smaller, *i.e.* the group F-ratio for path A was  $p < 0.06$  and for path B it was  $p < 0.10$ . Individual groups were aligned in the same pattern on times as they were for both types of errors on each phase of this test.

swimming ability or the startle reflex. The protective effect of VIL on learning was most evident and only significant in terms of acquisition of the maze problem (path A). The basis of the beneficial effects of VIL on *in utero* development remains to be determined. There is preliminary data suggesting that VIL may reduce fetal brain phenylalanine content in PKU-VIL rats (18).

No phenylalanine-only or PCPA-only exposed groups, no phenylalanine-VIL or PCPA-VIL groups, no VIL-only or VIL *ad libitum*-fed groups, and no unsupplemented *ad libitum*-fed groups were included in the present investigation for several reasons. First, the inclusion of such controls in an investigation prior to determining the existence of a PKU-VIL effect would be counterproductive. Second, the purpose of this investigation was not to test the VIL supplement against phenylalanine-alone or PCPA-alone, but to assess its effects compared to a specific and established model of induced PKU that utilizes a particular combination of phenylalanine and PCPA dosages. Third, as both phenylalanine and PCPA have been shown not to produce an adequate biochemical model of PKU when used separately, and as neither have ever been shown to produce the types of behavioral effects assessed herein, there was little to be gained by the inclusion of these extensive additional control groups. Given that the data now support the potential of the VIL supplement in a model of maternal PKU, we will conduct further experiments, one of which will be to determine whether any plausible alternative combination or permutation of treatments can produce a comparable outcome.

As effective as the VIL supplement was at preventing the untoward effects of maternal hyperphenylalaninemia on maze

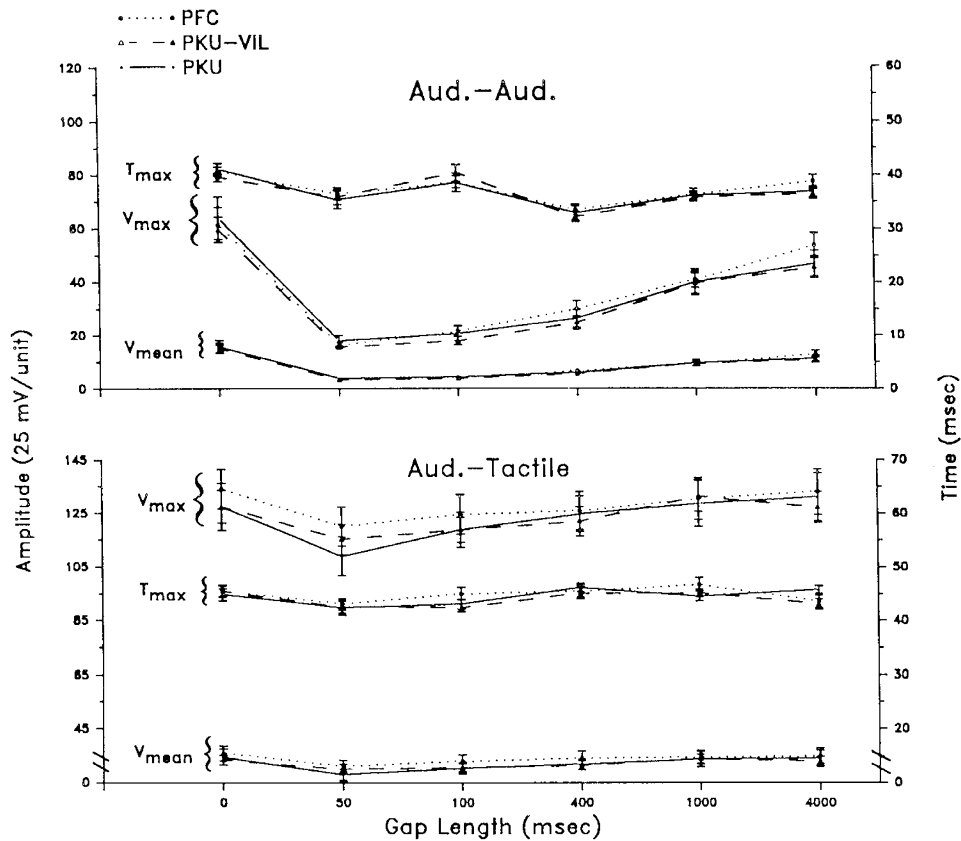


Fig. 3. Mean  $\pm$  SEM startle responses shown across various prepulse gap lengths (ms). *Upper*, responses to an auditory startle-eliciting stimulus presented following an auditory prepulse signal. *Lower*, responses to a tactile startle-eliciting stimulus presented following an auditory prepulse signal. Mixed model factorial ANOVA for each stimulus condition revealed no significant group or group-related interactions on any of the three dependent measures ( $V_{max}$ ,  $V_{mean}$ , or  $T_{max}$ ) recorded of the startle response.

acquisition, its effect on maze reversal learning was less clear. The PKU-VIL group did not commit significantly more errors on reversal than controls, suggesting a normalizing effect of the VIL supplementation. By contrast, the PKU-VIL group did not commit significantly fewer errors on reversal than the PKU group, suggesting no improvement. As the PKU and control groups differed from one another, it is not clear from these data whether the intermediate performance of the PKU-VIL group represents a partial effect of VIL or no effect. However, in a recently completed replication and extension of these data, a significant improvement in Olton radial-arm maze performance was found in the PKU-VIL group compared to the PKU group (Vorhees CV, Weisenberg KD, Acuff WP, and Berry HK, unpublished observations).

It remains to be seen whether a slightly different dose of the VIL mixture or a change in the proportions of the constituent branched-chain amino acids would be more effective than the present regimen. It also remains possible that combining VIL with other LNAA might enhance the efficacy of this general approach. An issue of equal importance is whether the cognitive improvements demonstrated here in the expanded Biel maze will generalize to other tests of cognitive performance, such as the Morris hidden-platform test of spatial relations learning.

The present data suggest a new approach for the treatment of maternal PKU. The data must be interpreted with caution, however, as a single test of rodent learning cannot assure that human cognitive development would be comparably protected, nor has the present research addressed the other sequelae of maternal PKU. Furthermore, the present data cover a period of rat development that would be comparable to only the first 8–12 wk of human development, *i.e.* the period of major organogenesis. This may not cover all the stages of CNS development

affected by maternal PKU, as maternal PKU results in chronic hyperphenylalaninemia, which extends from prior to conception to and beyond parturition. Maternal PKU also induces congenital heart defects, microcephaly, and intrauterine growth retardation, and research is underway on the effects of the current model on cardiogenesis and growth (19).

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