J Inherit Metab Dis (2007) 30:318–325
DOI 10.1007/s10545-007-0583-1

ORIGINAL ARTICLE

Regionally selective decreases in cerebral glucose metabolism in a mouse model of phenylketonuria

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Received: 13 February 2007 / Submitted in revised form: 6 March 2007 / Accepted: 8 March 2007 / Published online: 24 April 2007

Summary Impairment of cognitive function is characteristic of untreated phenylketonuria in humans and in the *pah*^{enu2} mouse model of phenylketonuria. We measured regional cerebral metabolic rate for glucose in the adult male *pah*^{enu2} mouse to determine the effect of PKU on functional activity in brain and to discern what, if any, brain areas are affected. Our results demonstrate selective decreases (17–21%) in regions thought to be involved in executive function. Regions most significantly affected include prelimbic, anterior cingulate, orbital frontal and perirhinal cortex. Sensory and motor areas of cortex and hippocampus were remarkably unaffected.

Abbreviations

DG	deoxyglucose
HMZ	homozygous
HTZ	heterozygous
i.v.	intravenous
PKU	phenylketonuria
$rCMR_{glc}$	regional cerebral metabolic rate for glucose
WT	wild-type

Communicating editor: Michael Gibson

Competing interests: None declared

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Introduction

Phenylketonuria (PKU) is an inherited disease of phenylalanine metabolism that results in profound mental retardation if treatment is not initiated very early in life (Scriver et al 1989). The classical form of PKU is caused by mutations in the gene for phenylalanine hydroxylase, the enzyme that catalyses the first step in the metabolism of phenylalanine. Patients with classical PKU have negligible phenylalanine hydroxylase activity in liver and elevated concentrations of phenylalanine in plasma and metabolic products of phenylalanine metabolism in urine. If phenylalanine in the diet is restricted beginning in infancy, the dire consequences of the mutation on mental function can be prevented.

A genetic mouse model of PKU (*pah*^{enu2}) was created in the BTBR strain by chemical germline mutagenesis (Shedlovsky et al 1993). The mutation is in the gene for phenylalanine hydroxylase, and it results in a loss of enzyme activity such that, in liver, phenylalanine hydroxylase activity is minimal. Plasma concentrations of phenylalanine in the *pah*^{enu2} mouse are 10–20 times normal and animals are hypopigmented. These characteristics are comparable to those seen in the human disease. Behavioural manifestations of the disease in the mouse indicate some impairment of cognitive function (Cabib et al 2003; Zagreda et al

1999). The cognitive deficits do not appear to interfere with the survival of the mice, at least in a laboratory setting. Whether the cognitive defects are equivalent to the profound mental retardation seen in the human disease is difficult to judge. In the present study we asked whether the functional activity of specific brain regions is affected by the mutation. To answer this question we have measured regional cerebral metabolic rates for glucose ($rCMR_{glc}$) in conscious, adult, male *pah*^{enu2} mice as an indication of the level of regional functional activity. Our results demonstrate regionally selective decreases in $rCMR_{glc}$ in the *pah*^{enu2} mutant mouse.

Materials and methods

Animals

All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and an animal study protocol approved by the NIMH Animal Care and Use Committee. *pah*^{enu2} mice with a F263S point mutation were created on a BTBR background (Shedlovsky et al 1993). *pah*^{enu2} breeding pairs (heterozygous (HTZ) females and homozygous (HMZ) males) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). HTZ and HMZ pups produced by the breeding pairs were weaned between 21 and 23 days of age and crossbred in pairs of HTZ females and HTZ males to provide offspring in three experimental groups: HMZ, HTZ, and wild-type (WT) mice. All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature with standard alternating 12 h periods of light and darkness. Food (NIH-31 rodent chow) and water were provided *ad libitum*. A total of 26 mature male mice between the ages of 4 and 5 months were studied.

Genotyping

Genomic DNA was extracted (Moore 1995) from a section of tail and exon 7 of the PAH gene was amplified as described previously (McDonald and Charlton 1997). The 132 bp PCR product was then subjected to restriction endonuclease digestion with *Alw*26I and incubated overnight at 37°C. The restriction fragments were separated by electrophoresis on a 20% polyacrylamide denaturing gel at 140 V for 16 h. The digestion fragments were: WT 82 and 50 bp; HTZ 82, 50, 48 and 34 bp; and HMZ 50, 48 and 34 bp.

Surgical preparation of animals

Mice were prepared for metabolic studies by insertion under light halothane anaesthesia of polyethylene catheters (PE-10) into one femoral artery and vein as previously described (Smith and Kang 2000). Mice were permitted to move freely throughout the 18–20 h recovery period and the metabolic study. Food and water were available *ad libitum*.

Physiological variables

Mean arterial blood pressure, haematocrit and arterial plasma glucose concentrations were measured to evaluate each animal's physiological state (Smith and Kang 2000). Rectal temperature and mean arterial blood pressure were monitored with a Model BAT-12 thermometer (Sensortek, Clifton, NJ, USA) and a Digi-Med Blood Pressure Analyzer (MicroMed, Louisville, KY, USA), respectively. Two mice were not included in the study due to hypoglycaemia.

Determination of $rCMR_{glc}$

$rCMR_{glc}$ was determined by the autoradiographic [^{14}C]deoxyglucose (DG) method as previously described for the rat (Sokoloff et al 1977), modified for use in the mouse (Qin et al 2002). The experimental period was initiated by an i.v. pulse injection of 120 mCi/kg of 2-deoxy-D-[1–^{14}C]glucose (specific activity 50–55 μCi/mmol; PerkinElmer Life Sciences, Inc., Boston, MA, USA) contained in ~40 μl of physiological saline. Timed arterial samples were collected during the following 45 min for determination of the time courses of the plasma glucose and [^{14}C]DG concentrations. At the end of the experimental interval, mice were killed with an i.v. injection of a lethal dose of sodium pentobarbital and brains were removed rapidly and frozen in isopentane (−40°C). Serial sections, 20 μm thick, were cut in a Leica 1850 cryostat (Leica, Deerfield, IL, USA) at −18°C, thaw-mounted on gelatin-coated slides, immediately dried in a stream of air, and exposed to EMC-1 film (Kodak, Rochester, NY, USA) along with calibrated [^{14}C]methylmethacrylate standards as previously described (Sokoloff et al 1977). Sections were then stained with thionin. Autoradiograms and stained sections were digitized with a pixel size of 11 μm by means of a 10-bit DVC-1310 digital camera (DVC Company, Austin, TX, USA). Images were aligned and analysed with an MCID Elite image processing system (Imaging Research, St. Catherine's, ON, Canada). Regions of interest were located and outlined on the Nissl-stained

Table 1 Physiological variables

	WT (7)	HTZ (9)	HMZ (10)
Age (days)	148±6	154±3	146±3
Body weight (g)	38±1	41±1	37±1
Body temperature (°C)	36.5±0.3	36.8±0.2	36.8±0.2
Haematocrit (%)	44±1	43±1	46±1
Arterial plasma glucose concentration (mmol/L)	7.5±0.4	6.9±0.4	6.9±0.2
Mean arterial blood pressure (mmHg)	84±4	89±2	88±2

Values are the means±SEM for the number of mice indicated in parentheses.

section by reference to a mouse brain atlas (Paxinos and Franklin 2001) and optical densities were measured on the autoradiograms of six to eight tissue sections. Measurements were made bilaterally. Concentrations of ^{14}C were determined from the optical density vs ^{14}C concentration curve determined from the calibrated plastic standards, and $rCMR_{glc}$ was calculated from the pixel-weighted average local tissue ^{14}C concentration and the time courses of [^{14}C]DG and glucose concentrations in arterial plasma by means of the operational equation of the method (Sokoloff et al 1977). We used the rate constants and the lumped constant determined in the normoglycaemic, conscious rat (Sokoloff et al 1977). The rate of glucose metabolism for the brain as a whole (CMR_{glc}) and brain volume were determined by analysis of autoradiograms of all sections of the entire brain digitized (42 μm pixel size) by means of a Multirad 850 Howtek Film Digitzer (Howtek, Hudson, NH, USA). Weighted average CMR_{glc} was determined as described above, and brain volume was determined from the total number of pixels and the calibrated pixel size.

Statistical analyses

Physiological variables and $rCMR_{glc}$ measurements were analysed for statistically significant differences between the WT mice and the HTZ and HMZ genotypes by two-tailed Dunnett's *t*-tests. Corrections for comparing multiple regions were made by means of the modified Hochberg procedure with the number of true null distributions estimated by the graphical P-plot method; familywise error was set at α=0.05 and 0.07 (Turkheimer et al 2001).

Results

Physiological measurements

Physiological variables (Table 1) were monitored during the measurement of $rCMR_{glc}$. The three groups of mice were well matched with respect to all of the physiological variables measured. Coat colour in WT and HTZ mice was black and tan, whereas in HMZ mice the coat colour was grey and tan, consistent with the hyperphenylalaninaemic phenotype.

Cerebral metabolic rate for glucose

Cerebral metabolic rates for glucose in the brain as a whole in both HTZ and HMZ mice (Table 2) were not statistically significantly different from that of WT, but brain weight and volume were reduced in HMZ mice by about 15% compared to WT. Mean values in HTZ mice were very similar to those of WT. $rCMR_{glc}$ were determined in 26 regions of brain (Fig. 1) and comparisons were made between WT and both HMZ

Table 2 Whole brain: weight, volume and mean CMR_{glc}

	WT (7)	HTZ (9)	HMZ (10)
Weight (g)	0.463±0.009	0.450±0.005	0.392±0.006*
Volume (cm^3)	0.422±0.011	0.419±0.010	0.367±0.007*
CMR_{glc}, (μmol/g per min)	0.499±0.026	0.477±0.022	0.449±0.025

Values are means±SEM for the number of mice indicated in parentheses except for brain weight in the WT and HTZ groups in which the numbers of mice were 5 and 8, respectively.
*Statistically significantly different from WT mice, $p<0.001$, Dunnett's *t*-test.

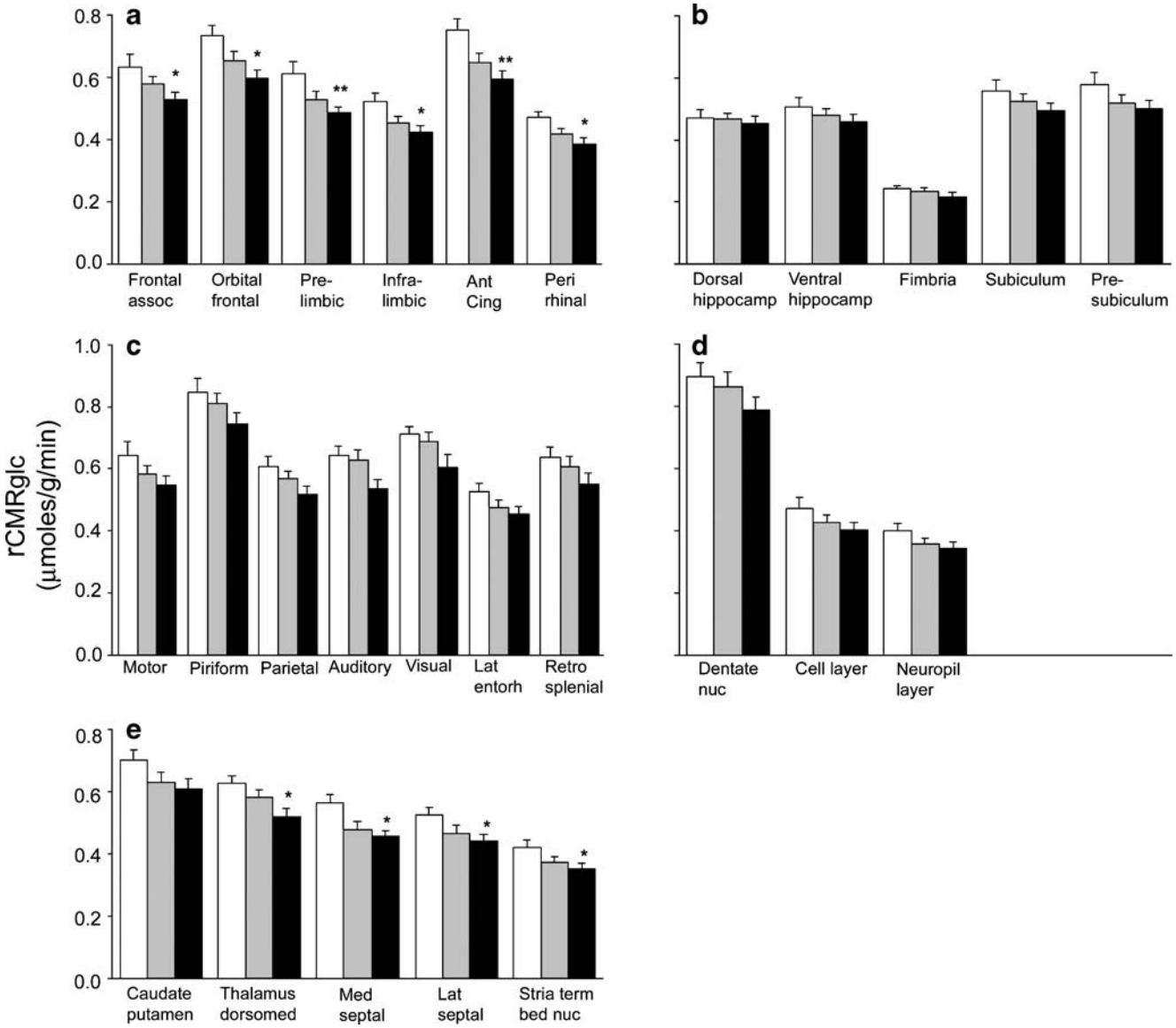

Fig. 1 Effects of a mutation in the gene for phenylalanine hydroxylase on $rCMR_{glc}$ in WT (open bars, n=7), HTZ (grey-filled bars, n=9) and HMZ (black-filled bars, n=10) mice. Bars represent the means±SEM of each group in regions of the prefrontal cortex (**a**), hippocampus (**b**), other areas of cortex (**c**), cerebellum (**d**), and subcortical regions (**e**). Means of the HTZ and HMZ groups were compared with WT by means of Student's t-tests with Dunnett's correction: *statistically significant difference compared with WT, $p<0.05$; **statistically significant difference compared with WT, $p<0.01$

and HTZ mice by Dunnett's t-tests. There were no statistically significant differences in $rCMR_{glc}$ between WT and HTZ mice in any of the regions examined, but HMZ mice were affected particularly in areas of the cortex (Figs. 1 and 2). We found statistically significant decreases (ranging from 17% to 21% compared with WT) in $rCMR_{glc}$ in frontal association, orbital, prelimbic, infralimbic, perirhinal and cingulate cortex; p-values ranged from 0.003 to 0.041. In subcortical areas we found statistically significant decreases (ranging from 15% to 19% compared with WT) in $rCMR_{glc}$ in thalamus, septal nuclei and bed nucleus of stria terminalis; p-values ranged from 0.010 to 0.049. $rCMR_{glc}$ values in the hippocampal formation were remarkably unaffected.

Because we analysed multiple brain regions in the same animals and the regional analyses are not totally independent measures, we applied the modified Hochberg procedure to the statistical results in the HMZ vs WT comparison to examine the effects of multiple comparisons. By the modified Hochberg method, results in the anterior cingulate, prelimbic, orbital frontal and perirhinal cortex and in the medial septal nucleus reached the 0.05 level of statistical significance and effects in the infralimbic cortex approached statistical significance at the 0.07 level.

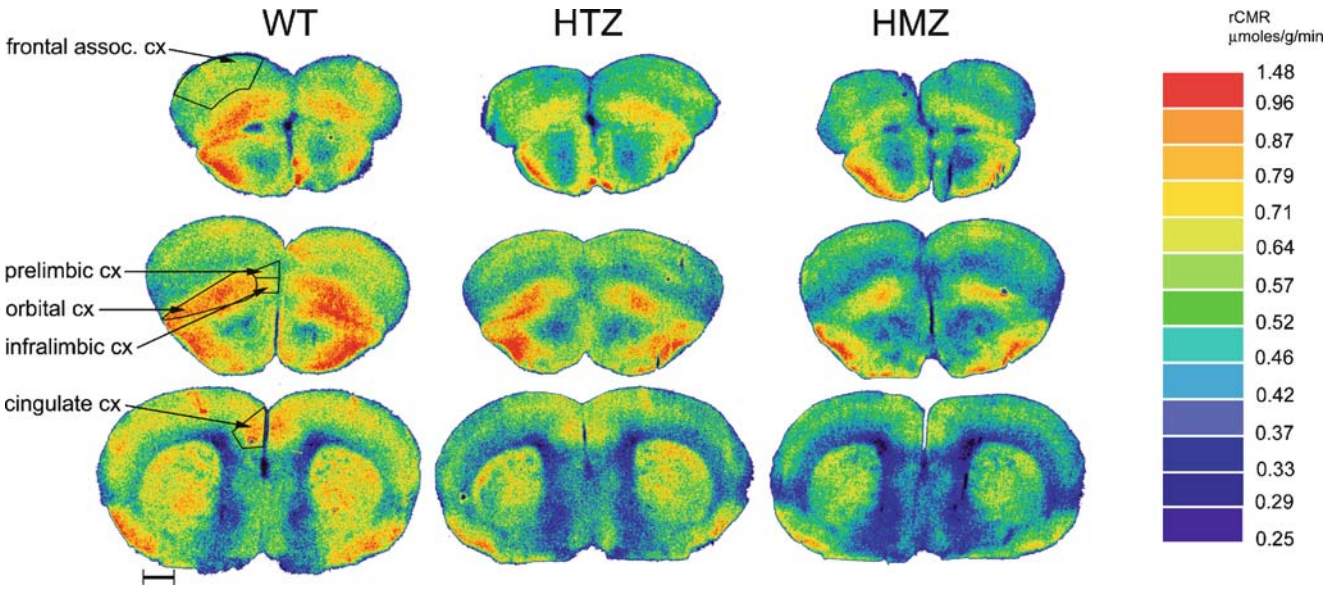

Fig. 2 Digitized [^{14}C]DG autoradiograms of coronal sections at the level of frontal association cortex (top row), prelimbic cortex (middle row), and anterior cingulate cortex (bottom row) from mice representative of the three genotypes studied: WT (left column), HTZ (centre column) and HMZ (right column). [^{14}C]DG autoradiograms were colour-coded for $rCMR_{glc}$ according to the colour bar on the right. The bar at the lower left is 1 mm

Discussion

The present study is, to our knowledge, the first study of regional brain functional activity in the *pah*^{enu2} mouse model of PKU. Our results show that in the adult male *pah*^{enu2} mouse, $rCMR_{glc}$ is decreased in selective brain regions. Cortical regions known to be involved in cognitive functions are particularly affected, whereas the hippocampal formation is spared. The effects on $rCMR_{glc}$ in prefrontal cortical areas are in accord with the cognitive impairments in PKU mice reported by Zagreda and colleagues (1999) and Cabib and colleagues (2003).

That *pah*^{enu2} mice in our study were homozygous for the mutation in the phenylalanine hydroxylase gene was confirmed by genotyping. We also noted that HMZ mice had a lighter coat colour compared with HTZ and WT, consistent with reduced tyrosine, the precursor for melanin production. In separate experiments on our *pah*^{enu2} mouse colony we measured amino acid concentrations in arterial plasma and found that compared with WT phenylalanine concentrations were 20-fold higher in HMZ mice and 38% higher in HTZ mice (Smith and Kang 2000). These observations confirm the biochemical phenotype of hyperphenylalaninaemia in our HMZ *pah*^{enu2} mice.

We applied the autoradiographic [^{14}C]DG method to determine $rCMR_{glc}$ as a measure of regional functional activity. The method was originally devised for use in the rat (Sokoloff et al 1977) and has been adapted for use in the mouse (Jay et al 1985; Qin et al 2002). In our calculations of $rCMR_{glc}$ we used the rate constants and lumped constant determined in the rat (Sokoloff et al 1977). The design of the method is such that it is relatively insensitive to the values of the rate constants, so inaccuracies in these values have negligible effects on the determined $rCMR_{glc}$. The $rCMR_{glc}$, however, is inversely proportional to the value of the lumped constant. Whereas there may be some difference between values of the lumped constant in mouse and rat, it is unlikely that there is a difference between *pah*^{enu2} mutants and WT mice. It has been shown that the lumped constant remains constant under all physiological conditions that have been studied and that changes occur only in pathophysiological states, such as severe hypoglycaemia (Sokoloff 1989). Therefore, it is highly unlikely that the relative changes in $rCMR_{glc}$ between *pah*^{enu2} and WT mice reported in our study are due to any methodological issues.

We have presented the statistical analysis of our results in two steps. The Dunnett's *t*-tests corrected for the fact that the WT group was used as a control for both HMZ and HTZ mice. With this analysis we found 10 regions in which $rCMR_{glc}$ was statistically significantly decreased (at the 0.01 to 0.05 level) in the HMZ mice. The Dunnett's correction of the *t*-statistic does not take into account that we analysed in the same animals multiple brain regions and that determinations of $rCMR_{glc}$ in the 26 regions are not independent of each other. We applied the modified Hochberg procedure with the number of 'true' null distributions estimated by the graphical P-plot method to account

for the comparison of multiple regions in the same animals. By the modified Hochberg procedure, decreases in $rCMR_{glc}$ in six regions in the HMZ mice were statistically significant (five at the 0.05 level and one at the 0.07 level). The decreases were all about 20% and five of the regions were areas of cortex, suggesting that effects in the PKU mice were very regionally specific.

In most brain regions our values for $rCMR_{glc}$ in WT mice (on a BTBR background) are in good agreement with those reported previously in mice on FVB/NJ (Qin et al 2002), and CFW (Wree et al 1989) backgrounds. For example, mean values for $rCMR_{glc}$ in auditory cortex were 0.64, 0.61, and 0.68 μmol/g per min in BTBR, FVB/NJ and CFW mice, respectively. In visual cortex, however, values differ such that the albino strain FVB/NJ has 25% lower $rCMR_{glc}$ than the pigmented strains, probably due to the retinal degeneration that often accompanies albinism.

Decreased $rCMR_{glc}$ in the *pah*^{enu2} mouse was not unexpected. Reduced functional activity is generally associated with a decreased metabolic rate (Sokoloff 1996). Other studies of diseases with cognitive deficits have reported decreased or unchanged $rCMR_{glc}$ in cretinism in adult rats (Dow-Edwards et al 1986) and in Down syndrome in humans (Shapiro et al 1990), respectively. New Zealand Black mice characterized by marked deficits in performance on tasks requiring learning and memory had widespread decreases in $rCMR_{glc}$ compared with control CFW mice (Wree et al 1989). In contrast to the *pah*^{enu2} mice and other mouse models of cognitive impairment, a mouse model of fragile X syndrome, another inherited form of mental retardation (Qin et al 2002), exhibited increases in $rCMR_{glc}$ in most brain regions examined, particularly in hippocampus. This effect in fragile X mice may be a function of the underlying pathology in this disease rather than diminished cognitive abilities per se. In a study of adult patients with PKU, $rCMR_{glc}$ was found to be normal in all regions examined except the anterior periventricular area (Hasselbalch et al 1996). The subjects of this study may not be equivalent to our HMZ mice because they had been treated with a phenylalanine-restricted diet very early in life and until the age of 15 years. In spite of the treatment, they had lower than normal IQ.

Regional selectivity is a striking feature in our study of the *pah*^{enu2} mouse. Effects on $rCMR_{glc}$ were statistically significant in regions of cortex involved in executive functions such as associative learning, working memory and decision-making. Particularly noteworthy were changes found in prelimbic, anterior cingulate, orbital, infralimbic and perirhinal cortex. Working memory in primates (Friedman and Goldman-Rakic 1994) and in rodents (Otto and Eichenbaum 1992) is identified with prefrontal cortical areas (Goldman-Rakic 1995). Lesions of the orbitofrontal cortex in rats and primates disrupt the ability to extinguish or reverse associations that have been learned through positive reinforcement (Bayliss and Gaffan 1991; Gallagher et al 1999; Jones and Mishkin 1972). In this regard our finding of a decrease in metabolic activity in orbital cortex is in agreement with the studies of Zagreda and colleagues (1999) in which *pah*^{enu2} mice were not impaired on acquisition of an odour discrimination task, but they required considerably more training to learn on reversals. Lesions of infralimbic cortex also disrupt reversal learning in rats, but the disruption may be attributable to an actual learning deficit, whereas the deficit after orbitofrontal cortex lesions appears to be a perseveration of the original association (Chudasama and Robbins 2003). In rodents, the anterior cingulate and perirhinal cortex have been implicated in evaluating reward–effort decision-making (Walton et al 2003) and emotional learning, respectively (Schulz et al 2004).

The lack of an effect on $rCMR_{glc}$ in the hippocampus in *pah*^{enu2} mice was surprising in view of the well-established role of the hippocampus in learning and memory (Cohen and Eichenbaum 1994). To address the possibility that a specific subregion of the hippocampus might be affected, we examined the dorsal hippocampus in much more detail (data not shown) and found that in all nine areas analysed there were no statistically significant effects. It may be that spatial memory function is preserved in the *pah*^{enu2} mouse and that the reported cognitive impairments (Cabib et al 2003; Zagreda et al 1999) do not require spatial memory.

The regional selectivity of the effects of PKU on $rCMR_{glc}$ contrasts with the global effects on cerebral protein synthesis (Smith and Kang 2000) and serotonin concentrations (Puglisi-Allegra et al 2000). Compared with WT, regional rates of cerebral protein synthesis (rCPS) were decreased by 20% throughout the brain in untreated adult *pah*^{enu2} mice (Smith and Kang 2000) and serotonin concentrations were reduced by 40–75% in all regions examined (Puglisi-Allegra et al 2000). An inhibition of amino acid transport at the blood–brain barrier due to hyperphenylalaninaemia in PKU may ultimately be responsible for these global effects. Specifically, reduced rCPS and serotonin concentrations may be due to the reduced brain concentrations of amino acids competing with phenylalanine for the neutral amino acid transporter in PKU mice (Smith and Kang 2000). Reduced branched-chain amino acid

concentrations and reduced monoamine precursors in brain may result in global effects on rCPS and serotonin concentrations, respectively. The regionally selective changes in $rCMR_{glc}$ that we report in this study are more likely a reflection of the functional deficits resulting from abnormal postnatal brain development in the presence of hyperphenylalaninaemia. It is possible that they are also associated with structural abnormalities such as decreases in dendritic arborization (Bauman and Kemper 1982) and dendritic spine densities. Pathways that develop postnatally may be selectively vulnerable, and in the mouse these pathways may differ from those affected in human patients with untreated PKU because of species differences in the timing of postnatal brain development.

Conclusions

The effects of a mutation in the gene for phenylalanine hydroxylase on cerebral functional activity in adult mice are remarkably regionally specific. It appears that functional activity in much of the brain, including the hippocampal formation, is spared, whereas prefrontal cortical functional activity is diminished. These findings further confirm the value of this animal model of PKU and suggest the regions of the brain in which hyperphenylalaninaemia during critical phases of brain development may cause changes that so profoundly affect cognitive function.

Acknowledgements We wish to thank Robert Wang for overseeing the breeding colony and Julia Kang Lee for surgical preparation of the mice. This research was supported by the Intramural Research Program of the NIMH, NIH.

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