Long-term changes in glutamatergic synaptic transmission in phenylketonuria


Summary
The cellular mechanisms that underlie impaired brain function during phenylketonuria (PKU), the most common biochemical cause of mental retardation in humans, remain unclear. Acute application of L-Phe at concentrations observed in the PKU brain depresses glutamatergic synaptic transmission but does not affect GABA receptor activity in cultured neurons. If these depressant effects of L-Phe take place in the PKU brain, then chronic impairment of the glutamate system, which may contribute to impaired brain function, could be detected as changes in postsynaptic glutamate receptors. This hypothesis was tested by using a combination of liquid chromatography–mass spectrometry, patch-clamp, radioligand binding and western blot approaches in forebrain tissue from heterozygous and homozygous (PKU) Pah<sup>enu2</sup> mice. Brain concentrations of L-Phe were nearly six-fold greater in PKU mice (863.12 ± 17.96 μmol/kg) than in their heterozygous counterparts (149.32 ± 10.23 μmol/kg). This concentration is significantly higher than the K<sub>B</sub> of 573 μM for L-Phe to compete for N-methyl-D-aspartate (NMDA) receptors. Receptor binding experiments with [3H]MK-801 showed significant up-regulation of NMDA receptor density in PKU mice. Consistent with the depressant effects of L-Phe, expression of NMDA receptor NR2A and (RS)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor Glu1 and Glu2/3 subunits was significantly increased, whereas expression of the NR2B subunit was decreased. There was no change in GABA α1 subunit expression. Given the role of the glutamatergic system in brain development and function, these changes may, at least in part, explain the brain disorders associated with PKU.

Keywords: phenylketonuria; NMDA receptor; AMPA receptor; Pah<sup>enu2</sup> mice; mental retardation

Abbreviations: AMPA = (RS)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; LC/MS/MS = liquid chromatography–tandem mass spectrometry; L-Phe = l-phenylalanine; NMDA = N-methyl-D-aspartate; PKU = phenylketonuria

Introduction
Glutamate, the main excitatory neurotransmitter, plays a crucial role in brain development and function (Johnston, 2003). Growing evidence indicates that impaired glutamatergic synaptic transmission may contribute to a diverse group of paediatric neurological disorders (e.g. fragile X syndrome, Rett syndrome, epilepsy) (Johnston, 2003; Loftis and Janowsky, 2003). Establishment of abnormalities of glutamatergic signalling in each disease state and elucidation of the molecular and cellular mechanisms mediating these alterations is important not only for developing novel therapeutic strategies but also for understanding the roles of glutamatergic synaptic transmission in brain functioning. This process is often complicated by the pathogenic complexity in many diseases. For a number of reasons, phenylketonuria (PKU) is an ideal model to investigate such mechanisms. PKU represents the most common biochemical cause of brain disorder in humans. Importantly, the cause of PKU is simple and its starting point is well-defined: namely, the elevated concentrations of the aromatic amino acid phenylalanine (L-Phe), which occur immediately after birth (Scriver and Kaufman, 2001). Furthermore, there is a murine model for PKU (Pah<sup>enu2</sup> mice) that closely resembles the metabolic and...
neurobiological phenotype of human PKU (Zagreda et al., 1999; Sarkissian et al., 2000; Cabib et al., 2003). PKU is a group of recessively inherited metabolic disorders characterized by impaired conversion of L-Phe to tyrosine (Scriver and Kaufman, 2001). In PKU and the related and less harmful hyperphenylalaninaemia, an autosomal recessive gene encodes phenylalanine hydroxylase with reduced or absent enzymatic activity, resulting in abnormally high concentrations of phenylalanine in body fluids. The symptoms of PKU manifest mainly in the brain. Early signs of PKU are mental retardation, microcephaly and epilepsy. In the second or third decade of life a progressive motor disorder sets in (Pennington et al., 1985; Scriver and Kaufman, 2001; Vallian et al., 2003). High concentrations of phenylalanine are especially harmful during early infancy. Untreated patients with PKU have lower brain weights, changes in myelin structure and less developed dendritic trees (Huttenlocher, 2000). Despite tremendous progress in the understanding of the molecular basis of PKU, the mechanism(s) whereby hyperphenylalaninaemia results in brain dysfunction are not known. We have recently demonstrated that acute applications of L-Phe, at a range of concentrations found in the PKU brain, selectively depress glutamate synaptic transmission in rat and mouse hippocampal and cerebrocortical cultured neurons by a combination of pre- and post-synaptic actions: (i) competition for the glycine-binding site of the N-methyl-D-aspartate (NMDA) receptors; (ii) attenuation of neurotransmitter release; and (iii) competition for the glutamate-binding site of (RS)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptor subtypes (Glushakov et al., 2002, 2003). In the PKU brain, glutamate receptors function under conditions of chronic hyperphenylalaninaemia. If the mechanisms that mediate the antigu glutamatergic effects of acute application of L-Phe in vitro are active in vivo in the PKU brain, then chronic impairment in functioning of the glutamate system could be evident at the level of glutamate receptor density and expression.

Therefore, we used a combination of liquid chromatography–tandem mass spectrometry (LC/MS/MS), patch-clamp, radioligand binding and western blot approaches to investigate the long-term changes at excitatory synapses in murine PKU (Pahenu2 mice).

Methods
All animal experiments were approved by the University of Florida Animal Care and Use Committee.

Pahenu2 mice
Two experimental groups were studied: (i) heterozygous and (ii) homozygous Pahenu2 (PKU) BTBR mice. Homozygous Pahenu2 mice were produced by crossing homozygous Pahenu2 fathers and heterozygous Pahenu2 mothers. Initial Pahenu2 breeding pairs (homozygous females and homozygous males) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained as a colony under specific pathogen free (SPF) conditions. The mice were on a normal (high L-Phe) diet and 6–10 weeks old at the time they were killed. Homozygous mice are initially distinguished from their heterozygous littermates by their lighter coat colour. The genotype is confirmed by determining concentrations of phenylalanine in plasma by LC/MS/MS and restriction fragment length polymorphism analysis. The Pahenu2 mutation creates a new Alw261 restriction site in exon 7 (McDonald and Charlton, 1997). Genomic DNA was extracted from blood samples and exon 7 of the Pah gene was amplified using primers in introns 6 and 7. The resulting 220-bp PCR product was then digested with Alw261. The restriction fragments were separated by electrophoresis on a 15% polyacrylamide gel at 90 V for 2 h.

Liquid chromatography–tandem mass spectrometry
The concentration of L-Phe in brain tissues was determined using LC/MS/MS (API 4000 LC/MS/MS system; Applied Biosystems, Foster City, CA, USA). Mice were deeply anaesthetized with Nembutal (80 mg/kg) and killed by cervical dislocation. Forebrains were quickly dissected on ice, frozen in liquid nitrogen and stored at −80°C. At the time of the experiment, brain tissue was homogenized in de-ionized water (0.5% w/v) and centrifuged at 10 000 g for 15 min. Samples were prepared from 200 μl of the supernatant together with 50 μl of 1.5 μM Phe.13C3 as internal standard. Proteins were precipitated with 800 μl of cold acidified acetonitrile (formic acid, trifluoroacetic acid, acetonitrile, v/v/v 0.4:0.2:100). The samples were vortex-mixed for 30 s and centrifuged at 10 000 g for 10 min. The clear supernatant was evaporated to dryness at 40°C. Dry residues were reconstituted in 200 μl of de-ionized water and aliquots (25 μl) were injected into the LC/MS/MS equipment for further analysis. Chromatographic separation was done on a C-18 column (4.5 × 100 mm, 3 μm particles, 24°C) using an isocratic mobile phase (1 mM trifluoroacetic acid in 1 1 H2O/acetonitrile, 93.1/6.9 v/v) at 0.6 ml/min. Detection was carried out in atmospheric pressure chemical ionization mode by multiple reaction monitoring of ion pairs mass/charge (m/z) 163.8/72.0 and m/z 170.1/152.6 for L-Phe and internal standard, respectively. Retention times were 3.45 min for both L-Phe and internal standard in processed samples. Quantitation of L-Phe was based on calibration curves generated by injecting known concentrations of L-Phe and internal standard. Quality control samples were included in every run.

Radioligand binding
On the day of the assay, tissue pellets were thawed in 5 mM Tris buffer (pH 7.4), washed three times (40 000 g, 30 min, 4°C), and resuspended in 5 mM Tris buffer. [3H]MK-801 (final concentration 2 nM) was used to selectively label the NMDA receptor complex. Maximal activation of the NMDA receptor complex was assured by the inclusion of 10 μM glutamate and 10 μM glycine. For saturation binding assays, 200 μl aliquots of [3H]MK-801-labelled membrane (100–150 μg protein) were incubated in the absence or presence of unlabelled MK-801 (0.6–25 nM) in a final volume of 0.5 ml 5 mM Tris buffer. Incubation time and temperature were 2 h and 25°C, respectively. Non-specific binding, defined as binding not displaced by an excess (50 μM) of unlabelled MK-801, typically represented <10% of total binding. Incubation was terminated by addition of 3 ml ice-cold buffer followed by vacuum filtration through Whatman GF/B glass-fibre filters presoaked in 0.3% (w/v) polyethyleneamine. Radioactivity trapped by the filters was determined using a liquid scintillation counter. Each assay was conducted in...
triplicate. The number of binding sites ($B_{\text{max}}$) and the dissociation constant ($K_d$) were determined using the radioligand binding analysis program GraphPad (v. 3.0; GraphPad Software, San Diego, CA, USA).

**Western blot analyses**

For membrane protein extraction, 50 mg samples of frozen forebrain tissue were rinsed twice with PBS, homogenized in 200 μl of extraction buffer (Mammalian Cell Lysis kit; Sigma, St Louis, MO, USA), and incubated for 30 min on ice. Tissue homogenates were centrifuged at 15 000 g for 10 min at 4°C. The supernatant was collected and stored at −80°C until the day of the experiment. The protein concentration of samples was determined using the BSA Protein Assay kit (Pierce, IL, USA). Protein concentrations were measured in triplicate.

Western blot analysis was used to detect and quantify proteins of interest. Membrane protein samples were heated to 100°C for 5 min in loading buffer containing 60 mM Tris–HCl, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 100 mM dithiothreitol and 0.01% (w/v) bromophenol blue, pH 6.8. Individual samples containing 20 μg of protein in 30 μl of loading buffer were subjected to electrophoresis on SDS–polyacrylamide 7.5% (w/v) Tris–HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA, USA) using a Bio-Rad Mini Protein apparatus. The protein samples were separated in the presence of running buffer containing 15 mM Tris base, 192 mM glycine and 20% (v/v) methanol, pH 8.3 (Bio-Rad Laboratories, Hercules, CA, USA). Protein transfer to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Little Chalfont, UK) was performed at 90 V for 1.5 h in the presence of ice-cold transfer buffer containing 25 mM glycine and 0.06% (w/v) SDS, pH 8.3, at room temperature for 1.5 h at 90 V. Protein transfer to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Little Chalfont, UK) was performed at 90 V for 1.5 h in the presence of ice-cold transfer buffer containing 25 mM Tris base, 192 mM glycine and 20% (v/v) methanol, pH 8.3 (Bio-Rad Mini Trans Blot system). Membranes were then treated with primary antibody at appropriate dilutions in PBST buffer with 5% milk overnight at 4°C. The primary antibodies (Upstate, Lake Placid, NY, USA) anti-Glu1, anti-Glu2/3, anti-NR2A, anti-NR2B and anti-GABA$_A$ α1 receptor were used in concentrations of 0.125–1 μg/ml. After washing three times in PBST buffer for 10 min, membranes were treated for 1.5 h at room temperature with goat anti-rabbit hors eradish peroxidase secondary antibodies (Upstate, NY, USA) diluted 1:10 000 in PBST buffer with 5% milk. Membranes were washed as above, incubated in Supersignal West Pico Chemiluminescent Substrate (Pierce) for 5 min at room temperature and developed using Kodak BioMax XR Film. Films were scanned using Digital Imaging System FluoroChem (Alpha Innotech Corporation, San Leonardo, CA, USA) and analysed (ImageJ v. 1.31; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Western blot analysis for tissue samples from each animal for each antibody treatment was repeated on three or four gels. The relative optical density of each antibody treatment of tissue samples from homozygous (PKU) animals with respect to the same antibody treatment in heterozygous animals was calculated according to equation 1. First, the mean value of optical densities of tissue samples from heterozygous animals was calculated for each antibody treatment for each gel (equation 2). Secondly, the relative optical density for each PKU tissue sample on separate gels was calculated by dividing the optical density of each PKU tissue sample by the mean optical density of tissue samples from heterozygous animals for this particular gel. Thirdly, the mean values of relative optical density for each PKU animal and for each antibody treatment was calculated for all gels. Fourth, the mean ± SEM value for all PKU animals for each antibody treatment was calculated. Because expression of GABA receptor α1 subunit was not significantly different between PKU and heterozygous mice, corrections for variations in loading between wells were made by dividing the equivalents for glutamate receptor subunits by the equivalents for GABA receptor α1 subunit from the same well.

$$\text{ROD} = 1 \frac{1}{n_{\text{PKU}}} \sum_{k=1}^{n_{\text{PKU}}} \left( \frac{1}{n_{\text{Gel}}} \sum_{j=1}^{n_{\text{Gel}}} \frac{PKU_{jb}}{Contr_{bj}} \right)$$

$$\text{Contr}_b = \sum_{k=1}^{n_{\text{Gel}}} Contr_{kb}$$

where ROD is the relative optical density, $n_{\text{PKU}}$ is the number of PKU animals, $n_{\text{Gel}}$ is the number of gels, PKU, is a tissue sample from a particular PKU animal, Contr$_b$ is the mean value for the tissue samples from all heterozygous animals on gel $b$ and $k$ is the number of tissue samples from heterozygous animals on gel $b$.

**Neuronal cultures**

Hippocampi were dissected from newborn rats, and treated with 0.25% trypsin to dissociate the cells using procedures previously described (Glushakov et al., 2002). Cells were resuspended in Neurobasal Medium containing B-27 serum-free supplement (Invitrogen Life Technologies, Carlsbad, CA, USA), and were plated in poly-l-lysine-coated 35 mm Nunc plastic tissue culture dishes (1.5 × 10$^6$ cells/dish/2 ml medium).

**Electrophysiological recordings**

Whole-cell voltage-clamp recordings of membrane ionic currents were conducted in neurons between 12 and 27 days in vitro as previously described (Glushakov et al., 2002, 2003). The basic extracellular solution contained (in mM): NaCl 140, KCl 4, CaCl$_2$ 2, MgCl$_2$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 10, and glucose 11. The pH of the extracellular solution was adjusted to 7.4 using NaOH. The main solution for filling the patch electrodes contained (in mM): Cs gluconate 135, NaCl 5, KCl 10, MgCl$_2$ 1, CaCl$_2$ 1, Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) 11, HEPES 10, Na$_2$ATP 2, and Na$_2$GTP 0.2 mM. The pH of the intracellular solution was adjusted to 7.4 using CsOH. The experiments were performed at room temperature (22–23°C) at a holding potential ($V_{\text{holding}}$) of −30 mV, and the extracellular solutions contained Mg$^{2+}$. $\text{Ni}_{\text{NMDA}}$ was recorded in response to a 6 s period of NMDA application. Various concentrations of NMDA, glycine and L-Phe were added to the extracellular solution according to the protocols described below. All compounds were purchased from Sigma. Current data were digitized on-line and analysed off-line using the software program pClamp9 (Axon Instruments, Foster City, CA, USA).

**General data analysis**

Values are reported as mean ± SEM. Prior to parametric testing, the assumption of normality was validated using the Kolmogorov–Smirnov test with Lilliefors’ correction (SSPS v. 10; SPSS, Chicago, IL, USA). Multiple comparisons among groups were analysed using analysis of variance (two- or one-way repeated measures with two- or one-way replication where appropriate) followed by Student–Newman–Keuls testing. Single comparisons were analysed using a two-tailed Student’s $t$ test. $P < 0.05$ was considered significant.
Results

Consistent with the genetic defect, \( Pah^{enu2} \) BTBR mice exhibited increased concentrations of L-Phe. Brain concentrations of L-Phe in homozygous \( Pah^{enu2} \) mice were almost six times higher than those in their heterozygous counterparts (Fig. 1). It is interesting that concentrations of L-Phe in the forebrains of heterozygous \( Pah^{enu2} \) mice were slightly higher than those in wild-type BTBR mice reported in the literature (Sarkissian et al., 2000).

To verify whether L-Phe inhibits glutamate receptor function at the range of concentrations found in the \( Pah^{enu2} \) mouse brain, we determined concentration–response relationships for glycine to activate \( I_{\text{NMDA}} \) in the presence of different concentrations of L-Phe (0, 1, 3, 10 and 30 mM). Increasing concentrations of L-Phe caused a parallel shift of the concentration–response relationship for glycine to activate \( I_{\text{NMDA}} \) towards greater concentrations of glycine (Fig. 2). At the same time, L-Phe neither decreased the maximal glycine-induced activation of \( I_{\text{NMDA}} \) nor altered the slope of the concentration–response relationships. Analysis of the concentration–response relationships by Schild regression yielded a linear response with a slope of 1.09 and an equilibrium constant (\( K_B \)) of 573 \( \mu \text{M} \). These findings are consistent with competitive antagonism of L-Phe at the glycine-binding site for glycine to activate \( I_{\text{NMDA}} \) in the presence of different concentrations of L-Phe (0.1, 3, 10 and 30 mM). Increasing concentrations of L-Phe caused a parallel shift of the concentration–response relationship for glycine to activate \( I_{\text{NMDA}} \) towards greater concentrations of glycine (Fig. 2). At the same time, L-Phe neither decreased the maximal glycine-induced activation of \( I_{\text{NMDA}} \) nor altered the slope of the concentration–response relationships. Analysis of the concentration–response relationships by Schild regression yielded a linear response with a slope of 1.09 and an equilibrium constant (\( K_B \)) of 573 \( \mu \text{M} \). These findings are consistent with competitive antagonism of L-Phe at the glycine-binding site.

Fig. 1 Measurement of the brain concentration of phenylalanine in heterozygous (+/−) and homozygous (−/−) \( Pah^{enu2} \) mice using LC/MS/MS. (A) Continuous bold lines represent the typical examples of the negative ion scan LC/MS/MS spectrum of phenylalanine extracted from the brain tissues of heterozygous (weight of the sample 38.6 mg) and \( Pah^{enu2} \) (weight of the sample 37 mg) mice, respectively. The narrow lines correspond to the multiple reaction monitoring pair of \( m/z \) 170.1 (parent ion) and \( m/z \) 152.6 (product ion) for the internal standard (\( ^{13}\text{C}_5 \) L-Phe). (B) Bar graphs showing the concentration of phenylalanine, measured in the forebrain of heterozygous (+/−) and homozygous (−/−) \( Pah^{enu2} \) mice. Data are means ± SEM of four to six brain samples. (−/−) versus (+/−): *\( P < 0.01 \).

Fig. 2 Inhibition of the NMDA (1 mM)-activated current by L-Phe. (A) Concentration–response curves to glycine (Gly) were carried out in rat cultured hippocampal neurons in the absence and presence of L-Phe (concentrations noted in figure). Peak \( I_{\text{NMDA}} \) was normalized to \( I_{\text{NMDA}} \) recorded at 10 \( \mu \text{M} \) Gly in the absence of L-Phe and plotted against the concentration of glycine [Gly]. Endogenous Gly in primary neuronal cultures explains the non-zero \( I_{\text{NMDA}} \) in the absence of added Gly. (B) Dose ratios (dr) were calculated from [Gly] producing equal responses (60% of activation) in the presence of L-Phe divided by [Gly] with the same response obtained without L-Phe. These dr(s) were then applied to the Schild equation: \( \log (\text{dr}-1) = \log [\text{L-Phe}] - \log K_B \), where \( K_B \) is the equilibrium dissociation constant for L-Phe.
site of the NMDA receptor. Given that the glycine-binding site of the NMDA receptors within a synapse is typically not saturated (Bergeron et al., 1998), L-Phe may effectively attenuate NMDA receptor function in the PKU brain. Together with the previously reported IC 50 of 0.98 mM for L-Phe to attenuate non-NMDA receptor function in cultured neurons (Glushakov et al., 2003), these results suggest that endogenous L-Phe may effectively depress glutamatergic synaptic transmission in the Pah enu2 mouse brain.

If L-Phe depresses glutamate receptor function in vivo, then chronic exposure to high levels of endogenous L-Phe should cause long-term changes in glutamate receptor density and expression. In order to test this hypothesis, receptor density and receptor subunit composition were studied by radioligand binding using [3H]MK-801 and by western blot analyses, respectively. The forebrain tissue used in these experiments was isolated from the same homozygous and heterozygous Pah enu2 mice in which measurement of brain concentration of L-Phe was performed. The maximum binding (B max) and affinity (K D) data for [3H]MK-801 binding sites of mouse forebrain for the two groups are presented in Fig. 3. The maximum binding for [3H]MK-801 (NMDA receptor density) was significantly greater in homozygous PKU mice than in heterozygous littermates (P < 0.005), whereas the affinity for the radioligand was not different between these groups (P = 0.1). These findings are consistent with compensatory up-regulation of NMDA receptors in the presence of the competitive antagonist L-Phe. In agreement with the radioligand binding data, expression of the NR2A subunit of the NMDA receptors in the forebrain of homozygous Pah enu2 mice was significantly increased compared with that of heterozygous Pah enu2 mice. Surprisingly, the expression of NMDA receptor NR2B subunits was diminished in PKU mice (Fig. 4A, B).

In neuronal cultures, acute application of L-Phe also significantly depresses glutamate release and functioning of non-NMDA glutamate receptors, but does not affect activity of GABA receptors (Glushakov et al., 2003). Therefore, it is plausible to expect that L-Phe, by depressing non-NMDA glutamate receptor functioning, may elicit a compensatory change in expression of these receptors in vivo in the PKU brain. GABA receptors, on the other hand, should be less affected during PKU. In order to test these possibilities, a western blot analysis of GluR1 and GluR2/3 subunits of AMPA receptors and α1 subunits of GABA receptors was performed. Consistent with the functional effects of L-Phe on non-NMDA glutamate receptors and GABA receptors in vitro (Glushakov et al., 2003), the expression of both GluR1 and GluR2/3 subunits was significantly increased in homozygous Pah enu2 mice (Fig. 4A, B), whereas the expression of GABA receptor α1 subunit was not different in the homozygous and heterozygous mice (Fig. 4C).

**Discussion**

The results obtained indicate that glutamatergic synaptic transmission in the hyperphenylalaninaemic PKU brain has undergone marked alterations. There is much in vitro, in vivo and clinical evidence that abnormal glutamatergic synaptic transmission contributes to a variety of brain disorders, which are phenotypically similar to those observed in PKU (e.g. cognitive impairment, seizures, microcephaly). Thus, the observed changes in the glutamatergic system in the Pah enu2 mouse brain provide new insights into the understanding of the cellular mechanisms that may mediate PKU-specific brain disorders.

Identification of the major causes of brain dysfunctions in PKU and an understanding of the cellular mechanisms that mediate these dysfunctions rely heavily on precise measurements of phenylalanine concentrations in the brains of PKU subjects. Measurements in early-treated patients indicate that,
following an oral load, the L-Phe concentration varies and may increase up to as much as 0.8 mM (Moller et al., 2003). Several lines of evidence suggest that the available measurements of brain phenylalanine concentration in PKU patients may be an underestimation (Pietz et al., 1999; Weglage et al., 2002). The difficulties of measuring brain phenylalanine concentrations in PKU patients are essentially overcome in Pahenu2 (PKU) mice. By using these laboratory animals, age, genetic, dietary and other experimental conditions can be controlled. In addition, the variability of the results obtained, due to insufficient signal-to-noise ratios, can be significantly diminished by performing steady-state measurements. Indeed, we found that brain concentrations of phenylalanine in all homozygous PKU mice were markedly increased and in agreement with concentrations reported by others (Sarkissian et al., 2000; Joseph and Dyer, 2003). This concentration of phenylalanine is close to the IC50 for L-Phe for depressing the frequency of AMPA/kainate receptor-mediated mEPSCs (980 ± 130 μM) (Glushakov et al., 2003) and substantially higher than the Kd of L-Phe to compete for the glycine-binding site of NMDA receptors (576 μM; Fig. 2). The changes in glutamatergic but not GABAergic transmission in PKU mice are in agreement with the depressant effect of L-Phe on glutamate receptor function and neurotransmitter release in the neuronal cultures (Glushakov et al., 2002, 2003), and correlate with the significant increase in brain concentration of phenylalanine in the same animals measured by LC/MS/MS (Fig. 1). These findings, together with the

![Fig. 4](image-url) Changes in protein expression of NMDA receptor NR2A and NR2B and AMPA receptor Glu1 and Glu2/3 subunits in the forebrain of homozygous (PKU) Pahenu2 mice. (A) Representative western blot (WB) images of NR2A, NR2B, Glu1 and Glu2/3 subunits in the forebrain of homozygous and heterozygous Pahenu2 mice; the left-hand set is from heterozygous (+/-) mice and the right-hand set is from the same brain area isolated from homozygous (-/-) Pahenu2 mice. Western blots for all receptor subunits were performed on forebrain tissue isolated from the same heterozygous and homozygous mice. (B) Histogram showing results of the densitometric analysis of western blots for NR2A, NR2B, Glu1 and Glu2/3 subunits. Densities of NR2A, NR2B, Glu1 and Glu2/3 blots from heterozygous mice were taken as 100%. (C) The results of western blot analysis for the α1 subunit of GABA_A receptor isolated from the same heterozygous and homozygous mice as for NMDA receptor NR2A and NR2B and AMPA receptor Glu1 and Glu2/3 subunits. Data are means ± SEM of four to six brain samples. (-/-) versus (+/-): *P < 0.01; †P < 0.05.
fact that the non-tyrosine metabolites of phenylalanine, at PKU-relevant concentrations, do not affect glutamatergic synaptic transmission in neuronal cultures (Glushakov et al., 2003), indicate that elevated L-Phe is probably a major cause of the changes in the glutamatergic system in the PKU brain observed in this study. It is important to stress that L-Phe itself is probably a major cause of brain dysfunction in PKU. Thus, PKU-specific neurological symptoms can largely be prevented by a strict diet that is low in phenylalanine, but not a relaxed diet containing elevated concentrations of tyrosine (Scriver and Kaufman, 2001).

The alteration in glutamate receptors in the PKU brain has been found at the level of NMDA receptor binding and of NMDA and AMPA receptor subunit expression. In particular, consistent with the depressant effect of L-Phe on glutamate receptor function in neuronal cultures, receptor binding experiments showed significant up-regulation of NMDA receptor density in PKU mice. The expression of NMDA receptor NR2A subunit and AMPA receptor subunits Glu1 and Glu2/3 were also significantly increased. In contrast, expression of the NR2B subunit was decreased. These differential changes in expression of NMDA receptor subunits NR2A and NR2B in the PKU mouse brain may be explained by the mechanism whereby L-Phe depresses NMDA receptor function. L-Phe competes for the glycine-binding site of NMDA receptors (Fig. 2) (Glushakov et al., 2002, 2003). The potency of glycine as a co-agonist at expressed NMDA receptors has been shown to be as much as 10-fold higher for receptors containing NR2B subunits than for those containing NR2A subunits (Priestley et al., 1995). Similarly, Kew and colleagues showed that NMDA receptors in cortical neurons of young rats, which express a relatively greater amount of NR2B, had higher affinity for glycine than receptors in neurons of older rats (Kew et al., 1998). Because L-Phe is a relatively weak antagonist of NMDA receptors at the glycine binding site, it is likely to depress more potently NMDA receptors containing NR2A subunits that have lower affinity for glycine. Greater depression by L-Phe of NR2A-containing NMDA receptors would cause a greater compensatory reaction, which would result in an increased expression of NR2A-containing NMDA receptors. In conditions of permanent depression of NR2A-containing NMDA receptors, the relative contribution of NR2B-containing receptors may increase. This may cause a compensatory decrease in NR2B receptor expression. During CNS development the NR2B subunit is more highly expressed in the embryonic and neonatal brain, and expression of the NR2A subunit increases as the brain matures (Watanabe, 1996). The increase in NR2A/NR2B proportion represents premature ‘ageing’ of NMDA receptors in the PKU brain, and may result in altered pharmacological and functional profiles of the NMDA system in the PKU brain. In particular, Tang and colleagues demonstrated that overexpression of NMDA receptor 2B in the forebrains of transgenic mice leads to enhanced learning ability and memory with respect to wild-type mice (Tang et al., 1999). Therefore, we may speculate that the decrease in NMDA NR2B receptor expression in the PKU brain may contribute to PKU-specific impairment of learning and memory.

It has been reported that the brain concentration of glycine in PKU infants is less than 17% of normal (Quentin et al., 1974a). The cerebrospinal fluid glycine concentration of older PKU children and adults was within the normal range (Quentin et al., 1974a, b). Therefore, NMDA receptor function can be more severely depressed by L-Phe in the brains of young PKU subjects, whereas this effect of L-Phe is lessened later on in life. A greater depressant effect of L-Phe on NMDA receptor function in the developing brain would be in an agreement with the observed greater damage caused by hyperphenylalaninaemia during early infancy in PKU (Scriver and Kaufman, 2001).

Our preliminary unpublished observations indicate that, despite the compensatory up-regulation of glutamatergic synaptic transmission in the continued presence of elevated concentrations of L-Phe, the total functional response of glutamate receptors in the PKU brain is still depressed. Therefore, the pathophysiological changes produced by chronic L-Phe exposure could involve effects induced by both exposure to and withdrawal of L-Phe. For example, we hypothesize that a combination of factors may occasionally lead to potentiation of glutamate receptor activity and the generation of epileptic activity, one of the brain-related disorders in PKU. These factors include: (i) increase in expression and density of glutamate receptors during chronic exposure to L-Phe; (ii) increase in concentrations of glycine in the brain of non-infant PKU patients; and (iii) a decrease in the brain concentration of L-Phe caused by variations in diet and/or age-dependent changes (withdrawal effect).

The alterations in the expression and function of NMDA and AMPA receptors in the developing hyperphenylalaninaemic brain may have a substantial impact on synaptic plasticity and brain function. The impairment of glutamate receptor activity by L-Phe may explain the observation that untreated patients with PKU have lower brain weights, reduced dendritic arborization and decreased numbers of dendritic spines (Huttenlocher, 2002). The cellular mechanisms of brain disorders characteristic of PKU are probably complex and multifaceted. Specific investigation of the role of L-Phe-induced alterations in glutamatergic synaptic transmission in the functional and morphological plasticity of synapses, involving the dopaminergic and other systems, and in the behavioural changes of PKU mice should advance understanding of this phenomenon and of the roles of glutamatergic synaptic transmission in brain functioning.

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