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The Effect of Hyperphenylalaninaemia on the Muscarinic Acetylcholine Receptor in the HPH-5 Mouse Brain

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Summary: Previous studies on the effect of hyperphenylalaninaemia on the development of the muscarinic acetylcholine receptor in the cerebrum of the rat, using α-methylphenylalanine-induced hyperphenylalaninaemia, have shown a gradual and steady decrease in the number of binding sites for this neurotransmitter. The HPH-5 mouse, a phenylalanine hydroxylase mutant, can be hyperphenylalaninaemic without the use of a hydroxylase inhibitor. By employing quantitative autoradiography using [^{3}H]quinuclinidylbenzilate to label muscarinic acetylcholine receptors, a refined analysis of this decrease in neurotransmitter binding sites can be made. The decrease was confirmed and is therefore due to the hyperphenylalaninaemia *per se* and not to the use of the inhibitor. Various areas of the brain reacted differently to hyperphenylalaninaemia, from no change (putamen) to a gradual decrease (external layer of the olfactory bulb, parietal, occipital and cingulate areas of the cerebral cortex, CA_1 and CA_3 layer of the hippocampus) to a decrease preceded by a transient increase (frontal area of the cerebral cortex, caudate nucleus).

The extent of these changes depends on the duration of exposure to hyperphenylalaninaemia as well as on the degree of brain maturation, but can even be observed in the brain of the adult mouse on a hyperphenylalaninaemic regimen for 11 days.

Since the hippocampus has been shown to be involved in the long-term storage of information, damage to this structure by hyperphenylalaninaemia may provide a clue to the global mental retardation observed in untreated PKU.

Phenylketonuria (PKU; L-phenylalanine, tetrahydrobiopterin: oxygen oxidoreductase (4-hydroxylating) (EC 1.14.16.1) deficiency, McKusick 261600) is an inborn error of metabolism associated with mental retardation when untreated. The relatively small number of brains of patients with PKU analysed show a decrease in myelin (Alvord

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et al 1950; Jervis 1954; Scholz 1957; Poser and Van Bogaert 1959; Crome 1962; Malamud 1966; Shah et al 1972; Baumann and Kemper 1982; Kornguth et al 1992), as well as more specific changes in certain areas as far as investigated, such as a reduction in the size of the Meynert pyramidal cells of the visual cortex and Bitz cells of the motor cortex (Baumann and Kemper 1982; Takashima et al 1991), neuronal losses in the right lateral geniculate nucleus, the visual cortex, the frontal cortex and the hippocampus (Kornguth et al 1992) and a reduction in the number of dendritic processes on the Purkinje cells (Kornguth et al 1992).

The application of magnetic resonance imaging (MRI) to treated PKU patients has shown abnormalities, predominantly in the white matter of the posterior cerebral hemispheres (Thompson et al 1990, 1991; Pearsen et al 1990; Bick et al 1991) even when these patients were on an optimally controlled dietary regimen (Lou et al 1992). Although it has been reported that there is no correlation between the degree of the MRI changes and the IQ scores (Pearsen et al 1990), deficits in pattern-recognition tests have been observed in well-controlled PKU patients (Lou et al 1992), suggesting that the white matter changes may be consequential for optimal neurophysiological performance.

A hypothesis that could explain these diverse observations has been proposed (Hommes and Matsuo 1987; Hommes 1991). Central in this hypothesis is the inhibition of phenylalanine of ATP-sulphurylase (ATP:sulphate adenylyltransferase, EC 2.7.7.4; Hommes, 1985; Hommes and Matsuo, 1988), leading to a decreased synthesis of sulphatides. A lower level of sulphatides provides a lower degree of protection for myelin basic protein towards proteolytic degradation, resulting in an increased turnover of myelin, not compensated by an increased rate of synthesis (Hommes and Moss 1992). Loss of neurons will be a consequence, thereby decreasing the degree of neuronal connectivity. Experimental evidence for each of the steps of the hypothesis is available (for review see Hommes and Matsuo (1987) and Hommes (1991)). The evidence for the last steps of the sequence of events leading to brain dysfunction in hyperphenylalaninaemia, i.e. fewer synaptic contacts, is the least liberal. Synaptic densities are difficult to quantitate by electron-microscopic methods (Huttenlocher and de Courten, 1987). Moreover, synaptic information transmission may not be the only mode of information flow; parasynaptic intercellular communication may play an important role as well (Herkenham 1987). Both systems use neurotransmitter receptors as part of the mechanism of information transmission. A determination of neurotransmitter receptor density in hyperphenylalaninaemia may therefore contribute to the evidence for the last steps of the proposed hypothesis. The present communication describes such experiments using an animal model of phenylalanine hydroxylase deficiency, the HPH-5 mouse (McDonald et al 1990). Some of the data have been presented in preliminary form (Hommes 1993).

MATERIALS AND METHODS

The HPH-5 mouse, obtained from J.D. McDonald (McDonald et al 1990), had unlimited access to food, consisting of Wayne Mouse Breeder Blox 8626 during breeding and gestation and Wayne Rodent Blox 8604 after weaning and during maintenance (Wayne Laboratory Animal Diets, Madison, WI, USA). The mice had

unlimited access to drinking water, which consisted of normal tap water during breeding and gestation and of a phenylalanine solution (25 g/L) to induce hyperphenylalaninaemia. The age at which treatment was started and the duration of treatment is given in the tables of the Results section. Mice were anaesthetized by intraperitoneal injection of pentobarbital (60 μg/g body weight) and decapitated; the brains were carefully removed and mounted for coronal sectioning on a cryostat chuck, using solid carbon dioxide. Blood was collected for amino acid analysis by quantitative column chromatography using a Beckman 7300 system. Quantitative autoradiography was carried out as described (Kuhar 1985).

Serial sections of 20 μm thickness were thaw-mounted on gelatin chromic alum-coated slides using a Bright cryostat. Chuck-mounted brains, when not sectioned immediately, were stored at -80°C. Slide-mounted sections, when not further processed immediately, were stored at -20°C and used within 3 days.

Binding to brain membrane fractions was carried out as described previously (Matsuo and Hommes 1988). To verify binding conditions further, a brain paste was made by homogenizing 1 part mouse brain with 1 part homogenizing buffer (w/v) containing 0.13 mol/L NaCl, 0.01 mol/L KCl, 0.002 mol/L $MgCl_2$, 0.01 mol/L Na_2HPO_4, pH 7.3 (buffer A) using a Dounce homogenizer. The paste was similarly mounted on a chuck and sectioned. Brain sections were incubated at room temperature in buffer A supplemented with 2 nmol/L [^{3}H]quinuclinidylbenzilate, QNB (specific activity 38.8 Ci/mmol; Amersham, Arlington Heights, IL, USA), in the absence or in the presence of 10 μmol/L atropine to determine non-specific binding. No measurable non-specific binding was observed under these conditions in the autoradiography experiments. The sections were rinsed twice in ice-cold buffer A and then incubated for 1.5 h at 0°C in buffer A. The brain paste sections were scraped from the slide and rinsed with 1 ml of distilled water into scintillation vials. After vigorous vortexing for 1 min, 0.3 ml was removed for protein assay by the method of Bradford (1976); the remainder was used for counting by liquid scintillation spectrometry.

The brain tissue sections were exposed to Hyperfilm-^{3}H (Amersham) for 1 week at 0°C, after which they were stained with the modified Harris-type haematoxylin stain (Sigma, St. Louis, MO, USA). Commercial tritium standards (Amersham) were included with each film for quantitation of the autoradiograms. Films were developed with developer D19 (Eastman Kodak, Rochster, NY, USA). The Zeiss IBAS/SEM system was used for quantitation of the autoradiograms. Identification of the anatomical areas of the brain was facilitated by the use of the atlas of Sidman et al (1971).

Dopamine D_1 receptors in brain membrane fractions were determined as described for the muscarinic acetylcholine receptor, using SCH 23390 (specific activity 72 Ci/mmol; Amersham, Arlington Heights) and 30 nmol/L spiperone (Research Diagnostics, Flanders, NJ, USA) to block both dopamine D_2 and serotonin-5HT2 receptors. Non-specific binding was assessed by carrying out the experiments in the presence of 10 μml/L fluphenazine, which was a gift of Bristol-Meyers-Squibb, Princeton, NJ, USA).

Statistical analyses were done by Student's *t*-test.

RESULTS

Binding of QNB to muscarinic acetylcholine receptors has been demonstrated in many studies (cf. Kuhar and Yamamura 1975, 1976; Pauly et al 1991). To verify the conditions of measurement, a brain membrane preparation was incubated with increasing concentrations of [^{3}H]QNB in the absence and in the presence of 10 μmol/L atropine to evaluate non-specific binding. The results are shown in Figure 1A for this binding for a brain membrane preparation of the HPH-5 mouse on normal drinking water and on phenylalanine-containing drinking water. When a Scatchard plot was constructed from these data, an association constant of 0.41 nmol/L was found for the membrane fraction prepared from the brains of the HPH-5 mouse on normal drinking water as compared to a value of 0.48 nmol/L for the same fraction prepared from the brain of the hyperphenylalaninaemic HPH-5 mouse (Figure 1B). Maximum binding was found to be 2570 fmol/mg protein and 2140 fmol/mg protein, respectively. The hyperphenylalaninaemic HPH-5 mouse therefore shows a decreased binding with the same association constant. Similarly, when brain paste sections were incubated with [^{3}H]QNB at various concentrations, saturation was observed at QNB concentrations higher than 1 nmol/L (Figure 2). A

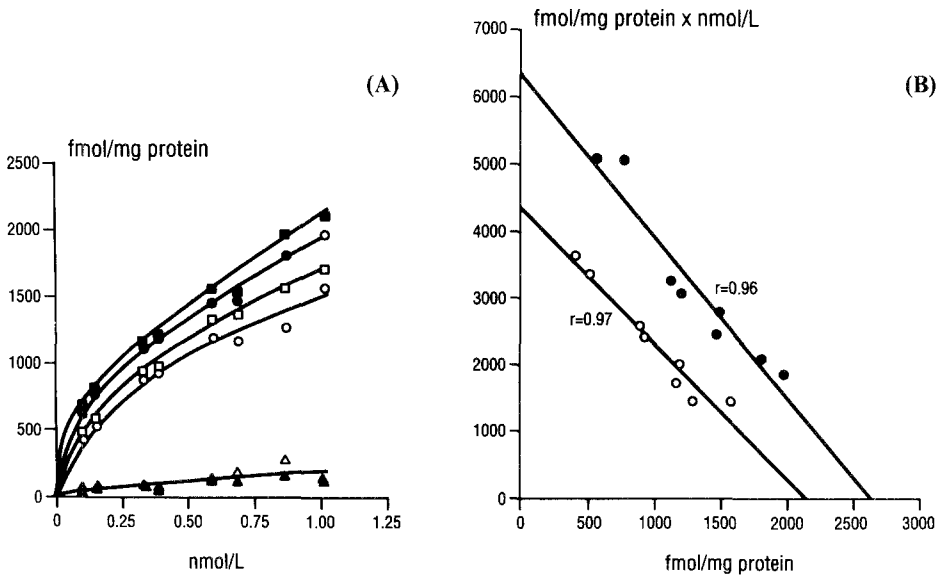

Figure 1 Binding of [^{3}H]QNB to brain membrane preparations of the 122-day-old HPH-5 mouse on normal drinking water (solid symbols) and of the 122-day-old HPH-5 mouse on phenylalanine-containing drinking water from age 22 days (open symbols). Brain membrane fractions were prepared from 5 mouse brains. Each point represents the mean of 3 experiments. (A) Total binding (□), non-specific binding (△) and the difference between total and non-specific binding (○). (B) Scatchard plot constructed from the data for specific binding. The straight line was obtained by the method of least squares. The association constant and maximum binding were 0.41 nmol/L and 0.48 nmol/L and 2570 fmol/mg protein and 2140 fmol/mg protein for normal HPH-5 and hyperphenylalaninaemic HPH-5 mouse, respectively

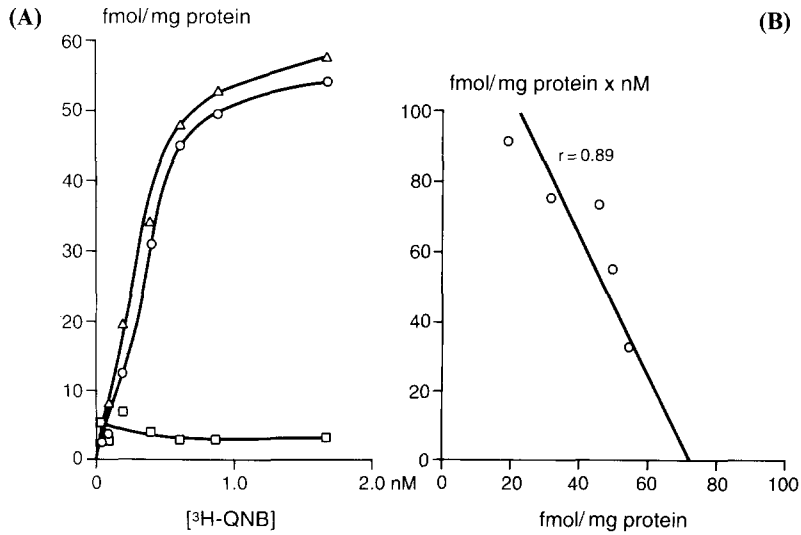

Figure 2 (A) Total binding (△), non-specific binding (□) and specific binding (○), i.e. the difference between total and non-specific binding, of [^{3}H]QNB to sections of whole brain paste of the HPH-5 mouse. Non-specific binding was determined in the presence of 10 μmol/L atropine. Each point represents the mean of 3 experiments. (B) Scatchard plot constructed from the data for specific binding. The straight line was obtained by the method of least squares. The association constant for binding was found to be 0.4 nmol/L, the maximum binding 309 fmol/mg protein

Scatchard plot for the specific binding showed an association constant of 0.4 nmol/L. A QNB concentration of 4 nmol/L was therefore chosen for the subsequent experiments. Saturation of the binding sites at this QNB concentration was obtained after 1.5–2 h of incubation, while loose binding of the label could be removed in 1.5 h of washing (Figure 3). An incubation time of 2 h for the labelling and a wash period of 1.5 h was therefore chosen for the subsequent experiments. These data are in agreement with previous observations on binding of QNB to the muscarinic acetylcholine receptor (cf. Kuhar 1985) and with data obtained on the α-methylphenylalanine-induced hyperphenylalaninaemia in rats (Matsuo and Hommes 1988). These experiments were carried out with a brain paste prepared from the HPH-5 mouse on normal drinking water. Since no difference was observed in the association constant with hyperphenylalaninaemic HPH-5 mouse, the experimental conditions apply equally to the hyperphenylalaninaemic HPH-5 mouse.

An example of the binding of [^{3}H]QNB in the brain of the HPH-5 mouse is shown in Figure 4. Differences in intensity of labelling are clearly visible, especially in the hippocampus. It has been shown that the hippocampus plays an essential role in long-term memory in humans, monkeys, rats and mice (Milner et al 1968; Morris et al 1982; Parkinson et al 1988; Eichenbaum et al 1989; Dias-Granadas and Amsch 1992). Table 1 shows that in particular the CA_3 layer of the hippocampus shows a significant decrease in muscarinic acetylcholine receptors as a result of exposure to a hyperphenylalaninaemic condition. It is not limited to the CA_3 layer but can also

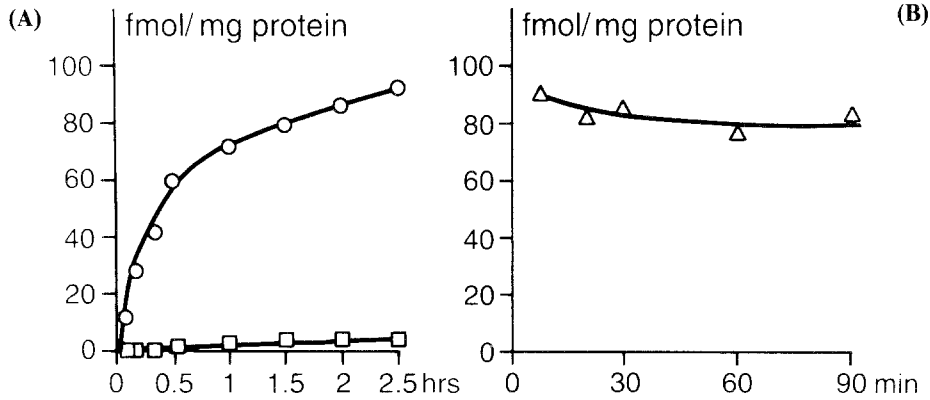

Figure 3 (A) Total binding (○) and non-specific (□) binding of [^{3}H]QNB to sections of whole brain paste of the HPH-5 mouse as a function of the incubation time. Non-specific binding was determined in the presence of 10 μmol/L atropine. Each point represents the mean of 3 experiments. (B) Specific binding as a function of the washing time. Sections of brain paste were first incubated for 2 h in the presence of [^{3}H]QNB at room temperature, then transferred to the same buffer without [^{3}H]QNB at 0°C. Each point represents the mean of 3 experiments

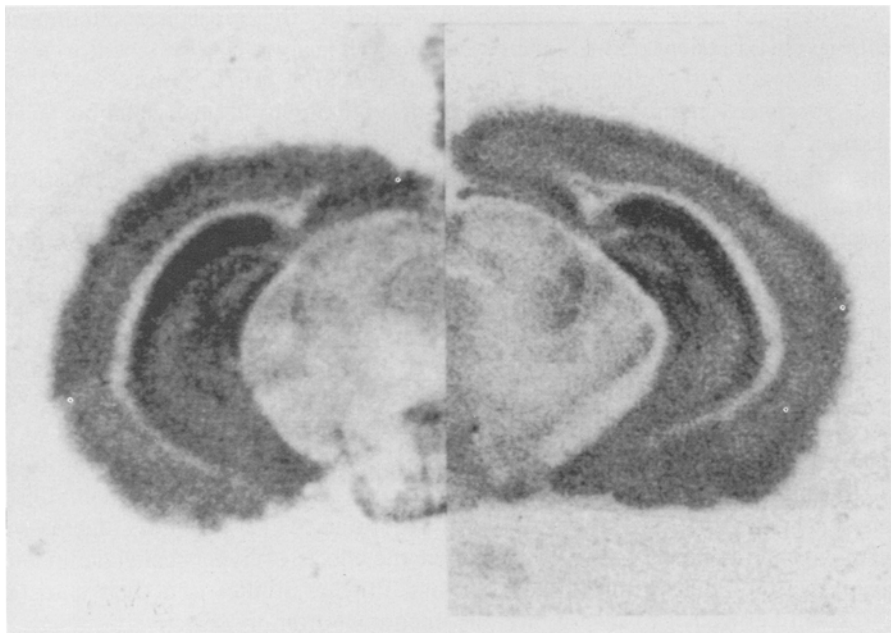

Figure 4 Binding of [^{3}H]QNB to a coronal section of the brain of the HPH-5 mouse, aged 98 days and raised on normal drinking water (left, blood phenylalanine 150 μmol/L) and aged 90 days and on phenylalanine-containing drinking water from age 22 days (right, blood phenylalanine 1092 μmol/L). This section corresponds most closely to section 361 of the atlas of Sidman et al (1971)

Table 1 Binding of [^{3}H]QNB to the hippocampus of the HPH-5 mouse[a]

	Age in days (days on phenylalanine)			
	52 (30)		*67 (45)*	
	Plasma phenylalanine (μmol/L)			
Area of hippocampus	*Experimental 540*	*Control 193*	*Experimental 779*	*Control 267*
CA_1 layer	485 ± 65 (5)	766 ± 78 (9)	738 ± 79 (12)*	807 ± 40 (6)
CA_2	668 ± 58 (10)	681 ± 117 (11)	628 ± 96 (11)	648 ± 31 (6)
CA_3 layer	624 ± 53 (15)*	704 ± 123 (14)	649 ± 72 (12)*	745 ± 38 (6)

[a]Values in fmol/mg are given as mean ± SD with number of measurements in parentheses
*$p < 0.05$ as compared to respective control by Student's t-test
Newborn mice were raised for 22 days under normal conditions, then had access to phenylalanine-containing drinking water. Two brains of experimental and of control of both ages were analysed

be observed in the CA_1 layer, although it apparently takes a longer time of exposure to hyperphenylalaninaemia to develop.

In some areas of the brain a decrease in muscarinic acetylcholine receptor density is preceded by a transient increase, as is illustrated in Table 2 for the frontal area of the cerebral cortex and the caudate nucleus, while still other brain structures, such as the putamen (Table 2) are seemingly unaffected by the hyperphenylalaninaemic condition. This transient phase of increased muscarinic acetylcholine receptor density may be age related. It could not be observed in the adult HPH-5 mouse for 11 days on the hyperphenylalaninaemic regimen (resulting in a plasma phenylalanine level of 1315 μmol/L).

There is a general decrease in the cerebral cortex in the density of muscarinic acetylcholine receptors (Table 3). Transient increases were not observed for these areas, although the time between initiation of the hyperphenylalaninaemic condition and sampling may have been too long.

A similar decease was observed in the external layer of the olfactory bulb (Table 2) without any apparent transient increase.

Such changes in neuroreceptor density are not necessarily limited to the muscarinic acetylcholine receptor (cf. Matsuo and Hommes 1988). The dopamine D_1 receptor shows an increase (Table 4). When the binding of SCH 23390 was measured in the absence of spiperone, the sum of the dopamine D_1, dopamine D_2 and serotonin-5HT2 receptor is measured. When the dopamine D_1 is subtracted from this, it becomes evident that dopamine D_2 plus serotonin-5HT2 receptors decrease in hyperphenylalaninaemia. This illustrates that the effects of hyperphenylalaninaemia can vary between neurotransmitter receptors. Further studies and their statistical analyses will be needed to investigate this phenomenon more selectively.

DISCUSSION

There are at least four, and possibly five, different muscarinic acetylcholine receptor genes (Bonner et al 1987; Peralta et al 1987; Wall et al 1992a). All subtypes, with the possible exception of m-5 (Wall et al 1992a), are expressed to similar extents in whole rat brain and all bind the antagonists QNB and atropine with binding constants well

Table 2 Binding of [^{3}H]QNB to areas of the brain[a]

	Age in days (days on phenylalanine)					
	43 (12)		*59 (28)*		*77 (46)*	
	Plasma phenylalanine (μmol/L)					
Brain area	*Experimental 1328*	*Control 139*	*Experimental 918*	*Control 137*	*Experimental 568*	*Control 223*
External layer of olfactory bulb	503 ± 160 (16)*	587 ± 206 (8)	ND	ND	448 ± 92 (17)	590 ± 120 (36)
Frontal cerebral cortex	528 ± 28 (4)	495 ± 21 (6)	642 ± 25 (4)**	544 ± 60 (6)	465 ± 35 (10)***	493 ± 33 (10)
Caudate nucleus	935 ± 96 (9)*	701 ± 97 (8)	634 ± 40 (40)	599 ± 58 (4)	501 ± 7 (5)*	607 ± 36 (6)
Putamen	593 ± 27 (12)	584 ± 41 (12)	711 ± 78 (7)	773 ± 63 (10)	742 ± 72 (20)	779 ± 60 (7)

[a]Values in fmol/mg are given as mean ± SD with number of measurements in parentheses
*$p < 0.01$, **$p < 0.02$, ***$p < 0.025$ as compared to respective control by Student's t-test
ND = not determined
Newborn mice ($n = 2$) were raised for 31 days under normal conditions, then placed on phenylalanine-containing drinking water. Two controls of same ages were also analysed

Table 3 Binding of [^{3}H]QNB to areas of the cerebral cortex of the HPH-5 mouse[a]

	Age in days (days on phenylalanine)			
	52 (30)		*67 (45)*	
	Plasma phenylalanine (μmol/L)			
Area of cerebral cortex	*Experimental 540*	*Control 193*	*Experimental 779*	*Control 267*
Parietal	627 ± 41 (30)*	694 ± 78 (24)	554 ± 47 (24)*	635 ± 78 (24)
Occipital	657 ± 48 (28)***	687 ± 63 (71)	593 ± 80 (36)*	663 ± 72 (34)
Cingulate	601 ± 67 (57)*	665 ± 87 (24)	574 ± 71 (38)**	628 ± 44 (16)

[a]Values in fmol/mg are given as mean ± SD with number of measurements in parentheses
*$p < 0.001$, **$p < 0.005$, ***$p < 0.01$ as compared to respective control by Student's t-test
For further details see the legend to Table 1

Table 4 Binding of SCH 23390 to membrane fractions of the HPH-5 mouse

	Experimental		*Control*	
Receptor	B_{max}	K_d	B_{max}	K_d
Dopamine D_1 + D_2 + serotonin 5HT2	470	0.46	520	0.46
Dopamine D_1	260	0.72	160	0.24
Dopamine D_2 + serotonin 5HT2	210		360	

Incubations (in triplicate) were carried out with the same brain membrane fraction as used in Figure 1, at a protein concentration of 0.15 mg/ml. SCH 23390 concentrations ranging from 0.1 to 2.5 nmol/L were used, in the absence and in the presence of 30 nmol/L spiperone to assay the sum of the dopamine D_1, D_2 and serotonin 5HT2 receptors and dopamine D_1 receptor, respectively. Non-specific binding was determined with 10 μmol/L fluphenazine. Maximum binding (B_{max}, in fmol/mg protein) and dissociation constants (K_d, in nmol/L) were determined from Scatchard plots

below the concentrations of these compounds used in the present study (Peralta et al 1987). The sum of the densities of all muscarinic acetylcholine receptor subtypes has therefore been determined in the present investigation. The HPH-5 mouse on normal drinking water was used as the control, rather than the BTB-R mouse from which the HPH-5 mouse was derived (McDonald et al 1990). The reason for using this control is the observation that interstrain differences in muscarinic cholinergic receptor density do exist (Schwab et al 1992). Any differences in muscarinic acetylcholine receptor density are therefore judged against the same genetic background and therefore are presumably due to the hyperphenylalaninaemic condition.

The long-term effect of hyperphenylalaninaemia seems to be a decrease in muscarinic acetylcholine binding sites in specific areas of the brain, while other areas are seemingly unaffected. Quantitative autoradiography using tritium-labelled ligands suffers from the problem of differences in absorption of the β-emission by various tissues. It is known that grey matter absorbs more than white matter (Geary et al 1985). Values for white matter therefore tend to be underestimated. Our previous studies have shown an increased turnover of myelin, not compensated by an increased rate of synthesis, resulting in a net loss of myelin (Berger et al 1980; Hommes et al 1982; Taylor and Hommes 1982), while many studies have shown decreases in lipids, both in human PKU as well as in animal models (cf. Gaull et al 1975). Removal of

lipids eliminates the regional quenching differences (Herkenham and Sokoloff 1984). The decreases in density of the muscarinic acetylcholine receptors as observed in the present study are therefore unlikely to be due to increased quenching in the brains of the hyperphenylalaninaemic mice. The present study confirms, therefore, the earlier studies on homogenates of cerebral cortex of the α-methylphenylalanine-induced hyperphenylalaninaemic rat (Matsuo and Hommes 1988) and demonstrates that the changes observed earlier are due to the hyperphenylalaninaemic state *per se* and not to the inhibitor of the phenylalanine hydroxylase used to induce the hyperphenylalaninaemic state. In addition, it provides more anatomical detail as to where these decreases do occur.

Preceding the decrease in some areas of the brain was a transient overexpression of the receptor. Upregulation of receptor gene expression as a response to interference with synaptic transmission has been demonstrated for the muscarinic acetylcholine receptor. Harrison et al (1991) documented an increased muscarinic acetylcholine receptor mRNA in Alzheimer's disease, although it is predominantly the nicotinic acetylcholine receptor that is decreased in this condition (Whitehouse et al 1982). Chronic atropine treatment upregulates the muscarinic m1 receptor mRNA (McKinney and Robbins 1992) as well as the other muscarcinic receptor mRNAs (Wall et al 1992b). The precise biological substrates for mental dysfunction such as can be observed in untreated or poorly treated PKU are unknown. They are most probably manifold. Learning and memory are important components of normal brain function. In particular, the hippocampus has been implicated in the acquisition of and the long-term storage of information (Madison et al 1991). It is therefore of particular significance that prolonged exposure to hyperphenylalaninaemia results in a loss of muscarinic acetylcholine receptors of this structure. This loss is not necessarily limited to this neurotransmitter receptor, but may extend to other neurotransmitter receptors as well, as illustrated in Table 4.

Neuronal connectivity is thought to be the basis of complex behaviours (Getting 1989). Interruption of this process at a crucial position such as the hippocampus may contribute to global mental retardation. Other areas of the brain are likewise affected by the hyperphenylalaninaemia. These areas are more concerned with specialized brain functions and thus may augment the damage at a more central position.

The present study contributes to understanding of the possible cause of the damaging effect of hyperphenylalaninaemia in that it identifies brain structures most vulnerable to the hyperphenylalaninaemic condition. A decrease in neurotransmitter receptor density, and therefore possibly a decrease in the number of synapses and thus a decreased connectivity, is a consequence of the proposed mechanism of damage to neurons by hyperphenylalaninaemia, mediated through an increased myelin turnover (Hommes and Matsuo 1987; Hommes 1991). The present observations are consistent with this proposed mechanism.

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