

RESEARCH ARTICLE

Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice

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Classical phenylketonuria (PKU) is a metabolic disorder caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). If untreated, accumulation of phenylalanine will damage the developing brain of affected individuals, leading to severe mental retardation. Here, we show that a liver-directed PAH gene transfer brought about long-term correction of hyperphenylalaninemia and behavioral improvement in a mouse model of PKU. A recombinant adeno-associated virus (AAV) vector carrying the murine PAH cDNA was constructed and administered to PAH-deficient mice (strain PAH^{enu2}) via the portal vein. Within 2 weeks of treatment, the hyperphenylalaninemic phenotype improved and completely normalized in the animals treated with higher vector doses. The therapeutic effect persisted for

40 weeks in male mice, while serum phenylalanine concentrations in female animals gradually returned to pretreatment levels. Notably, this long-term correction of hyperphenylalaninemia was associated with a reversal of hypoactivity observed in PAH^{enu2} mice. While locomotory activity over 24 h and exploratory behavior were significantly decreased in untreated PAH^{enu2} mice compared with the age-matched controls, these indices were completely normalized in 12-month-old male PKU mice with lowered serum phenylalanine. These results demonstrate that AAV-mediated liver transduction ameliorated the PKU phenotype, including central nervous system dysfunctions.

Gene Therapy advance online publication, 1 April 2004;
doi:10.1038/sj.gt.3302262

Keywords: phenylketonuria; adeno-associated virus vector; hyperphenylalaninemia; behavioral recovery

Introduction

Classical phenylketonuria (PKU; McKusick OMIM 261600) is an autosomal recessive disorder resulting from a deficiency of the liver enzyme phenylalanine hydroxylase (PAH; EC 1. 14.16.1).¹ PAH converts phenylalanine (Phe) to tyrosine with the aid of tetrahydrobiopterin (BH₄), and a deficiency of this enzyme causes accumulation of Phe and abnormal metabolites in the body fluids. If untreated, this condition irreversibly damages the central nervous system (CNS) of the patient, resulting in severe mental retardation. Conventional therapy for PKU consists of dietary restriction of Phe, which can prevent neuronal damage if initiated very early in life. However, the strict and complicated diet is often associated with poor compliance, particularly in adolescents and young adults. Premature termination of the diet leads to declined neuropsychological function, and noncompliance in pregnant women with PKU can produce devastating defects in the offspring referred to

as 'maternal PKU syndrome'. A permanent cure is therefore awaited to liberate patients from dietary restrictions, and gene therapy is an attractive novel approach to this goal.

However, previous preclinical studies of PKU gene therapy have revealed that a long-term cure of PKU is a formidable task. Generally, recombinant retroviral vectors cannot deliver the normal PAH gene to the liver at sufficient levels to overcome hyperphenylalaninemia.^{2,3} Adenoviral-mediated PAH gene transfer achieved a complete reduction of serum Phe in PKU animals, but the therapeutic effects did not persist and the vector was not effectively readministered due to immune responses against the virus.^{4,5} On the other hand, adeno-associated virus (AAV) vectors comprise another class of gene delivery vehicles, which have been shown to stably transduce nondividing cells such as hepatocytes, muscle fibers and neurons.^{6–8}

In this study, we evaluated a recombinant AAV vector carrying the PAH gene in a mouse model of PKU (PAH^{enu2} strain).^{9–11} A missense mutation (F263S) in the PAH gene was introduced into BTBR mouse strain by chemical mutagenesis, resulting in a loss of enzyme activity. Consequently, the homozygous PAH^{enu2} mice share many phenotypic characteristics with human PKU

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Received 24 December 2003; accepted 11 February 2004

patients, such as profound hyperphenylalaninemia (>20 mg/dl; normal 1–2 mg/dl), behavioral disturbances and hypopigmentation. Previous work suggested that at least 10% of normal PAH activity would be required to prevent hyperphenylalaninemia in PKU mice.^{4,5}

Results

Construction of the recombinant AAV vector

We first evaluated vectors derived from AAV serotypes 1 through 5. Recombinant AAV vectors containing the mouse erythropoietin (Epo) gene were infused into the mouse portal vein, and the serum Epo levels were determined. Among them, the AAV5-derived virion yielded the highest Epo concentration (unpublished results).^{12,13} Next, we tested several promoters to drive the Epo gene in the context of AAV5. We found that the CAG promoter was the strongest in transgene expression in the liver (unpublished results).¹⁴ This promoter consists of the human cytomegalovirus (CMV) immediate-early enhancer, the chicken β -actin promoter, and a chicken β -actin/rabbit β -globin composite intron.

Based on these results, we constructed an AAV vector as shown in Figure 1 (AAV5/CAG-mPAH). A recombinant AAV plasmid pAAV5/CAG-mPAH was comprised of the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal flanked by the AAV5 inverted terminal repeats (ITRs shown as hairpin loops in Figure 1). The vector DNA was then packaged into the AAV5 capsid through an adenovirus-free, transient transfection protocol.¹⁵

Correction of hyperphenylalaninemia

For liver-targeted gene transfer, the vector was injected into 5–7-week-old PAH^{enu2} mice via the portal vein. We injected male PKU mice with 3×10^{12} vector genomes (vg) ($n=3$), 1×10^{13} vg ($n=4$), 3×10^{13} vg ($n=3$) or 1×10^{14} vg ($n=3$) of AAV5/CAG-mPAH per animal. Female PKU mice were infused with 1×10^{13} vg ($n=4$), 3×10^{13} vg ($n=4$) or 1×10^{14} vg ($n=5$) per animal.

Serum Phe levels were determined prior to the infusion, biweekly until 12 weeks postinfusion, and every 4 weeks thereafter (Figure 2). Before gene transfer (week 0), all PAH-deficient mice showed profound hyperphenylalaninemia (33.7 ± 3.4 mg/dl; range 29.3–43.5 mg/dl; $n=27$). The degree of hyperphenylalaninemia was not significantly different between males (33.2 ± 2.6 mg/dl; $n=14$) and females (34.3 ± 4.1 mg/dl; $n=13$). Figure 2a shows the kinetics of blood Phe in male PKU mice receiving different doses of AAV5/CAG-mPAH. A striking decrease in serum Phe was observed 2–4 weeks after gene transfer. With the lowest vector dose

(3×10^{12} vg), serum Phe was only slightly lowered after 2 weeks (from 35.0 ± 1.6 to 28.1 ± 7.0 mg/dl; $P=0.18$ by paired *t*-test), but was significantly lowered after 4 weeks (15.6 ± 6.9 mg/dl; $P=0.027$ by paired *t*-test). With higher vector doses (1×10^{13} , 3×10^{13} and 1×10^{14} vg), the serum Phe level was clearly lowered ($P=0.001$, 0.006 and 0.002 by paired *t*-test, respectively) to a therapeutic range (<10 mg/dl) in 2 weeks. At 4 weeks postinfusion, each cohort of male mice recorded the lowest serum Phe. In particular, it was completely normalized in the mice treated with 3×10^{13} vg (1.4 ± 0.5 mg/dl) and 1×10^{14} vg (1.2 ± 0.5 mg/dl) of AAV5/CAG-mPAH.

The reduced serum Phe levels were stably maintained for 40 weeks. Complete correction of hyperphenylalaninemia (<2 mg/dl) persisted in the mice treated with the highest vector dose (1×10^{14} vg), and the mice receiving

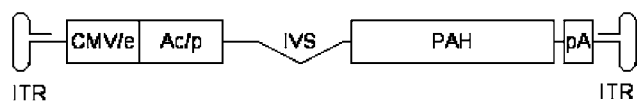


Figure 1 Structure of the AAV5/CAG-mPAH vector. The vector consisted of a CMV immediate-early enhancer (CMV/e), the chicken β -actin promoter (Ac/p), a chicken β -actin/rabbit β -globin composite intron (IVS), the 1.4 kb murine PAH cDNA (PAH) and the SV40 late polyadenylation signal (pA) flanked by the AAV5 inverted terminal repeats (ITRs shown as hairpin loops).

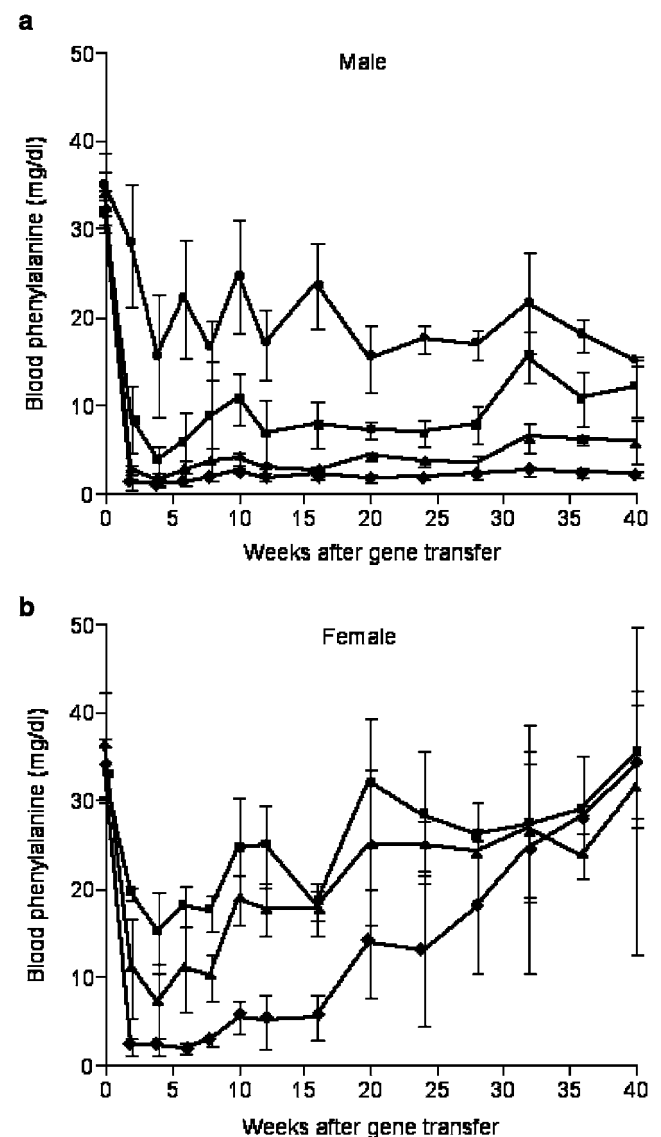


Figure 2 Persistence of the recombinant AAV-mediated correction of hyperphenylalaninemia in male (a) and female (b) PKU mice. Serum Phe concentration was determined prior to vector infusion (week 0) and periodically after gene transfer, and represented as the mean \pm s.d. for each treatment group. The applied vector dose was 3×10^{12} vg (circles), 1×10^{13} vg (squares), 3×10^{13} vg (triangles) or 1×10^{14} vg (diamonds) per animal.

the second highest dose (3×10^{13} vg) stayed in a well-controlled therapeutic range (<6 mg/dl). Mice receiving lower vector doses (3×10^{12} and 1×10^{13} vg) showed moderate correction of hyperphenylalaninemia, with significant long-term efficacy of the single AAV infusion.

Figure 2b shows the kinetics of serum Phe in female PKU mice after receiving 1×10^{13} , 3×10^{13} or 1×10^{14} vg of AAV5/CAG-mPAH. The vector administration was effective in the female PKU mice, too, but the dose-response and duration were different from the male mice; that is, about three times more vector was required for the female mice to exhibit an equivalent reduction in serum Phe (Figure 3). At 4 weeks postinfusion when the reduction was at its maximum, 1×10^{13} vg of AAV5/CAG-mPAH lowered serum Phe by 50% in the female mice, while the same level of reduction was achieved by 3×10^{12} vg in the males. Similarly, an 80% reduction was achieved by 3×10^{13} vg in the females, whereas only 1×10^{13} vg were required in the males. Complete correction of hyperphenylalaninemia was achieved by 1×10^{14} vg in the females, while it was achieved by 3×10^{13} vg as well as 1×10^{14} vg in the males. As for duration, the therapeutic effect did not persist in the female PKU mice as seen in the males. Serum Phe levels in each female cohort remained low until 8 weeks post-gene transfer, but gradually rose thereafter. With vector doses of 1×10^{13} and 3×10^{13} vg, serum Phe was greater than 20 mg/dl at 20 weeks, and returned to the pretreatment level at 40 weeks. With the highest dose (1×10^{14} vg), serum Phe was kept below 10 mg/dl until 16 weeks, then gradually increased and returned to the pretreatment level at 40 weeks.

Although we did not kill the animals for enzyme assay, previous studies on adenoviral-mediated gene transfer to PAH^{enu2} mice allowed us to estimate the PAH activity accomplished by our vector. These studies showed, in good agreement, that the threshold PAH activity to correct hyperphenylalaninemia was about 10% of normal mice.^{4,5} As shown in Figure 3, male PKU mice given 3×10^{12} vg and females given 1×10^{13} vg of the vector showed 50–60% reduction in serum Phe; we speculate that these mice would express about 5% of normal PAH activity. On the other hand, male PKU mice given 3×10^{13} or 1×10^{14} vg and females given 1×10^{14} vg completely recovered from hyperphenylalaninemia,

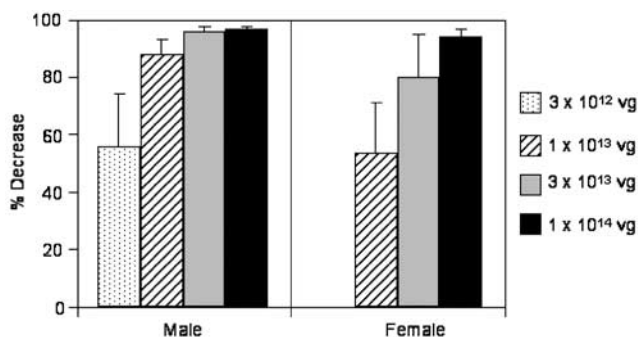


Figure 3 Vector dose-dependent reduction of serum Phe in PKU mice. Percent reduction of serum Phe was calculated by the following formula: $\{(\text{serum Phe at week 0}) - (\text{serum Phe at 4 weeks})\} \times 100 / (\text{serum Phe at week 0})$. Bars represent the mean \pm s.d. of % reduction of serum Phe in PKU mice treated with 1×10^{12} vg (dotted bar), 1×10^{13} vg (hatched bar), 3×10^{13} vg (gray bar), or 1×10^{14} vg of AAV5/CAG-mPAH (black bar).

hence their liver PAH activities would be 10% of normal or greater. Male PKU mice given 1×10^{13} vg (ca. 90% reduction in serum Phe) and females given 3×10^{13} vg of AAV (ca. 80% reduction) would have 5–10% of normal PAH activity.

Correction of hypopigmentation

Associated with extended reductions in serum Phe, hypopigmentation in the AAV-treated PKU mice was ameliorated. While the coat color of untreated mice remained grayish brown, hair darkening in the mice receiving higher vector doses was observed 2 weeks post-transduction, and the mice grew black hair in 4 weeks which was indistinguishable from that of wild-type (WT) BTBR mice (Figure 4). Male PKU mice with reduced serum Phe retained black hair throughout the observation period, while female PKU mice lost pigmentation as the therapeutic effect diminished.

Recovery from hypoactivity following PAH gene transfer

Along with persistent correction of hyperphenylalaninemia and hypopigmentation, we observed behavioral

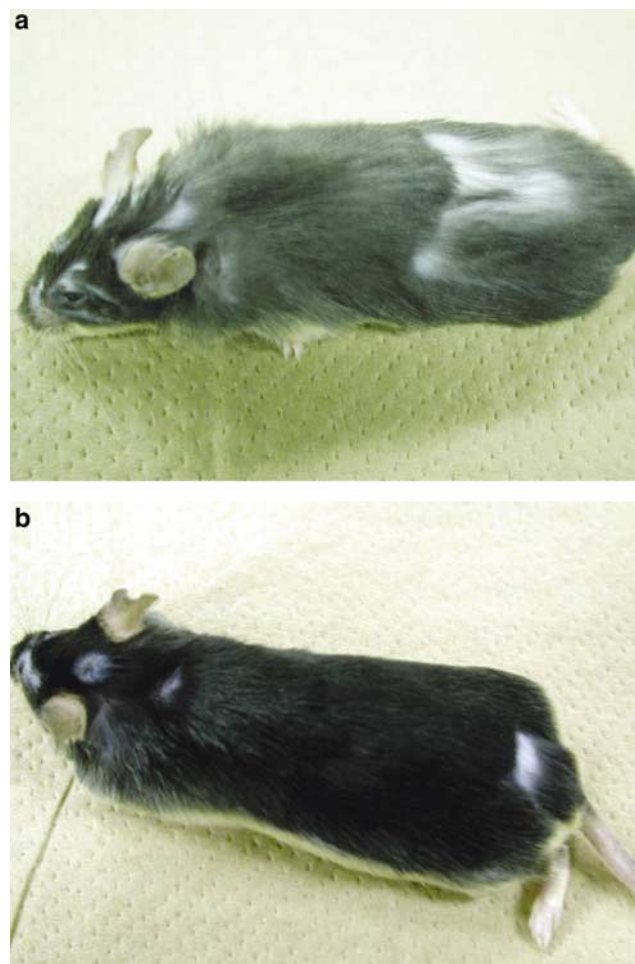


Figure 4 Correction of hypopigmentation in PKU mouse following PAH gene transfer. (a) Untreated PKU mouse showing grayish brown hair, easily distinguished from wild-type and PAH^{+/-} heterozygous BTBR mice. (b) By 8 weeks after PAH gene transfer, the PKU mouse with complete correction of hyperphenylalaninemia recovered black coat color and was indistinguishable from normal BTBR mice.

recovery in AAV-treated PKU mice. Consistent with previous studies showing abnormal behavior and cognitive deficits in PAH^{mut2} mice,^{9,16,17} we found that untreated PKU (PAH^{-/-}) mice were relatively hypoactive compared with WT (PAH^{+/+}) and heterozygous carrier (PAH^{+/-}) animals. The hypoactivity became apparent with aging, and the difference was significant among animals aged 10 months or older. Figure 5 shows the results of behavior tests on the 12-month-old animals. As for total locomotion over 24 h, the untreated PKU mice displayed about 70% of normal activity (Figure 5a, $P < 0.01$ by Student's *t*-test). On the other hand, the AAV-administered male mice without hyperphenylalaninemia exhibited significantly higher 24-h locomotion than the untreated mice (Figure 5a, $P = 0.001$ by Student's *t*-test). Indeed, the AAV-treated animals showed a normal activity level in this test.

Similarly, PAH gene transfer improved the PKU animals' exploratory activity in a novel environment.

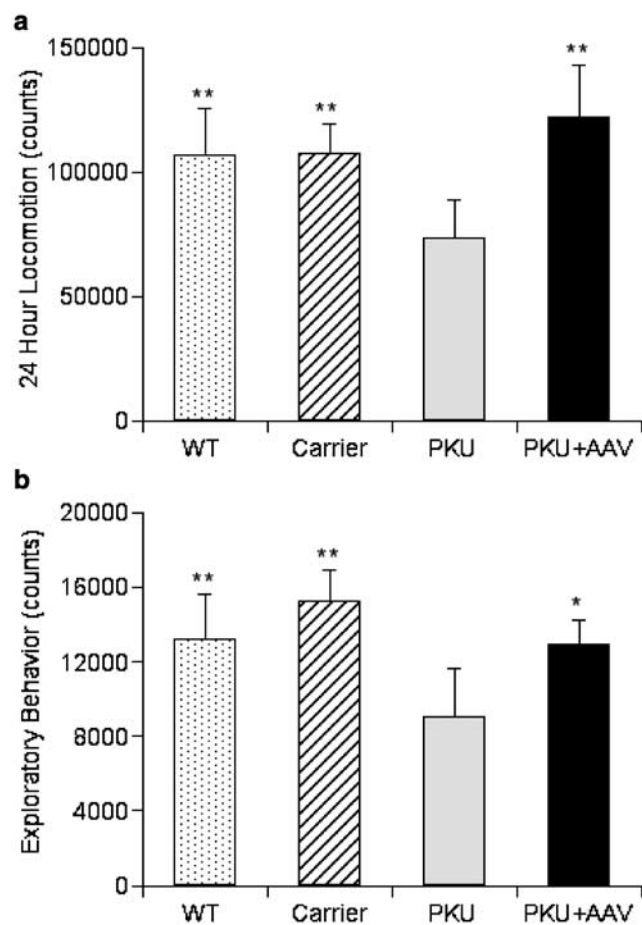


Figure 5 Recovery from hypoactivity following PAH gene transfer. (a) Total locomotion over 24 h. Mice were placed under an infrared sensor and ambulatory activity was recorded consecutively for 24 h. Wild-type (WT), heterozygous and AAV-treated PKU mice exhibited significantly higher locomotory activity than untreated PKU mice (** $P < 0.01$ by Student's *t*-test). (b) Exploratory behavior. Mice were placed in a novel cage under a sensor and ambulatory activity was quantified during the first 2 h in the chamber. This test showed significantly higher performance by WT, heterozygous and AAV-treated PKU mice than untreated PKU animals (** $P < 0.01$ and * $P < 0.05$ by Student's *t*-test). Bars represent the mean \pm s.d. of WT mice (WT; dotted bar), heterozygous mice (Carrier; hatched bar), untreated PKU mice (PKU; gray bar), and AAV-transduced PKU mice (PKU + AAV; black bar).

When settled in a novel cage, the untreated PKU males showed 60–70% of normal exploratory activity (Figure 5b, $P < 0.01$). On the other hand, PKU mice that had recovered from hyperphenylalaninemia explored as vigorously as WT animals, and their activity level was significantly greater than that of untreated PKU mice ($P = 0.015$). These results clearly indicate that the PAH gene transfer improved the CNS function of PKU mice in addition to correction of hyperphenylalaninemia.

Discussion

In this study, we demonstrated that AAV-mediated transduction of the PKU mouse liver brought about a long-term cure of the disease. A single infusion of AAV5/CAG-mPAH completely normalized the hyperphenylalaninemic phenotype in male PKU mice, and the longevity of the therapeutic effect was superior to any other gene delivery vehicle thus far. Although not thoroughly investigated, the result suggests that the transgene was transcriptionally active during observation, and that no significant immune response was elicited against the transduced hepatocytes in the animals. In addition, infusion of very large amounts of AAV did not show any toxicity in the treated mice. The vector safety and viability may be further improved by adopting recently developed purification methods, such as iodixanol gradient, affinity or ion-exchange chromatography.^{18–21}

A major problem we encountered in this study was that the same AAV vector was less effective in female PKU mice. About three times more vector was required to achieve an equivalent reduction of serum Phe seen in males, and the therapeutic effect was shorter in duration. The underlying mechanisms for these female-specific phenomena are currently unknown. Davidoff *et al*²² recently reported similar observations that AAV2- and AAV5-derived vectors less efficiently transduced livers of female mice than males. They suggested that the difference was due to an androgen-dependent pathway for augmenting hepatocyte transduction, but its mode of action is undetermined. Since precise molecular events involved in recombinant AAV-mediated transduction remain obscure, the critical step accounting for the observed sex difference is also a mystery. Androgen may augment the uptake of AAV particles into the cell or traffic them to the nucleus; alternatively, it may stabilize the AAV genome in an episomal state, or enhance vector integration into the host chromosome. Of these possibilities, the last one is less likely, because only a small fraction (<10%) of recombinant AAV genome was reportedly integrated into the mouse hepatocytes.²³ If there is an androgen-dependent mechanism to retain the AAV genome in an episomal state in the liver, lack of such machinery would allow gradual loss of the vector DNA in females, thereby transgene-derived PAH activity would descend over time as we observed. Other possibilities accounting for the lower therapeutic efficacy include transcriptional silencing and an immune response against AAV-transduced hepatocytes, although the latter is unlikely to occur only in female mice.

In genetic treatment of autosomal and acquired disorders, sex-dependent transduction raises a novel issue. Development of more efficient vectors may over-

come this problem, or other approaches can be considered. In terms of PKU, the disease-associated pathology is caused by accumulated Phe in the body fluids. Thus, it can be prevented by 'heterologous gene therapy', ie targeting tissues other than hepatocytes. Several investigators have exploited this strategy because of difficulties with liver transduction and safety concerns. Christensen *et al*²⁴ transduced primary keratinocytes with genes for PAH and GTP cyclohydrolase I, which is the rate-limiting enzyme in BH₄ biosynthesis. They showed that the cells cleared excess Phe in the culture medium, and suggested that engraftment of enough of these cells may function as a metabolic sink for detoxification. Harding *et al*²⁵ investigated the potential of skeletal muscle as a PAH-expressing organ. Using a transgenic technique, they created mice expressing PAH in the skeletal muscle but not in the liver. These mice showed hyperphenylalaninemia at baseline, but serum Phe significantly decreased when the animals were supplemented with BH₄. A similar approach to bone marrow cells was unsuccessful,²⁶ and careful consideration is required in translating these transgenic studies into human applications.

A novel finding in this study was that AAV infusion lead to behavioral improvement in addition to correction of hyperphenylalaninemia and hypopigmentation. To our knowledge, this is the first demonstration that a gene-based approach to PKU actually benefited CNS function. It has been reported that free amino acid and amine contents are dramatically reduced in the PAH^{enu2} mouse brain, as in untreated human PKU patients.^{27,28} Presumably, the observed hypoactivity in older PKU mice was associated with the abnormal synthesis of biogenic amines, whereas the abnormality was reversed in AAV-treated PKU animals with normal serum Phe. We speculate that the behavioral recovery in these mice represents an analogous situation in which dietary restriction of Phe can improve some neuropsychiatric symptoms in untreated PKU patients. It is of particular interest whether an earlier genetic intervention can prevent irreversible neuronal defects in PKU and preserve more sophisticated CNS function such as memory. The AAV vectors and PAH^{enu2} mice will provide an attractive system to address such prompting questions.

Materials and methods

AAV vector construction

To isolate murine PAH cDNA (GenBank Accession # NM008777), liver mRNA was prepared from a C57BL/6J mouse (from Clea Japan, Tokyo, Japan) with Isogen reagent (Nippon Gene, Toyama, Japan) and an mRNA Purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The PAH cDNA was cloned by reverse transcriptase-directed polymerase chain reaction using a Superscript II cDNA synthesis kit (Invitrogen, Grand Island, NY, USA). The CAG promoter was derived from pCAGGS (a gift from Dr J Miyazaki, Osaka University, Osaka, Japan).¹⁴ The AAV5 vector plasmid pAAV5LacZ and a helper plasmid 5RepCapA were generous gifts from Dr JA Chiorini (National Institutes of Health, Bethesda, MD, USA).¹² To construct a recombinant AAV5 vector plasmid for PAH expression, the expression

cassette of pAAV5LacZ was replaced with the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal, and the plasmid was referred to as pAAV5/CAG-mPAH (Figure 1).

Recombinant AAV stocks were propagated according to an adenovirus-free, three-plasmid transfection protocol described previously.¹⁵ Briefly, subconfluent 293 cells (4×10^8 cells per 10 trays) in Cell Factories 10 (Nunc, Roskilde, Denmark) were cotransfected with 650 µg of the vector plasmid pAAV5/CAG-mPAH, 650 µg of the AAV helper plasmid 5RepCapA and 650 µg of the adenoviral helper plasmid pLadeno1 (identical to pVAE2AE4-2 in Matsushita *et al*,¹⁵ kindly provided by Avigen, Alameda, CA, USA) by using the calcium phosphate precipitation method for a period of 6 h. Cells were harvested 72 h after transfection and lysed by three freeze-thaw cycles. The crude viral lysate was incubated with Benzonase (Merck KGaA, Darmstadt, Germany) and centrifuged. Finally, the clear supernatant was subjected to two rounds of CsCl density-gradient ultracentrifugation for purification. The physical titer of the viral stock was determined by DNA dot blot and hybridization with the murine PAH cDNA probe, along with plasmid standards. Typically, we obtained 5×10^{13} vg of AAV5/CAG-mPAH from a culture container (10 trays).

Transduction of mouse liver

All animal experiments were carried out in accordance with our institutional guidelines. PAH^{enu2} mice were generous gifts from Dr T Shiga (University of Tsukuba, Tsukuba, Japan), and a colony was established at Jichi Medical School (Tochigi, Japan). PKU mice used for *in vivo* gene transfer were 5–7 weeks of age. Mice were anesthetized with isoflurane inhalation followed by laparotomy. A 300 µl of saline suspension containing 3×10^{12} – 1×10^{14} vg of AAV5/CAG-mPAH was slowly injected into the portal vein using an insulin syringe with a 29-gauge needle (Terumo, Tokyo, Japan).

Serum Phe assay

Serum Phe was measured by an enzymatic microfluorometric assay using an Enzaplate PKU-R kit (Bayer Medical, Tokyo, Japan). Mice were tail phlebotomized and the blood was spotted onto a mass-screening grade paper filter (#545, provided by Advantec Toyo, Tokyo, Japan). A 3 mm diameter disc was punched out from the dried blood spot and placed in a 96-well plate. Phe was eluted from the disc and incubated with Phe dehydrogenase, an NAD-dependent enzyme, and resazurin. The enzyme reaction produces NADH, which in turn converts resazurin to resorufin with the aid of diaphorase. The resultant resorufin was measured on a Fluoroskan Ascent plate reader (Labsystems, Helsinki, Finland) with a 544/590 nm filter set.

Mouse behavior tests

Mice were tested at 12 months of age. To measure locomotory activity over 24 h, the home cage of the mouse was placed under an infrared sensor that detects thermal radiation from animals (Supermex; Muromachi Kikai, Tokyo, Japan).²⁹ Ambulation was scored by a personal computer interfaced to the sensor. Alternatively, exploratory behavior was tested by placing the mouse in a novel cage under the infrared sensor.

Ambulatory activity was quantified during the first 2 h in the chamber.

Acknowledgements

We are grateful to Dr JA Chiorini for pAAV5LacZ and 5RepCapA, Dr J Miyazaki for pCAGGS, Dr T Shiga for PAH^{enu2} mice, Avigen for pLadeno1, and Advantec Toyo for #545 filter paper. We also thank Dr Y Hakamata for technical assistance in the animal experiments. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Japan.

References

- 1 Scriver CR, Kaufman S. Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The Metabolic and Molecular Basis of Inherited Diseases*. McGraw-Hill: New York, 2001, pp 1667–1724.
- 2 Liu T-J, Kay MA, Darlington GJ, Woo SLC. Reconstitution of enzymatic activity in hepatocytes of phenylalanine hydroxylase-deficient mice. *Somat Cell Mol Genet* 1992; **18**: 89–96.
- 3 Eisensmith RC, Woo SLC. Gene therapy for phenylketonuria. *Eur J Pediatr* 1996; **155** (Suppl 1): S16–S19.
- 4 Fang B et al. Gene therapy for phenylketonuria: phenotypic correction in a genetically deficient mouse model by adenovirus-mediated hepatic gene transfer. *Gene Therapy* 1994; **1**: 247–254.
- 5 Nagasaki Y et al. Reversal of hypopigmentation in phenylketonuria mice by adenovirus-mediated gene transfer. *Pediatr Res* 1999; **45**: 465–473.
- 6 Nathwani AC et al. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* 2002; **100**: 1662–1669.
- 7 Kay MA et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–261.
- 8 Muramatsu S et al. Behavioral recovery in a primate model of Parkinson's disease by triple transduction of striatal cells with adeno-associated viral vectors expressing dopamine-synthesizing enzymes. *Hum Gene Ther* 2002; **13**: 345–354.
- 9 Shedlovsky A, McDonald JD, Symula D, Dove WF. Mouse models of human phenylketonuria. *Genetics* 1993; **134**: 1205–1210.
- 10 McDonald JD, Charlton CK. Characterization of mutations at the mouse phenylalanine hydroxylase locus. *Genomics* 1997; **39**: 402–405.
- 11 McDonald JD et al. The phenylketonuria mouse model: a meeting review. *Mol Genet Metab* 2002; **76**: 256–261.
- 12 Chiorini JA, Kim F, Yang L, Kotin RM. Cloning and characterization of adeno-associated virus type 5. *J Virol* 1999; **73**: 1309–1319.
- 13 Mingozzi F et al. Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J Virol* 2002; **76**: 10497–10502.
- 14 Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**: 193–200.
- 15 Matsushita T et al. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Therapy* 1998; **5**: 938–945.
- 16 Zagreda L et al. Cognitive deficits in a genetic mouse model of the most common biochemical cause of human mental retardation. *J Neurosci* 1999; **19**: 6175–6182.
- 17 Cabib S et al. The behavioral profile of severe mental retardation in a genetic mouse model of phenylketonuria. *Behav Genet* 2003; **33**: 301–310.
- 18 Hermens WT et al. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 1999; **10**: 1885–1891.
- 19 Zolotukhin S et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy* 1999; **6**: 973–985.
- 20 Auricchio A, O'Connor E, Hildinger M, Wilson JM. A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Mol Ther* 2001; **4**: 372–374.
- 21 Kaludov N, Handelman B, Chiorini JA. Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum Gene Ther* 2002; **13**: 1235–1243.
- 22 Davidoff AM et al. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood* 2003; **102**: 480–488.
- 23 Nakai H et al. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. *J Virol* 2001; **75**: 6969–6976.
- 24 Christensen R, Kolvraa S, Blaese RM, Jensen TG. Development of a skin-based metabolic sink for phenylalanine by overexpression of phenylalanine hydroxylase and GTP cyclohydrolase in primary human keratinocytes. *Gene Therapy* 2000; **7**: 1971–1978.
- 25 Harding CO et al. Metabolic engineering as therapy for inborn errors of metabolism – development of mice with phenylalanine hydroxylase in muscle. *Gene Therapy* 1998; **5**: 677–683.
- 26 Harding CO et al. Expression of phenylalanine hydroxylase (PAH) in erythrogenic bone marrow does not correct hyperphenylalaninemia in *Palr^{enu2}* mice. *J Gene Med* 2003; **5**: 984–993.
- 27 Puglisi-Allegra S et al. Dramatic brain aminergic deficit in a genetic mouse model of phenylketonuria. *NeuroReport* 2000; **11**: 1361–1364.
- 28 Pascucci T, Ventura R, Puglisi-Allegra S, Cabib S. Deficits in brain serotonin synthesis in a genetic mouse model of phenylketonuria. *NeuroReport* 2002; **13**: 2561–2564.
- 29 Masuo Y, Matsumoto Y, Morita S, Noguchi J. A novel method for counting spontaneous motor activity in the rat. *Brain Res Protoc* 1997; **1**: 321–326.