The phenylketonuria mouse model: a meeting review

J. David McDonald, a,* Maria Andriolo, b Francesco Calì, b Mario Mirisola, c Stefano Puglisi-Allegra, d Valentino Romano, c Christineh N. Sarkissian, e and Carolyn B. Smith f

a Department of Biological Sciences, Wichita State University, Wichita, Kansas, USA
b OASI Istituto per la ricerca sul ritardo mentale e l’involuzione cerebrale, Troina, Sicily, Italy
c Department of Biopathology and Biomedical Methodology, University of Palermo, Palermo, Sicily, Italy
d Department of Psychology, University of Rome, and Fondazione Santa Lucia IRCCS, Rome, Italy
e McGill University—Montreal Children’s Hospital Research Institute, Montreal, Que., Canada
f Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bethesda, MD, USA

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The BTBR-Pah c mu2 PKU mouse line has been characterized to a degree that enables its use as a reliable model for human phenylketonuria (PKU). Numerous studies documenting the genetics, biochemistry, and phenotype of this PKU mouse strain have demonstrated striking similarities to the human disease. Recently, a meeting was held in Troina, Sicily entitled “Mouse Models in Phenylketonuria Research.” The presentations at this meeting were descriptions of the usage of this disease model to investigate different aspects of the PKU syndrome, to reveal previously unknown aspects of the molecular etiology of PKU, and to explore new treatment regimens. The meeting concluded with a roundtable discussion with the intent to articulate issues arising from current research with the mouse PKU model and to point to potentially fruitful areas for future research. It is hoped that the proceedings of this meeting will provide the interested reader both with an overview of most of the current scientific projects and an enhanced understanding of the experimental possibilities afforded by this very accurate disease model. For the purpose of this article, a brief summary of each presentation will be made, including a statement of specific issues raised and suggestions for certain future experimental directions stemming from the experimental results. The article will then conclude with a broader statement of current research, troubling issues, and future directions that arose during the roundtable discussion.

Dr. J. David McDonald of Wichita State University opened up the meeting with a description of his research to characterize the modeling of the maternal PKU syndrome displayed by this mouse strain [1]. He described how a defined diet deficient in phenylalanine (Phe), used in conjunction with different levels of Phe in the drinking water, allowed the manipulation of the blood Phe levels of pregnant PKU females. This dietary manipulation can be used to produce maternal blood Phe levels, emblematic of any of the three classes of dietary treatment of classical PKU: untreated (highly elevated blood Phe), treated (normal blood Phe), or partially treated (elevated blood Phe). With this system, he was able to show with very high statistical significance that three fetal morphometric parameters (i.e., head circumference, body weight, and body length) were all reduced in the offspring of PKU females with elevated blood Phe levels during pregnancy compared to those offspring of PKU females with normal blood Phe levels. Because these morphometric parameters are likewise affected in human maternal PKU, this result serves to verify that this model, already known to model the neonatal aspects of PKU, also models the maternal PKU syndrome. Further, the defined diet should permit a more thorough investigation of the relationship between maternal blood Phe and fetal birth defects.

In this study, both test and control pregnancies were set up to produce litters composed of half-mutant and half-nonmutant fetuses. A direct PCR-based genotyping assay was used to identify the genetic status of each fetus.
so that the question could be asked as to whether either class was more severely affected [2]. The results showed that, in this mouse model system as in the human disease, fetal genotype does not appear to be a significant factor in teratology. In both systems, the important factors are maternal genotype and maternal diet.

Another aspect of the maternal PKU syndrome that this research revealed to be similar between mice and humans is the approximately twofold Phe concentration gradient across the placental barrier into the fetal compartment. Both in mice and humans, this concentration gradient amplifies the exposure of the developing fetus to blood Phe and, in doing so, is likely to exacerbate the metabolic imbalance of PKU by interfering with the transport of other metabolites that use the same transport protein at the placental barrier.

An especially compelling question that arises from this line of research is the extent and the types of birth defects that arise as a result of switching maternal blood Phe levels during pregnancy. Although switching from elevated to normal and from normal to elevated would be of interest, the former type of switch is likely to be of greatest immediate biomedical interest because it mimics the common occurrence in human maternal PKU pregnancies in which the PKU female is already several months into a pregnancy before dietary treatment has begun. Other pressing issues that can be addressed with this maternal PKU modeling system is the intensive investigation of the biochemical, molecular, and cellular factors that may be responsible and warrant further investigation. For example, measuring brain levels of the large neutral amino acids tyrosine (Tyr) and tryptophan (Trp), the respective precursors of catecholamine and indolamine neurotransmitters, showed that they were reduced. This suggests that their reduction leads to the observed neurotransmitter reduction. However, these researchers also noted that the reduction in brain Trp levels was not severe and they made another observation that suggests a different reason for the observed reduction.

To assess cognitive ability, Dr. Puglisi-Allegra and his colleagues sought out tests that would not use explicit (positive or negative) reinforcement and would avoid the effects of a lengthy training component and thus provide analogous tests of recognition given to human subjects. They elected to use two non-associative tasks: the Spatial Novelty Test (SNT) and the Object Recognition Test (ORT). These two tests take advantage of the spontaneous preference that rodents display for novel stimuli. In SNT, animals are expected to explore objects that have been displaced from a previous position [5]. In ORT, animals are expected to explore a novel object that is similar to a previously explored sample [6]. The SNT is designed to estimate the ability of rodents to encode spatial relationships while ORT has been developed as a variant of the delayed non-matching to sample tasks. The homozygous PKU mutants failed both tests by exhibiting very poor processing of spatial and non-spatial information compared to their nonmutant siblings. These observations support the view that cognitive deficits promoted by hyperphenylalaninemia might depend on neurodevelopmental disturbances involving late developing brain structures.

Dr. Carolyn Smith from NIMH presented research in which she compared the rates of cerebral protein synthesis in adult PKU mutant and nonmutant animals and found a 20% reduction in the average cerebral protein synthesis rate [7]. Her investigations also revealed that the high levels of arterial Phe in mutant animals produce an elevated level of brain Phe and also lead to significant reductions in the brain levels of other large neutral amino acids (viz., tyrosine, tryptophan, leucine, isoleucine, histidine, methionine, threonine, and valine). It has long been speculated that a reduced rate of protein synthesis in brain is a major etiological factor in the development of mental retardation in untreated PKU. The decrease in protein synthesis is thought to be the simple and direct result of the reduction in the influx of
large neutral amino acids. Dr. Smith’s results suggest that the situation is actually more complex and that the relationship of the reduction in protein synthesis to the neuropathology of PKU is still unclear. Her results suggest that the brain compensates for a reduction in the influx of large neutral amino acids with amino acids liberated from intracellular protein pools. Thus, although she found a 20% reduction in the intracellular level of leucine, there was no significant reduction in the concentration of leucine bound to tRNA. Further, experimental results indicated that in the mutant mice most of the leucine bound to tRNA was derived from protein degradation within the brain; in the nonmutant animals, the fraction from protein degradation was less than half. Thus, while this research confirms a long-held belief about the effect of the PKU metabolic imbalance on brain metabolite levels and protein synthesis rates, it forces a reexamination of the precise role that these events play in the molecular basis of the neuropathology associated with untreated PKU. These results clearly indicate that in the adult brain amino acids derived from protein degradation may provide a buffer against limitations in amino acid transport at the blood–brain barrier.

These findings suggest a number of future experiments, aimed at revealing the precise relationship between the metabolic imbalance of PKU and the neuropathology that follows from this imbalance. Does the compensation provided by protein degradation come with a cost to the tissue or does it merely represent a shifting of existing tissue pools? Are particular proteins targeted for degradation to produce amino acids or is this a more global phenomenon within the central nervous system? Another important area of research is the further understanding of the decreased rate of protein synthesis. Is there a particular factor or step in the process that is governing the change or is the effect on protein synthesis secondary to other cellular changes? The question of the reversibility of the observed effect merits study. If proteins are degraded to overcome a reduction in amino acid influx, are those proteins replaced when the metabolic imbalance is relieved? This is an important question because, while the brain is in many respects an organ that demonstrates compensation, it is well established that brain damage incurred in untreated PKU is irreversible. Among other questions, it will likely be very important to use the model to identify the presence of especially sensitive developmental windows and to examine the dose–response relationship within those windows. It is likely that such studies will lead to a greater understanding of the molecular etiology of mental retardation in PKU and it is hoped that this will help reduce the incidence of this pathological feature of the disease.

Dr. Valentino Romano and his colleagues Drs. Mario Mirisola, Maria Andriolo, and Francesco Calif from the University of Palermo and OASI Institute per la ricerca sul ritardo mentale e l’involuzione cerebrale in Sicily presented proteome research in which the protein expression profiles, obtained by two-dimensional protein electrophoresis and digital imaging [8,9], from the brains of age- and sex-matched mutant and nonmutant animals were compared. These maps display striking differences in the type, number, and levels of expressed genes between these two classes.

Dr. Romano found about 30% similarity between the two proteome profiles, while the differences that were revealed fell into both of the two possible classes: (1) proteins present in the PKU mice but not in the nonmutant animals and (2) proteins present in the nonmutant animals but not in the PKU mice. Their first round of analysis focused on the first group of proteins. They compared the two-dimensional gel mobility of the proteins with that of other known proteins for similarity. So far in this analysis, a number of proteins show no similarity to known proteins for these two parameters. However, there are proteins within this group that share apparent molecular weight and isoelectric point with specific proteins known to play a role in other neurodegenerative pathologies such as Down's syndrome and senile dementia. Dr. Romano’s group seeks to extend the analysis of the proteins showing differences between mutant and nonmutant classes through protein sequencing and comparison with protein sequence in the SWISSPROT database.

Dr. Romano’s group also reported the discovery of a specific Phe hydroxylase mRNA in adult mouse brain (manuscript in preparation). Phe hydroxylase is encoded by a single gene in both mouse and human. The enzyme converts Phe to Tyr by a hydroxylation reaction. The expression of the Phe hydroxylase gene has been demonstrated in human and mouse liver and, to a lesser extent, in mouse and human kidneys. The presence of Phe hydroxylase activity in the brain tissue of both species has been hypothesized but never demonstrated [10,11]. The level of this newly discovered transcript in brain tissue is comparable to liver tissue as judged by Northern blot analysis. However, no Phe hydroxylase activity can be detected in whole brain crude extract by using the 14C-Phe-hydroxylation assay. Furthermore, they were unable to detect Phe hydroxylase protein in crude brain extracts by Western blot analysis with monoclonal antibody directed against Phe hydroxylase.

To explain the apparent discrepancy between RNA and protein data, they analyzed Phe hydroxylase mRNA from both tissues. That analysis revealed a significant modification in the brain Phe hydroxylase transcript. The discovery of a Phe hydroxylase transcript that does not appear to be expressed in adult brain raises the possibility that Phe hydroxylase may be ex-
pressed at some other time during the development of the mouse brain. Further analysis will be undertaken to examine earlier developmental stages for the presence of Phe hydroxylase protein.

Dr. Christineh Sarkissian of McGill University presented research results describing the use of the mouse PKU models to evaluate the efficacy of oral enzyme substitution therapy with Phe Ammonia Lyase (PAL) as adjunct alternative therapy for PKU, independent of artificial diets. She also described ways in which she and her colleagues have mimicked various degrees of hyperphenylalaninemia. They have controlled Phe levels in BTBR-Pah^emo2 homozygotes to model reproducible euglycemic phenylalaninemia and predictable untreated classical PKU. They have also made use of a mildly affected mouse model for PKU, BTBR-Pah^emo1 [12] in conjunction with BTBR-Pah^emo2 to produce BTBR-Pah^emo2/BTBR-Pah^emo1 compound heterozygotes, which model mild hyperphenylalaninemia [13].

The current low-Phe dietary treatment has achieved its primary goals of preventing mental retardation and providing a positive pregnancy outcome for PKU human mothers [14–16]. However, long-term compliance has proven to be very difficult. This is, in part, because of the arduous nature of the dietary regimen, requiring a major alteration in lifestyle, and because of the poor organoleptic qualities of the diet itself [17]. Dr. Sarkissian’s research group has chosen to investigate PAL for enzyme substitution therapy; a form of treatment that could potentially allow PKU patients to consume a more normal diet.

PAL was chosen for a number of reasons: (1) unlike Phe hydroxylase, PAL is a robust autocatalytic protein without the need for the very costly Phe hydroxylase cofactor. It is also an enzyme that stoichiometrically converts l-Phe to the harmless metabolites trans-cinnamic acid and trivial amounts of ammonia [18–20]; (2) the proposed route of protected PAL administration is oral as it will deplete the Phe pool in the intestine, whether the Phe source is dietary or from the endogenous run out of free Phe from the bound pool [21]; and (3) cloning technology (Ibex Technologies, Montreal, Canada) has enabled the production of an economical supply of PAL [22].

Dr. Sarkissian reported that her research group has been successful at reducing blood Phe levels with PAL and, in doing so, has demonstrated both the proof of pharmacological and physiological principles. First, they verified the efficacy of blood Phe abatement by intraperitoneal injection of PAL into PKU mice and showed that it acted to lower ambient blood Phe levels and that the effect persisted over 24 h. Second, they showed that PAL could be administered orally. This second result took two forms: (1) protected free PAL, enclosed within Escherichia coli cells and delivered orally, allowed free access to the enzyme while protecting PAL from the action of digestive enzymes, which were unable to penetrate the cells and (2) PAL was orally delivered with the protease inhibitor aprotinin [22].

This work has demonstrated that oral enzyme substitution therapy with PAL has significant promise for future treatment of PKU. The application of this form of therapy in humans should control the Phe pool size without drastic lifestyle restriction. Various oral protein drug strategies are currently being implemented with the aim of improving PAL protection, bioavailability, and producing a formulation that is fit for repeated administration.

The meeting concluded with an extended roundtable dialog by all participants. This discussion focused largely on two areas: remaining problems that need to be solved to extend the utility of the PKU mouse model; and other areas of fruitful future research that had not been addressed elsewhere in the presentations by meeting participants.

The use of current methods of dietary manipulation (i.e., feeding a Phe-deficient diet, addition of Phe to the drinking water) yields blood Phe levels that are significantly erratic, a problem that seems to arise due to the fast-release nature of the purified Phe consumed. This is an issue for several of the experimental pursuits, especially maternal PKU studies and PAL supplementation studies. The following were suggested methods for reducing these fluctuations: (1) addition of solid Phe to the defined diet during formulation (this option may ultimately require the Phe to be supplied by a complex protein source, which will be gradually broken down during digestion); (2) an implantable pump to deliver a steady dose rate over time (a more elaborate option); and (3) frequent injection or oral gavage of a Phe solution (a more invasive and labor-intensive option).

One current shortcoming that can hamper learning and behavior studies is that there is presently no way to produce completely unaffected mutant animals. Such mice are typically protected by their birth mother’s metabolism in utero but must nurse to survive after birth. Unfortunately, the Phe content of the nursing diet produced by a female, even on a low-Phe diet, is far too high for a mutant neonate and, presumably, a significant amount of brain damage is incurred during the nursing period before the mutant animal can be weaned onto a low-Phe dietary condition. More elaborate ways to solve this problem include successful perinatal implementation of a PAL-type therapy or somatic gene therapy treatment. A less elaborate option is the pup-in-a-cup protocol in which a neonate is intubated and fed a low Phe nursing diet while being incubated at mouse body temperature for a period of 2–3 weeks [23].

Acquiring PKU mice can be difficult and costly. Until recently, interested parties have been able to obtain the BTBR-Pah^emo1 and BTBR-Pah^emo2 mice from the Jackson Laboratory. This is, in principle, an ideal situation in
which all users obtain their animals from the same source and changes introduced by genetic drift are thereby minimized. However, the Jackson Laboratory recently decided that, due to insufficient demand, these mutant lines will be cryopreserved, an option for preservation that implies greatly increased cost and time to supply animals in the future. This decision leaves practicing PKU mouse researchers as the sole remaining inexpensive source for mutant animals. A possible solution to this quandary is to seek out new commercial sources for archiving, production, and distribution of mutant animals that all users may access quickly and easily.

Finally, in addressing areas of future fruitful research we discussed gene therapeutic correction of the PKU phenotype in these mice. This disease model seems especially promising for this purpose for three reasons. It confers a decided hypopigmentation on mutant animals, which have high blood levels of Phe. Anything that reduces the blood Phe levels will increase the ability of the mutant animal to produce pigment. This yields an easily visible coat-color gauge, indicating therapeutic levels of gene expression. Further, it is clear from working with the mild BTBR-Pahenu<sup>+/−</sup> strain that 5% of normal Phe hydroxylase activity is enough to reverse the mutant phenotype. Lastly, the disease PKU has a well-defined and accessible target organ, the liver. Although there have been several attempts in the past with older generation retroviral and adenoviral gene transfer systems that yielded only temporary correction of the PKU phenotype, the newer adeno-associated virus gene transfer systems seem to hold much greater promise for long-term correction. It is hoped that successful application of these vectors to this PKU model system will not only provide new treatment options for humans afflicted with PKU but, inasmuch as PKU is a paradigm of many other inborn errors of metabolism, will also provide new options for other human heritable diseases.

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References


