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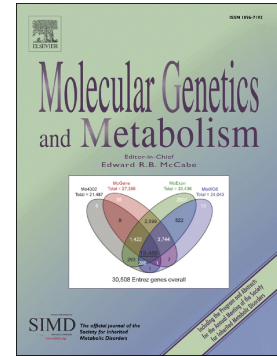
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Partial rescue of neuropathology in the murine model of PKU following administration of recombinant phenylalanine ammonia lyase (pegvaliase)

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Key words: phenylketonuria, recombinant phenylalanine ammonia lyase, tyrosine hydroxylase

Abstract: Pegylated recombinant phenylalanine ammonia lyase (pegvaliase) is an enzyme substitution therapy being evaluated for the treatment of phenylketonuria (PKU). PKU is characterized by elevated plasma phenylalanine, which is thought to lead to a deficiency in monoamine neurotransmitters and ultimately, neurocognitive dysfunction. A natural history evaluation in a mouse model of PKU demonstrated a profound decrease in tyrosine hydroxylase (TH) immunoreactivity in several brain regions, beginning at 4 weeks of age. Following treatment with pegvaliase, the number of TH positive neurons was increased in several brain regions compared to placebo treated ENU2 mice.

Introduction: PKU is an inborn error of metabolism, caused by a genetic deficiency of phenylalanine hydroxylase (PAH), the enzyme responsible for the conversion of phenylalanine (Phe) to tyrosine (Tyr) (Scriver et al., 2001). In the absence of PAH, plasma Phe levels are elevated, ultimately leading to neurocognitive dysfunction (Al Hafid & Christodoulou, 2015). Neurocognitive dysfunction has also been causally linked to reduced plasma Tyr concentrations (de Groot et al., 2010). Pegvaliase (recombinant phenylalanine ammonia lyase, rAvPAL: CAS Number 4.3.1.5) is derived from *Anabaena variabilis*, expressed in *E.coli* and PEGylated. Pegvaliase converts systemic Phe to *trans*-cinnamic acid and trace amounts of ammonia and is currently in clinical development as an enzyme substitution therapy for PKU (Sarkissian et al., 2008). The BTBR*Pah*^{enu2} (ENU2) mouse model of PKU was developed by germline ethylnitrosourea mutagenesis followed by a Phe clearance screen to isolate a mutant mouse line deficient in PAH activity (Shedlovsky et al., 1993). The ENU2 mouse model recapitulates many of the clinical phenotypes of PKU patients, including hyperphenylalanemia, hypopigmentation, and cognitive defects (Shedlovsky et al., 1993). In the ENU2 mouse model, dopamine, catecholamine and serotonin concentrations are reduced in multiple brain regions, similar to observations in human PKU patients (de Groot et al., 2010). Several hypotheses exist to explain this finding, including competition between Phe and Tyr for hydroxylation by TH, reduced synthesis of TH, or reduced BBB transport of Tyr (de Groot et al., 2010). Previous studies have demonstrated that chronic administration of pegvaliase to ENU2 mice results in normalization of plasma Phe levels, body weight, and fur pigmentation (Sarkissian et al., 2008). The objective of the current study was to characterize Tyr expressing neuron populations, astrocytosis, and microglial activation in the hypothalamus, midbrain, and pons of untreated ENU2 mice, and then to evaluate the effects of pegvaliase on select neuropathological parameters in female ENU2 mice following subcutaneous injections given three times weekly for 5, 8 or 12 consecutive weeks.

Materials and Methods: All experimental procedures were approved by the University of Florida's Animal Care and Use Committee. A natural history study was first conducted on untreated ENU2 mice aged 4, 20, 36 and 52 weeks (n= 3-5/age group). Subsequently, forty-four female mice (5-67 weeks old at study initiation, weighing 13-36 grams), consisting of 29 ENU2 mice and 15 wild type (WT) control mice were assigned to 9 groups (5 mice/group) and administered a bolus injection of either placebo vehicle or pegvaliase three times per week (Monday, Wednesday, Friday) for 5, 8, or 12 weeks. Body weights were recorded for all animals pre-study and prior to dose administration for the duration of the

study. Blood samples for Phe analysis were obtained at 4 pm on pre-study and Study Days 7, 14, 28, 42, and 70 by nicking the tail vein. Plasma Phe was determined using a modified fluorimetric assay (McCaman & Robins, 1962). To survey the survival of dopaminergic neurons and to assess the degree of astrocytic and microglial activation, a one in six series of sections was immunohistochemically stained for the TH (PV), glial fibrillary associated protein (GFAP, astrocytes), or the microglial markers CD68 and Iba-1 via previously published protocols (Kielar et al., 2007, Macauley et al., 2012; Vuilleminot et al., 2015; Weimer et al., 2007). To visualize neuronal cytoarchitecture and permit stereological analyses of total neuron number, a one in six series of 40 μ m coronal sections from each forebrain was mounted onto gelatin-chrome alum coated microscope slides and Nissl stained with cresyl fast violet (Kielar et al., 2007).

Results: In the natural history study, optical fractionator estimates of the number of TH positive neurons in the arcuate nucleus, dorsomedial hypothalamic nucleus and area A8 of the pontine reticular formation in WT and untreated ENU2 mice at 4, 20, 36, and 52 weeks of age revealed a significant reduction in the number of TH positive neurons in all three types of nuclei, beginning at 4 weeks of age in untreated ENU2 mice. In marked contrast, counts of Nissl stained neurons in the same brain nuclei revealed no significant change in the number of stained neurons in any nuclei at any age. Staining for glial fibrillary associated protein (GFAP) as a marker of astrocytosis or CD68 as a marker for microglial activation revealed no obvious differences between WT and untreated ENU2 mice at any age. Immunostaining for Iba-1 as an additional marker for microglial activation revealed only subtle differences in microglial morphology, with microglia appearing slightly more darkly stained and with a marginally larger cell body than seen in WT mice at 9 and 13 months of age. However, thresholding image analysis determined these differences in Iba-1 staining were not significant at either age.

In the subsequent study, pegvaliase treatment was well tolerated for the duration of the study, and treated animals had a sleek and healthy appearance, with a fur coat that returned to the dark color of the WT background strain. In contrast, untreated ENU2 mice maintained the characteristic hypopigmented coat, and unhealthy appearance associated with PAH deficiency. Plasma phenylalanine decreased in treated mice within the first 7 days of treatment. A probable immune response was observed at approximately day 14, as evidenced by increasing plasma phenylalanine levels. Upon tolerization, plasma Phe returned to levels similar to WT animals by approximately Day 28, and remained at or below WT levels for the remainder of the treatment period. Following treatment with pegvaliase for 5, 8, or 12 weeks, optical fractionator counts obtained from the arcuate nucleus and dorsomedial hypothalamic nucleus revealed a significant increase in the number of TH positive neurons compared to placebo treated mice (Figure 1).

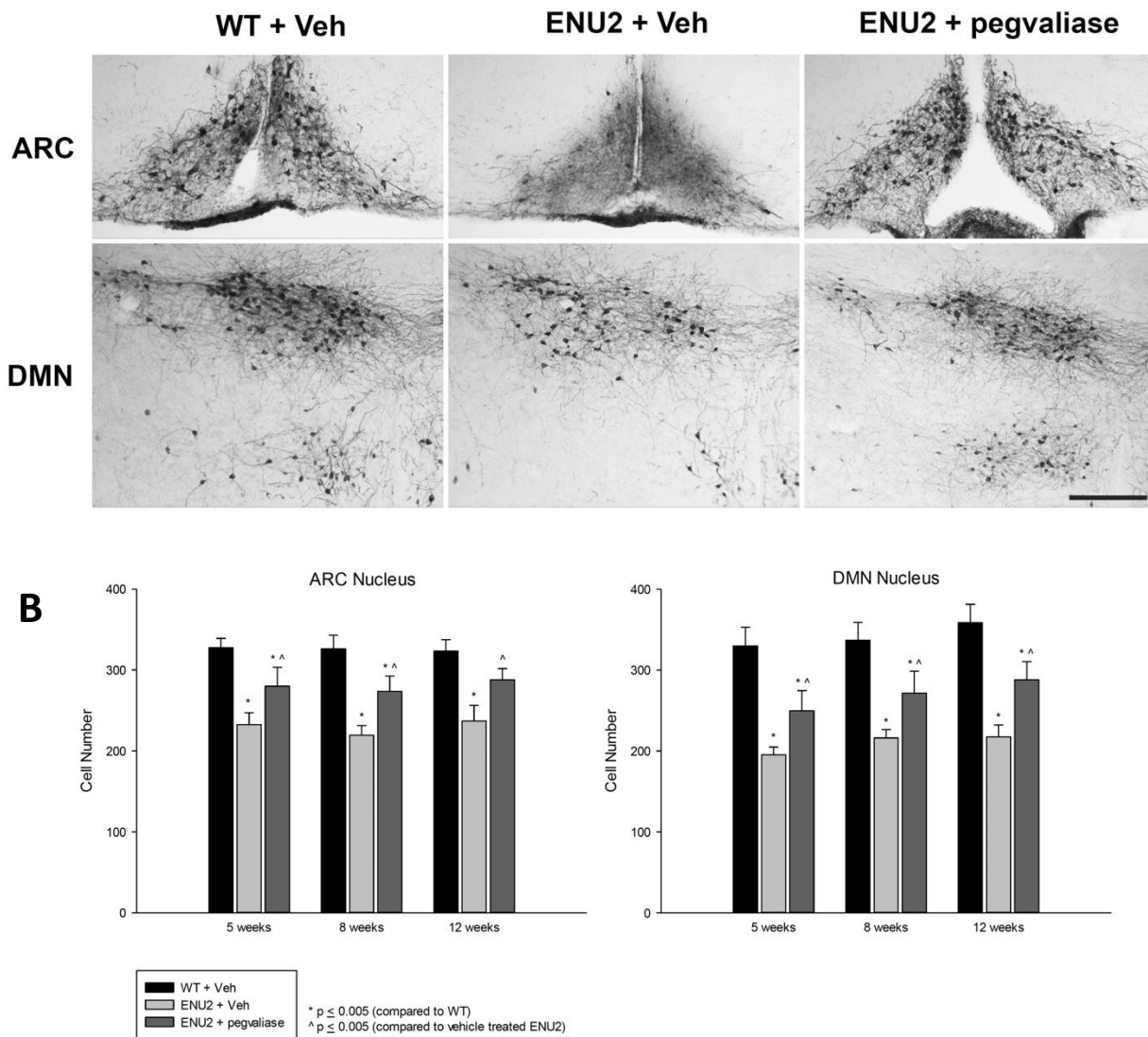


Figure 1: Effects of pegvaliase upon immunohistochemically detectable tyrosine hydroxylase (TH) in ENU2 mice. (A) Compared to 12 week old wild type mice (WT), staining for the dopaminergic marker tyrosine hydroxylase (TH) revealed markedly less intense TH immunostaining in the arcuate nucleus (ARC) and dorsomedial hypothalamic nucleus (DMN) age-matched ENU2 mutant mice, together with an apparent decrease in the number of neurons in both nuclei. In pegvaliase treated ENU2 mice of the same age the intensity of TH staining was largely restored, although not to the levels seen in control WT mice. Scale bar = 200 μm . (B) Optical fractionator counts of TH stained neurons in these nuclei confirmed there to be a significant reduction in both ARC and DMN in untreated ENU2 compared to WT control mice at all ages examined. This phenotype was partially reversed by 5-12 weeks of pegvaliase treatment, which significantly increased the number of neurons in both nuclei, albeit not to WT levels.

Discussion: The natural history assessments in untreated ENU2 mice revealed a dramatic and early onset downregulation of TH in multiple brain areas of ENU2 mice, accompanied by subtle microglial activation. This finding is consistent with reports implicating monoamine neurotransmitter deficiency as

partially responsible for the neurocognitive symptoms commonly observed in PKU. Although the particular mechanism of neurotransmitter deficiency has not been fully elucidated, it is generally accepted to be the result of chronically elevated Phe in PKU patients and may be the result of competition between Phe and Tyr for hydroxylation by TH, reduced synthesis of TH, or reduced BBB transport of Tyr (de Groot et al., 2010; Harding, 2014). Nissl staining of neurons in untreated mice in the natural history study indicated that TH expressing neurons are still present in the brain, but have simply downregulated TH expression to the point that they are no longer detectable by immunostaining. As such these cells may be amenable to intervention, and the therapeutic effects of pegvaliase upon TH neuron number suggest that this is the case. As expected given the dramatic reduction in plasma Phe, treatment with pegvaliase resulted in a significant increase in TH positive neurons. The mechanism by which pegvaliase influences TH levels is currently unclear, and needs further investigation. This effect is observed after 5 weeks of treatment with pegvaliase, and is maintained during 12 weeks of treatment. Although the effect of pegvaliase did not fully restore TH positive neurons to WT levels, the data indicates that pegvaliase is capable of rescuing TH deficiency in ENU2 mice within the first 5 weeks of treatment, and can maintain this effect throughout long-term treatment. Given that the reduction in TH staining occurs to a similar extent in ENU2 mice irrespective of age, our data suggest that rather than there being a therapeutic window of opportunity, pegvaliase treatment may have therapeutic efficacy across a broad age range. It will be important to test this hypothesis experimentally.

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Highlights of Manuscript MGM_2017_97

- Phenylketonuria (PKU) is characterized by neurocognitive dysfunction
- Pegvaliase, an enzyme substitution therapy, may potentially treat PKU
- Pegvaliase administration largely reversed dopaminergic deficits in PKU mice
- Pegvaliase may have therapeutic efficacy across a broad age range