NOVEL THERAPEUTICS AND PATHOMECHANISMS IN HUMAN MENDELIAN DISORDERS:
PHENYLKETONURIA, MAPLE SYRUP URINE DISEASE, AND SUCCINIC SEMIALDEHYDE DEHYDROGENASE DEFICIENCY

By

KARA RAIN VOGEL

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
College of Pharmacy
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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of KARA RAIN VOGEL find it satisfactory and recommend that it be accepted.

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DEHYDROGENASE DEFICIENCY

Abstract

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AUGUST 2014

The body of this dissertation is focused on understanding the pathomechanisms and paving the way for new treatment paradigms in human metabolic disease, particularly phenylketonuria (PKU), maple syrup urine disease (MSUD), and succinic semialdehyde dehydrogenase (SSADH) deficiency.

Phenylketonuria and MSUD are heritable aminoacidopathies displaying aberrant cerebral transport of large neutral aminoacids. This work presents evidence that non-physiological amino acids (NPAAs) have pharmacodynamic efficacy in selective exclusion of phenylalanine from the brain of phenylketonuric mice. Data is presented for feeding and intraperitoneal injection studies of various NPAA’s including methyl-aminoisobutyric acid (MAIB), and some selected MAIB-related alkanoic acid analogues. My data indicates that MAIB is the most selective phenylalanine transport inhibitor identified thus far. Regional brain amino acid studies in intermediate MSUD mice fed low (6%) and high (19%) protein chow suggest that despite varying improvements in the pathophysiological branched-chain
amino acids (leucine, isoleucine and valine) in serum, glutamine, aspartate, glutamate, gamma-aminobutyric acid (GABA), asparagine, citrulline, and serine levels remained unchanged in the brain, demonstrating that dietary correction of MSUD monitored in blood does not accurately reveal corrections in brain biochemistry, providing important insights for human patients. Moreover, I have documented similar findings in PKU mice.

The final chapters of this work contain a review of the treatment prospects for SSADH disorder (a defect in GABA metabolism), and our collaborative work with the University of California focused on hyperphysiological GABA’s on mTOR-driven selective autophagy. SSADH-deficient mouse studies utilizing electron microscopy to quantify mitochondria in liver and brain tissues suggest mitophagic inhibition may play a causal role in the findings of oxidative stress in patients and mice. The impact of these findings are discussed from a pharmacological viewpoint including the scope of treatment of hyperGABAergic disorders.

Lastly, I have included my literature characterization of hepatocyte transplantation (HTx) for inborn errors of metabolism which suggests that we can attempt therapeutic HTx in a murine model of a new disease, transaldolase deficiency, with a goal of gaining almost complete hepatic repopulation with gene-replete (wild-type) cells. My final article is a prelude to future postdoctoral work in the area of liver repopulation and novel therapeutic approaches.
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Dedication

To my Beautiful Boys, who give my life purpose.
Introduction

“The Croonian Lectures are prestigious lectureships given at the invitation of the Royal Society and the Royal College of Physicians. Among the papers of William Croone at his death in 1684, was a plan to endow one lectureship at both the Royal Society and the Royal College of Physicians. His wife provided the bequest in 1701 specifying that it was ‘for the support of a lecture and illustrative experiment for the advancement of natural knowledge on local motion, or (conditionally) of such other subjects as, in the opinion of the President for the time being, should be most useful in promoting the objects for which the Royal Society was instituted’. One lecture was to be delivered by a fellow to the Royal College of Physicians and the other, on the nature and laws of muscular motion to be delivered before the Royal Society. The Royal Society lecture series began in 1738 and that of the Royal College of Physicians in 1749” (Wikipedia “Croonian Lecture” 2014; taken verbatim).

In 1908, Archibald Garrod, an English physician, delivered a series of lectures before the Royal College of Physicians in the Croonian Lectureship series. Professor Garrod provided his clinical insight, vignettes and biochemical findings on what were then considered quite rare disorders in man, including albinism, alkaptonuria, cystinuria and pentosuria, and others. Garrod was the first to intuit and speculate on the chemical disruptions that were the basis of these disorders, and he described their incidence and inheritance patterns. Today, Sir Archibald Edward Garrod KCMG, FRS (1857-1936) is widely considered the father of the field of “inborn errors of metabolism”, the latter primarily autosomal-recessively inherited disorders that disrupt specific metabolic pathways in mammals. Garrod also discovered
alkaptonuria, understanding its inheritance, and providing a rapid mechanism to its identification through the observation of the development of “black urine” when an alkaptonuric’s urine specimen would sit at room temperature for any length of time (Fernández-Cañón et al. 1996).

Well before the presentation of Garrod’s seminal work, another English physician, William Harvey, described the basic tenets of a fundamental process of humans, the blood circulation. William Harvey (1 April 1578 – 3 June 1657) was an English physician. He was the first to completely describe the systemic circulation and properties of blood being pumped to the brain and body by the heart, though earlier writers had provided precursors of the theory (Wikipedia “William Harvey” 2014). In 1657, Harvey was quoted as having said: “Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature by careful investigation of cases of rarer forms of disease”.

In the last 30-odd years, the Gibson laboratory has embraced the tenets and concepts put forth by both Garrod and Harvey, seeking to understand the pathomechanisms and pathophysiology associated with several inborn errors of metabolism, including succinic semialdehyde dehydrogenase (SSADH) deficiency, the most prevalent disorder of GABA degradation, as well as the large neutral amino acidurias phenylketonuria (PKU) and maple syrup urine disease (MSUD). Dr. Gibson discovered the primary enzymatic defect in patients with SSADH deficiency in 1983 (a primary topic of his dissertation), and since that time has described the phenotype and genotype, developed a murine model of the disease, and
evolved his laboratories' work into several ongoing and planned clinical trials. The Gibson laboratory employs pharmacological, cellular and dietary treatment paradigms to study the disorders listed above, encompassing molecular and neurobiological approaches, neuropharmacology and genetics, and various analytical techniques. I was fortunate to join the Gibson laboratory in 2010 while Dr. Gibson was Chair of Biological Sciences at Michigan Technological University, and I have had the opportunity to advance our understanding of the mechanisms and biology of these disorders, which I will describe briefly in the next few paragraphs.

Since his time as a faculty member at the University of Pittsburgh School of Medicine, Dr. Gibson and colleagues have had a keen interest in therapeutic hepatocyte transplantation (HTx) as a treatment paradigm in several inborn errors of metabolism, but most importantly MSUD (Skvorak et al. 2009; Skvorak et al. 2013). Liver-related morbidity is the 12th most common cause of death in the United States, and in 2011 over 16,000 people awaited organ donation (Kochanek et al. 2011), highlighting the expanding need for liver-directed therapeutics. Both cell-culturing techniques and cryopreservation methodologies are showing constant improvements, thereby broadening the potential utility of HTx. Nonetheless, limitations remain in the routine clinical application of HTx: 1) the maintenance of cellular viability post-transplant; 2) immune response/rejection and graft vs. host disease; and 3) engraftment/expansion of transplanted cells into the host liver.

Dr. Markus Grompe, Professor of Pediatrics and Molecular and Medical Genetics at the Oregon Health & Sciences University, Portland, OR, was one of the first investigators to verify that therapeutic HTx could be achieved pre-clinically in the mouse (Karnezis et al. 2001;
Grompe and colleagues successfully exploited fumarylacetoacetate hydrolase (fah) deficiency in the distal portion of the tyrosine metabolic pathway to provide an engraftment advantage for exogenously administered fah+/+ cells to fah-deficient mice. In human tyrosinemia type I, patients invariably progress to early morbidity from hepatocellular carcinoma, a finding recapitulated in the murine model. Intervention with nitisinone, an inhibitor of the upstream formation of homogentisic acid in the tyrosine pathway, prevents the formation of hepatotoxic fumarylacetoacetic acid in patients and mice, a life-saving intervention in both. To achieve successful HTx in the murine model, fah-deficient mice were reared with concomitant NTBC treatment, and fah+/+ hepatocytes applied via intrasplenic administration with staged removal of nitisinone intervention. With this approach, the liver of fah-deficient mice could be ~90% repopulated with fah+/+ cells via a single exogenous cell administration (Hamman, Winn, and Harding 2011; Al-Dhalimy et al. 2002). These studies have been extended in several other models, thereby confirming that HTx could provide corrective potential for a large spectrum of inborn errors of metabolism, a relevant observation in the face of growing donor organ shortages. Of interest, a clinical trial is currently recruiting for PKU patients at the University of Pittsburgh School of Medicine for a study of therapeutic hepatocyte transplant (www.clinicaltrials.gov: NCT NCT01465100).

Together with colleagues at the University of Pittsburgh School of Medicine, Dr. Gibson and colleagues have explored the relevance of HTx in a murine model of MSUD, using direct application of either gene-replete hepatocytes or placental pluripotent stem cells into the hepatic mass for repopulation (Skvorak et al. 2009; Skvorak et al. 2013). To attempt to
gain an engraftment advantage, newborn MSUD mice were employed, in which the liver was actively growing and expanding. Whereas significant metabolic correction of the ensuant hyperleucinemia associated with MSUD was observed in conjunction with 3-5% of engraftment of gene-competent cells, long-term correction has been difficult to attain. My future plans are to explore a different mouse model for HTx, the TALDO (transaldolase)-deficient mouse (Hanczko et al. 2009; Engelke et al. 2010). Transaldolase (TALDO) deficiency is a rare metabolic disease in the pentose phosphate pathway, which manifests as a severe, early-onset multisystem disease. The murine taldo model represents a faithful replica of the human disorder, the latter rare and described in only about 30 patients, yet a majority of taldo-deficient mice eventually succumb to hepatocellular disease (Hanczko et al. 2009). Only recently has the finding of hepatocellular carcinoma been described in a TALDO-deficient patient (Leduc et al. 2014). My hypothesis is that taldo-deficient mice will represent an outstanding platform for therapeutic HTx, akin to what was observed by Grompe and coworkers in the fah-deficient mouse model. If this hypothesis is correct, I would propose moving these studies forward with a goal of deleting the TALDO gene in other larger mammals, such as pig, in order to develop a system in which to develop humanized liver in a larger mammal, through repopulation of wild-type human hepatocytes. Whereas xenografts from pig to human are almost universally ineffective, the appropriate immunosuppression the converse (HTx from human to pig) may be mechanistically feasible, especially with the progressive demise of endogenous taldo-negative hepatic cells in porcine liver. “Humanized” liver tissue derived from an animal model of the dimensions of the pig may facilitate development of a much larger pool of transplantable organs for future needs in humans.
The Gibson laboratory is also actively interested in studying specific molecules that can block the entry of phenylalanine into the brain of both PKU patients, and *pah* (phenylalanine hydroxylase)-deficient mice. Patients with PKU are treated with dietary restriction of protein for life, and the diet is both challenging to maintain, unpalatable and problematic for maintenance, especially during the adolescent period. Any pharmacotherapeutic liberalizing the dietary restrictions in PKU patients would significantly advance the treatment of this inherited amino aciduria. The laboratories’ interest in this line of study springboarded from the work of Zinnanti and colleagues, in which it was shown that feeding the non-physiological amino acid norleucine to *msud*-deficient mice restricted the accumulation of leucine in the brain, and led to measurable improvements in both neuropathology and behavior (Zinnanti et al. 2009). Along these lines, the Harper group at the University of Wisconsin had previously demonstrated that selected non-physiological amino acids, such as aminoisobutyrate, could retard the transport of phenylalanine across the blood-brain transporter responsible for the movement of large neutral amino acids (Tews and Harper 1986; Tews, Repa, and Harper 1990; Tews, Repa, and Harper 1991; Tovar et al. 1988). My work in this area has targeted two lead compounds, aminoisobutyrate and methylaminoisobutyrate, that have promise in the selective capacity to restrict phenylalanine transport into the brain of PKU mice. My work with the Gibson lab in this area enabled Dr. Gibson to obtain a two-year grant from the National PKU Alliance that supported my studies, and a more recent ITHS (Institute for Translational Health Sciences)-collaborative grant with the University of Washington, the target of which is to develop sufficient preclinical safety and toxicological data to facilitate a phase 0 trial of methylaminoisobutyrate. I am also
embarking on overexpression studies of several plant proteins that are allosterically activated by phenylalanine, with the goal of eventually developing an at-home blood phenylalanine monitoring device for PKU patients. The latter would fill a major deficiency in gauging the metabolic control of PKU through blood monitoring, since these analyses can take several days to complete.

SSADH deficiency has been a primary focus of the Gibson laboratory for decades (Gibson et al. 1983). SSADH-deficient mice represent a relevant phenocopy of human SSADH deficiency (Hogema et al. 2001), although on the more severe end of the phenotypic spectrum. The attenuated lifespan of this model associates with a seizure profile that evolves from absence through generalized tonic-clonic convulsions, and eventual lethal status epilepticus (Cortez et al. 2004; Buzzi et al. 2006; Wu et al. 2006), associated with chronic elevations of both GABA and the GABA, gamma-hydroxybutyric acid (GHB). Premature lethality facilitated characterization of a number of potent pharmacotherapeutics that were hypothesized to have utility in phenotype rescue (Hogema et al. 2001; Gupta et al. 2002). The high content of taurine in dam’s milk prompted Hogema and colleagues (2001) to examine this non-physiological amino acid as an intervention in SSADH-deficient mice, and these investigators observed significant extension of truncated lifespan. An open-label intervention in SSADH deficiency with taurine was pursued following these findings, and in conjunction with therapeutic efficacy observed in a single SSADH-deficient patient (Pearl et al. 2009). Although biomarker studies are still in progress, neurobehavioral parameters in patients were not improved with taurine intervention (Pearl et al. 2014). Additionally, rescue of the lethal murine phenotype with the GABAB receptor antagonist CGP-35348 has led to the
genesis of a double-blind, placebo-controlled trial of the SGS-742, also a GABA\(_B\) receptor antagonist, that is currently enrolling subjects.

The most successful intervention for rescue of premature lethality in SSADH-deficient mice was NCS-382, a specific, high-affinity GHB receptor antagonist. The latter produced the first solid data indicating a role for GHB in the pathophysiology of SSADH deficiency. Although extensively employed to characterize the location, structure and activity of GHB receptors in brain, NCS-382 has not been characterized \textit{in vivo}. Thus, the goal of potentially intervening in SSADH-deficient patients with NCS-382 awaits several key achievements in our laboratory, which I am beginning to explore: 1) development of pilot toxicology, safety and tolerability, as well as pharmacokinetic data, on NCS-382 in animals; 2) development of an FDA IND for the use of NCS-382 in a phase 0 trial; and 3) successful intervention in healthy adults with NCS-382. Nonetheless, I believe that NCS-382 could be developed for potential clinical use in SSADH deficiency, while also providing a useful therapeutic in other forms of high-dose chronic GHB intoxication, such as the use of GHB as a recreational drug (Bay et al. 2014).

Prior to my joining the laboratory, earlier studies from the Gibson laboratory identified altered biomarkers indicative of oxidative stress in SSADH-deficient mice, including elevated liver and cerebellar superoxide dismutase (mitochondrial) and hypothalamic catalase (peroxisomal), associated with depletion of the key intracellular antioxidant glutathione (GSH; Latini et al. 2007; Sauer et al. 2007). In early 2011, the Gibson laboratory was contacted by Prof. Suresh Subramani and colleagues from the University of California San Diego. Professor Subramani is a well-respected investigator who has authored multiple publications outlining the mechanisms by which selected proteins are imported into mitochondria and peroxisomes.
(Nazarko et al. 2014), employing yeast as a model system. In their continuing studies focused on mechanistic regulation of autophagy in yeast, Dr. Subramani and colleagues identified a role for the inhibitory neurotransmitter, GABA, in selectively blocking autophagic processes associated with mitophagy and pexophagy (Lakhani et al. 2014), employing yeast mutants in the GABA degradative pathway that recapitulated comparable biochemical outcomes to that of higher mammals. Subramani and colleagues further demonstrated that over-accumulation of organelles correlated with a higher degree of oxidative metabolism (mitochondria, peroxisomes) increasing intracellular oxidative damage.

Dr. Subramani and colleagues sought to determine if these autophagic disruptions were recapitulated in mammalian systems, and specifically in the hyperGABAergic SSADH-deficient mouse. My work with the UC San Diego group was able to confirm elevated mitochondrial numbers in SSADH-deficient brain and liver, expanded size of liver mitochondria, and enhanced parameters of oxidative stress associated with these anomalies. Importantly, treatment with the autophagy-inducing drug rapamycin normalized the majority of these abnormalities. These data have opened up completely unsuspected roles for GABA in cell signaling, novel treatment paradigms for SSADH-deficient patients, and insight into other hyperGABAergic states and the ensuing role of oxidative damage. The impact of these studies is reflected in the recent award of an R21 grant to the Gibson laboratory to more fully explore the link(s) between GABA and autophagy, and the role of pharmacotherapeutics in mitigating those processes.

Finally, in very recent work submitted for publication, I have examined the effect of protein intake on blood and brain amino acid levels in a murine model of MSUD. As is the
case for PKU, metabolic control of MSUD is gauged through examination of blood amino acids, and the goal is to maintain blood leucine levels in an appropriate target range, usually between 250-400 micromole/liter. The Gibson laboratory has advanced the hypothesis that blood leucine is not necessarily a faithful surrogate for the concentration of leucine (and other amino acids) in the brain. This hypothesis stems from the observation that even well-controlled MSUD patients (control based upon blood leucine level) show long-term neurocognitive deficits in IQ, executive function, and others (Muelly et al. 2013). I have shown that dietary intervention with reduced protein intake in MSUD mice, which results in normalization of blood leucine levels, still left major anomalies in brain amino acid homeostasis in these mice (Vogel et al, submitted). Importantly, these deficits were predominantly in amino acids involved in neurotransmission, including glutamate, glutamine, aspartate and GABA. These data, and similar data being obtained in mice that model PKU, argue strongly that gauging metabolic control in these heritable amino acidurias is a very poor surrogate for metabolic control in the brain, underscoring the urgent need for more targeted therapeutics.

In the ensuing chapters of this dissertation, I will highlight novel pathophysiological concepts and associated treatment considerations for SSADH deficiency, PKU and MSUD. Following in the footsteps of both Garrod and Harvey, it is my hope that I have advanced our understanding of these disorders, improved the treatment horizons for patients, and thereby provided an lasting impact on our knowledge of these, and other, inborn errors of metabolism.
Bibliography


CHAPTER TWO
Non-physiological amino acid (NPAA) therapy targeting brain phenylalanine reduction: pilot studies in PAHENU2 mice.

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Abstract

Transport of large neutral amino acids (LNAA) across the blood brain barrier (BBB) is facilitated by the L-type amino acid transporter, LAT1. Peripheral accumulation of one LNAA (e.g., phenylalanine (phe) in PKU) is predicted to increase uptake of the offending amino acid to the detriment of others, resulting in disruption of brain amino acid homeostasis. We hypothesized that selected non-physiological amino acids (NPAAs) such as DL-norleucine (NL), 2-aminonorbornane (NB; 2-aminobicyclo-(2,1,1)-heptane-2-carboxylic acid), 2-aminoisobutyrate (AIB), and N-methyl-aminoisobutyrate (MAIB), acting as competitive inhibitors of various brain amino acid transporters, could reduce brain phe in Pah enu2 mice, a relevant murine model of PKU. Oral feeding of 5 % NL, 5 % AIB, 0.5 % NB and 3 % MAIB reduced brain phe by 56 % (p < 0.01), -1 % (p = NS), 27 % (p < 0.05) and 14 % (p < 0.01), respectively, compared to untreated subjects. Significant effects on other LNAAAs (tyrosine, methionine, branched chain amino acids) were also observed, however, with MAIB displaying the mildest effects. Of interest, MAIB represents an inhibitor of the system A (alanine) transporter that primarily traffics small amino acids and not LNAAAs. Our studies represent the first in vivo use of these NPAAs in Pah enu2 mice, and provide proof-of-principle for their further preclinical development, with the long-term objective of identifying NPAA combinations and concentrations that selectively restrict brain phe transport while minimally impacting other LNAAAs and downstream intermediates.

Introduction

Phenylketonuria (OMIM 261600) represents a heritable amino aciduria in which supraphysiological accumulation of phe is predicted to alter large neutral amino acid (LNAA)
transport into brain. Depletion of specific brain LNAAs may have particularly deleterious effects, since these are precursors of critical downstream intermediates. These include S-adenosylmethionine (SAMe) derived from methionine, and the monoamine neurotransmitters dopamine and serotonin, derived from tyrosine and tryptophan, respectively. Among multiple mammalian amino acid transporters, at least four transport LNAAs (including phe (phe; F), tyrosine (tyr; Y), tryptophan (trp; W), leucine (leu; L), isoleucine (ile; I), valine (val; V), and methionine (met; M)) across brain capillaries (e.g., the blood brain barrier (BBB)) and intestinal mucosa (Choi and Pardridge 1986; Smith et al 1987). These transporters (LAT 1-4; all Na+-independent) comprise the system L nutrient transport system (Chrostowski et al 2009; Lin et al 2004; Christensen 1990; Table 1), originally characterized in Ehrlich ascites tumor cells as a system susceptible to 2-aminonorbornane (NB; Fig. 1) inhibition. Collectively, these LATs promote LNAA movement down a concentration gradient from blood, via endothelia, to the extracellular fluid (ECF) side and serve to insure homeostatic maintenance of brain LNAA levels. Under pathological situations (e.g., PKU), elevated levels of phe will overwhelm LAT homeostasis and increase self-uptake to the detriment of other LNAAs. A schematic diagram of the amino acid transport systems at the BBB, and their corresponding amino acid specificity, is depicted in Fig. 2.
<table>
<thead>
<tr>
<th>Transporter</th>
<th>Expression&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acids transported&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAT-2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Je, Ile, Ki, Pl, Br, Te, SM</td>
<td>Y,F,W,T, N,I,C,S,L,V,Q (H,A,M,G)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Segawa et al 1999</td>
</tr>
<tr>
<td>LAT-3</td>
<td>Pa, Li (fetal, adult), SM</td>
<td>I,L,V,F</td>
<td>Babu et al 2003</td>
</tr>
<tr>
<td>LAT-4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Pl, Ki, Leuc</td>
<td>F,L,I,M</td>
<td>Bodoy et al 2005</td>
</tr>
</tbody>
</table>

<sup>a</sup>(tissue abbreviations) = Li, liver; BM, bone marrow; Br, brain; Pl, placenta; Te, testes; Je, jejunum; Ile, ileum; Ki, kidney; SM, skeletal muscle; Pa, pancreas; Leuc, leucocytes

<sup>b</sup>(amino acid single letter codes) = H, his; T, thr; N, asn; C, cys; S, ser; Q, gln; A, ala; G, gly (see text for other amino acid abbreviations)

<sup>c</sup>isoform of LAT-1,

<sup>d</sup>lower affinity for amino acids in parentheses,

<sup>e</sup>isoform of LAT-3

---

**Figure 1:** NPAAs evaluated in the current study. A, norleucine (NL); B, 2-aminoisobutyric acid (AIB); C, N-methyl-2-aminoisobutyric acid (MAIB); D, 2-aminonorbornane (NB). MAIB is a selective competitive inhibitor of the system A transport system (see Fig. 2). Structures were obtained from web resources ([www.wikipedia.com](http://www.wikipedia.com); [www.sigma-aldrich.com](http://www.sigma-aldrich.com))
Several NPAAs have been employed as inhibitors of different brain amino acid transport systems (Fig. 2; Tovar et al 1988; Tews and Harper 1986; Tews et al 1991; Tews et al 1990; McKean et al 1968). The prototypical LAT inhibitor, NB, is a bridged compound with high specificity for the LAT-1 system. Tews and Harper (1986) demonstrated that dietary...
supplementation of NL (Fig. 1) effectively lowered brain phe levels in the rat, while Ennis and coworkers (1994) presented evidence that AIB (Fig. 1) could compete with phe for transport on the LAT-1. Methyl-aminoisobutyrate (MAIB; Fig. 1) represents a specific inhibitor of the system A transporter with preference for small amino acids, although it can traffic glutamine, threonine, and methionine, the latter two generally considered LNAAs (Pisoni et al 1987). The preceding reports, and others documenting competition between LNAAs themselves for access to the brain (Christensen et al 1948; Knudsen et al 1995) have led to the concept of LNAA supplementation as an approach to lowering brain phe in PKU patients. Along these lines, Pietz and coworkers (1999) demonstrated that LNAA supplementation could decrease phe levels, and others (Matalon et al 2006; Michals-Matalon et al 2007) modified this diet with successful outcomes in PKU patients. Nonetheless, long term use of these LNAA-derived diets may be hampered by negative nitrogen balance (Dotremont et al 1995; van Spronsen and Enns 2010), which highlights the potential utility of applying NPAAAs that are predicted to be non-metabolizable.

A systematic evaluation of NPAA intervention, targeting LNAA transport, has not been presented in any mammalian system. Zinnanti and coworkers (2009) provided proof-of-principle for this therapeutic approach employing dietary supplementation of NL in mice with intermediate maple syrup urine disease. NL feeding in these animals (in which leu, ile and val accumulate in response to the primary defect in branched-chain ketoacid dehydrogenase) extended lifespan while improving both brain biochemistry and neurobehavioral outcomes in affected animals. We have extended the work of Zinnanti and coworkers by implementing a
preliminary characterization of the NPAAs described in Fig. 1 in a murine model of PKU, Pah\textsuperscript{enu2} mice. Outcome measures included body weights, dietary consumption and movement, and metabolic measures including LNAAs in brain and blood, monoamine neurotransmitters and methionine analogues in brain, and blood chemistries under selected dietary regimens. The current report summarizes the findings of our pilot studies.

**Materials and Methods**

**Animal husbandry and subject number**

For breeding, breeder pairs were established with male Pah\textsuperscript{enu2} and female heterozygous Pah\textsuperscript{enu2} subjects. Offspring were genotyped employing genomic DNA derived from tail clips obtained at weaning (20 days of life). Subsequently, PCR and restriction endonuclease digestion with BsmAl was performed, followed by visualization using 4% agarose gel electrophoresis (Zagreda et al 1999). Since our breeding scheme did not provide wild-type (WT) controls, and we sought to employ heterozygous Pah\textsuperscript{enu2} subjects as controls (thereby obviating the need for additional husbandry to generate WT subjects), we characterized monoamines and amino acids in WT subjects (of identical genetic background, C57Bl6) and compared to heterozygous Pah\textsuperscript{enu2} mice (Table 2). No significant differences were observed for eight of 13 metabolites. Conversely, 5-HT, HVA, 5-HT turnover, total BCAA, and Met did differ significantly, although there was overlap of ranges (for all but HVA) and mean values were within 2 SD of each other. Based upon these comparative data, we felt justified in our pilot studies employing heterozygous Pah\textsuperscript{enu2} mice as internal controls.
Table 2

Monoamines and amino acids in the brain of heterozygous Pah
enu2 (T) subjects and wild-type (WT) controls

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>T mice</th>
<th>WT mice</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>8.10 ± 1.03 (n = 12; 6.11–10.24)</td>
<td>8.80 ± 1.24 (n = 7; 6.62–10.21)</td>
<td>ns</td>
</tr>
<tr>
<td>5-HT</td>
<td>7.60 ± 4.34 (n = 12; 2.92–15.36)</td>
<td>3.09 ± 0.23 (n = 7; 2.65–3.35)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.70 ± 0.06 (n = 5; 0.63–0.81)</td>
<td>0.66 ± 0.08 (n = 7; 0.54–0.79)</td>
<td>ns</td>
</tr>
<tr>
<td>3-MT</td>
<td>0.56 ± 0.09 (n = 12; 0.43–0.71)</td>
<td>0.51 ± 0.25 (n = 7; 0.25–0.85)</td>
<td>ns</td>
</tr>
<tr>
<td>HVA</td>
<td>1.08 ± 0.10 (n = 5; 0.97–1.23)</td>
<td>1.71 ± 0.24 (n = 7; 1.41–2.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>3.01 ± 1.78 (n = 12; 0.91–5.40)</td>
<td>1.99 ± 0.48 (n = 7; 1.39–2.80)</td>
<td>ns</td>
</tr>
<tr>
<td>DA turnover</td>
<td>0.28 ± 0.09 (n = 12; 0.20–0.55)</td>
<td>0.27 ± 0.06 (n = 7; 0.24–0.41)</td>
<td>ns</td>
</tr>
<tr>
<td>5-HT turnover</td>
<td>0.39 ± 0.09 (n = 12; 0.31–0.56)</td>
<td>0.65 ± 0.15 (n = 7; 0.46–0.84)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DA release</td>
<td>0.06 ± 0.01 (n = 5; 0.05–0.07)</td>
<td>0.06 ± 0.02 (n = 7; 0.03–0.09)</td>
<td>ns</td>
</tr>
<tr>
<td>Phe</td>
<td>66 ± 16 (n = 12; 31–87)</td>
<td>59 ± 33 (n = 8; 32–124)</td>
<td>ns</td>
</tr>
<tr>
<td>Tyr</td>
<td>64 ± 17 (n = 5; 45–88)</td>
<td>52 ± 42 (n = 8; 13–120)</td>
<td>ns</td>
</tr>
<tr>
<td>BCAA</td>
<td>155 ± 30 (n = 5; 108–189)</td>
<td>104 ± 21 (n = 7; 77–144)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Met</td>
<td>71 ± 14 (n = 12; 57–95)</td>
<td>38 ± 16 (n = 8; 14–63)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values shown are mean ± 1 SD, with number of animals and range in parentheses. BCAA represents total BCAA (the sum of valine, isoleucine and leucine) with 5-HT turnover representing turnover of serotonin; DA turnover = (DOPAC + HVA)/DA; 5-HT-turnover = 5-HIAA/5-HT; DA release = 3-MT/DA. Statistical analyses employed an unpaired t test.
A sample size of five facilitated simple parametric statistical analysis (t test, ANOVA).

This sample size was based upon power calculations utilized in another study in which hepatocyte transplantation was the study parameter. In that work, an n = 5 sample size (hepatocyte-transplanted imsud mice vs. PBS-transplanted imsud mice; Skvorak et al 2009) enabled detection of a 20 % reduction in alloisoleucine level with a significance of p < 0.05. Accordingly, this number of subjects was adopted in the current study. The interventional protocol was approved by the institutional IACUC (protocol L0214).

Diet preparation and intervention

Dietary NPAA administration was chosen to most closely mimic the clinical setting.

NPAAAs included 5 % 2-aminoisobutyric acid (AIB), 5 % DL-norleucine (NL), 0.5 % norbornane (NB; 2-amino-1-bicyclo(2,2,1)heptane-2-carboxylic acid), and 3 % N-methyl-aminoisobutyric acid (MAIB) (all obtained from Sigma Aldrich), and control chow (2018 Teklad Global Rodent Chow, 18 % protein, or extruded sterilizable chow 2018X). We characterized 5 % (w/w) NL and AIB based upon the previous report of Zinnanti et al (2009) employed in the imsud murine model. The expense of NB (~$350/g) prohibited its use at 5 %; accordingly, we chose 0.5 % w/w (a serendipitous choice). Two experiments were performed, with the first evaluating NL, NB and AIB in regular 18 % Teklad mouse chow (18R), and the second utilizing MAIB in sterilizable, extruded 18 % protein chow. Following our initial trial with NL, AIB and NB intervention, our mouse colony suffered a Helicobacter infection, likely associated with our unsterilized 18 % diet. Accordingly, for MAIB intervention we were constrained to employ an extrudable,
sterilizable diet of identical protein content. We chose to initially examine a regular protein chow (as opposed to a low-protein chow) in order to gauge the potential for eventual dietary relaxation of protein intake in the clinical setting.

Chow was made in batches of 300 g. 18R was made with 300 mL of filtered water and mixed with drug and dehydrated 24 h at 46 °C. 18X chow was made with 500 mL of autoclaved water and dehydrated 20–24 h at 46 °C. Drugs were not autoclaved, and assumed stable at 46 °C. Dietary intervention was begun at 3.5 weeks of age (post weaning and genotyping) and maintained for 3 weeks. Animals were housed under a 12 h light/dark cycle with ad libitum access to food and water. Mouse weights were recorded three times weekly, and food consumption calculated weekly. Brain halves and sera were collected into 1.5 mL tubes at sacrifice. Brain halves were flash-frozen with liquid nitrogen prior to storage at −80 °C.

Analyses of LNAA including SAMe and SAH

We used stable-isotope dilution liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to determine SAM, SAH, methionine (met), tryptophan (trp), phenylalanine (phe), tyrosine (tyr), isoleucine (ile), leucine (leu) and valine (val) in serum and brain tissue from Pah emu2 mice. Stable isotope internal standards (2H3-SAMe, 2H3-methionine, 2H3-tryptophan, 2H3-phenylalanine, 2H7-tyrosine, 13C6-isoleucine, 2H3-leucine and 2H8-valine) were obtained from either CDN Isotopes or Cambridge Isotope Laboratories. Isotopically labeled SAH was not commercially available and 2H4-SAH was prepared by enzymatic synthesis utilizing an Axis Homocysteine EIA kit (Axis-Shield Diagnostics Ltd, UK). Composition of the reaction mixture were as follows: 900 μl phosphate buffer (reagent A), 50 μl adenosine, dithiothreitol and citric acid (reagent B), 50 μl recombinant S-adenosyl-L-
homocysteine hydrolase (reagent C) and 100 μl 1 mmol/L ²H₄-D,L-homocysteine. The mixture was incubated at 37 °C for 30 min. Upon completion of the reaction, ²H₄-SAH was purified by HPLC with ultraviolet absorbance at 260 nm by injecting 25 μl fractions and the eluate fractions containing ²H₄-SAH were pooled. ²H₄-SAH was separated with a Spheroclon ODS2 analytical column (250 × 4.6 mm, 5 μm; Phenomenex) with H₂O-methanol (90:10, by volume), containing 500 μL/L formic acid as mobile phase at a flow rate of 1.5 mL/min. The concentration of ²H₄-SAH was estimated by LC-MS/MS. The obtained ²H₄-SAH stock solution was combined with additional internal standards in eluent A. Calibrators and stable isotope internal standards were included in each analytical run for calibration. Stock standards of S-adenosylmethionine (SAMe) and S-adenosylhomocysteine (SAH) were prepared in 0.1 M PCA at a concentration of 1 mmol/L and stored at −80 °C. An amino acid standard solution (500 μmol/L in 0.1 M HCL; Fluka) containing methionine (met), tryptophan (trp), phenylalanine (phe), tyrosine (tyr), isoleucine (ile), leucine (leu) and valine (val) was purchased from Sigma (St. Louis, MO, USA).

Preparation details of half-brain tissue samples obtained from Pah ᵇᵐᵘ² mice were as follows. Calibration stock solutions of each standard were diluted in type 1 water to perform a 6-point calibration curve (Table 3). Pah ᵇᵐᵘ² half-brain tissue samples that were previously deproteinized 1:5 with ice-cold 0.1 M PCA were thawed and spun. Samples were prepared by the addition of 180 μL mobile phase A containing approximately 10–50 μmol/L of each internal standard to 20 μL of blank, standard, QC or brain extract and mixed by vortex. Prepared sample was loaded into a 96-well microtiter plate and 5 μl was injected for analysis.
Table 3

Mass transitions, retention times, and specific calibration curves

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Analyte MRM (m/z)</th>
<th>Labeled isotope</th>
<th>Labeled isotope MRM (m/z)</th>
<th>Retention time</th>
<th>Serum calibration curve</th>
<th>Tissue calibration curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td>399 → 250</td>
<td>$^2$H$_3$-SAMe</td>
<td>402 → 250</td>
<td>7.1</td>
<td>400–12.5 nmol/L</td>
<td>10–0.31 μmol/L</td>
</tr>
<tr>
<td>SAH</td>
<td>385 → 136</td>
<td>$^2$H$_4$-SAH</td>
<td>389 → 138</td>
<td>6.8</td>
<td>400–12.5 nmol/L</td>
<td>10–0.31 μmol/L</td>
</tr>
<tr>
<td>met</td>
<td>150 → 104</td>
<td>$^2$H$_3$-met</td>
<td>153 → 107</td>
<td>6.5</td>
<td>250–7.8 μmol/L</td>
<td>200–6.25 μmol/L</td>
</tr>
<tr>
<td>trp</td>
<td>205 → 188</td>
<td>$^2$H$_3$-trp</td>
<td>208 → 191</td>
<td>8.0</td>
<td>250–7.8 μmol/L</td>
<td>200–6.25 μmol/L</td>
</tr>
<tr>
<td>phe</td>
<td>166 → 120</td>
<td>$^2$H$_3$-phe</td>
<td>171 → 125</td>
<td>7.6</td>
<td>250–7.8 μmol/L</td>
<td>200–6.25 μmol/L</td>
</tr>
<tr>
<td>tyr</td>
<td>182 → 136</td>
<td>$^2$H$_7$-tyr</td>
<td>189 → 143</td>
<td>6.5</td>
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<td>200–6.25 μmol/L</td>
</tr>
<tr>
<td>ile</td>
<td>132 → 86</td>
<td>$^{13}$C$_6$-ile</td>
<td>138 → 91</td>
<td>7.3</td>
<td>250–7.8 μmol/L</td>
<td>200–6.25 μmol/L</td>
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<td>Analyte</td>
<td>Analyte MRM (m/z)</td>
<td>Labeled isotope</td>
<td>Labeled isotope MRM (m/z)</td>
<td>Retention time</td>
<td>Serum calibration curve</td>
<td>Tissue calibration curve</td>
</tr>
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</tr>
<tr>
<td>leu</td>
<td>132 → 82</td>
<td>$^2$H$_3$-leu</td>
<td>135 → 85</td>
<td>7.5</td>
<td>250–7.8 μmol/L</td>
<td>200–6.25 μmol/L</td>
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<tr>
<td>val</td>
<td>118 → 72</td>
<td>$^2$H$_8$-val</td>
<td>126 → 80</td>
<td>5.9</td>
<td>250–7.8 μmol/L</td>
<td>200–6.25 μmol/L</td>
</tr>
</tbody>
</table>

Preparation details of serum samples obtained from Pah<sup>enu2</sup> mice were as follows. Stock solutions of each standard were diluted in type 1 water to perform a 6-point calibration curve (Table 3). Sample preparation utilized microcentrifugal filter units, Amicon Ultra-0.5 ml, 10 kDa NMWL (Millipore, USA). Samples were prepared by the addition of 100 μl mobile phase A containing 10–50 μmol/L labeled-isotope internal standards to 20 μl of blank, standard, serum, QC or unknown serum followed by vortex. Microcentrifugal filter units were centrifuged for 20 min at 14,800×g at 4 °C. Sample filtrate was transferred to a microtiter plate and 5 μl was injected for analysis.

Chromatographic separation was achieved on an EZ-faast 250 × 2.0 mm 4 μm AAA-MS analytical column (Phenomenex) maintained at 36 °C at a flow of 250 μL/min with a binary gradient and a total run time of 12 min. Eluents for HPLC were: (A) 4 mM ammonium acetate, 0.1 % formic acid, 0.1 % heptafluorobutyric acid (pH = 2.5); (B) 100 % methanol and 0.1 % formic acid. The initial gradient condition was 75 % A: 25 % B and was increased in a linear fashion to 100 % B in 6 min and held constant for 1 min. At 7.1 min the eluents were reset to initial conditions for 5 min. The flow from the column was delivered to the ion source from the
period of 3 to 8 min, otherwise the flow was diverted to waste. The compounds were detected by multiple reaction monitoring (MRM) using positive ESI with a dwell time of 30 ms. The curtain gas was set at 15 L/min, and source gas 1 and 2 were set at 60 L/min. The heater was set to 700 °C with an ionspray voltage of 5000 V and CAD gas (nitrogen) was set at $3.5 \times 10^{-5}$ Torr. Sample separation and injection was performed by a Shimadzu Prominence LC System interfaced with a 4000 Q TRAP® LC-MS/MS (ABSciex). All data were collected using Analyst software version 1.4.2.

**Analyses of monoamines in brain**

Monoamine metabolites in Pah$^{enu2}$ and heterozygous Pah$^{enu2}$ half-brain were quantified by reverse-phase HPLC with electrochemical detection. Flash-frozen tissues were homogenized in 0.1 M perchloric acid supplemented with dithioerythritol and diethylenetriamine pentaacetic acid (Ogburn et al 2006). A schematic diagram of the pathways of degradation for the monoamine neurotransmitters, dopamine (DA) and 5-hydroxytryptamine (5-HT; serotonin) is shown in Fig. 3. Note that the turnover of DA is described as the sum (DOPAC + HVA)/DA, while that for 5-HT is represented by 5-HIAA/5-HT. The ratio of 3-methoxytyramine (3-MT) to DA represents an estimate of the release of DA into the synaptic cleft.

![Figure 3](image_url)

**Figure 3:** Schematic of dopamine (DA) and serotonin (5-hydroxytryptamine; 5-HT) synthesis and metabolism. Schematic of dopamine (DA) and serotonin (5-hydroxytryptamine; 5-HT) synthesis
and metabolism. Abbreviations: Tyr, tyrosine; Trp, tryptophan; L-DOPA, L-
dihydroxyphenylalanine; 5-HTP, 5-hydroxytryptophan; DOPAC, 3,4-dihydroxyphenylacetic acid;
HVA, homovanillic acid; 3-MT, 3-methoxytyramine (a DA metabolite released into the synaptic
cleft); 5-HIAA, 5-hydroxyindoleacetic acid

**Blood chemistries**

Comprehensive serum chemistry was obtained using the serum pathology profile
provided by IDEXX RADIL (Columbia, MO, USA; [http://www.idexxradil.com](http://www.idexxradil.com)). Brain estimation of
NL content

During initial evaluation of NPAA administration with NL, we employed the MassTrak system
([http://www.waters.com](http://www.waters.com)). With this system, we found perfect coelution of NL with phe,
precluding accurate quantitation of either species. However, employing LC-MS/MS analysis for
quantitation of phe in later studies, we could employ both systems to estimate brain/blood NL
levels by differential, thus providing an estimate of NL levels during intervention. The absence
of stable isotopically labeled internal standard for NL precluded our capacity to quantitate NL
levels using LC-MS/MS.

**Statistical analyses**

Metabolite data was grouped with respect to genotype (Pah<sup>enu2</sup> and heterozygous Pah<sup>enu2</sup>) in order to correlate the effects of NPAAs under normo- and hyperphenylalaninemic
conditions. A column statistical approach (ANOVA with Tukey post-hoc) facilitated significant
reduction of data analysis with this grouping. For studies employing MAIB and those comparing
metabolites for wild-type (WT) and heterozygous Pah<sup>enu2</sup> mice, a two-way t test was employed.
Analysis was performed with the Graphpad Prism V5 program, and significance set at the 95th percentile.

**Results**

**Diet consumption and general health**

Food and water consumption, and body weights, were recorded during each 3-week trial. Food and water consumption, and body weights, did not differ significantly with respect to control cohorts (heterozygous Pah $enu^2$) as a function of treatment intervention (data not shown). There were no obvious phenotypic differences for subjects consuming AIB, NB or MAIB with regard to movement. Conversely, subjects consuming 5% NL displayed significant motor impairment (also seen with 3% NL consumption; data not shown). This motor impairment is best described as impaired levation, specifically an inability to raise the abdomen from the cage bottom, inability to climb, and a slow irregular gait. These findings were prominent in Pah $enu^2$ subjects but not observed in heterozygous Pah $enu^2$ subjects.

**LNAA levels and methionine metabolites in brain extracts**

NL, NB, and MAIB intervention significantly lowered brain phe levels in Pah $enu^2$ subjects, with NL displaying the largest reduction (56% vs. 27% for NB; Fig. 4), whereas 5% AIB intervention did not reduce brain phe. Additionally, NL feeding significantly reduced phe even in heterozygous Pah $enu^2$ subjects. No dietary intervention significantly altered trp levels (not shown), although all diets (AIB, NL and NB) manifested pronounced effects on tyr, total BCAAs and met (Fig. 4). Tyr levels were significantly decreased in both genotypes with NL and NB feeding, and these diets had comparable effects on total BCAA levels in both genotypes.
Significant effects of all dietary interventions were observed on met levels (Fig. 4), and met was the only LNAA for which 5 % AIB intervention led to a significant reduction (both genotypes). Dietary intervention with 3 % MAIB also significantly decreased phe in Pah enu2 mice (14 % reduction; Fig. 5), while slightly (but significantly) reducing tyr levels as well. Conversely, there was no significant alteration on brain total BCAAs nor met levels (Fig. 5), and there were no changes in brain trp levels (data not shown).

Figure 4: Selected brain LNAAs amino acids as a function of diet and genotype
Selected brain LNAAs amino acids as a function of diet and genotype (T, heterozygous Pah enu2 mice; M, Pah enu2 mice; trp levels not shown). Amino acid levels shown as nmol/g (gr) wet weight tissue. Parenthetical values represent the number of mice studied. Total branched chain amino acids (BCAA) represent the sum of ile, val and leu. Statistical analysis (one-way ANOVA
with Tukey post-hoc) compared cohorts with and without NPAAs, and within genotype only (*p < 0.05 compared to control, no drug intervention)

![Graphs showing the effects of dietary intervention on brain LNAAs](image)

**Figure 5**: Selected brain LNAAs as a function of dietary intervention with 3% MAIB

Selected brain LNAAs as a function of dietary intervention with 3% MAIB. For abbreviations, see Fig. 4 legend. Statistical analysis employed a two-way t test, comparing dietary intervention only within the same genotype.

We extended brain LNAA characterization to an examination of downstream metabolites, S-adenosylmethionine (SAMe) and S-adenosylhomocysteine (SAH) (Fig. 6). For SAMe, 5% NL feeding had a significant lowering effect in both genotypes, while 3% MAIB intervention significantly reduced SAH, but not SAMe, levels (Fig. 6).
Figure 6: S-adenosylmethionine (SAMe) and S-adenosylhomocysteine (SAH) levels as a function of dietary intervention. For abbreviations, see Fig. 4 legend. Statistical analysis (one way ANOVA with Tukey post-hoc) compared cohorts with and without NPAAs (for AIB, NL and NB), and within genotype only (*p < 0.05 compared to control). Statistical analysis for MAIB intervention employed a two-way t test, comparing dietary intervention only within genotype.
LNAA levels in sera as a function of dietary intervention

We examined LNAA levels in sera to estimate the potential of our dietary intervention to restrict gut uptake and absorption. Dietary intervention with NL, NB and MAIB had no significant effect on sera levels of phe, tyr, met, trp or total BCAA levels (data not shown). Of interest, 5 % AIB feeding significantly reduced blood trp and met levels (data not shown), the latter consistent with the isolated effect of AIB on brain met (Fig. 4).

Brain monoamines as a function of dietary intervention

Since tyr and trp represent precursors of the monoamine neurotransmitters dopamine and serotonin (Fig. 3), respectively, we characterized monoamine neurotransmitters and associated metabolites in brain extracts. For AIB, NL and NB feeding, there were no differences for DOPAC, HVA and DA release (the latter quantified as the 3-MT/DA ratio) with respect to either genotype (data not shown). The most significant effects on monoamine levels was observed with 0.5 % NB intervention, which significantly decreased DA and 5-HT levels, in both genotypes, while simultaneously lowering 3-MT levels (Fig. 7). A significant effect with NL intervention was only observed in heterozygous Pah enu2 mice for serotonin and its metabolite, 5-HIAA. NB intervention drastically enhanced turnover of both DA and 5-HT, in both genotypes (Fig. 7). The significant depletion of serotonin levels in Pah enu2 mice in the absence of drug intervention, and its downstream metabolite 5-HIAA, was consistent with our previous findings (Arning et al 2009). Feeding of 5 % AIB showed no effect on any monoamine metabolite.
Figure 7: Monoamine neurotransmitters and metabolites (see Fig. 3) in brain extracts of Pah enu2 and heterozygous Pah enu2 mice as a function of diet. Statistical analyses and abbreviations as described in legends to Figs. 3 and 4. Not shown are DOPAC, HVA, and the ratio of 3-MT/DA (dopamine release), which showed no significant differences for either genotype with any diet, with the exception of MAIB (see Fig. 8).

Although 3 % MAIB feeding did not significantly reduce either DA or 5-HT concentrations in Pah enu2 mice (Fig. 8), significant reductions in DOPAC, HVA and 3-MT levels were seen in both genotypes. As well, there was a significant reduction in the 5-HT metabolite, 5-HIAA, in Pah enu2 subjects. Finally, the turnover of both DA and 5-HT was significantly decreased in Pah enu2 mice with MAIB feeding, while the release of DA (measured as the ratio of 3-MT/DA) was significantly decreased in both genotypes (Fig. 8). These results for DA and 5-HT were opposite to those observed for NB feeding (Fig. 7).
Figure 8: Monoamine neurotransmitters and associated metabolites in brain extract of Pah enu2 and heterozygous Pah enu2 mice as a function of 3 % MAIB feeding. Abbreviations and statistical analyses as described in Figs. 3 and 5

Effect of NL feeding on blood chemistries

Heterozygous Pah^enu2^ mice were supplemented with 5 % NL (n = 7) or unsupplemented chow (n = 6) for 3 weeks, at which time sera was isolated for blood chemistries. The latter included glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, phosphorus, Na⁺, Cl⁻, K⁺, total CO₂, cholesterol, triglycerides, Ca²⁺, total bilirubin, alkaline phosphatase (ALP),
alanine aminotransferase, and gamma-glutamyltransferase. No significant differences were observed between cohorts, with two exceptions: BUN (5.43 ± 0.30 mg/dL (+NL) vs. 3.83 ± 0.17 (no NL) (p = 0.001; two-tailed t test); and ALP (41.6 ± 4.0 U/L (+ NL) vs. 57.7 ± 2.3 (no NL) (p = 0.0065). An increased BUN level with NL intervention suggested endogenous metabolism of NL.

**Estimation of NL levels in brain and blood**

To verify uptake, absorption and transport across the BBB, NL levels were estimated in terminal samples of sera and brain. In five subjects, the sera NL concentration was 3.11 ± 0.41 mM (SD; range 2.57–4.75) while that in brain was 822 ± 27 nmol/gr tissue (assuming a tissue density approximating water, ~0.82 μM). These data support absorption, transport and uptake into brain. Specific methodology has not been developed to accurately quantify NB, AIB and MAIB.

**Discussion**

The objective of the pilot studies presented was to provide proof-of-principle supporting the use of dietary NPAA intervention in Pah enu^2^ mice as a potential treatment strategy targeting brain phe levels. These initial interventional studies represent the first application of NL and AIB in Pah enu^2^ mice, and one of the first in vivo applications of NB in a mammalian system (Han et al 2012). MAIB was previously employed to study the transport of amino acids across the BBB in the rat, where it was found to have an absence of inhibitory effects on phe influx using intravenous injection (Wadhwani et al 1990). Overall, the phe-reducing capacity of NL, NB and MAIB in the brain of Pah enu^2^ mice provides a rationale for their further characterization in this mouse model. At the concentrations employed, however, neither NL nor NB was selective for
phe, with significant effects on other LNAAs, methionine analogues and monoamine metabolites. Nonetheless, the different L system transporters described in mammals (Table 1), their differential locations and amino acid specificities, suggests that appropriate NPAA concentrations can be formulated (either alone or in combination) that may maximally restrict brain phe transport with minimal effects on other LNAAs.

A novel finding in our work was the effect of 3% MAIB feeding on LNAA transport. MAIB is reported to be a specific A system inhibitor (Bröer and Brookes 2001), yet its consumption in Pah enu2 mice led to significant reduction of both brain phe and tyr, and concomitant effects primarily on the dopamine system. The mild effects of 3% MAIB feeding on LNAA levels (normal BCAA, met, trp), coupled with an absence of effect on DA and 5-HT levels in Pah enu2 mice, suggests that MAIB deserves further consideration as a candidate molecule targeting the reduction of phe-specific transport into brain in Pah enu2 mice. Another interesting observation from our studies was the met-specific effect of AIB. Generally, AIB had minimal effects on most LNAAs, with the exception of a specific reduction of met in both brain and sera, and serum trp levels. Earlier studies revealed that AIB shares a transport system in common with alanine, cysteine, glycine, methionine, serine and proline (Bröer and Brookes 2001; Lepley and Mukkada 1983), and other investigators have demonstrated the capacity of met to inhibit AIB transport in both rat liver and mammary gland (Crawhall and Purkiss 1973; Shennan and McNeillie 1994). In the latter study by Shennan and McNeillie, trp was also a potent inhibitor of AIB transport, consistent with our findings of reduced blood met and trp during AIB intervention.

Structural evidence suggests that AIB, NB and MAIB are unlikely to be extensively metabolized in mammals, yet systematic metabolic analyses is lacking. The use of any NPAA as
a competitive inhibitor of phe transport, in either Pah enu2 or PKU patients, will be thwarted if there is a significant contribution to the nitrogen pool resulting from consumption and metabolism. Negative nitrogen balance represents one of the mitigating factors against the long term use of LNAA supplements in PKU patients as a mechanism to restrict phe transport into brain (van Spronsen et al 2010). As secondary and methylated amines, respectively, neither AIB nor MAIB are expected to contribute significantly to the nitrogen load. The bridged nature of NB also argues against a significant component of metabolism. However, NL is a primary amine that may be readily transaminated in vivo, consistent with our results. As well, NL may be metabolized by intestinal bacteria. For example, NL is transaminated by Candida in gut, and it may be a substrate for amino acid oxidase (Der Garabedian and Vermeersch 1987). AIB has been shown to be a substrate for at least one bacterial species (Aaslestad and Larson 1964). Our initial blood chemistries for NL suggest that careful consideration be given to nitrogen load during our NPAA interventions, and we are examining the use of both D- and L-norleucine individually.

We visually observed motor impairment in Pah enu2 subjects receiving NL, which was not observed in animals receiving AIB, MAIB or NB. We speculate that the movement abnormalities associated with NL are associated with depletion of SAMe and SAH (Fig. 6), which was not observed with NB and AIB feeding, although MAIB did significantly lower SAH levels. Since SAMe is the methyl donor for a number of reactions of dopamine metabolism (including catechol-O-methyltransferase and phenylethanolamine-N-methyltransferase), as well as epinephrine and norepinephrine metabolism, this may provide some insights into the motor dysfunction seen with NL feeding (Kurian et al 2011). Moreover, it remains to be explained why
Pah enu2 mice have such a striking depletion of serotonin, which we have seen in both the current and previous studies (Arning et al 2009). Interventions geared to replenish 5-HT levels in Pah enu2 subjects would seem prudent (e.g., 5-hydroxytryptophan), and may have treatment relevance to patients with PKU, especially with respect to the long-term cognitive dysfunction observed (Enns et al 2010). This is supported by mounting evidence that the serotoninergic system plays an important role in cognition and learning (Geldenhuys and Van der Schyf 2011).

Utilizing NPAAs to reduce cerebral phe transport is pertinent to the long-term objective of employing oral NPAA intervention(s) in PKU patients for whom dietary adherence to low-protein intake is suboptimal, and potentially to identify NPAA interventions that would facilitate normal dietary protein intake for patients. Additional preclinical studies are required to optimize the concentration and possible combinatorial dosages of NPAAs, in order to produce a selective inhibition of phe transport into brain. Studies along these lines are actively underway in our laboratory.

Acknowledgements

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Conflict of interest

None
References


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Characterization of 2-(methylamino)alkanoic acid capacity to restrict blood–brain phenylalanine transport in Pahenu2 mice: Preliminary findings

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Highlights

- Select 2-(methylamino)alkanoates restrict Phe uptake and transport in PKU mice.
- Aminoisobutyrate (AIB) restricts accretion of Phe in brain of PKU mice.
- AIB, N-methyl AIB, and isoalnine lower blood Phe in PKU mice.
- Computer modeling of the mammalian large neutral amino acid transporter is underway.

Abstract

Background

Our laboratory seeks a pharmacotherapeutic intervention for PKU that utilizes non-physiological amino acids (NPAAs) to block the accumulation of phenylalanine (Phe) in the brain. In previous studies (Vogel et al. 2013), methylation of the amino group of 2-aminoisobutyrate (AIB) provided an enhanced degree of selectivity for Phe restriction into the brain of Pahenu2 mice in comparison to unmethylated AIB, leading to the hypothesis that 2-(methylamino)alkanoic acid analogs of AIB might represent targeted inhibitors of Phe accretion into the brain.

Methods

Pahenu2 and control mice were intraperitoneally administered (500–750 mg/kg body weight, once daily; standard 19% protein diet) AIB, methyl AIB (MAIB), isoalnine, and two MAIB analogs, 2-methyl-2-(methylamino)butanoic (MeVal) and 3-methyl-2-(methylamino)pentanoic (MePent) acids for one week, followed by brain and blood isolation for amino acid analyses using UPLC.
Results

In the brain, AIB significantly reduced Phe accretion in Pahenu2 mice, while MeVal significantly improved glutamine and aspartic acids. Four of five test compounds improved brain threonine and arginine levels. AIB, MAIB and IsoVal significantly reduced blood Phe, with no effect of any drug intervention on other sera amino acids.

Conclusions

Further evaluation of AIB and the 2-(methylamino)alkanoic acids as inhibitors of brain Phe accumulation in Pahenu2 mice is warranted, with more detailed evaluations of route of administration, combinatorial intervention, and detailed toxicity studies.

Abbreviations

- PKU, phenylketonuria;
- Pahenu2 mice, murine model of PKU developed with ethynitrosourea mutagenesis;
- LNAA, large neutral amino acids;
- A transporter, system A (alanine) transport system (small neutral amino acids);
- ASC transporter, system ASC (alanine-serine-cysteine) transport system;
- X transporter, system X (acidic amino acid) transporter;
- Y transporter, system Y (basic amino acid) transporter;
- LAT 1-4, LNAA transport system, system L transporter;
- NPAA, non-physiological amino acid;
- DOL, day of life;
• BBB, blood–brain barrier;
• Phe, phenylalanine;
• Tyr, tyrosine;
• Met, methionine;
• His, histidine;
• Thr, threonine;
• Leu, leucine;
• Ile, isoleucine;
• Val, valine;
• Arg, arginine;
• Ala, alanine;
• Trp, tryptophan;
• GABA, gamma-aminobutyric acid;
• Glu, glutamic acid;
• Gln, glutamine;
• Asp, aspartic acid;
• Gly, glycine;
• BCAA, branched chain amino acids (Leu, Ile, Val);
• AIB, 2-aminoisobutyrate (also 2-methyl-2-aminopropionic acid);
• MAIB, methylaminoisobutyrate (also 2-methyl-2-(methylamino)propionic acid);
• IsoVal, isovaline (also 2-methyl-2-aminobutanoic acid);
• MeVal, methylvaline (also 3-methyl-2-(methylamino)butanoic acid);
Introduction

Contemporary therapy for phenylketonuria (PKU) requires restriction of dietary phenylalanine (Phe) intake and supplementation with special medical foods, which prevents the developmental delays associated with long-term, untreated hyperphenylalaninemia [1]. Nonetheless, rigid dietary intervention presents challenges, including institution and maintenance of diet, stigmatization of patients, and emerging evidence of mild neurocognitive deficits even with good metabolic control [2], [3] and [4]. Moreover, overrestriction of dietary amino acid intake may have untoward consequences. In the last decade, a number of novel therapeutic approaches to PKU have emerged, including cofactor intervention (KuvanR; sapropterin, Biomarin), enzyme therapy (phenylalanine ammonia lyase; PEG-PAL), which degrades circulating Phe into non-toxic trans-cinnamic acid, and glycomacropeptide intervention (a cheese byproduct devoid of Phe), as well as gene therapy and hepatocyte transfer to the liver (www.clinicaltrials.gov). Despite these advances, a targeted pharmacological approach to PKU treatment remains undeveloped.

More than 60 years ago, the demonstration of competition between large neutral amino acids (LNAAs) for uptake across the blood brain barrier (BBB) led to the hypothesis that LNAA supplements could decrease Phe levels and replenish depleted LNAAs in PKU [5] and [6].
Those studies suggested that therapeutic competition for amino acid transport into the brain of individuals with PKU might be feasible [7] and [8]. At least four mammalian amino acid transporters have been identified that are responsible for movement of LNAAs (Phe, Tyr, Trp, Leu, Ile, Val, Met, as well as His and Thr) across the BBB and intestinal mucosa [9], and these LNAA transporters (so-called LNAA transporters, or LATs 1–4) constitute the system L transport system [10] and [11]. Despite the specificity of the LATs for LNAAs, considerable evidence indicates that most brain amino acid transport systems (including system L (the LATs), small neutral amino acids (transported on the A (alanine) and ASC (alanine–serine–cysteine) transport systems)), and even the acidic and basic amino acid transporters share considerable overlap in amino acid trafficking[12]. Moreover, as demonstrated by Pardridge and Oldendorf [13] nearly 50 years ago, the Km values for the system L transporter vary considerably, with the highest affinity for Phe and Trp (~ 30–50 μM), somewhat lower affinity for Met, Tyr and Leu (~ 80–90 μM) and the lowest affinity for Ile, Val and His (~ 140–170 μM). The preceding data suggests that selected therapeutic agents may have treatment relevance in competing against LNAAs (e.g., Phe in PKU) from uptake into the gut and brain of patients with PKU.

Zinnanti and colleagues [14] were the first to examine this type of competition study in a murine model of a large neutral aminoacidopathy. These investigators demonstrated that feeding of 5% d,l-norleucine (NL; a non-physiological amino acid (NPAA)) to mice genetically engineered to recapitulate MSUD (a large neutral amino aciduria, like PKU) resulted in significant behavioral and neurometabolic improvements with concomitant lowering of brain Leu levels. Our laboratory extended these studies by piloting a study of selected NPAAs in a murine model of PKU. The NPAAs evaluated included d,l-norleucine; 2-aminoisobutyric acid
(AIB); 2-methyl-2-(methylamino)propionic acid (also referred to as methyl-aminoisobutyrate (MAIB); and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)), compounds possessing differential affinities for various brain amino acid transporters [15]. This study, with MAIB and BCH (the prototypical LAT-1 competitive inhibitor) was the first exploration of these compounds in a mammalian species. Using the genetically-engineered Pahenu2 mouse model as experimental platform [16], we found that all species employed (except AIB), at dietary intake levels of 3–5% w/w mouse chow, resulted in significant reduction of brain Phe, yet also resulted in reductions of other LNAAs as well. Only MAIB, fed to Pahenu2 mice at a dietary concentration of 3%, led to reasonably specific Phe reduction (accompanied by a modest reduction in brain Tyr). This finding was of interest, since MAIB is purported to be specific for the system A transporter [15], which has overlap with the L system transporter [12]. The absence of an effect with AIB in our initial feeding studies, with a more specific and significant effect from the methylated species (MAIB), prompted us to hypothesize that 2-(methylamino)alkanoic acids might represent potentially valuable competitive inhibitors of the L system (LAT-1). In the current report, we have examined this hypothesis in the Pahenu2 mouse model.

2. Materials and methods

2.1. Design and rationale

In previous studies, unsaturated d,l-norleucine species was effective at inhibiting accretion of Phe into the brain of Pahenu2 subjects [15]. This finding, coupled with results for AIB and MAIB feeding (see above) suggested that longer-chain species whose 2-amino group
was methylated might be selective inhibitors of brain Phe transport. Accordingly, along with MAIB (2-methyl-2-(methylamino)propionic acid), we examined 3-methyl-2-(methylamino)butanoic acid (methyl valine, or MeVal) and 3-methyl-2-(methylamino)pentanoic acid (MePent), alkanoic acid species one and two methylene groups longer than MAIB, respectively (Fig. 1). As control for the methylated nitrogen, we employed 2-amino-2-methylbutanoic acid (isovaline, or IsoVal). The expense of these compounds precluded their use in mouse chow, as previously employed [15], and accordingly we opted for once-daily i.p. administration (see below). Moreover, i.p. administration ensured uptake and the potential to explore blood and brain uptake of dietary amino acids. Since our design included i.p. administration of drug, we repeated studies with AIB and MAIB as well, which had previously been examined using dietary intervention. All drugs (except AIB; Sigma-Aldrich, St. Louis, MO) were obtained from MolPort (SIA MolPort, Riga, Latvia) by custom synthesis, and purity exceeded 99% in all cases.
Figure 1: Structures of the non-physiological amino acids (NPAAs) evaluated in the current study. A, 2-aminoisobutyric acid (AIB; also 2-methyl-2-aminopropionic acid); B, 2-methyl-2-(methylamino)propionic acid (MAIB); C, isovaline (IsoVal; also 2-methyl-2-aminobutanoic acid); D, 3-methyl-2-(methylamino)butanoic acid (also methylvaline (MeVal)); E, 3-methyl-2-(methylamino)pentanoic acid (MePent).

2.2. Animal husbandry and drug administration

Heterozygous Pahenu2 mice were bred monogamously and maintained under a 14:10 light to dark cycle. In an earlier study [15], we directly compared metabolite levels between homozygous normal (Pahenu2+/+) and heterozygous (Pahenu2+/−) mice, and observed no statistically significant differences. Accordingly, to increase our control data range in this pilot evaluation, metabolic findings from both homozygous unaffected and heterozygous mice were pooled as controls. Diet consisted of Harlan Global Teklad 2019 (19% protein) pelleted rodent chow with ad libitum access to food and water. Animals were genotyped by two primer PCR amplification, restriction enzyme digestion (BbSI and BsmAI) and 4% agarose gel electrophoresis, as previously reported [15]. Mice were injected for one week from age 3 to 10 days, and animals of both genders were included. Entire litters were injected with 50 μL total volume of PBS (vehicle control), AIB (500 mg/kg), MAIB (500 mg/kg), MeVal (500 or 750 mg/kg), IsoVal (500 mg/kg) or MePent (500 mg/kg). These dosages were chosen based upon previous feeding and dietary consumption characteristics for this strain [15]. Higher dosages and studies of dose response are in progress. Our calculations indicated that daily consumption of diet (powdered) containing approximately 2–3% w/w of drug equated to an approximate injection dosage of 500–750 mg/kg drug, and accordingly we chose this dose range for preliminary
evaluation. Drugs were dissolved in PBS at 100 mg/mL and stored at −20 °C. Drug administration commenced on the third day of life and continued once daily for 7 days based upon body weight. Because we had no information concerning potential drug toxicity, we limited our evaluations of subjects to a one week duration of study. On DOL 10 animals were sacrificed, and following cardiac puncture to obtain blood for sera collection, the brain was rapidly excised, divided sagittally, snap frozen and stored at −80 °C. Serum was collected following a 10 minute low-speed centrifugation at 4 °C and stored at −80 °C. All animal work was approved by the Washington State University IACUC (AFS 4232; 4276).

2.3. Amino acid determinations

Amino acids were quantified by UPLC and tandem mass spectrometry as previously described [15].

2.4. Statistical analyses

Data is presented in column analysis format, with mean and SEM (error bars). Statistical evaluations were comprised of a two-tailed t test between genotypes (grouped wild-type and heterozygous subjects compared to mutant (Pahenu2) subjects). Previous metabolic studies had verified that it was acceptable to group wild-type and heterozygous subjects, and this greatly improved animal numbers (n value) in the current study [15]. When there was a significant difference between control and Pahenu2 subjects, we applied ANOVA and Tukey post-hoc [15] in the Pahenu2 group to determine if drug intervention led to significant alteration in metabolite levels as compared to untreated (PBS vehicle) Pahenu2 subjects. To indicate when ANOVA was applied in the Pahenu2 group, the letter “A” appears above the
vehicle-treated Pahenu2 subjects (in the brain, this value was p = 0.052 for aspartate, and the ANOVA evaluation was still applied in only this instance). In the absence of a significant difference between vehicle-treated control and Pahenu2 subjects, a two-tailed t test was performed between control and mutant only within the respective treatment group. The n values for blood measurements were smaller than those for brain evaluations since the volume of sera obtained was below the limit required (0.02–0.03 mL) for comprehensive amino acid studies in some animals.

3. Theory

Pilot studies [15] revealed that non-physiological amino acids (NPAAs) could both selectively and non-selectively lower Phe accretion in the brain of Pahenu2 mice, a murine model of PKU. In those studies, methylation of the amino group of 2-aminoisobutyrate (AIB) provided an enhanced degree of selectivity for Phe restriction into the brain using a dietary feeding intervention as compared to the unmethylated parent compound. This observation resulted in the hypothesis that 2-(methylamino)alkanoic acid analogs of AIB might represent highly specific inhibitors of Phe accretion in the brain of this mouse model, potentially providing additional evidence for a pharmacotherapeutic approach to PKU treatment.

4. Results

Findings for quantitative amino acid analysis in brain extracts are depicted in Fig. 2 and Fig. 3. The large neutral amino acids (LNAA) are shown in Fig. 2, and for the brain this includes both His and Thr. Met was omitted as there were no significant differences by genotype across the treatment groups. In Fig. 3, additional amino acid data is depicted, notably including the
amino acid neurotransmitters (GABA, Glu, Asp, Gly and the Glu precursor Gln), as well as other amino acids where significant differences were observed, including Arg and Ala. The abnormalities observed with these brain amino acids were generally consistent with our previous studies on the brain extracts of untreated Pahenu2 mice [17]. The data of Fig. 4 and Fig. 5 depict results from quantitative sera amino acid analyses corresponding to the same subjects from whom brain data had been obtained. Fig. 4 depicts all large neutral amino acids, with the exception of His and Thr, which are shown with blood Gln levels in Fig. 5.
Figure 2: Large neutral amino acids (LNAAs) in brain extracts as a function of diet and genotype. Amino acid identity is shown in three letter code on the y-axis (units, nmol/g tissue wet weight). Parenthetical values on the x-axis represent the number of animal subjects evaluated. Abbreviations: C = control, including both wild-type and heterozygous Pahenu2 mice; M = Pahenu2 mice. Statistical analysis as described in the text (*p < 0.05; **p < 0.01; ***p < 0.001).
Figure 3: Selected brain amino acids as a function of diet and genotype.

For abbreviations and other descriptions, see Fig. 1 legend.
**Figure 4:** Large neutral amino acids (LNAAs) in sera as a function of diet and genotype. For abbreviations and other descriptions, see Fig. 1 legend.

**Figure 5:** Selected sera amino acids as a function of diet and genotype. For abbreviations and other descriptions, see Fig. 1 legend.

5. Discussion and conclusions

5.1. Brain amino acid characterization

Under normal protein intake (19% diet), our studies revealed that AIB significantly lowered brain Phe using an i.p. administration approach. This was at variance with earlier findings (dietary feeding) in which AIB administration was ineffective at lowering brain Phe [15]. For MAIB, i.p. administration revealed a tendency toward lower brain Phe which failed to achieve significance (p = 0.114), consistent with earlier data in which MAIB feeding significantly lowered brain Phe [15]. There was no effect on brain Phe levels with administration of IsoVal, MeVal nor MePent (Fig. 2). Intraperitoneal administration of MAIB, IsoVal, MeVal and MePent significantly increased Thr in Pahenu2 mice as compared to PBS-treated subjects, while MAIB intervention resulted in a further lowering of Tyr (which showed the most dramatic reductions of all the LNAAs in Pahenu2 mice), consistent with our previous studies using MAIB applied via dietary administration [15].
With regard to other LNAAs, His was significantly increased in Pahenu2 subjects in comparison to controls, while the branched chain amino acids (Leu, Ile and Val) and Trp were significantly decreased overall, and there was no effect of drug administration on any of these LNAAs (Fig. 2) [15] and [17]. These reductions did not always achieve significance as a function of treatment, particularly when a selected drug administration led to lowering of the control level of amino acid, or when the SEM was large (as often found with administration of both MeVal and MePent). Met (data not shown) was not significantly different by genotype, but there was a significant decrease in its concentration in control subjects treated with AIB intervention, consistent again with previous studies employing dietary administration [15].

For the neurotransmitter amino acids (including Asp, Glu, GABA, Gly and the Glu precursor Gln), we observed a significant decrease in Gln as previously observed [17], and this decrease in Pahenu2 mice was exacerbated by AIB intervention and significantly improved with MeVal intervention (as was also the case for Asp; Fig. 3). For both GABA and Glu, there was a trend toward decreased levels in Pahenu2 subjects which failed to achieve statistical significance, and there was little effect from drug intervention [17].

We found unusual results for three additional amino acids in brain extracts. As depicted in Fig. 3, Arg was significantly decreased in mutant brain as we had previously observed [17], and its levels were significantly improved with AIB, MAIB, IsoVal and MePent intervention. Additionally, Pahenu2 mice were hyperglycinemic, as previously observed, and there was no effect with any drug intervention [17], while Ala levels were low which was at variance with the previous finding of its elevation in Pahenu2 mouse brain. Our results for Asp, Arg, His, Gly and
Ala are of interest if we consider the various transporters in the brain and their overlapping specificities [12]. For example, the system L transporter (LAT) has considerable overlap with the A and ASC transporters, and even overlap with the Y transporter (basic amino acids). Conversely, Asp is trafficked only on the X transporter (acidic amino acids), which has no overlap with the L transporter. It is possible that increased phenylalanine levels are altering (competing with) these transporters, even the system X transporter, but it is challenging to explain why His and Gly are increased as opposed to decreased, suggesting an inverse effect (akin to agonism) by elevated Phe on their transport [18] and [19]. Conversely, there is the potential for unidentified transporters yet to be described for brain amino acid transport.

5.2. Blood amino acid characterization

The results of blood amino acid determinations are shown in Fig. 4 and Fig. 5. Overall, the n values were lower, which resulted in more data variation. Nonetheless, despite often low n values, we pursued blood amino acid analyses to glean insights on drug interventions on the peripheral circulation.

Amino acid analysis revealed that AIB, MAIB and IsoVal all resulted in significant reductions in blood Phe (Fig. 4). For the remainder of LNAAs, the trend was for decreased levels in blood, which was somewhat more pronounced with Tyr and Trp, a finding consistent with results for brain amino acid profiling. More consistent reductions in Gln and His were found in blood, the former consistent with the brain results while the latter was the opposite of the brain results. With the exception of Phe, drug intervention did not correct any blood amino acid. As was the case for brain extracts, there was considerable variation for animals receiving
MeVal in addition to IsoVal (Fig. 4 and Fig. 5), pointing to interindividual variation and possibly toxicity. Any further studies with these species (IsoVal, MeVal and MePent) will certainly require dosing and toxicity analyses moving forward.

5.3. Large neutral aminoacidopathies, in silico modeling and future considerations

The large neutral aminoacidopathies, PKU and MSUD, share many similarities in metabolic neuropathology, yet some rather distinct features [20] and [21]. In both disorders, as modeled in knockout mice, disturbances in brain amino acid neurotransmitters follow similar patterns, with elevated Gly and depleted Gln, Glu and Asp. On the other hand, results for Arg, His and Thr are quite different with respect to disease, with depleted Arg in Pahenu2 mice and elevated brain Arg in mice modeling msud [20], elevated His in the brain of Pahenu2 mice (no demonstrable abnormality in msud mice), and depleted Thr in Pahenu2 mouse brain (elevated in msud mice). Another important observation is that the remaining LNAAs (e.g., Tyr, Trp, Met) are not significantly depleted in murine msud while quite decreased (at least for Tyr and Trp) in the Pahenu2 model. This might be explained by the much higher Km of Phe for the LAT-1 as compared to the Km values for the BCAAs. Some of these differences in metabolic neuropathology likely relate to accumulation of the BCAA ketoacids in msud mice (and patients), especially 2-oxoisocaproic acid, which is expected to gain brain access on the monocarboxylate transporter [22]. The preceding observations suggest that pharmacological intervention in PKU and MSUD, targeting restriction of the offending amino acid from entry into the brain, will require quite different approaches.
To begin to address the latter consideration, we have begun exploring the development of further compounds using an in silico approach for ligand docking on the L transporter (LAT-1). To this end, we have used the Schrodinger small molecule drug discovery suite (www.schrodinger.com). As a preliminary step, we have simulated LAT-1 folding. Since the LAT1 crystal structure remains unsolved, the folding must be simulated with the Prime component of Schrodinger. To accomplish this, we have modeled the input sequence from a high homology match of the LAT-1 (Lat1: light chain, NM_003486.5), as depicted in Fig. 6. As predicted from this initial modeling, the LAT-1 is composed entirely of α-helical strands which form a perfect pore for LNAA transport. This preliminary modeling data is being refined and will be used to identify potential ligand inhibitors that can be examined in our murine models.

Figure 6: Simulation of LAT1 folding with Prime modeling software (www.schrodinger.com). Comparative modeling of the input sequence (Lat1: light chain, NM_003486.5) via fold recognition was performed with the highest identity homolog (3G19C) imported from the default Find Homologs BLAST query of the pre-loaded Prime PDB library. This particular LAT1
structure was built using the knowledge-based (as opposed to energy-based) model-building method within Prime.

In conclusion, our pilot studies employing the 2-(methylamino)alkanoic acids in the current study provide further supportive evidence for more detailed examinations of AIB and MAIB in Pahenu2 mice, with a particular focus on routes of administration, toxicity and regional brain characterization. Ongoing studies will work to determine optimal concentrations for these compounds, or those to be identified by in-silico modeling, that will eventually produce a high degree of specificity for restriction of Phe accumulation in the brain. For the longer chain species, namely MeVal and MePent, toxicity may pose a concern and safety considerations will be extensively characterized prior to further in vivo studies with these compounds.

Conflict of interest statement

There is no conflict of interest.

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CHAPTER THREE
BRAIN-BLOOD AMINO ACID CORRELATES FOLLOWING PROTEIN RESTRICTION IN MURINE MAPLE SYRUP URINE DISEASE

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Abstract

**Background:** Conventional therapy for patients with maple syrup urine disease (MSUD) entails restriction of protein intake to maintain acceptable levels of the branched chain amino acid, leucine (LEU), monitored in blood. However, no data exists on the correlation between brain and blood LEU with protein restriction, and whether correction in blood is reflected in brain. **Methods:** To address this question, we fed intermediate MSUD mice\[1,2\] diets of 19% (standard) and 6% protein, with collection of sera (SE), striata (STR), cerebellum (CE) and cortex (CTX) for quantitative amino acid analyses. **Results:** LEU and valine (VAL) levels in all brain regions improved on average 28% when shifting from 19% to 6% protein, whereas the same improvements in SE were on average 60%. Isoleucine (ILE) in brain regions did not improve, while the SE level improved 24% with low-protein consumption. Blood branched-chain amino acids (LEU, ILE, and VAL in SE) were 362-434 μM, consistent with human values considered within control. Nonetheless, numerous amino acids in brain regions remained abnormal despite protein restriction, including glutamine (GLN), aspartate (ASP), glutamate (GLU), gamma-aminobutyric acid (GABA), asparagine (ASN), citrulline (CIT) and serine (SER). To assess the specificity of these anomalies, we piloted preliminary studies in hyperphenylalaninemic mice [3, 4], modeling another large neutral aminoacidopathy, employing an identical dietary regimen, we found remarkably consistent abnormalities in GLN, ASP, and GLU. **Conclusions:** Our results suggest that blood amino acid analysis may be a poor surrogate for assessing the outcomes of protein restriction in the large neutral amino acidopathies, and further indicate that chronic neurotransmitter disruptions (GLU, GABA, and ASP) may contribute to long-term neurocognitive dysfunction in these disorders.
Keywords

Maple syrup urine disease (MSUD)
Branched-chain keto-acid dehydrogenase (BCKDH) complex
Mouse model
Large neutral amino acid transporter (LAT-1)
Branched-chain amino acids (BCAAs)
Phenylketonuria (PKU)
Large neutral aminoacidopathies
Dietary protein restriction

Abbreviations

SE, sera; CB, cerebellum; STR, striata; CTX, cortex; W6, wild-type mice receiving 6% protein intake; M6, mutant mice receiving 6% protein intake; W19, wild-type mice receiving 19% protein intake; M19, mutant mice receiving 19% protein intake; insud−/+; intermediate maple syrup urine disease mice; Pahenu2−/−, phenylketonuric mice; LEU, leucine; ILE, isoleucine; VAL, valine; ALLO, allosisoleucine; SER, serine; CIT, citrulline; THR, threonine; ALA, alanine; LYS, lysine; HIS, histidine; GLN, glutamine; ASP, aspartate; GLU, glutamate; GABA, 4-aminobutyrate; PHE, phenylalanine; TYR, tyrosine; MET, methionine; TRP, tryptophan; ASN, asparagine;
Background

Maple syrup urine disease (MSUD), branched-chain ketoacid dehydrogenase (BCKDH) deficiency) and phenylketonuria (PKU) comprise the large neutral aminoacidopathies. Untreated MSUD results in accumulation of the branched-chain amino acids (BCAAs) leucine (LEU), valine (VAL) and isoleucine (ILE). Additionally, induction of catabolism will induce release of BCAAs from muscle and increase the BCAA’s corresponding α-keto acids (BCKAs), including α-ketoisocaproate (KIC, from LEU), α-keto-β-methylvalerate (KMV, from ILE) and α-ketoisovalerate (KIV, from VAL), all of which can freely traverse the blood-brain barrier (BBB) and transaminate to yield the corresponding BCAAs [5]. Treatment of MSUD requires strict protein restriction combined with BCAA supplementation to levels compatible with adequate development and growth. Although dietary intervention prevents the severe developmental delays associated with chronic hyperleucinemia, it nonetheless presents challenges that include maintenance and adherence to diet, and mounting evidence for long-term deficits in neurocognition despite acceptable metabolic control determined in blood [6-16]. Additionally, over restriction of dietary amino acid intake may have untoward consequences. The preceding discussion suggests that while prudent, dietary treatment alone for MSUD may be suboptimal.

The pathophysiology of MSUD has been extensively reviewed [17]. Episodic increases in blood BCAAs occur when muscle degrades protein in response to physiological stress [18-20], also increasing the level of corresponding BCKAs. Increased muscle BCKAs induces reversal of cytosolic transaminases and depletes tissue levels of other amino acid nitrogen donors. As LEU exits muscle and other tissues via the large neutral amino acid transporter (LAT-2) [21],
heteroexchange mechanisms stoichiometrically drive import of other amino acids and increase the relative LEU blood concentration. At the BBB, elevated BCAAs may saturate the LAT-1 (see below) transporter and may block uptake of other large neutral amino acids (LNAAs; including phenylalanine (PHE), tyrosine (TYR), methionine (MET); tryptophan (TRP); and histidine (HIS)). Increased blood BCKAs enter the brain via the monocarboxylate transporter (MCT) and reverse flux through cerebral transaminases, depleting brain glutamate (GLU), glutamine (GLN) and gamma-aminobutyric acid (GABA) (among others), while enhancing production of LEU and \( \beta \)-ketoglutarate [5, 22-25]. These disruptions in brain amino acid homeostasis may also be accompanied by disruptions of oxidative phosphorylation, elevated cerebral lactate level and oxidative damage [20, 26].

As noted above, LNAAs cross the BBB on the LAT-1 transporter, and accumulation of offending amino acids such as the BCAAs in MSUD can result in exclusion of other LNAAs from the brain, although this has not been rigorously evaluated in either patients or an animal model of MSUD. Despite the specificity of the LAT-1 for LNAAs, most brain amino acid transport systems display broad overlap in the amino acids transported [3, 4, 27, 28]. Here, we have employed a murine model of MSUD to address the question of whether control of BCAA level, as monitored in blood, is reflected by similar correction in the brain. Several novel therapeutic approaches to PKU have emerged recently [29], yet with the exceptions of phenylbutyrate administration (which selectively lowers circulating BCAAs) [30] or orthotopic liver transplantation for the most severe forms of MSUD [8, 15, 31, 32], few therapeutic advances have emerged in MSUD. The current report addresses our hypothesis, and for the first time
verifies in a murine model of MSUD that assessment of metabolic control using blood studies is a poor surrogate for disturbances of amino acid homeostasis in the brain.

Methods

Animal subjects

The intermediate MSUD murine model (\textit{imsud} mice) has been described, and manifests \textasciitilde 6\% of residual BCKDH enzyme function [1, 2, 33, 34]. Animal subjects were genotyped by PCR amplification. Subjects of both genders were employed, ages 10-20 days of life. Subjects were allowed \textit{ad libitum} access to chow and water throughout experimentation. All animal experimentation was performed with IACUC approval (ASAF 4232-007).

Diets employed

We performed pilot studies with the Teklad TD.90016 diet (6.9\% casein=6\% protein; low protein) since it has been employed previously for maternal protein restriction in rodents [35]. In the latter studies, the induction of serine-metabolizing enzymes was characterized in mice receiving 2, 6 and 18\% protein. Based upon empiric considerations, we felt that 2\% protein would be too stringent in \textit{imsud} mice and lead to catabolic crisis. Teklad custom diets are isocaloric and balanced for nitrogen level. The corresponding Teklad TD.91353 diet (19\% protein) was used as control diet. Animals were on diet for 8-18 days prior to sacrifice. Power analyses indicated that \( n=6 \) subjects of each genotype (\textit{imsud}^{+/+} and \textit{imsud}^{-/-}) would be sufficient to obtain statistically significant outcomes. For brain region analyses (see below), we frequently had \( n=5-6 \) subjects per diet and genotype, but the smaller size of mutant subjects often led to insufficient blood for analytical studies, thereby leading to smaller \( n \) values for sera
(SE) and enhanced data variability. At study conclusion, subjects per genotype were n=3-7 for SE and n=4-8 for brain.

Tissue harvesting and analytical methodology

At appropriate time points, animal subjects were euthanized, and blood collected by cardiac puncture. The brain cavity was rapidly accessed on an ice-cold glass plate, with parietal cortex (CTX), striata (STR), and cerebellum (CB) rapidly excised. Harvested tissues and sera following centrifugation were stored at –80 °C until analyses. LNAAs and other amino acids were determined with ultraperformance liquid chromatography (UPLC) MassTrak amino acid system as previously described [36]. All values in SE are presented in units of μ+mol/L, whereas all values in brain regions are presented in units of nmol/gr tissue.

Statistical analyses

In prior studies, we had observed depletion of GLU, GLN, ASP, serine (SER), alanine (ALA) and GABA, with elevated glycine (GLY) in brain of imsd mice. However, those studies were performed in whole brain [1, 2, 33, 34], potentially leading to artifact from mixing of regions with high vs. low metabolite levels. In the current study we assessed discrete brain regions. Accordingly, we had no insight whether to expect brain regional amino acids to be constant or different. Thus, we initially employed a two-way ANOVA to assess correlations between brain region and protein intake, and interaction of these parameters, accompanied by a Holm-Sidak’s multiple comparisons test. Our primary statistical analyses employed a two-tailed t test within genotype as a function of protein consumption. Both statistical evaluations are presented in all
figure legends. Comparisons of amino acid concentrations between blood and brain assume a density of brain tissue of ~1 g/ml [37].

Results

For *imsud* mice, all BCAAs were significantly increased in comparison to littermate controls, verifying the utility of the model (Fig. 1). With decreased protein consumption, LEU in mutant SE decreased from 1072 (504) (standard deviation shown in parentheses after mean) to 436 (107) μmol/L, while the same decreases in mutant were 1026 (444) to 723 (415) in CB, 1147 (535) to 853 (648) in STR and 1017 (516) to 755 (452) in CTX (brain regions: nmol/gr tissue). ILE changes in mutant mice with decreased protein were 488 (115) to 362 (41) μmol/L in SE, 555 (84) to 585 (226) in CB, 625 (132) to 687 (349) in STR and 583 (126) to 636 (235) in CTX (nmol/gr tissue). For VAL, comparable changes were 1007 (441) to 434 (71) μmol/L in SE, 950 (347) to 651 (288) in CB, 1071 (421) to 758 (453) in STR and 894 (448) to 661 (351) in CTX (nmol/gr tissue).

![Figure 1](image.png)

**Figure 1.** Branched chain amino acids in sera (SE), cerebellum (CB), striata (STR) and cortex (CTX) as a function of protein intake (6 or 19%) and genotype (w, wild-type; m, mutant). Two-
way ANOVA (parameters: protein intake, tissue), p=ns for tissue and interaction, p<0.05 for protein intake. Two-tailed t-test, p<0.05 for all mutant vs. wild-type (not shown).

Within tissues (SE, CB, STR and CTX), there was no significant improvement in BCAAs with lowered protein intake, although the trend in SE BCAAs approached significance (t-test, 2-tailed, p=0.074-0.125 vs. CB, STR and CTX, p=0.210-0.789), but variation in SDs (occasional low SE quantity for selected animals) was problematic. Alloisoleucine (ALLO, a pathognomonic biomarker for MSUD) showed similar trends (Fig.2). The changes for ALLO in mutant with decreased protein consumption were 39 (22) to 12 (3) μmol/L in sera, 66 (37) to 37 (24) in CB, 75 (44) to 44 (31) in STR, and 41 (29) to 27 (15) in CTX (brain values, nmol/gr protein). As expected, ALLO was undetectable in wild-type mice with either diet. There was no significant difference in any tissue with decreased protein intake for ALLO in mutant mice.
Figure 2. Alloisoleucine levels (see Fig. 1 for details). Two-way ANOVA, p<0.05 for protein intake, p=ns for tissue and interaction. Two-tailed t test, p=ns between mutant and wild-type as a function of protein intake.

We then turned our attention to the other LNAAs (MET, PHE, TYR and TRP) in the same regions (Fig. 3). For TYR, changes for mutant mice with decreased protein intake were 94 (19) to 134 (10) μmol/L in SE, 98 (13) to 143 (44) in CB, 57 (n=2)-92 (26) in STR and 102 (17) to 139 (46) in CTX (brain, nmol/gr tissue). For TRP, the same values for mutant were 49 (5) to 57 (12) μmol/L in SE, 28 (4) to 31 (8) in CB, 28 (7) to 32 (10) in STR and 25 (4) to 26 (9) in CTX. For MET, these values were 77 (16) to 82 (27) μmol/L in SE, 132 (41) to 93 (29) in CB, 121 (40) to 113 (28) in STR and 146 (11) to 143 (29) in CTX. For PHE, the corresponding values were 94 (15) to 86 (19) μmol/L in SE, 104 (13) to 118 (13) in CB, 99 (17) to 124 (41) in STR and 100 (12) to 101 (14) in CTX (nmol/gr tissue).

Elevated BCAA levels had no substantial effect on TRP, MET and PHE levels in any brain region, although SE TYR was improved (increased in mutant mice; Fig. 3) with decreased protein consumption (without a corresponding correction in brain regions). This finding lends credence to the hypothesis that correction of blood amino acid content may not reflect corresponding corrections in the brain. Conversely, compared to control mice receiving identical diet, levels of TYR or TRP were depleted in CB, STR and CTX with high protein intake, but these depletions were not consistent across different brain regions (Fig. 3).
**Figure 3.** Other large neutral amino acids (tyrosine, tryptophan, methionine, phenylalanine) as a function of protein intake and genotype (see Fig. 1 for details). Two-way ANOVA, p<0.05 for both protein intake, tissue and interaction. Two-tailed t-test (*p<0.05), comparing M19 and W19 in CTX and CB (TYR) and STR (TRP). Also, p<0.05 for TYR in SE (M6 vs M19).

We next surveyed all remaining physiological amino acids across brain regions, and observed significant abnormalities in GLN, ASP, GLU, GABA, ASN (asparagine); CIT (citrulline), SER and ALA (Figs. 4-6)). For comparison purposes, concentrations for GLN, ASP and GLU are not depicted for SE because of the much higher brain levels, which would graphically dwarf SE values, and GABA is not quantifiable in SE. Changes in GLN in mutant brain regions with decreased protein consumption were 2590 (726) to 3021 (477) in CB, 1575 (516) to 2342 (503) in STR and 1862 (739) to 2064 (336) in CTX (nmol/gr tissue). For ASP, the same changes with decreased protein were 1818 (303) to 2206 (420) in CB, 1662 (517) to 1829 (183) in STR and 1808 (420) to 2021 (187) in CTX; for GLU, 4973 (774) to 5045 (1263) in CB, 6238 (1264) to 6554 (407) in STR and 7019 (1226) to 7246 (134) in CTX; and for GABA, 1920 (207) to 2317 (378) in
CB, 1958 (316) to 2364 (409) in STR and 2595 (171) to 2789 (329) in CTX (nmol/gr tissue).

Figure 4. Neurotransmitter amino acids (glutamine, aspartate, glutamate and GABA) as a function of protein intake and tissue (see Fig. 1 Legend). Two-way ANOVA, p<0.05 for tissue, protein intake and interaction. Two-tailed t-test, *p<0.05 for all mutant vs. wild-type under both protein intake regimens.

With the exception of GABA (which improved with lower protein intake), GLN, ASP and GLU were not improved with lowered protein consumption (Fig. 4). Conversely, lowered protein intake normalized the level of ASN in CB and the concentration of CIT in STR and CTX, while both amino acids were significantly increased vs. control during high-protein intake (Fig. 5). Similar findings were observed for SER and ALA, in which correction with lowered protein consumption was observed in CB, but not in STR or CTX (Fig. 6). Threonine (THR) is often considered a member of the LNAA subgroup, but we found no consistent changes in brain regions, with the exception of a single instance in striata (Fig. 7). Conversely, LYS is not considered a LNAA, yet its values were significantly impacted during high-protein consumption (Fig. 7).
Figure 5. Asparagine and citrulline level as a function of protein intake and tissue. Two-way ANOVA, \( p<0.05 \) for protein intake and interaction, \( p=\text{ns} \) for tissue. Two-tailed \( t \)-test, \(*p<0.05\) between genotype with identical protein intake.

Figure 6. Serine and alanine levels as a function of protein intake and tissue. Two-way ANOVA, \( p<0.05 \) for protein intake, tissue and interaction. Two-tailed \( t \)-test, \(*p<0.05\) for genotype with identical protein intake.
Figure 7. Threonine and lysine levels as a function of protein intake and tissue. Two-way ANOVA, p<0.05 for protein intake, tissue and interaction. Two-tailed t-test, *p<0.05 for genotype with identical protein intake.

Since concentrations of several amino acids are much lower in SE vs. brain regions (e.g., Figs. 1-3), we did not present them graphically, as noted above. We observed no significant changes for GLU and GLN in SE (p>0.05), yet found brain disruptions that were uncorrected with lowered protein intake (Fig. 4). Conversely, in SE we observed a significant difference for ASP (19% diet: imsd<sup>−/−</sup>, 59 ± 17 (n=5) vs. imsd<sup>+/+</sup>, 104 ± 21 (n=7) (p<0.05), which corrected with 6% diet (imsd<sup>−/−</sup>, 78 ± 37 (n=3) vs. imsd<sup>+/+</sup>, 136 ± 90 (n=5) (p=ns). ASN in SE was within normal limits for imsd subjects, despite significant alterations in brain. For CIT, elevations in SE approximated those seen in brain regions (data not shown). SER levels in SE were not abnormal in mutant mice (although they were disrupted in brain regions; Fig. 6). Conversely, disruptions of ALA levels in SE mirrored the abnormalities observed in brain (data not shown). Of interest, abnormalities of LYS in SE mirrored the disruptions observed in brain (Fig. 7), especially in the
instance of high-protein intake, perhaps suggesting that some portion of LYS is trafficked on the large-neutral amino acid transporter.

Depletion of brain GLN, GLU and GABA have been suggested to associate with transamination via accumulated KIC in the brain [5]. Accordingly, we predicted that $Pah^{enu2}$ mice (representing the large neutral aminoacidopathy PKU [3, 4]) would have normal GLN, GLU and GABA since KIC is not elevated in this disorder. To address this hypothesis, we developed cohorts of $Pah^{enu2/-}$ mice and parallel controls and fed them diets identical to those given to $imsud$ mice. We first verified the utility of our PKU model through quantitation of brain PHE levels, which was significantly increased (and not corrected even with 6% protein intake; Fig. 8). Unexpectedly, alterations in brain regions for ASP, GLN and GLU in $Pah^{enu2/-}$ mirrored those observed in $imsud$ mice (Fig. 9). These changes in $Pah^{enu2/-}$ mice with decreased protein intake were: GLN: 3675 (381) to 4500 (258) in CB, 1964 (261) to 2308 (208) in STR and 1557 (280) to 1876 (297) in CTX (nmol/gr tissue); ASP: 2468 (443) to 2335 (98) in CB, 1758 (293) to 1661 (109) in STR and 2216 (456) to 2044 (306) in CTX; GLU: 6781 (559) to 6833 (320) in CB, 6041 (461) to 5777 (363) in STR and 6427 (810) to 6164 (637) in CTX; GABA: 2152 (279) to 2470 (283) in CB, 1735 (264) to 1470 (168) in STR and 2304 (233) to 2271 (308) in CTX (all nmol/gr tissue). The striking similarity in abnormalities between $imsud^{-/-}$ and $Pah^{enu2/-}$ mice in different regions of the brain, as a function of protein intake (Fig. 9), suggests that other factors, in addition to brain keto-acid accumulation, may be at play in the brains of animals with large neutral aminoacidopathies, and most likely in patients as well.
**Figure 8.** Phenylalanine levels as a function of protein intake and tissue in phenylketonuric (PKU) mice. Two-way ANOVA, p<0.05 for protein intake, tissue and interaction. All values in mutant mice were significantly increased in comparison to control mice (p<0.05, two-tailed t-test; not shown on graph).

**Figure 9.** Glutamine, aspartate, glutamate and GABA as a function of protein intake and tissue in phenylketonuric mice. Two-way ANOVA, p<0.05 for tissue, protein intake and interaction. Two-tailed t-test, *p<0.05 for mutant vs. wild-type. Note: For GABA in CTX, GABA was also significantly decreased in mutant vs. wild-type mice. In only a single instance did lower protein result in significant correction between mutant populations (GLN in CB).
Discussion

The range of means for BCAAs in SE of imsd mice receiving 6% dietary protein was 362-434 μM (Fig. 1), consistent with the range of LEU for 13 intermediate MSUD patients under dietary intervention (379 ± 147 μM; Hoffmann et al 2006) and 282 ± 277 μM for classical MSUD patients [5] considered to be under “metabolic control”. Based on these human data, 6% protein consumption normalized blood BCAAs, yet we observed significant disruption of several other amino acids in brain despite apparent metabolic control manifested in SE. We hypothesized that reduced levels of GLN, GLU, GABA and ALA in brain regions might be partially explained by KIC accumulation [5; see Fig. 7; not measured in the current study], yet similar anomalies in PKU mice argues against this hypothesis (Fig. 9). CIT accumulation in imsd brain regions likely reflects depletion of ASP, the latter necessary for condensation with CIT to form argininosuccinate within the urea cycle.

The original studies of Pardridge and Oldendorf [38] in rat indicated that PHE, TRP, MET, TYR, LEU, ILE, VAL and HIS were transported into the brain on the LAT-1, with $K_m$ values ranging from ~ 25 to 200 μM (highest affinity, PHE; lowest affinity HIS). Limited data suggests that THR and LYS may also traffic on LAT-1 [3, 4, 39, 40], yet we found no evidence for disrupted THR levels. Conversely, we did detect decreased LYS in brain regions of imsd subjects. LYS would be expected to be transported predominantly via the B transporter [3], although there is considerable redundancy in many of the blood-brain transporters.

Long-term neurocognitive deficits are observed in MSUD, even with acceptable metabolic control (as determined in blood), and both animal and human data suggests that these deficits correlate with exclusion of the monoamine precursors TYR and TRP from the
brain due to chronic elevations of BCAAs [1, 2, 25, 33, 34]. While our studies revealed significant effects on brain TYR with diet in imсуд subjects, we found no consistent effect on TRP (although we did not quantify monoamine neurotransmitters). Alternatively, our data supports the premise that long-term neurocognitive deficits in MSUD patients may also correlate with depletion of amino acid neurotransmitters (ASP, GLU and GABA). Based upon the preliminary studies in the current report, our next objectives are to investigate LEU-depleted diets in imсуд+/− mice that are appropriately supplemented with ILE and VAL, a diet corresponding to that used in MSUD patients, and to again investigate the amino acid outcomes both regionally in brain and blood. Such studies will yield a better overview of the expected outcomes for a diet more closely resembling that utilized in the clinic. The main conclusions drawn from the current report include:

• The first detailed cross-correlation assessment of amino acids in brain and blood, as a function of total protein intake, and the first analysis of amino acids in discrete regions of the brain of imсуд mice, including cerebellum, cortex and striata

• A clear demonstration that blood amino acid analysis, and the assignment of metabolic control with respect to BCAA levels, represents only a partial picture of brain amino acid homeostasis

These results further support the concept that dietary intervention in MSUD is likely to be insufficient to avoid neurocognitive deficiencies in patients, underscoring the urgent need for more focused therapeutics in MSUD.
Authors’ contributions
KRV performed all animal studies, including breeding and dietary intervention, dissection, and assisted with development of the manuscript: EA, BW and TB performed all analytical studies and assisted in drafting the manuscript; SM performed all data analysis, including data evaluation, statistical modeling, and assisted in drafting the manuscript; KMG designed the study, evaluated amino acid data, derived the hypothesis for testing and developed the first draft of the manuscript.

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Competing Interests
The authors declare that they have no competing interests.
References


CHAPTER FOUR
Review

Thirty years beyond discovery—Clinical trials in succinic semialdehyde dehydrogenase deficiency, a disorder of GABA metabolism

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Abstract

This review summarizes a presentation made at the retirement Symposium of Prof. Dr. Cornelis Jakobs in November of 2011, highlighting the progress toward clinical trials in succinylsemialdehyde dehydrogenase (SSADH) deficiency, a disorder first recognized in 1981. Active and potential clinical interventions, including vigabatrin, L-cycloserine, the GHB receptor antagonist NCS-382, and the ketogenic diet, are discussed. Several biomarkers to gauge clinical efficacy have been identified, including cerebrospinal fluid metabolites, neuropsychiatric testing, MRI, EEG, and measures of GABAergic function including (11 C)flumazenil positron emission tomography (PET) and transcranial magnetic stimulation (TMS). Thirty years after its discovery, encompassing extensive studies in both patients and the corresponding murine model, we are now running an open-label trial of taurine intervention, and are poised to undertake a phase II trial of the GABAB receptor antagonist SGS742.

Abbreviations

ABAS-II adaptive behavior assessment scale II

ACTH adrenocorticotropic hormone

ADHD attention deficit hyperactivity disorder

AED antiepileptic drug

BBB blood brain barrier

CSF cerebrospinal fluid

DA dopamine
EEG electroencephalography

FDA Food and Drug Administration (of the USA)

FMZ flumazenil (GABAAR ligand benzodiazepine binding site)

GABA 4-aminobutyric acid

GABA\textsubscript{A}R GABA\textsubscript{A} receptor

GABA\textsubscript{B}R GABA\textsubscript{B} receptor

GABA-T GABA-transaminase

GAD glutamic acid decarboxylase

GHB 4-hydroxybutyric acid

GHBR GHB receptor

IND investigational new drug

IQ intelligence quotient

IS infantile spasms

KD ketogenic diet

LCS L-cycloserine

MCI mild cognitive impairment

MRI magnetic resonance imaging
NCS-382 (6,7,8,9-tetrahydro-5-hydroxy-5 H-benzocyclohept-6-ylidene)acetic acid

SGS742 phosphinic acid (3-aminopropyl)(n-butyl) phosphinic acid

SSADH succinic semialdehyde dehydrogenase (or ALDH5A1 aldehyde dehydrogenase 5a1)

TMS transcranial magnetic stimulation

TCA tricarboxylic acid

VGB vigabatrin (gamma-vinyl GABA; SabrilR (Lundbeck Corporation))


Prelude

It is doubtful that any reader of the Journal would question the concept that the care and treatment of patients with inborn errors of metabolism functions optimally through a “team” approach. The team is generally defined as the clinician, supported by clinical chemists, dieticians, physical and/or occupational therapists, clinical psychologists, nurse practitioners, and other colleagues. This “team” approach represents the foundation of the current article, when a young analytical chemist named Cornelis Jakobs came to the University of California, San Diego, to pursue his Doctoral studies in stable-isotope dilution mass spectrometry with Dr. Lawrence Sweetman and Dr. William Nyhan. Jakobs had identified three patients who excreted gamma-hydroxybutyric acid in urine, and he hypothesized that the defect in these patients was
in succinic semialdehyde dehydrogenase (SSADH, or ALDH5A1 (aldehyde dehydrogenase 5a1) (Jakobs et al 1981). What he needed was an enzymologist to test this hypothesis, and the senior author was fortunate enough to have been that individual (Gibson et al 1983). The path to the current paper highlights the team approach, with clinicians, molecular biologists, mammalian geneticists, neurophysiologists, and so many other scientists that it is challenging to identify (much less remember) them all. The good news is that we now possess sufficient knowledge of the pathophysiology of this disorder to institute clinical trials! Where these trials take us remains to be seen, but we can say with certainty that without Cornelis, we would never have started. The senior author will miss the daily interactions by e mail, but a 30-year friendship is not easily lost or forgotten.

Introduction

The treatment of inborn errors of metabolism presents a host of challenges for the clinician, some of which include compartmentalization of the primary defect, alternative pathways for metabolism, interlinked metabolic pathways (e.g., oxidative phosphorylation with fat oxidation), the confounds of first-pass drug metabolism in the liver, the blood brain barrier, and many others. When considering neurometabolic disorders, these challenges magnify as we must entertain the largely anabolic and inaccessible brain. Does the primary metabolic lesion affect mainly the putamen, the amygdala, the striata or cortex, or cerebellum? Is the defect global or localized, and are there effective treatments that will cross the blood brain barrier? Are metabolites that form in the brain able to enter the peripheral metabolism, or are they intracellularly trapped? Adding to the complexity are neuronal-glial interactions, brain remodeling, and a number of other physiological parameters associated with brain metabolism.
such that it becomes a remarkable achievement that we can ever effectively treat a neurometabolic disorder.

SSADH deficiency mirrors all of these treatment challenges, and has posed additional considerations when entertaining effective treatment strategies. A defect of GABA metabolism, SSADH deficiency features accumulation of two neuromodulators, GABA and GHB, and paradoxically represents a hyperGABAergic seizure disorder (Fig. 1) (Wong et al 2004; Maitre 1997; Pearl et al 2011). One pathophysiological mechanism includes GABAA receptor downregulation, shown on positron emission tomography with 11 C-flumazenil (Pearl et al 2009a). Clinical reports on dozens of patients reveal a neurological disorder that may often be misdiagnosed as autism or autism spectrum, oppositional defiance, ADHD or other behavioral disorders (Pearl et al 2003). Most therapies have been symptomatic, targeting occasional seizures, behavioral dysfunction, or other clinical manifestations, but few interventions target the primary lesion in this disease. Further complicating the situation is the fact that some GABA receptors in neural tissue are excitatory during development (as opposed to inhibitory, the common role for GABA in brain) (Rheims et al 2008; Herlenius and Lagercrantz 2001), the observation that GHB produced in the periphery (liver has about 70 % of the SSADH activity of brain; Chambliss et al 1995) can freely traverse the blood brain barrier (BBB), and the probable main neurotoxin, GABA, can only be removed by enhanced metabolism or conjugation.
Figure 1: The GABA metabolic pathway. The block in SSADH deficiency is indicated by the crosshatched box. Upward arrows indicate metabolite elevations in patients and aldh5a1-/- mice. High levels of GHB will inhibit presynaptic DA (dopamine) release and enhance turnover. The site of VGB acting on GABA-transaminase (GABA-T) is indicated. Additional abbreviations: GAD, glutamic acid decarboxylase; TCA, tricarboxylic acid
Figure 2: Chemical structures of the compounds discussed. in the text, including the species accumulating to pathological levels in SSADH deficiency, GHB and GABA (bottom)

In this review, a synopsis of interventional strategies for SSADH deficiency is presented, highlighting what has been attempted, and what appears to be on the horizon (Knerr et al 2007). Most of the therapies that are planned, or studies in progress with patients, have springboarded from preclinical findings in the murine model of SSADH deficiency, aldh5a1 −/− mice (Gibson et al 2002). Brief reference will be made to important findings in the mouse model that have revealed novel treatment concepts.
Treatment strategies for SSADH deficiency

Vigabatrin

Perhaps the most widely employed intervention for SSADH deficiency, at least in Europe, has been vigabatrin (VGB; gamma-vinylGABA; Sabril®; Lundbeck Corp.). VGB, an irreversible inhibitor of GABA-transaminase (Figs. 1 and 2), acts pharmacologically to increase brain GABA. VGB is employed for infantile spasms (IS) (especially in tuberous sclerosis patients), as well as refractory complex partial seizures. In the case of SSADH deficiency, the use of VGB is predicted to lead to decreased production of succinic semialdehyde (Fig. 1), the probable precursor of GHB (Gropman 2003). High-dose VGB in the aldh5a1 −/− model led to significant extension of lifespan, although metabolic measurements (brain GHB/GABA) were not performed (Hogema et al 2001). Along these lines, the results of clinical intervention support decreased production of GHB, but a concern with VGB is the potential to further increase GABA in an already hyperGABAergic disorder (Pearl and Gropman 2004; Tables I & II).

Despite a clear rationale for VGB in SSADH deficiency, surprisingly few literature reports have been presented, and no blinded, controlled trial has been published. The case reports of clinical improvements with VGB (Table 1) have been tempered by other reports of worsening of symptoms, including seizure control (Matern et al 1996). Although approved in Europe since the 1980s for intervention in IS, VGB was not approved until 2009 by the US FDA for treatment of IS in pediatric patients and uncontrolled complex partial seizures in adults. The delays in FDA approval related to early studies in animals, including rats and dogs, which suggested edema in myelin tracts and vacuolation of white matter (Butler et al 1987; Qiao et al 2000; Peyster et al...
VGB may have particular utility for IS in patients under 2 years of age, since these seizures are particularly challenging, and a common intervention for IS, ACTH (adrenocorticotropic hormone), may be difficult to obtain. Moreover, VGB showed adverse effects on visual fields in approximately 1/3 of patients receiving it for epilepsy (Krauss et al 1998; Spence and Sankar 2001; Vanhatalo et al 2002). The mechanism of retinal toxicity remains undefined, although reduced ornithine aminotransferase (OAT) activity observed with VGB consumption may elevate retinal ornithine and induce a form of gyrate atrophy (De Biase et al 1991; Sorri et al 2010). Moreover, GABA is a feedback regulator of OAT (Daune and Seiler 1988). These observations suggest that monitoring ornithine levels during VGB intervention would be prudent. In addition, eight of 23 patients taking VGB had new-onset, reversible MRI T(2)-weighted hyperintensities and restricted diffusion in thalami, globus pallidus, dentate nuclei, brainstem, or corpus callosum (Pearl et al 2009b). Because of significant concerns, the FDA permits the use of VGB only for clinical instances in which the potential benefit against seizure outweighs the risk of visual loss. As well, VGB has a number of “black-box” warnings, and the FDA requires routine ocular exams/visual field testing.

Table 1

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Sex</th>
<th>VGB (months)</th>
<th>Clinical improvement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>F</td>
<td>20-100 mg/kg (4 mo)</td>
<td>none</td>
<td>Gibson et al (1989)</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>75 mg/kg/d (16 mo)</td>
<td>ataxia, EEG (swd)²</td>
<td>Jaeken et al (1989)</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>500-3500 mg/d (9 mo)</td>
<td>ataxia, IQ</td>
<td>Jakobs et al (1992)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>Sex</td>
<td>VGB (months)</td>
<td>Clinical improvement</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>-----</td>
<td>--------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>1500 mg/d</td>
<td>hypotonia</td>
<td>Uziel et al (1993)</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>25-75 mg/kg/d</td>
<td>cognition, behavior (low dose)</td>
<td>Matern et al (1996)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>1500 mg/d (6 mo)</td>
<td>hypotonia, myoclonus</td>
<td>Al-Essa et al (2000)</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>22-34 mg/kg/d (57 mo)</td>
<td>hyperactivity, agitation behavior</td>
<td>Ergezinger et al (2003)</td>
</tr>
<tr>
<td>2.5</td>
<td>F</td>
<td>20 mg/kg/d (12 mo)</td>
<td>hypotonia</td>
<td>Escalera et al (2010)</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>25 mg/kg/d (9 mo)</td>
<td>communication</td>
<td>Casarano et al (2011)</td>
</tr>
</tbody>
</table>

*When reported, CSF GHB decreased 14-72 % and CSF GABA increased 2.3 to 6-fold during VGB intervention (Ergezinger et al 2003); bElectroencephalogram, spike wave discharge. Two additional reports indicated clinical improvement in ~1/3 of patients treated with VGB, without clinical details (Rahbeeni et al 1994; Gibson et al 1997).

Receptor studies in aldh5a1-/- mice have shown significant alterations of GABAB and GABAA receptors (GABABR and GABAAR), yet no alterations in GHB receptor (GHBR) binding or number (Cortez et al 2004; Buzzi et al 2006; Wu et al 2006; Mehta et al 2006). These data suggest that GHB is not necessarily the primary neurotoxin in SSADH deficiency, a role more likely fulfilled by GABA. The absence of GHBR alterations may associate with the capacity of GHB to freely traverse the blood-brain barrier, which GABA cannot. The cumulative data suggests that elevating brain GABA levels via VGB intervention in SSADH deficiency might not be prudent. Additionally, GABA-T exists in the periphery, including liver, kidney and even blood (Tillakaratne et al 1995), and the kinetic characteristics of the neural enzyme are different from those of GABA-T in the periphery. Thus, incomplete inactivation of peripheral GABA-T’s by VGB may still result in elevated intracerebral GHB. This hypothesis is supported by metabolic
findings in patients receiving VGB (Table 2; Gibson et al 1995). Four patients treated with VGB (40-100 mg/kg/d) for 1-12 months underwent lumbar puncture for CSF collection. During treatment, there were variable improvements in cerebellar signs, concentration, hyperactivity, agility and cognitive function. As predicted, CSF total GABA increased and GHB decreased, although the absolute decrease in GHB was never as great as the GABA increase. Single body fluid measures such as these (Table 2) are limited by an absence of parallel measurements in other fluids, including plasma and urine. The fact that GHB levels did not decrease to the extent seen for total GABA increase (Table 2) is consistent with the hypothesis of peripheral resupply of GHB due to incomplete inactivation of peripheral GABA-T’s by VGB.

Table 2

Cerebrospinal fluid (CSF) metabolites during VGB intervention in four SSADH-deficient patients

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Patient</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>1110 : 22.4</td>
<td>708 : 32.1</td>
<td>-36 : +143</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>615 : 18.3</td>
<td>457 : 38.8</td>
<td>-26 : +212</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>525 : 22.1</td>
<td>384 : 33.6</td>
<td>-27 : +152</td>
</tr>
</tbody>
</table>

All metabolites in units of μmol/L. CSF control ranges: GHB, 0-2.6 μmol/L (n = 10); total GABA, 4.7-11.8 μmol/L (n = 10). Total GABA refers to free and “bound” GABA (e.g., homocarnosine, and other GABA compounds) (reprinted from Gibson et al 1995).
**L-cycloserine**

It is rational to attempt inhibition of GABA-T in patients with SSADH deficiency, as described above for VGB. However, if the primary source of pathology in this disease is elevated GABA, the expected decrease in GHB formation may be thwarted by further GABA elevations. L-Cycloserine (LCS) provides another potential inhibitor of GABA-T that warrants examination.

Cycloserine exists as two enantiomers: 1) the D-isomer (also known as Seromycin), used to treat tuberculosis and selected neurological disorders (associated with its potent NMDA receptor agonist activity (Lowther et al 2010)); and 2) the synthetic L-isomer. The latter, LCS, has been characterized in a number of model systems, yet the literature presents somewhat contradictory and conflicting findings. Early data highlighted the capacity of LCS to inactivate GABA-transaminase (see Fig. 1) as well as alanine aminotransferase (ALA-T; Beuster et al 2011). Linked to its capacity to raise intracerebral GABA levels (Fig. 1), LCS has been piloted in several seizure models, including petit-mal seizures in the rat (Marescaux et al 1985) and audiogenic seizures in mice (Polc et al 1986). Nonetheless, clinical development of LCS has been hampered because it also inhibits the first enzyme of sphingolipid formation, 3-ketodihydrosphingosine synthase (serine palmitoyltransferase; Cho 2007). The capacity of LCS to elevated intracerebral GABA is dose-dependent, with smaller doses (10-30 mg/kg p.o. or i.p. in rodents) reducing hyperexcitability, while higher doses (30-100 mg/kg) evoke central depressant actions (Polc et al 1986). As well, the literature indicates that inhibition of sphingomyelin formation may be dose-dependent, and may not be substantial at low pharmacologic doses of LCS.
Despite several rodent studies, there has been no report of LCS intervention in higher mammals or humans, which would make interventional studies in SSADH-deficient patients a very distant goal. Nonetheless, LCS may provide inhibition of GABA-T without the associated ocular side effects found with VGB, and preclinical studies in aldh5a1 -/- mice would be of interest.

**SGS742**

SGS-742 (originally CGP-36742) is one of a long line of phosphinic-acid analogues of GABA that are antagonists of the GABABR (Farlow 2009). These antagonists were originally developed by Ciba-Geigy Corporation (Froestl et al 2004), and eventually the proprietary rights moved to other corporations. As mentioned above, studies in aldh5a1 -/- mice have provided compelling evidence for use-dependent alterations of both GABAAR and GABABR (Gupta et al 2002; Cortez et al 2004; Wu et al 2006; Buzzi et al 2006). Accordingly, antagonists may prove beneficial as a treatment strategy in SSADH deficiency through blockade of supraphysiological agonist (GABA) levels. In preliminary studies in aldh5a1 -/- mice, high-dose CGP 35348 (an early phosphinic acid progenitor of SGS742) provided significant extension of lifespan for mice that succumbed to early status epilepticus (Hogema et al 2001; Gupta et al 2002). The phosphinic acid analogue SGS742 was subsequently introduced by Saegis Pharmaceuticals and Novartis Corporation, and proposed for clinical trials in patients with mild cognitive impairment (MCI; Bowery 2006; Bullock 2005; Froestl et al 2004). The rationale for SGS742 in MCI patients centers on the known inhibition of learning and memory associated with functional activation of the GABABR by its ligand, GABA. SGS742 has undergone phase I/II safety and tolerability evaluation in humans, and has shown efficacy in MCI patients (Froestl et al 2004), yet extended
trials have not been reported. Currently, the proprietary rights to SGS-742 have been obtained by the National Institutes of Health of the United States.

Pilot trials of SGS742 in aldh5a1 -/- mice have shown promising results. Moderate levels (30-100 mg/kg/d, i.p. administration) reduced spike-wave discharge, and led to near normalization of the electrocorticograph (Pearl et al 2009c). These data formed the foundation of a planned clinical intervention with SGS742 in SSADH-deficient patients. Two important hurdles must be cleared prior to undertaking this trial, however: 1) all safety and tolerability studies of SGS742 were performed in adults, and there are many adolescent patients with SSADH deficiency which could be enrolled in a blinded clinical trial; and 2) an application to the US Food and Drug Administration (FDA) for an IND (Investigational New Drug). Despite these challenges, we envision undertaking a blinded, placebo-controlled trial of SGS742 in SSADH-deficient patients shortly.

Ketogenic diet

Nylen and colleagues (2008, 2009; Stewart et al 2008) posited that early lethality in aldh5a1 -/- mice occurred proximal to the weaning period. These investigators proposed that the high-fat content of dam’s milk was protective to the pups, and hypothesized that the use of the ketogenic diet (KD), a broadly employed dietary intervention for refractory epilepsy (Cusmai et al 2012; McNally and Hartman 2012), would lead to beneficial outcomes in the murine model (Nylen et al 2008, 2009; Stewart et al 2008). Implementation of a 4:1 (fat:carbohydrate) KD in aldh5a1 -/- mice led to a significant improvement in seizure profiles, body weights and energy production, as well as extended lifespan. These findings have led a number of clinicians to
consider using the KD in SSADH-deficient patients for whom control of convulsions has proven challenging. Nonetheless, no clinical report on its use has been presented. The other major drawback to use of the KD is the fact that most convulsions in SSADH-deficient patients can be controlled with standard antiepileptics (AEDs), such as lamotrigine, levetiracetam, or topiramate, and the KD is both challenging to implement and maintain. Thus, conventional seizure control has predominantly employed standard AEDs (Pearl et al 2011).

NCS-382

As noted above, one of the paradoxical findings in aldh5a1−/− mice is the absence of GHBR alterations despite high levels of GHB (Wu et al 2006; Mehta et al 2006). Nonetheless, in early studies on aldh5a1−/− mice, the drug most effective in rescuing these animals from early lethality was NCS-382 (6,7,8,9-tetrahydro-5-hydroxy-5 H-benzocyclohept-6-ylidene acetic acid), a pure GHBR antagonist (Hogema et al 2001; Gupta et al 2002). These data suggest some role for GHB (and its receptor) in the process of early lethality, yet this remains paradoxical in the face of normal GHBR pharmacology observed in vivo (Mehta et al 2006). Despite positive outcomes with NCS-382 in aldh5a1−/− mice, no detailed studies on its use have been performed in these mice, and NCS-382 is not an FDA-approved drug. Considerable data on the safety and tolerability of NCS-382 would be required, not only in higher mammals (dog, primate, etc), but minimally phase I/II safety and tolerability data in humans, before any consideration of this compound could be undertaken in SSADH-deficient individuals.
Taurine

The high content of taurine in dam’s milk prompted Hogema and colleagues (2001) to examine this non-physiological amino acid as an intervention in aldh5a1 -/- mice, and these investigators observed significant extension of truncated lifespan. Efficacy of taurine intervention in aldh5a1 -/- mice prompted Saronwala and colleagues (2008) to perform a pilot investigation in an SSADH-deficient patient. The patient, a 2-year old male, was treated with 200 mg/kg/day taurine over a 12 month period, and tolerated it well. Plasma taurine levels ranged from 2-8 fold normal, and there was no correlation between taurine level and GHB excretion. By 9 months of treatment, marked behavioral improvements were noted, as well as improved peer interactions, level of activity and coordination. Repeat MRI obtained at 12 months of treatment was interpreted as resolution of prior restricted diffusion abnormality of the globus pallidus, with a very small residual T2 signal MRI abnormality.

Taurine is an abundant free amino acid in various tissues, and has important roles in neuromodulation and osmoregulation (Olive 2002). Taurine is known to interact with both GABAARs and GABABRs, and increases chloride conductance in excitable tissues. The neuroprotective action of taurine against beta-amyloid and glutamate receptor agonists in chick retinal neurons is blocked by the GABAAR antagonist picrotoxin (Louzada et al 2004). There is evidence that the taurine transporter is able to utilize GABA as a substrate, and that GABA competitively inhibits this transporter (Tomi et al 2008). There appears to be a reciprocal relationship between GABA and taurine, as taurine transporter null mice show increased GABAAR densities in the hippocampal dentate gyrus and in the cerebellum (Oermann et al...
Thus, since taurine appears to modulate GABA transmission, there may be beneficial competitive effects with respect to GABA in SSADH-deficient patients.

We extended the single-patient evaluation of taurine described above by enrolling patients into an open label trial in order to collect baseline data while preparing for a controlled trial with biomarkers used to evaluate this population without pharmacologic intervention. Subjects with confirmed SSADH deficiency were eligible for recruitment. Following informed consent, subjects were titrated weekly from 50 mg/kg/day until reaching a target dose of 200 mg/kg/day to a maximum dosage of 16 grams/day. The adaptive behavior assessment scale-II (ABAS-II) was administered at baseline and requested at 6 and 12 months of therapy. The ABAS II measures adaptive skills from conceptual, social, and practical domains in study participants. This scale has normative data and is offered for the age ranges 0-5, 5-21, and 16-89 years. Patients initiated at age 16 years and older were administered the adult version. We used linear longitudinal modeling to compare ABAS scores during the period pre- to the time-averaged scores in the period during taurine use. The model adjusted variance estimates, analogous to a paired t-test, were used to account for the correlation of scores within the same participant.

Sixteen patients were recruited and provided baseline data: 6 M/10 F, age range at enrollment 2-28 yrs (mean 12 yrs). One patient had a serious adverse event on 16 grams/day (200 mg/kg): hospitalization for hypersomnia. This led to a dose lowering paradigm with a new maximum daily dose of 10 grams. The taurine has been otherwise well-tolerated. Six subjects have completed the trial; three withdrew early due to perceived lack of efficacy. For patients who provided follow-up data during active therapy, there were no significant changes between
pre- and Rx-composite scores in general adaptive, conceptual, practical, or social skills. However, a borderline lower score was observed on the conceptual \((p = 0.1)\) index during taurine use. Our open-label pilot trial of taurine in patients with SSADH deficiency did not reveal demonstrable improvement in adaptive neurobehavioral functioning (Pearl et al 2012). Nonetheless, additional biomarker studies during taurine intervention are in progress.

**Other potential interventions in SSADH deficiency**

As mentioned above, interventions targeting reduced GHB production through inhibition of GABA-T may be complicated by concomitant GABA elevations. One approach to mitigating this side-effect may be co-application of 3-mercaptopyruvate, an inhibitor of glutamic acid decarboxylase (Netopilová et al 1997; Roberts et al 1978). Such studies could be piloted in aldh5a1 -/- mice, but the intracerebral levels of both glutamate and GABA would require cautious evaluation. A further mechanism to mitigate GABA elevation in SSADH deficiency, in the presence or absence of drug intervention, might be supplementation with L-histidine with the objective of enhancing homocarnosine (the GABA:L-histidine dipeptide). Homocarnosine is a major component of excitable tissues, with roles in neuromodulation, osmoregulation, and neuroprotection (Bauer 2005). Increasing intracerebral homocarnosine in SSADH deficiency may have additional therapeutic benefit, in relation to its capacity to quench 4-hydroxy-2-nonenal (4-HNE), a major lipid peroxidation product and cytotoxic carbonyl agent (Aldini et al 2005). Since SSADH is the major aldehyde dehydrogenase responsible for degradation of 4-HNE in mammals (Murphy et al 2003), the quenching role of homocarnosine might have therapeutic relevance. Quantitation of 4-HNE levels in neural tissue of aldh5a1 -/- mice, or fluids/tissues derived from SSADH-deficient patients, however, has not been reported.
Outcome measures for clinical trials

A challenge for any effective clinical trial is the identification of surrogate biomarker outcomes that can be quantified to gauge efficacy. Along these lines, numerous studies in aldhl5a1-/- mice, and SSADH-deficient patients, have highlighted potential biomarkers, some of which have already been clinically validated. Along these lines, GABAergic anomalies (both GABAB and GABAAR) in aldhl5a1-/- mice have led to corollary studies of GABA receptor function in patients (Pearl et al 2009a; Reis et al 2012). For example, (11 C)flumazenil (FMZ) binding (a marker for GABAAR function) is decreased throughout all brain regions in patients with SSADH deficiency. Similar anomalies have been documented for GABAB function in patients employing transcranial magnetic stimulation (TMS), a technique in which depolarization or hyperpolarization of the neurons is induced using weak electric currents from a magnetic field (de Vries et al 2012). Both (11 C)FMZ binding and TMS will be considered as outcome measures in clinical trials of SSADH-deficient patients.

Additional standard clinical measures that can be employed for clinical trials include neurobehavioral evaluation (as described for taurine intervention), as well as EEG and MRI. Several reports of EEG in SSADH-deficient patients have revealed slowing in both hemispheres and spike-wave discharges (Vanadia et al 2012), an observation consistent with the effect of GHB on EEG (Snead 1978; 2000; Snead and Gibson 2005). As well, a consistent finding in patients has been hyperintensity on the T1-weighted MRI images in the globus pallidi bilaterally (Pearl et al 2009a; Yalcinkaya et al 2000; Ziyeh et al 2002). This finding is non-specific, and most likely represents localized cytotoxicity associated with metabolite accumulation and/or downstream metabolic disruptions. Nonetheless, brain MRI remains a valid clinical biomarker
to test therapeutic efficacy, and was employed as an outcome marker in the index patient treated with taurine (Saronwala et al 2008). Optimally, a phase II trial (targeting tolerability, dosing, safety, and other features without assessing efficacy) could be employed to identify which of the biomarkers outlined above is the most responsive and reproducible for interventional evaluation in SSADH-deficient patients.

**Pathophysiological considerations in SSADH deficiency**

Most data from the aldhl5a1 -/- mouse model points to GABA as the primary mediator of neuropathology in SSADH deficiency (Pearl et al 2009c). Insights into discrete metabolic effects (GABA, GHB) might be gleaned, therefore, by contrasting the phenotypes of SSADH and GABA-T deficiencies. The index siblings with GABA-T deficiency presented with severe psychomotor retardation, hypotonia, hyperreflexia and growth acceleration (Jaeken et al 1984), while a recently described third patient (Tsuji et al 2010) presented with intractable seizures. SSADH deficiency features intellectual disability with disproportionate deficit in expressive language, hypotonia, ataxia, infrequent seizures, and a component of neuropsychiatric morbidity in adolescence and adulthood. The severity of GABA-T deficiency supports a prominent neuropathological role for increased GABA in both diseases, although both disorders feature other metabolic disturbances, such as elevated β-alanine and homocarnosine in GABA-T deficiency, and increased 4,5-dihydroxyhexanoic acid, D-2-hydroxyglutaric acid, homocarnosine and succinic semialdehyde in SSADH deficiency.
Conclusions

One of many challenges with SSADH deficiency that will remain to be negotiated includes the fact that both GHB and GABA accumulate, and likely each plays a role in the pathophysiology of the disorder. Along these lines, combinatorial therapy (e.g., VGB/SGS742, or NCS-382/SGS742, etc) may be optimal, if regulatory hurdles can be cleared. Peripheral therapy (whether cell-based or gene therapeutic, targeting the liver) could have value, and preclinical studies in aldh5a1 -/- mice using adenoviral hepatic gene therapy have shown efficacy (Gupta et al 2004a, b). Intracerebral cell or gene therapy also represents an attractive objective in SSADH deficiency, especially since the phenotype is primarily neurological. Finally, some consideration must be given to in utero therapy, since studies in the murine model have revealed that GABA and GHB accumulate in early aldh5a1 -/- embryos, and the fact that the GABAAR is excitatory during early development (Jansen et al 2008; Waagepetersen et al 1999; Herlenius and Lagercrantz 2001). Thus, consideration may need to be given to the earliest possible intervention that can mitigate long-term neurological complications.

Looking back on the progress made in SSADH deficiency, it has clearly not been fast enough for the benefit of the patients. Nonetheless, once we traverse into the realms of both animal and human research, multiple regulatory considerations come into play. Today, however, we have a validated animal model, an IRB-approved patient registry, and multiple websites that have enabled patients and parents to interact and exchange information (www. pndassoc.org; www.waveofhope-ssadh.org; www.liamslinks.org). The Pediatric Neurotransmitter Disease Association, a parent support group focused solely on disorders of neurotransmitter metabolism, is supporting research through a competitive grant program. Our
goal remains to continue working as a team, focused on identifying better, long-term therapies for patients and families with SSADH deficiency, so that all can enjoy an enhanced quality of life.

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**Conflict of interest**

None.
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Defects in GABA metabolism affect selective autophagy pathways and are alleviated by mTOR inhibition

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Abstract

In addition to key roles in embryonic neurogenesis and myelinogenesis, γ-aminobutyric acid (GABA) serves as the primary inhibitory mammalian neurotransmitter. In yeast, we have identified a new role for GABA that augments activity of the pivotal kinase, Tor1. GABA inhibits the selective autophagy pathways, mitophagy and pexophagy, through Sch9, the homolog of the mammalian kinase, S6K1, leading to oxidative stress, all of which can be mitigated by the Tor1 inhibitor, rapamycin. To confirm these processes in mammals, we examined the succinic semialdehyde dehydrogenase (SSADH)-deficient mouse model that accumulates supraphysiological GABA in the central nervous system and other tissues. Mutant mice displayed increased mitochondrial numbers in the brain and liver, expected with a defect in mitophagy, and morphologically abnormal mitochondria. Administration of rapamycin to these mice reduced mTOR activity, reduced the elevated mitochondrial numbers, and normalized aberrant antioxidant levels. These results confirm a novel role for GABA in cell signaling and highlight potential pathomechanisms and treatments in various human pathologies, including SSADH deficiency, as well as other diseases characterized by elevated levels of GABA.

Synopsis

Defects in GABA metabolism lead to its accumulation, which can cause severe neurological and behavioral disorders like SSADH deficiency. By activating mTOR/Tor, GABA inhibits selective autophagy pathways, suggesting a therapeutic application for mTOR inhibitors in GABA metabolic disorders.

- Novel role for GABA in cell signaling
- Elevated GABA increases mTOR activity
• Inhibition of mitophagy and pexophagy caused by elevated GABA increases oxidative stress
• Mice that accumulate GABA in tissues have increased mitochondria in the brain and liver
• The mTOR inhibitor rapamycin can reduce mitochondria numbers in mice with elevated GABA and thereby reduce oxidative stress

Introduction
The non-protein amino acid γ-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter and is present in concentrations of between 1–10 mM in the brain (Young & Chu, 1990). It is also present in tissues outside the central nervous system (CNS) (Watanabe et al, 2002). GABA has been detected in the peripheral nervous and endocrine systems and is widely found in non-neuronal tissues, where it displays diverse physiological roles (Tillakaratne et al, 1995). Disorders due to defects in GABA metabolism cause severe neurological and neuromuscular symptoms. The autosomal-recessive metabolic disorder, succinic semialdehyde dehydrogenase (SSADH) deficiency, is the most common of the inherited disorders of GABA metabolism (Pearl et al, 2006). The clinical features of SSADH deficiency encompass developmental delay, psychomotor retardation, hypotonia, seizures, ataxia, and other behavioral problems (Gibson et al, 1997). Other diseases caused by defects in GABA metabolism include GABA transaminase deficiency, where patients have elevated levels of GABA in serum and cerebrospinal fluid causing abnormal development and seizures (Pearl et al, 2006; Tsuji et al, 2010). A chronic excess of GABA may also cause sleep abnormalities (Arnulf et
al, 2005; Kim et al, 2008; Pearl et al, 2009). However, the exact role of GABA in the pathophysiology of these disorders remains unclear.

The GABA shunt is a closed-loop metabolic pathway that bypasses two steps of the tricarboxylic acid (TCA) cycle, converting α-ketoglutarate to succinate, which feeds back into the TCA cycle (Supplementary Fig S1). The bypass step occurs via the transamination of α-ketoglutarate to glutamate, which undergoes decarboxylation by glutamate decarboxylase to GABA. Next, the mitochondrial GABA transaminase converts GABA to the metabolic intermediate succinic semialdehyde (SSA), which is either oxidized to succinate to enter the TCA cycle, or reduced to γ-hydroxybutyric acid (GHB) (Bach et al, 2009). During normal physiological conditions, the GABA shunt allows constant replenishment of both the GABA and glutamate neurotransmitters.

It has been over thirty years since the first report of SSADH deficiency in a patient with neurological abnormalities (Jakobs et al, 1981). Later studies proved that the reduction or absence of SSADH activity was due to mutations in the ALDH5A1 gene which encodes the SSADH enzyme, leading to increased levels of GABA and its metabolite, GHB, in patients (Gibson et al, 2003). Due to the variety and severity in symptoms of the disease and difficulties in diagnosing patients, SSADH deficiency may be significantly under-diagnosed in clinical settings (Pearl et al, 2003).

Currently, although the metabolic pathway of SSADH deficiency is known, how the accumulation of GABA contributes to the clinical manifestation of the disease is not known, and there is no established or universally effective treatment for the disease. Management of
SSADH deficiency tends to treat the seizures, behavioral problems, and other symptoms associated with the disorder (Kim et al, 2011). However, these symptoms may be secondary to the main cause of the disease. The murine model of SSADH deficiency represents a relevant phenocopy of the human disease, with seizures and evidence of oxidative stress in tissues, along with increased levels of the peroxisomal enzyme catalase in the brain and elevated superoxide dismutase in the brain and liver (Latini et al, 2007).

In the current report, we evaluated the hypothesis that GABA impacts autophagy-related pathways. Toward this goal, we use yeast as a novel model system to elucidate the underlying mechanism by which GABA regulates autophagy-related pathways and translate the salient findings to a murine model of the disease.

Autophagy is a major catabolic pathway involved in the targeting and degradation of intracellular proteins and organelles to the lysosome/vacuole in a tightly regulated process (Yang & Klionsky, 2009) that is highly conserved from yeasts to humans (Meijer et al, 2007). During this process, which allows cells to adapt to various environmental changes, a double-membrane vesicle known as an autophagosome sequesters organelles or cytosolic proteins and then fuses with the lysosome/vacuole, releasing its contents into the lumen, where they are degraded and recycled.

Autophagy can be either a non-selective process, gathering a bulk portion of the cytosol for degradation, or it operates specifically to degrade particular proteins or organelles, such as peroxisomes (pexophagy) (Till et al, 2012), mitochondria (mitophagy) (Kanki & Klionsky, 2008), or ribosomes (ribophagy) (Kraft et al, 2008). Much of the same core machinery used for general
autophagy also overlaps in the selective autophagy pathways. Defects in autophagy-related pathways have already been implicated in a wide variety of diseases, ranging from cancer and neurodegenerative disorders to aging (Mizushima et al, 2008), reflecting the theme that autophagy and autophagy-related pathways play essential roles in cellular homeostasis and quality control.

In this study, we find an unexpected role of GABA as a regulator of mitophagy and pexophagy. When GABA levels are increased, yeast cells are unable to specifically degrade mitochondria and peroxisomes via these selective autophagy pathways during starvation conditions, leading to oxidative stress due to the accumulation of these organelles. These effects mimic the oxidative stress observed in humans and the murine model of SSADH deficiency. The GABA-induced defects can be overridden by the Tor1 inhibitor, rapamycin, via signaling pathways we have identified in this study. Furthermore, we show that rapamycin can reduce elevated mitochondrial numbers and normalize aberrant antioxidant levels found in the murine model of the disease. These results demonstrate a proof of concept for using autophagy-inducing or mTOR-inhibiting drugs as treatment for disorders characterized by elevated levels of GABA.

Results

Increased levels of GABA inhibit pexophagy and mitophagy, but not other autophagy-related pathways

Patients with SSADH deficiency have up to a threefold increase in GABA levels (Gibson et al, 2003). Previous research in yeast mutants of the GABA shunt pathway has demonstrated that
deletion of the yeast SSADH, uga2, also increases intracellular GABA levels threefold (Kamei et al, 2011), which is comparable with the human form of the disease. Mutants deleted for other genes in the GABA shunt, including uga1 and gad1, have similar intracellular GABA levels as wild-type (WT) cells (Kamei et al, 2011). We probed whether increased levels of GABA affect autophagy-related pathways in yeast. In S. cerevisiae, autophagy-related pathways can be monitored by transferring cells to starvation medium that lacks nitrogen and amino acids (SD-N) to induce autophagy, for which organelle-specific markers can be followed to monitor specific selective autophagy pathways. We found that the UGA2, but not the UGA1 mutant of the GABA shunt pathway, partially inhibited pexophagy compared to the WT, as shown by the delay in degradation of the peroxisomal matrix protein, Pot1, at the 12-h time point (Supplementary Fig S2).

The addition of GABA to the starvation medium also inhibited autophagy-related pathways, because 10 mM GABA showed a severe defect in both pexophagy (Fig 1A) and mitophagy (Fig 1B and C). Both pexophagy and mitophagy assays assess the degradation of superfluous organelles upon nutrient limitation. The defect in pexophagy was shown by the delay in degradation of the peroxisomal matrix protein, Pot1, fused to GFP (Pot1-GFP, Fig 1A). In this standard assay, WT cells are first grown in oleate medium for 15 h to increase peroxisome number and then transferred to starvation conditions, wherein pexophagy is activated and detected by the appearance of free GFP. The defect in mitophagy was shown by the delay in the degradation of the mitochondrial outer membrane protein, Om45, fused to GFP (Om45-GFP, Fig 1B). In this assay, WT cells are grown in YPL medium, which contains lactic acid as a carbon source for 12-14 h to increase mitochondrial number and then transferred to
starvation conditions, where mitophagy is detected by the appearance of free GFP. An alternative mitophagy assay using fluorescence microscopy showed a large number of mitochondria labeled by OM45-GFP outside of the vacuole after 12 h in YPL medium. After transferring cells to starvation medium for 24 h, mitochondria were delivered to the vacuole as seen by GFP clearly located inside the vacuole lumen. However, when GABA was added to the starvation medium, OM45-GFP-labeled mitochondria remained outside of the vacuole (Fig 1C).

**Figure 1:** Increased levels of GABA inhibit pexophagy and mitophagy, but not other autophagy-related pathways

1. **Peroxisomes** were induced by growing the WT strain expressing Pot1-GFP in oleate medium to mid-log-phase, then transferred to SD-N starvation medium with or without GABA to trigger pexophagy for 6 h. GFP cleavage was analyzed at the indicated time points by immunoblotting.

2. **Mitochondria** were induced by growing the WT strain expressing OM45-GFP in YPL medium to mid-log-phase and subsequently transferring cells to either SD-N with or without GABA to
trigger mitophagy for 12 h. GFP cleavage was analyzed at the indicated time points by immunoblotting.

3. Mitophagy was monitored by fluorescence microscopy using a WT strain expressing OM45-GFP grown in YPL medium for 12 h to mid-log-phase in the presence of FM4-64, and transferred to either SD-N medium with or without GABA for 24 h. Bar, 5 μm.

4. The Cvt pathway was monitored using the WT strain in SD medium with or without GABA, grown to mid-log-phase, after which samples were analyzed for Ape1 maturation.

5. Ribophagy was monitored by growing the WT strain expressing Rpl25-GFP in SD medium to mid-log-phase and transferring cells to SD-N either with or without GABA for 24 h.

6. Autophagy was monitored by growing the WT strain expressing GFP-Atg8 in SD medium to mid-log-phase and transferring cells to SD-N either with or without GABA for 6 h.

Interestingly, the addition of 10 mM GABA did not block other selective autophagy pathways such as the biosynthetic Cvt pathway, which was monitored by the maturation of the vacuolar aminopeptidase, Ape1, in growth conditions. This maturation of Ape1 was unaffected by elevated levels of GABA in the medium (Fig 1D). Similarly, ribophagy, which was monitored by the degradation of the ribosomal fusion protein, Rpl25-GFP, in starvation conditions, remained unaffected by the addition of GABA. Free GFP accumulated at the same level as that seen in untreated cells (Fig 1E). The non-selective general autophagy pathway also remained unaffected by the addition of 10 mM GABA, as judged by the normal degradation of the GFP-Atg8 fusion protein (Fig 1F). Fluorescence microscopy confirmed that bulk autophagy was unaffected, because when WT cells were placed in starvation conditions for 6 h, GFP-Atg8 localized to the vacuole whether 1 mM or 10 mM GABA was added to the nutrient-limited medium. As expected, the autophagy-deficient atg1Δ strain was blocked in GFP-Atg8 localization to the vacuole (Supplementary Fig S3).
As GABA functions as a nitrogen source in S. cerevisiae, we asked whether GABA blocked pexophagy and mitophagy in strains that cannot use GABA as a nitrogen source. Previous studies in S. cerevisiae have shown that strains deficient in either UGA1 (utilize GABA) or UGA2 cannot grow in medium with GABA as the source of nitrogen (Coleman et al, 2001). Both pexophagy and mitophagy were blocked when 10 mM GABA was added to test uga1Δ mutants (Supplementary Fig S4A and B). We also tested SSA, a GABA metabolite formed downstream of GABA by the GABA transaminase (Uga1). Neither pexophagy nor mitophagy was inhibited by elevated levels of SSA (Supplementary Fig S5A and B).

These results show that the inhibition of pexophagy and mitophagy occurs even when cells cannot utilize GABA as a source of nitrogen, implicating a signaling process involving GABA itself, but not its metabolite(s).

The GABA-induced block in pexophagy and mitophagy is overridden by rapamycin

Rapamycin is a pleiotropic bacteria-derived drug molecule that is commonly used to trigger autophagy pathways via its ability to inhibit the pivotal kinase Tor1 (target of rapamycin) (Raught et al, 2001). We tested whether rapamycin could override the GABA-induced block in pexophagy and mitophagy by performing the above-mentioned assays in the presence of rapamycin and GABA.

Rapamycin did override the block in pexophagy caused by the addition of GABA (Fig 2A). An alternative pexophagy assay using fluorescence microscopy showed a large number of peroxisomes labeled by Pot1-GFP outside of the vacuole, and low levels of free GFP in the vacuole at 6 h after GABA addition to the starvation medium. However, when both rapamycin
and GABA were added to the starvation medium, the number of Pot1-GFP-labeled peroxisomes decreased dramatically and GFP was clearly located in the vacuole (Fig 2B). Rapamycin also overrode the block in mitophagy caused by the addition of GABA, as measured by OM45-GFP degradation to yield free GFP (Fig 2C).

Figure 2: The GABA-induced block in pexophagy and mitophagy is overridden by rapamycin
1. Peroxisomes were induced in oleate medium and pexophagy was monitored as described for Fig 1.
2. Pexophagy was monitored by fluorescence microscopy using a WT strain expressing Pot1-GFP grown in oleate medium to mid-log-phase in the presence of FM4-64, and transferred to either SD-N medium with or without GABA or to SD-N with GABA and rapamycin for 6 h. Bar, 5 μm.
3. Mitochondria were induced in YPL medium and mitophagy was assessed as described for Fig 1.
Increasing GABA levels endogenously also inhibits pexophagy and mitophagy and these defects are overridden by rapamycin

Cellular GABA levels were increased genetically using a high copy number plasmid to over-express the glutamate decarboxylase gene, GAD1, which catalyzes the conversion of glutamate to GABA (Coleman et al, 2001). Experiments were performed in the uga2Δ background strain to keep GABA levels elevated in the cells by slowing down GABA catabolism. Mitophagy was significantly inhibited when GAD1 was over-expressed compared to WT strain (Fig 3A). Pexophagy was also significantly delayed compared to WT strain (Fig 3B). However, autophagy was unaffected (Fig 3C). Much like the exogenous addition of GABA, the defects in mitophagy and pexophagy found in the GAD1 over-expression strains could be rescued with the addition of rapamycin (Fig 3A and B).
Figure 3: Increasing GABA levels endogenously also inhibits pexophagy and mitophagy, and these defects are suppressed by rapamycin

1. WT cells expressing OM45-GFP, along with the uga2Δ strain over-expressing the GAD1 gene and expressing OM45-GFP were grown in YPL medium to mid-log-phase. To monitor mitophagy, strains were transferred to SD-N starvation medium (with or without rapamycin).

2. WT strain along with the uga2Δ strain over-expressing the GAD1 gene was grown in oleate medium and pexophagy was monitored as described in Fig 1, with or without rapamycin. Samples were taken at the indicated time points, and Pot1 degradation was analyzed by immunoblotting (45 kD).

3. To monitor autophagy, WT cells expressing GFP-Atg8 along with the uga2Δ strain over-expressing the GAD1 gene and expressing GFP-Atg8 were grown in SD medium and transferred to SD-N.

Increased GABA levels activate Tor1 while inhibiting pexophagy and mitophagy through Sch9

We wanted to see whether the increase in GABA levels affected Tor1 activity during nutrient limitation, because Tor1 is the mechanistic target of rapamycin (Raught et al, 2001). In starvation medium, autophagy and autophagy-related pathways are induced by the inactivation of Tor1 (Kamada et al, 2004). Tor1 activity can be measured by the phosphorylation of the S6 ribosomal protein, as inhibition of autophagy and phosphorylation of the S6 ribosomal protein are controlled by the same signal transduction pathway (Blommaart et al, 1995).

Despite starvation conditions that normally inhibit Tor1, the increase in GABA partially activated Tor1 during pexophagy and mitophagy. As expected, when cells were transferred to starvation medium for 6 h to induce pexophagy, the levels of S6 phosphorylation were markedly decreased. However, in the same conditions with the addition of GABA, there was a small increase in S6 phosphorylation, suggesting that GABA activated Tor1 (Fig 4A). This was also observed during
mitophagy conditions, where S6 phosphorylation was absent in starvation conditions but increased upon the addition of GABA after 6 h (Fig 4B).

Figure 4: Increased GABA levels activate Tor in starvation conditions and inhibit pexophagy and mitophagy by acting through Sch9.

WT and sch9Δ strains were grown, as described earlier, for pexophagy or mitophagy assays with or without GABA.

- **A,B** WT cells were cultured under pexophagy (A) or mitophagy (B) conditions with or without GABA and rapamycin. S6 phosphorylation at the indicated time points was analyzed by immunoblotting with a loading control.
- **C.** Samples were analyzed for Pot1 degradation by immunoblotting (45 kD).
- **D.** GFP production during mitophagy was analyzed by immunoblotting.
- **E.** Proposed model for the regulation of pexophagy and mitophagy by GABA. Elevated GABA activates Tor1 in starvation conditions and inhibits pexophagy and mitophagy by activating Sch9.
The addition of rapamycin to inhibit Tor1 function leads to the induction of autophagy even in nutrient-rich conditions (Noda & Ohsumi, 1998). We found that rapamycin overrides the increase in Tor1 activity caused by the addition of GABA to the starvation medium, as seen by the complete reduction in S6 phosphorylation during both pexophagy and mitophagy conditions (Fig 4A and B).

To confirm that GABA acts through Tor1, we tested the tor mutant strain tor1Δ tor2ts and found that the addition of GABA lost its inhibitory effect on selective autophagy in this strain, whereas GABA inhibited the WT strain (Supplementary Fig S6).

The Ser/Thr kinase, Tor1, has a number of potential downstream targets. The AGC family protein kinase, Sch9, which is analogous to the mammalian TORC1 substrate S6K1, is directly phosphorylated by TORC1 at multiple sites to activate the protein kinase (Urban et al, 2007). Therefore, we wanted to see whether the inhibition of pexophagy and mitophagy caused by elevated GABA functioned through Sch9. We tested the sch9Δ strain and found that the addition of GABA lost its inhibitory effect on both pexophagy (Fig 4C) and mitophagy (Fig 4D) even though both pathways were blocked when GABA was added to WT strains. The inhibition of selective autophagy by GABA in the atg13Δ strain (Supplementary Fig S7) suggests that GABA does not operate through the Atg1/Atg13 complex. Therefore, mechanistically our model suggests that elevated GABA activates Tor1 in starvation conditions and inhibits pexophagy and mitophagy through Sch9 (Fig 4E).
Varying levels of Tor activity may inhibit specific autophagy-related pathways

Previous research has shown the role amino acids play in mTOR activation in mammalian cells (Hara et al, 1998), as well as the involvement of amino acids in the regulation of the mTOR pathway, whereby the addition of amino acids inhibited autophagy and increased S6 phosphorylation in rat hepatocytes (Blommaart et al, 1995). The over-activation of the mTOR signaling pathway has been implicated in many types of cancer (Guertin & Sabatini, 2007), tissue hypertrophy (Lee et al, 2007), and other diseases (Inoki et al, 2005). However, the molecular mechanism of amino acid signaling in mTOR activation is only just emerging (Kim & Guan, 2011).

To test the hypothesis that there may be a threshold of Tor1 activity required to inhibit non-selective autophagy compared to mitophagy and pexophagy, we predicted that autophagy would be inhibited by increasing the concentration of GABA, which would increase Tor1 activity. We found that 10 mM GABA partially activated Tor1 in autophagy conditions (Fig 5A), to similar levels as those observed in pexophagy (Fig 4A) and mitophagy (Fig 4B) conditions. 50 mM GABA showed a much larger increase in Tor1 activity compared to 10 mM GABA (Fig 5A). However, 10 mM GABA did not inhibit autophagy, as shown by the normal degradation of the GFP-Atg8 fusion protein, comparable to WT (Fig 5B), whereas 50 mM GABA did inhibit autophagy, as shown by the delay in GFP-Atg8 degradation, compared to WT. Thus, a partial activation of Tor1 activity by GABA is enough to inhibit mitophagy and pexophagy, but higher levels of Tor1 activity may be required to inhibit autophagy.
Figure 5: Increasing GABA concentration further increases Tor activity and inhibits autophagy. WT cells were cultured under autophagy conditions with or without GABA for 6 h.

1. S6 phosphorylation after 6 h in SD-N was analyzed by immunoblotting with a loading control.
2. GFP production monitoring autophagy at the indicated time points was analyzed by immunoblotting.

The GABA-induced block of pexophagy and mitophagy increases reactive oxygen species levels that can be reduced by rapamycin.

Mitochondria are the main source of cellular reactive oxygen species (ROS) (Wallace, 2005), and aberrant and dysfunctional mitochondria increase ROS levels in the cell (Giaime et al, 2012) leading to oxidative stress (Zuin et al, 2008). Defective peroxisomes are also known to increase ROS levels (Bonekamp et al, 2009), and impaired pexophagy promotes oxidative stress in
mammalian cells (Vasko et al, 2013). We hypothesized that the GABA-induced block in pexophagy and mitophagy may cause an increase in intracellular ROS levels.

Relative intracellular ROS levels of WT cells in four different treatment conditions were tested under both pexophagy and mitophagy conditions: WT cells, WT with GABA, WT with GABA plus the antioxidant glutathione, and WT with GABA plus rapamycin. We chose glutathione as an antioxidant as it has previously been shown to be taken up by yeast when supplemented to the medium (Yano et al, 2009; Ayer et al, 2010). After 24 h in starvation medium, cells were assayed for intracellular ROS levels using the redox-sensitive fluorescent probe dihydrorhodamine-123 (DHR-123). Propidium iodide was used to distinguish living cells from dead cells (Zuin et al, 2008).

In the presence of GABA, we observed significant increases (**P < 0.01) in intracellular ROS, compared to the untreated WT strain in both pexophagy and mitophagy conditions (Fig 6A and B). The addition of the antioxidant glutathione along with GABA could significantly reduce ROS levels (*P < 0.05). However, the addition of rapamycin along with GABA reduced ROS levels much further (**P < 0.01) compared to glutathione (Fig 6A and B). The increase in intracellular ROS caused by elevated levels of GABA was also confirmed employing an alternative assay using the redox-sensitive fluorescent probe 2’,5’-dichlorofluorescein diacetate (DCFH-DA) (**P < 0.01). The enhanced ROS levels could be significantly decreased by rapamycin (**P < 0.01) (Supplementary Fig S8A). In addition, the atg32Δ strain, which is defective in mitophagy (Kanki et al, 2009), was also tested under mitophagy conditions and was also found to have increased ROS levels compared to the WT strain (**P < 0.01). Again, rapamycin was able to significantly reduce elevated ROS levels in the atg32Δ strain (**P < 0.01) (Supplementary Fig S8B). These results show that the
block in pexophagy and mitophagy caused by elevated levels of GABA increases cellular oxidative stress, probably due to the presence of longer-lived or damaged peroxisomes and mitochondria.

**Figure 6:** The GABA-induced block of pexophagy and mitophagy increases reactive oxygen species levels that can be reduced by rapamycin

- **A,B** WT, WT with GABA, WT with GABA and 10 mM GSH and WT with GABA and 200 nM rapamycin were tested for intracellular ROS levels under (A) pexophagy and (B) mitophagy conditions. After 24 h incubation, cells were stained with DHR-123 and propidium iodide for 1 h. Living cells were analyzed for DHR-123 fluorescence by flow cytometry. Data represent mean ± s.d. (n = 3). *P < 0.005, **P < 0.01

- **C,D** Yeast cells stained with 5 μM propidium iodide were used to differentiate between living and dead cells under (C) pexophagy or (D) mitophagy conditions. Significant differences between the treatments and strains were determined using an unpaired two-tailed t-test. **P < 0.01.

- **E.** Pexophagy assay was monitored by the degradation of Pot1-GFP and analyzed for GFP cleavage by immunoblotting.
• F. Mitophagy assay was monitored by the degradation of Om45-GFP and analyzed for GFP cleavage by immunoblotting.

It is also noteworthy that we observed significantly increased cell death induced by elevated GABA after 24 h, when comparing the number of live cells to dead cells by propidium iodide uptake in both pexophagy and mitophagy conditions compared to the WT strain (**P < 0.01) (Fig 6C and D) using a gate for high signals in the propidium iodide-specific channel (Supplementary Fig S9A). This effect was significantly reversed by parallel treatment with rapamycin (**P < 0.01) (Fig 6C and D). The percentage of dead cells positively correlated with increased ROS levels in live cells, suggesting a mechanistic link between GABA-induced redox stress and cell death (Supplementary Fig S9B and C).

To evaluate whether it was the block in selective autophagy pathways that caused the accumulation of ROS, or whether selective autophagy pathways are blocked as a consequence of increased ROS levels, we aimed to assess whether the inhibition of selective autophagy pathways caused by GABA could be suppressed by reducing ROS levels using glutathione. However, glutathione did not override the block in pexophagy or mitophagy caused by GABA, the way rapamycin did (Fig 6E and F), suggesting that it is the block in selective autophagy pathways that contributes to the increased levels of ROS.

Elevated GABA inhibits mitophagy in mammalian cells

To determine whether elevated GABA could also inhibit basal mitophagy in mammalian cells, we performed an image-based in vitro mitophagy assay using a tandem fluorochrome protein (mito-RFP-GFP) in human HeLa cells over-expressing human Parkin (Allen et al, 2013; Kim
et al, 2013; Lazarou et al, 2013), whereby fluorescently tagged mitochondria undergo a color change upon delivery to the lysosome after 3 days in DMEM (Fig 7A and B). HeLa cells were either untreated or treated with 1 mM GABA, with or without rapamycin. We found that 1 mM GABA significantly inhibited mitophagy (**P < 0.01) as quantified by the percentage of cells, displaying mitophagy compared to untreated cells, and that rapamycin was able to significantly mitigate the inhibition of mitophagy caused by elevated levels of GABA (**P < 0.01) (Fig 7C).

Figure 7: Elevated GABA inhibits mitophagy in mammalian cells
- A,B Example images of Parkin-expressing HeLa cells analyzed using a tandem fluorochrome protein (mito-RFP-GFP) mitophagy assay under (A) control conditions or (B) displaying mitophagy depicted by the red mitochondrial structures localized to lysosomes. Bar, 10 μm.
- C. Percentage of cells displaying mitophagy + s.d., **P < 0.01 using an unpaired two-tailed t-test, n > 80.
SSADH-deficient mice have increased numbers of mitochondria and aberrant antioxidant levels that can be normalized by rapamycin

In order to elucidate the evolutionary conservation of the mechanism found in yeast and its potential role in a clinical setting, we assessed the role of rapamycin treatment in a murine model of SSADH deficiency that represents a viable model for the human disease, characterized by elevated levels of GABA in physiological fluids and tissues up to threefold higher than WT mice (Hogema et al, 2001).

Using transmission electron microscopy (TEM) images of SSADH-deficient mice homozygous for a targeted mutation of the aldehyde dehydrogenase family 5, subfamily A1 gene (Aldh5a1−/−) and WT liver cells, we noticed morphological differences between mitochondria in Aldh5a1−/− mice compared to WT, whereby mitochondria appeared significantly larger, as judged by mitochondria area (**P < 0.01) (Fig 8A and B). In humans, mitochondrial proliferation often expresses as mitochondrial DNA depletion. We found that mitochondrial DNA was not depleted in Aldh5a1−/− mice compared to WT mice (Supplementary Fig S10). Our results from the yeast model indicated that SSADH-deficient mice should possess increased numbers of mitochondria. Indeed, we found significantly increased numbers of mitochondria in Aldh5a1−/− mice compared to WT mice in both the liver (Fig 8C) and brain (Fig 8D) (**P < 0.01). Moreover, rapamycin treatment lowered mitochondria numbers to levels not significantly different from WT mice (Fig 8C and D).
Figure 8: SSADH-deficient mice have increased numbers of mitochondria and aberrant antioxidant levels that can be normalized by rapamycin

1. Electron microscopy images of mitochondria from WT ($n = 44$) and SSADH-deficient mice ($\text{Aldh5a1}^−/−$) ($n = 80$) were calculated for area size.

2. Electron microscopy images showing typical sizes of WT and $\text{Aldh5a1}^−/−$ mice liver mitochondria. Bar, 0.5 μm.
3. Quantification of mitochondrial numbers from electron microscopy images of liver from WT ($n = 31$) and Aldh5a1−/− mice treated with vehicle ($n = 39$) or rapamycin ($n = 34$) (5 mg/kg body weight per day) via intraperitoneal injections for 3 successive days starting at day 7 of life.

4. Quantification of mitochondrial numbers from electron microscopy images of brain from WT ($n = 23$) and Aldh5a1−/− mice treated with vehicle ($n = 30$) or rapamycin ($n = 41$) (5 mg/kg body weight per day) via intraperitoneal injections for 3 successive days starting at day 7 of life.

5. Aldh5a1−/− mice were treated with vehicle or rapamycin (10 mg/kg body weight per day) via intraperitoneal injections for 10 successive days starting at day 10 of life. WT mice served as non-disease controls (set to 1). After sacrifice, liver homogenates were used to measure SOD enzyme activity using a colorimetric SOD activity assay.

6. Mitochondrial SOD2 protein levels were quantified from liver microsections using immunofluorescence microscopy and automated image analysis (WT set to 1).

7. Immunofluorescence images showing typical nuclear staining (DAPI, blue) and SOD2 staining (red) from WT, Aldh5a1−/− mice treated with vehicle and Aldh5a1−/− mice treated with rapamycin. Bar, 10 μm.

Data information: **$P < 0.01$, *$P < 0.05$ using an unpaired two-tailed t-test. Data represent average + s.d.

Previous studies using the SSADH-deficient mouse model have shown increased levels of the antioxidant superoxide dismutase (SOD) in the liver compared to WT mice (Latini et al, 2007). Our data also found the same trend, whereby SOD enzyme activity was elevated in the liver of Aldh5a1−/− mice by 25% compared to WT mice. We found that rapamycin treatment could significantly reduce elevated SOD levels in the Aldh5a1−/− mice (*$P < 0.05$) compared to diseased mice treated with the vehicle alone (Fig 8E). Next, to see whether the elevated SOD levels were specifically associated with mitochondrial SOD (SOD2), the level of this protein was quantified from microsections of liver biopsies using immunofluorescence microscopy and automated image analysis. SOD2 levels in the Aldh5a1−/− mice also increased by 25% compared to WT mice, and rapamycin treatment significantly reduced SOD2 levels in the Aldh5a1−/− mice (**$P < 0.01$).
compared to diseased mice treated with the vehicle alone (Fig 8F). Raw data from at least 10 individual images per treatment were normalized to nuclear staining (DAPI, blue) to measure SOD2 staining (red) for differences between WT, Aldh5a1−/− mice, and Aldh5a1−/− mice with rapamycin treatment (Fig 8G).

We predicted that the mechanism of Tor1 activation by elevated GABA levels in yeast should also be found in SSADH-deficient mice, as measured by S6 phosphorylation. We found the same trend in mice liver and brain samples as we did in yeast. On average when normalized, SSADH-deficient mice showed a 58% increase in S6 phosphorylation levels in the liver (Fig 9A and B) and a 20% increase in S6 phosphorylation levels in the brain compared to WT mice (Fig 9C and D), indicating increased levels of mTOR activity. Rapamycin treatment significantly reduced the elevated S6 phosphorylation levels in SSADH-deficient mice (Fig 9A–D). This demonstrates that SSADH deficiency in mammals may follow the same mechanistic pathway to inhibit selective autophagy pathways as shown in yeast (Fig 4E), and further supports the model that the increased levels of mTOR activity, as well as the accumulation of mitochondria associated with elevated GABA levels, can be reversed by mTOR inhibition.
Figure 9: SSADH-deficient mice have increased levels of S6 phosphorylation compared to WT mice that can be reduced by rapamycin treatment.

*Aldh5a1−/−* mice were treated with vehicle or rapamycin (10 mg/kg body weight per day) via intraperitoneal injections for 10 successive days starting at day 10 of life. WT mice served as non-disease controls. After sacrifice, homogenates were used to measure S6 phosphorylation.

1. Quantification of S6 phosphorylation of liver lysates from WT (*n* = 5) and *Aldh5a1−/−* mice treated with vehicle (*n* = 4) or rapamycin (*n* = 5) after normalization (WT set to 1).
2. S6 phosphorylation of liver lysates analyzed by immunoblotting.
3. Quantification of S6 phosphorylation of brain lysates from WT (*n* = 2) and *Aldh5a1−/−* mice treated with vehicle (*n* = 3) or rapamycin (*n* = 3) after normalization (WT set to 1).
4. S6 phosphorylation of brain lysates analyzed by immunoblotting.

Data information: **P < 0.01, *P < 0.05, using an unpaired two-tailed t-test. Data represent average ± s.d.
Discussion

Many disorders with varying symptoms present with increased levels of GABA in the brain as well as outside the CNS. These include sleep abnormalities (Arnulf et al, 2005) to more severe diseases such as SSADH deficiency (Gibson et al, 2003) and GABA transaminase deficiency (Tsuji et al, 2010). Our results show for the first time how elevated levels of GABA inhibit the selective degradation of both peroxisomes and mitochondria, but not general autophagy cargo (Fig 1).

These findings are in line with previous work conducted on the murine model of the SSADH deficiency disease which found significantly higher levels of the peroxisomal enzyme catalase in the thalamus, as well as increased levels of SOD (a mitochondrial enzyme) in the liver and cerebellum (Latini et al, 2007), suggesting that there could be similar defects in peroxisomal and mitochondrial turnover in human cells, but this hypothesis remains to be tested directly.

Interestingly, we also found that the block in both of these selective autophagy pathways caused by increased levels of GABA can be overridden with the autophagy-inducing drug and Tor1 inhibitor, rapamycin (Fig 2).

Previous studies in a plant model of SSADH deficiency showed the accumulation of ROS (Bouché et al, 2003), but the physiological reason was unclear. Our results demonstrate that the increase in GABA levels increases Tor1 activity, leading to the inhibition of both pexophagy and mitophagy, probably causing the retention of longer-lived and damaged peroxisomes and mitochondria, which could be the underlying cause for a concomitant increase in ROS levels (Fig
Mitochondria are well known to be the primary source of cellular ROS, which could potentially cause severe oxidative stress to the cell (Wallace, 2005).

Consistent with our hypothesis that the block in selective autophagy is the cellular cause of ROS increase, the GABA-induced ROS elevation is also reversed with rapamycin or partially with an antioxidant (Fig 6A and B). We also found that the increase in oxidative stress caused by elevated GABA significantly increased cell death in both pexophagy and mitophagy conditions and that rapamycin, by overriding the inhibition of selective autophagy caused by GABA, significantly reduced cell death (Fig 6C and D). While the antioxidant glutathione reduced ROS levels significantly, it could not override the block in pexophagy or mitophagy caused by GABA, whereas rapamycin could (Fig 6E and F). As rapamycin reduced ROS levels more than glutathione, and is known to induce pexophagy and mitophagy, we suggest that rapamycin overcomes the underlying cause of the disorder by reducing elevated mTOR activity to induce autophagy, thus clearing away old and damaged peroxisomes and mitochondria causing the high levels of ROS.

We find that the mechanism causing the inhibition of pexophagy and mitophagy is that increased GABA levels cause the partial activation of Tor1 during starvation conditions, which in turn inhibits the selective autophagy pathways through Sch9, the yeast homolog of the mammalian S6K1 kinase (Urban et al, 2007). Our data show that both pexophagy and mitophagy are not blocked by increasing GABA levels in the sch9Δ strain (Fig 4).

During starvation conditions when Tor1 is inactive, non-selective autophagy is initiated. We believe that there may be a certain threshold value of Tor1 activity that must be surpassed before autophagy and autophagy-related pathways are inhibited, but these thresholds may be
different. A partial increase in Tor1 activity caused by the increase in GABA levels may be enough to block specific selective autophagy pathways such as mitophagy and pexophagy, but a higher level of Tor1 activity may need to be reached before autophagy and other selective autophagy pathways are inhibited (Fig 5).

How GABA only inhibits mitophagy and pexophagy in nutrient-limited medium, but not ribophagy and general autophagy, is not yet clear. In proposing an explanatory model, we note that only Atg11-dependent pathways are blocked by the addition of 10 mM GABA, as neither ribophagy nor general autophagy requires Atg11. In yeast, the phosphorylation-dependent interaction of the mitophagy receptor, Atg32, and the pexophagy receptor, Atg36, with Atg11 is essential for the degradation of mitochondria and peroxisomes, respectively. Therefore, it is possible that Tor1, through Sch9, is regulating these interactions by an unknown mechanism, and only a fully inactive Tor1 will induce the association of these selective autophagy receptors with their cargo, using the scaffold protein Atg11. Given that Tor1 and Sch9 are kinases, modulation of phosphoregulation of the selective autophagy receptors is a possibility.

Translating our results from yeast to mammalian cells, we found that elevated levels of GABA could also inhibit mitophagy in HeLa cells and that this inhibition could be mitigated by rapamycin (Fig 7). In the murine model of SSADH deficiency, we found as predicted, increased numbers of mitochondria in SSADH-deficient mice liver (Fig 8C) and brain (Fig 8D) compared to WT mice, probably due to a defect in mitophagy, and that the elevated numbers of mitochondria could be normalized to levels not significantly different from WT upon rapamycin treatment (Fig 8C and D). We also found that mitochondria were on average larger in the Aldh5a1−/− mice liver.
compared to WT (Fig 8A and B). We show that the elevated levels of SOD previously reported in Aldh5a1−/− mice (Latini et al, 2007), and mitochondrial SOD2, can be significantly reduced with rapamycin treatment (Fig 8E and F), which may have important treatment relevance for the human disorder.

SSADH-deficient mice also have increased levels of S6 phosphorylation in the liver (Fig 9A and B) and brain (Fig 9C and D) compared to WT mice, indicating increased levels of mTOR activity. Rapamycin treatment reduced elevated S6 phosphorylation levels in SSADH-deficient mice significantly (Fig 9A-D). This trend follows the same mechanistic pathway as we found in yeast (Fig 4A and B).

We demonstrate for the first time the applicability of yeast to study the molecular mechanisms linked to human SSADH deficiency, as well as other disorders caused by defects in GABA metabolism. Our data indicate a pivotal role of the induction of selective autophagy pathways for restoring cellular organelle homeostasis in this disease setting. Interestingly, autophagy has emerged as a promising target mechanism for the treatment of a variety of neurological disorders, including Huntington's disease (Ravikumar et al, 2004), Alzheimer's disease, and Parkinson's disease (Mizushima et al, 2008; Chong et al, 2010). The data presented here further emphasize the vital role of tightly regulated autophagy for cellular homeostasis and provide a proof of principle for using autophagy-inducing drugs or mTOR inhibitors for the treatment of SSADH deficiency and other disorders characterized by elevated levels of GABA.

Further work would be required to identify whether mammalian cells follow the same mechanistic pathway as we have described in yeast. However, as the autophagy pathway is
conserved from yeast to mammals, the unexpected role of GABA as a regulator of selective autophagy pathways may be an evolutionarily conserved one.

**Materials and Methods**

**Yeast strains and growth conditions**

Yeast strains and plasmids used in this study are listed in Supplementary Tables S1 and S2, respectively. *S. cerevisiae* strains were grown in rich medium (YPD; 1% yeast extract, 2% peptone, and 2% glucose) or defined synthetic medium (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, and auxotrophic amino acids as required) at 30°C on a shaker set at 250 rpm. For the induction of peroxisomes, cells were transferred to oleate medium (1% oleate, 5% Tween-40, 0.25% yeast extract, 0.5% peptone, and 5 mM phosphate buffer). For the induction of mitochondria, cells were grown in lactate medium (YPL; 1% yeast extract, 2% peptone, and 2% lactate, pH 5.5). Pexophagy, mitophagy, ribophagy, and autophagy were induced by transferring cells to SD-N medium, which contained no nitrogen or amino acids (0.17% yeast nitrogen base without ammonium sulfate or amino acids and 2% glucose).

**Reagents**

1 M stock solution of GABA (Acros Organics) was dissolved in water and diluted down to either 1 mM, 10 mM or 50 mM in SD-N medium. 200 nM rapamycin (Sigma-Aldrich) was added to SD-N to induce autophagy. 5 μg/ml of FM 4-64 (Life Technologies) diluted from a 1 mg/ml stock solution in DMSO was added to label the vacuolar membrane. Succinic semialdehyde (SSA) was added to SD-N from a 1.5 M stock solution (Aldrich). 50 mM of the redox-sensitive dye
Measurement of intracellular reactive oxygen species

Intracellular ROS levels were measured using a modification of a protocol previously described (Zuin et al, 2008). Relative ROS levels were analyzed using either the redox-sensitive fluorescent probe, dihydrorhodamine-123 (DHR-123), or 2′,5′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes). Propidium iodide (PI, Sigma-Aldrich) was used to distinguish living from dead cells. After treatment, cells were incubated in medium containing 50 mM DHR-123 or DCFH-DA and 5 μM PI for 1 h. Fluorescence was detected using a FACScalibur flow cytometer (Beckton-Dickinson). PI signal was detected in channel FL3 (deep red fluorescence, Ex 488 nm, Em 670LP), and DHR-123/DCFH-DA was detected in channel FL1 (green fluorescence, excitation wavelength 488 nm, emission wavelength 535/30 nm). Only living cells were used to quantify DHR-123/DCFH-DA fluorescence by gating for PI-negative cells. At least 20,000 living cells per sample were analyzed. Specific fluorescence in channel FL1 was normalized to cells lacking DHR-123/DCFH-DA (background control). Each treatment was done in triplicate and repeated at least twice. Data are represented as mean ± s.d. Unpaired two-tailed t-test was used to calculate P values between the treatments (*P < 0.05, **P < 0.01).

Fluorescence microscopy

For the autophagy assay, cells were cultured to log-phase (A600 ~ 0.8/ml) in SD medium containing 5 μg/ml FM 4-64 to stain the vacuole membrane and transferred to SD-N, with or without GABA. For the pexophagy assay, cells were cultured to log-phase (A600 ~ 0.8/ml) in oleate
medium containing 5 μg/ml FM 4-64 and transferred to SD-N, with or without GABA and rapamycin. Images were captured at room temperature using a Plan Apochromat 100 × 1.40 NA oil immersion objective on a motorized fluorescence microscope (Axioskop 2 MOT plus; Carl Zeiss, Inc.) coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss, Inc.) and processed using AxioVision software (version 4.5; Carl Zeiss, Inc.).

**Immunoblotting**

Samples were prepared by precipitation with trichloroacetic acid and A600 0.1 equivalent was resolved using 12% SDS–PAGE followed by Western blotting with anti-Pot1 (1:5,000; Subramani Laboratory), anti-Ape1 (1:5,000; Klionsky Laboratory), anti-GFP (1:2,000; Roche), anti-phospho-S6 (1:2000 Ser235/236), anti-S6 (1:1,000) and anti-Vinculin (1:1,000) all from Cell Signaling Technology. Secondary antibodies were either anti-rabbit or anti-mouse polyclonal (both 1:10,000; Roche) followed by enhanced chemiluminescence (GE Healthcare).

**In vitro mammalian mitophagy assay**

The assay to quantify basal mitophagy in mammalian cells is based on differential stability of a tandem protein (mito-RFP-GFP) as previously described (Kim et al, 2013). In short, HeLa cells over-expressing human Parkin (Lazarou et al, 2013) were quantified for basal mitophagy. Transfection was performed on cells growing on cover slips using XtremeGene 9 (Invitrogen) according to the manufacturer's recommendations (1 μg plasmid + 3 μl XtremeGene in 200 μl serum-free medium for transfection of one well of a 6-well plate). 1 day after transfection, cells were either left untreated or treated with 1 mM GABA with or without 0.05 μM rapamycin (1:20,000 dilution of a 1 mg/ml stock) in DMEM for 3 days. Next, cells were fixed in 4%
PFA/PBS and analyzed by fluorescence microscopy. Cells were defined to display signs of mitophagy when areas of red fluorescence caused by lysosomal delivery of mitochondria were detected within the cytosol. The percentage of cells showing red structures among all transfected cells (as evident from red + green mitochondrial signals) was used to quantify basal mitophagy. At least 80 cells were analyzed per treatment.

**Animals**

Monogamous breeding colonies were established with heterozygous breeders of the B6.129-Aldh5a1tm1KMG/J mouse model, which is an established model representing SSADH deficiency. Tail snips were collected at day of life 15 for DNA extraction, and genotyping was performed with 3 primer 2 reaction polymerase chain reaction and 1.5% agarose gel electrophoresis, as described previously (Hogema et al, 2001). Animal work in the present study was approved by the animal use and care committee at Washington State University.

**Rapamycin treatment for mice to measure superoxide dismutase and S6 phosphorylation**

Rapamycin (LC Laboratories) stock was prepared at 25 mg/ml in DMSO (Sigma) and delivered to mice comprising both sexes daily via intraperitoneal injections of 10 mg/kg body weight with a final injection volume of 100 μl. 100 μl aliquots of stock solution were stored at −20°C. Mice were injected every 24 h beginning at the 10th day of life and continuing for 10 successive days. After 10 days of injection, the animals were euthanized by CO2 anesthesia and cervical dislocation. The livers and brains from all experimental animals were collected and snap-frozen in dry ice.
**Rapamycin treatment for mice for transmission electron microscopy**

Rapamycin was delivered to mice via intraperitoneal injection. Litters comprising both sexes were injected with either DMSO (vehicle) or 5 mg rapamycin per kg body weight diluted with 1X PBS to a final injection volume of 100 μl. Mice were injected intraperitoneally from day 7 to day 10 of life (3-day duration). On the eleventh day, animals were anesthetized with ketamine/xylazine and underwent perfusion fixation through the left ventricle. Postflush with physiological saline (0.9 M NaCl), 4% paraformaldehyde (in PBS) was circulated for 4 min at 15 mL/min with a peristaltic pump. Liver and brain tissues were collected into 2% glutaraldehyde/2% paraformaldehyde (in 0.1 M phosphate buffer) overnight. The next day the samples were rinsed in phosphate buffer, and the median lobe of the liver and the cerebral cortex were sectioned and cut into 1-mm cubic pieces.

**Tissue processing for transmission electron microscopy**

Samples were rinsed in 0.1 M phosphate buffer three times for 10 min and then fixed with 1% osmium tetroxide for 5 h. Tissues were again rinsed once each with 0.1 M phosphate and then 0.1 M cacodylate buffers for 10 min each. Next, tissue was dehydrated with a 7-step ethanol series for 10 min each, 100% acetone, and left in 1:1 acetone:SPURRs overnight. The next day the tissue was placed in 100% SPURRs resin for 3 days and then polymerized in an oven at 65°C for 24 h. Thin sections were obtained on a Reichert-Jung ultramicrotome (Leica) set to 60 nm and collected onto formvar-coated copper grids. In a grid stick pipette, samples were immersed in 2 ml of filtered 4% uranyl acetate and 10 μl KMnO4 for 10 min in the dark and subsequently rinsed thirty times in three separate beakers with filtered DI H2O. The samples were then rinsed once
with 0.1 N NaOH before staining with Reynold’s lead (pH = 12) for 10 min and rinsed with DI water as above. Grids were dried under a heat lamp for 30 min before viewing with the TEM.

**Mitochondrial quantification**

Images were taken with a FEI Tecnai G2 from at least three separate sections positioned to capture the cytoplasm of random cells (excluding the nucleus). 9.6 K images were collected for WT, Aldh5a1−/−, and Aldh5a1−/− mice treated with rapamycin. Mitochondria were counted based on evidence of the double membrane and cristae. The number of mitochondria was counted and averaged across multiple micrographs of equal areas from comparable regions of mouse liver or brain regions.

**Calculation of mitochondrial areas**

AxioVision software was used to outline TEM 9.6K images of individual mitochondria and calculate area (μM2) of whole mitochondria from WT (n = 44 micrographs) and Aldh5a1−/− (n = 80 micrographs) mice and taking an average of each group. Unpaired two-tailed t-test was used to calculate the P value between the two groups of mice (**P < 0.01).

**Methods for quantifying mitochondria-to-nuclear DNA ratio**

Genomic DNA was extracted from the tails of WT and Aldh5a1−/− mice and re-suspended in water. Concentrations of DNA were calculated using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Novaquant Mouse Mitochondrial to Nuclear DNA Ratio Kit (Novagen) was used as per manufacturer’s directions, using 1 ng of isolated genomic DNA. qPCR was performed using Fast Sybr Green Mastermix (Applied Biosystems) with Step One Plus Real
Time PCR System (Applied Biosystems) with the following program: 95°C × 10 min; 95°C × 3 s; 60°C × 30 s; 95°C × 15 s; 60°C × 1 min for 45 cycles. Experiments were performed in duplicate.

Quantification of mtDNA relative copy number to nuclear DNA was done by averaging the copy numbers calculated from trLEV/BECN1 gene pair and the 12s/NEB pair. The counts (Cts) from the trLEV gene were subtracted from BECN1 Ct to obtain ΔCt1, and 12 s Ct was subtracted from NEB to obtain ΔCt2. To calculate the copy number, the average of the two sets of gene pairs (trLEV/BECN1) and (12s/NEB) was used. Calculation of the individual ratios used the formula N = 2^ΔCt where Embedded Image and Embedded Image. Lastly, the average of the two copy number results was taken and difference between the two groups calculated using an unpaired two-tailed t-test.

**Enzymatic assay for SOD activity**

Livers were halved and homogenized 1:5 (w/v) with ice-cold 1 mM EDTA dissolved in 1X PBS (pH = 7.8) with an Omni TH tissue homogenizer. Homogenates were centrifuged at 14,000 rpm for 10 min at 8°C and supernatant harvested. SOD colorimetric-based activity assays were performed according to the methods described by the vendor (Cell Biolabs, Inc. STA-340). Absorbance was measured on a Synergy HT microplate reader (Biotek) for duplicate samples and standards. A 7-point standard curve was used to determine the optimal absorbance range. The relative SOD activity was determined by the inhibition of chromogen reduction by free xanthine/xanthine oxidase producing superoxide anions. An unpaired two-tailed t-test was used to compare genotype and treatment groups (GraphPad Prism 5) (*P < 0.05). Enzyme assays for liver were processed for enzyme activity within two weeks of homogenization.
**Immunofluorescence of mice biopsies**

Liver tissue sections (5 microns) were obtained from snap-frozen biopsies using a Leica CM1800 Cryostat. Staining of mitochondrial marker SOD2 was performed using rabbit anti-SOD2 (NB100-1969, Novus Biologicals) at 1:200, followed by incubation with a biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.) and Cy3-streptavidin (Jackson ImmunoResearch Lab. Inc.). Tissues were counterstained for DNA using DAPI (Roche Diagnostics Corp.). Images were captured and processed as described for immunofluorescence microscopy. Automated image analysis for the quantification of subcellular SOD2 protein content was performed using the CellProfiler software (http://www.cellprofiler.org) (Carpenter et al, 2006) and a self-made analysis pipeline. Briefly, channel intensity was calculated for at least 10 individual images per treatment for the DAPI (blue) channel and for the Cy3 (red) channel. Specific signal intensity for SOD2 staining was calculated by normalizing the Cy3 channel intensity to DAPI intensity. Data were normalized to the WT signal (set as 1) and are depicted as an average + s.d. Unpaired two-tailed t-test was used to calculate P values between the treatments (**\( P < 0.01 \)).

**Experimental work**

All yeast experiments were repeated 2–3 times. Mammalian experiments were performed once.

**Author contributions**

RL designed, performed, and analyzed all yeast experiments and designed and analyzed mammalian and mice experiments and data. KRV performed all mice experiments and analyzed data. AT performed ROS experiments in yeast and mouse immunohistochemistry experiments and
analyzed the mammalian data. JL helped with yeast and mammalian experiments. SFB performed qPCR of mice samples. SS and KMG supervised the project through all stages. RL and SS wrote the manuscript with help from the other co-authors.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Supplementary Figures**

*Figure S1*: Metabolic pathway of GABA.

The GABA shunt showing the formation and degradation of GABA in the yeast *S. cerevisiae* including the major enzymes involved in GABA metabolism.
Figure S2: Deletion of the yeast UGA2 gene encoding SSADH partially inhibits pexophagy but deletion of UGA1 does not affect pexophagy.

Deletion of the yeast UGA2 gene encoding SSADH partially inhibits pexophagy but deletion of UGA1 does not affect pexophagy. Pexophagy assay was monitored by the degradation of Pot1 after transferring cells from oleate to SDN for 24 h and analyzed by immunoblotting (45kD).
Figure S3: Elevated levels of GABA do not inhibit autophagy.

Autophagy analyzed by fluorescence microscopy in the presence of the vacuolar membrane dye, FM4-64. The differential interference contrast (DIC), FM4-64 and GFP-Atg8 images are shown. Bar, 5 μm.
**Figure S4:** Increased GABA levels inhibit pexophagy and mitophagy even in mutants that cannot utilize GABA as a source of nitrogen.

(A) Utilization of GABA mutant uga1Δ was subjected to pexophagy conditions as described in Figure 1. Samples were monitored for Pot1 degradation. (B) The uga1Δ strain expressing OM45-GFP was subjected to mitophagy conditions and analyzed for GFP cleavage.

**Figure S5:** Increased levels of succinic semialdehyde (SSA) do not inhibit pexophagy or mitophagy.

(A) Pexophagy in the Pot1-GFP wild-type strain (+/- SSA) was analyzed for GFP cleavage by immunoblotting. (B) Mitophagy assays with and without SSA analyzed for GFP cleavage by immunoblotting.
Figure S6: Increased GABA levels inhibit selective autophagy by acting through Tor.

WT and tor1Δ tor2ts strains were cultured under pexophagy conditions at 37°C for 72 h with or without GABA and samples were analyzed for Pot1 degradation by immunoblotting (45kD).

Figure S7: GABA does not act through Atg13.

WT and atg13Δ strains were cultured under pexophagy conditions with or without GABA and samples were analyzed for Pot1 degradation by immunoblotting (45kD).
Figure S8: The GABA induced block in mitophagy increases reactive oxygen species levels that can be mitigated by rapamycin treatment.

(A) WT, WT with GABA and WT with GABA and rapamycin were tested for intracellular ROS levels under mitophagy conditions for 24 h, along with (B) WT and the mitophagy mutant strain atg32Δ with and without rapamycin. After incubation, cells were stained with DCFH-DA and PI for 1 h. Living cells were analyzed for DCFH-DA fluorescence by flow cytometry. Data represent mean (n=4) plus standard deviation and are normalized to the WT condition (set to 1). (**p<0.01 using a two-tailed t-test). Data represent average + SD.
**Figure S9**: Strategy for parallel assessment of cell death and ROS levels in live cells with correlation analysis.

Yeast cells were stained with 5 μM of propidium iodide (PI) to differentiate between living and dead cells along with 50mM of the ROS sensitive dye DHR-123. (A) A high signal in fluorescence channel FL3 (high pass >670nm) was used to create a gate for quantifying dead cells. Only live cells (outside of the dead cell gate) were used to measure intracellular ROS levels in fluorescence channel FL1 (band pass filter 530/40). The percentage of dead cells (PI positive cells) positively correlates with increased intracellular ROS levels (as measured by DHR-123) of live cells in both (B) pexophagy and (C) mitophagy conditions. R2; correlation coefficient.
**Figure S10**: The increased size of liver mitochondria in Aldh5a1^-/- mice is not due to the depletion of mitochondria DNA.

WT (n=5) and Aldh5a1^-/- (n=5) mice DNA was quantified to determine the ratio of mitochondrial to nuclear DNA relative copy numbers. Data represent average ± SD.

**Table S1**

List of strains used in this study

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sch9Δ  |  MATa; trp1, his3, ura3, leu2, rme1, sch9::kanMX4  |  (Urban et al., 2007)

Table S2

List of plasmids used in this study

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<td>GFP-ATG8</td>
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The paper explained

Problem

GABA is the primary inhibitory neurotransmitter in the brain and is also found in tissues outside of the CNS, including the liver and kidneys. Defects in GABA metabolism lead to the accumulation of GABA, which can cause severe neurological and behavioral problems. Diseases caused by inborn errors of metabolism such as SSADH deficiency and other human pathologies are characterized by elevated levels of GABA and oxidative stress. However, the pathophysiological role of GABA in these disorders remains unclear, and there are no established or universally effective treatments for these diseases.

Results

Elevated levels of GABA inhibit the selective degradation of two organelles in yeast, mitochondria and peroxisomes, by activating the kinase Tor1, leading to oxidative stress, all of which can be alleviated by the Tor1 inhibitor, rapamycin. Translating our work to the murine
model of SSADH deficiency, mutant mice have increased numbers of mitochondria in the brain and liver, expected with a defect in the degradation of mitochondria, and morphologically abnormal mitochondria. Rapamycin administered to SSADH-deficient mice reduced mTOR activity, decreased elevated mitochondrial numbers, and normalized aberrant antioxidant levels.

**Impact**

We confirm a novel role for GABA in cell signaling and demonstrate the use of mTOR inhibitors to treat disorders characterized by elevated levels of GABA to restore cellular organelle homeostasis. We also demonstrate the use of yeast as a model organism to study the molecular mechanisms linked to SSADH deficiency and other diseases caused by defects in GABA metabolism.
References


Meijer WH, van der Klei IJ, Veenhuis M, Kiel JA (2007) ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 3: 106 – 116


CHAPTER FIVE
Review

Therapeutic hepatocyte transplant for inherited metabolic disorders: functional considerations, recent outcomes and future prospects

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Abstract

The applications, outcomes and future strategies of hepatocyte transplantation (HTx) as a corrective intervention for inherited metabolic disease (IMD) are described. An overview of HTx in IMDs, as well as preclinical evaluations in rodent and other mammalian models, is summarized. Current treatments for IMDs are highlighted, along with short- and long-term outcomes and the potential for HTx to supplement or supplant these treatments. Finally, the advantages and disadvantages of HTx are presented, highlighted by long-term challenges with interorgan engraftment and expansion of transplanted cells, in addition to the future prospects of stem cell transplants. At present, the utility of HTx is represented by the potential to bridge patients with life-threatening liver disease to organ transplantation, especially as an adjuvant intervention where severe organ shortages continue to pose challenges.

Abbreviations

AGT Alanine glyoxylate transaminase
APOLT Auxiliary partial orthotopic liver transplant
ASL Argininosuccinate lyase
ASS Argininosuccinate synthetase
BCAA Branched-chain amino acids
BCKA Branched-chain keto acids
BCKDH Branched-chain ketoacid dehydrogenase complex
CNSI Crigler-Najjar syndrome type I
CPSI Carbamoyl phosphate synthetase I
Introduction

Inherited metabolic diseases (IMDs; also referred to as inborn errors of metabolism) represent often rare conditions characterized by accumulation of metabolic intermediates in organs and physiological fluids. Characterization of metabolite accumulation may facilitate identification of the defective pathway(s), many of which primarily manifest in the liver (i.e., α1-antitrypsin
(AAT), tyrosinemia type I, maple syrup urine disease (MSUD), phenylketonuria (PKU), galactosemia, progressive familial intrahepatic cholestasis (PFIC), neonatal hemochromatosis, Wilson’s disease, among others). Conversely, other IMDs will manifest additional extrahepatic features (i.e., familial amyloidotic polyneuropathy, primary hyperoxaluria type I (PH), Crigler Najjar syndrome type I (CNSI), familial hypercholesterolemia, urea cycle disorders). While orthotopic liver transplant (OLT) holds significant corrective value for many of these disorders, the outcomes of liver transplantation in IMDs with extrahepatic defects may not be as promising (i.e., erythropoietic protoporphyria, hereditary hemochromatosis, Gaucher disease, nonalcoholicsteatohepatitis) (Weiss et al 2007; Zhang et al 2007; Li 2007). Trials with transplantation into recipient liver (i.e., HTx) of the liver’s primary functional cell type (hepatocytes) have demonstrated corrective value in clinical and preclinical models of acute liver failure, end-stage liver disease, and in IMDs (Fig. 1).
Liver-related morbidity is the 12th most common cause of death in the United States, and in 2011 over 16,000 people awaited organ donation (Kochanek et al 2011), highlighting the expanding need for liver-directed therapeutics. Both cell-culturing techniques and cryopreservation methodologies are showing constant improvements, thereby broadening the potential utility of HTx. Nonetheless, limitations remain in the routine clinical application of HTx: 1) the maintenance of cellular viability post-transplant; 2) immune response/rejection and
graft vs. host disease; and 3) engraftment/expansion of transplanted cells into the host liver. Nevertheless, HTx could provide corrective potential for a large spectrum of IMDs, and this potential becomes increasingly relevant in the face of growing donor organ shortages. Clinical studies in disorders such as glycogen storage disorders, and urea cycle defects have already highlighted the corrective capacity of HTx to improve clinical outcomes, and it is reasonable to assume that additional advances with HTx can be expected (Dhawan et al 2004; Ribes-Koninckx et al 2012; Horslen et al 2003; Wan et al 2013; Chistiakov 2012; Yu et al 2012).

**HTX for inherited metabolic diseases**

**Tyrosinemia type I (Fumarylacetoacetic acid hydrolase (FAH) deficiency)**

Mice with genetically-engineered deletion of the fah gene represent perhaps the first instance of a genetic disorder in which the feasibility of HTx was documented (Karnezis et al 2001; Erker et al 2010; Paulk et al 2012). Grompe and colleagues successfully exploited fah deficiency in the distal portion of the tyrosine metabolic pathway to provide an engraftment advantage for exogenously administered fah +/- cells to fah-deficient mice. In human tyrosinemia type I, patients invariably progress to early morbidity from hepatocellular carcinoma, a finding recapitulated in the murine model. Intervention with nitisinone, an inhibitor of the upstream formation of homogentisic acid in the tyrosine pathway, prevents the formation of hepatotoxic fumarylacetoacetic acid in patients and mice, a life-saving intervention in both. To achieve successful HTx in this model, fah-deficient mice were reared with concomitant NTBC treatment, and fah +/- hepatocytes applied via intrasplenic administration with staged removal of nitisinone intervention. With this approach, the liver of fah-deficient mice could be ~90 % repopulated with fah +/- cells via a single exogenous cell.
administration (Hamman et al 2005; Al-Dhalimy et al 2002). In an FAH-deficient newborn, HTx successfully bridged the patient to OLT (Ribes-Koninckx et al 2012).

**Phenylketonuria (Phenylalanine hydroxylase (PAH) deficiency)**

Phenylketonuria (PKU) results from mutations in the enzyme phenylalanine hydroxylase (PAH) located on the long arm of chromosome 12. High levels of phenylalanine (PHE) have been suggested to saturate the large neutral amino acid (LNAA) transporter, LAT1, at the blood brain barrier. The monoamine neurotransmitters dopamine and serotonin may be depleted due to insufficient levels of their essential precursors, tyrosine and tryptophan, which are also LNAAAs employing the LAT1 for brain access. Dopamine and serotonin regulate important biological processes including movement, mood, speech, and appetite, as well as many other processes. Moreover, depletion of tyrosine can impact noradrenergic neurotransmitters, including epinephrine and norepinephrine. Additionally, oligodendrocytes responsible for assembly and maintenance of the myelin sheath surrounding neurons are sensitive to high brain PHE, and the hypomyelination, demyelination, lesions, white matter abnormalities, and cognitive impairment observed in PKU have been attributed to oligodendrocyte damage associated with hyperphenylalaninemia (Dyer 1999).

**Current treatment for PKU**

Standard intervention requires strict adherence to a protein restricted, low phe diet supplemented with low PHE formula for protein supplementation (MacDonald 2000). PHE is an essential amino acid, and even diet-adherent patients may experience metabolic flux and suboptimal outcomes such as cognitive deficits. A number of new therapies are currently in
phase I-III clinical trials in patients (www.clinicaltrials.gov), including sapropterin (KuvanTM; PhenoptinTM), phenylalanine ammonia lyase (PAL) enzyme therapy, and dietary administration of glycomacropeptide, a whey by-product devoid of PHE (LaClair et al 2009; Bélanger-Quintana et al 2011). Sapropterin essentially represents cofactor therapy, and is effective only in a portion of PKU patients undergoing intervention (Harding 2010).

**Clinical and preclinical HTx in PKU**

Hamman and colleagues (2005) demonstrated that 5–10 % repopulation of double mutant fah −/−/Pah enu2 mice was incapable of completely correcting PHE levels. As described above, the fah background was employed to provide an engraftment advantage (Orejuela et al 2008). Transplant of double mutant subjects with fah +/+/pah +/+ hepatocytes was predicted to substantially enhance engraftment (Al-Dhalimy et al 2002; Overturf et al 1999). Partially corrected mice revealed significant reduction of serum PHE levels (<700 μM) with hepatocyte engraftment exceeding 5 %. Repopulation levels of 20 % corrected PHE in a single subject (85–175 μM). The authors also employed heterozygous (Pah enu2+/−) donors and found similar levels of engraftment correlated with partial serum PHE correction (Hamman et al 2005). In a follow-up study (2011), the same group transplanted the double mutant model with 1–4x105 fah +/+/pah +/+ hepatocytes administered intrasplenically. These investigators found that 3–10 % engraftment partially corrected blood PHE levels in deficient mice, while >10 % transplant engraftment resulted in normalization. Hamman and colleagues concluded that the absolute PAH activity/cell was a less critical variable than the actual number of PAH expressing cells (Hamman et al 2011).
Only limited clinical studies of HTx have been piloted in PKU patients. In a study by Stéphenne et al (2012), a 6-year-old boy diagnosed with tetrahydrobiopterin non-responsive PKU was referred for HTx due to chronic dietary non-compliance. Blood PHE was 564 ± 264 μmol/L (exceeding the prescribed upper limit of 360 μmol/L). He received domino transplant with hepatocytes isolated from the explant of a patient with glycogen storage disease type 1 (GSD1a; see below), but this failed to achieve clinical improvement. A second cellular infusion with hepatocytes derived from a “non-disease” liver was more successful, decreasing the half-life of PHE from 43 to 19 h and leading to reduction in PHE levels for a duration of 3 months (Stéphenne et al 2012). Of interest, an active trial of HTx in phenylketonuria is open and currently recruiting (www.clinicaltrials.gov), sponsored by the University of Pittsburgh and Children’s Hospital of Pittsburgh of UPMC.

Maple syrup urine disease (MSUD)

The branched-chain amino acids leucine, isoleucine, and valine undergo oxidative metabolism beginning with two steps common to all three amino acids: 1) transamination via branched-chain amino acid aminotransferase; and 2) subsequent oxidative decarboxylation catalyzed by branched-chain α-ketoacid dehydrogenase complex (BCKDH). Patients with MSUD harbor autosomal-recessively inherited mutations in any of three subunits involved in the oligomeric BCKDH complex: a) decarboxylase (E1 subunit); b) transacetylase (E2 subunit); or c) lipoamide dehydrogenase (E3 subunit). Defects in the E3 subunit also lead to inherited deficiencies in pyruvate and α-ketoglutarate dehydrogenase deficiencies, since E3 is a subunit common to these enzyme complexes as well. BCKDH deficiency results in accumulation of both BCAA and branched-chain keto acids in blood and tissues of MSUD patients. Increased
concentrations of BCAA/BCKA, especially leucine and its transamination product α-ketoisocaproic acid (KIC), are particularly toxic in brain (Zinnanti et al 2009; Strauss et al 2010), and may associate with neurotransmitter deficiency, neurological dysfunction and other pathomechanisms (Schönberger et al 2004). As is the case for PKU, the BCAAs are also LNAAs that can lead to saturation of the LAT-1 system when accumulated to high levels in the peripheral blood (Zinnanti et al 2009). Accumulation of KIC enhances transamination of alanine (leading to pyruvate and lactate accumulation) and glutamate (increasing generation of α-ketoglutarate). Glutamate is the direct precursor of inhibitory GABA, which may also be depleted (Zinnanti et al 2009). As with PKU, MSUD treatment is composed of dietary protein restriction and formula supplementation to ensure adequate intake of BCAAs necessary for somatic growth and brain development. Orthotopic liver transplantation (OLT) is particularly beneficial in classical MSUD where residual BCKDH activity is very low and outcomes quite poor (Strauss et al 2006a; Mazariegos et al 2012).

**Preclinical characterization of HTx in msud mice**

Homanics and coworkers developed murine models of MSUD (2006). Complete ablation of the E2αsubunit of BCKDH led to a model of classical MSUD with extremely truncated survival (Zinnanti and Lazovic 2012). This model was attenuated through transgenic knock-in of the human E2αsubunit, resulting in a model termed intermediate MSUD (imsud), with ~5–7 % of residual liver BCKDH and an extended lifespan (Homanics et al 2006). The observation that OLT was essentially corrective in classical MSUD led Skvorak and colleagues to preclinically examine HTx in imsud mice. These investigators injected BCKDH-replete hepatocytes directly into the hepatic mass of neonatal imsud pups, employing the rapid expansion characteristics of
neonatal liver as a potential mechanism to favor engraftment. Enhanced expression of BCKDH activity in liver of transplanted imsd subjects was associated with improved biochemical parameters and extended lifespan. Notably, significant corrections were observed in brain neurotransmitter abnormalities as well (Skvorak et al 2009a, b). The same authors subsequently extended these studies employing human amniotic epithelial cells in imsd subjects, taking advantage of the stem cell-like characteristics of these cells (Skvorak et al 2013a, b).

**Urea cycle disorders**

The urea cycle disorders encompass carbamoyl phosphate synthetase I (a mitochondrial regulatory enzyme), ornithine transcarbamoylase (also mitochondrial), argininosuccinate synthetase and lyase, and arginase (cytosolic localization). Deficiency of any of these enzymes blocks urea production, resulting in hyperammonemia and accumulation of other nitrogenous intermediates. Much of the pathology related to these disorders is associated with hyperammonemia, including lethargy, tremors, athetosis, convulsions, spastic (para)plegia, and ataxia. Biochemical and anthropomorphic findings in these patients include protein intolerance/avoidance, growth retardation, respiratory alkalosis and vomiting, and particularly in infants, an inability to regulate body temperature and/or breathing. While dietary and pharmacological treatment of urea cycle disorders significantly improves prognosis (estimated incidence 1:8,000) (Meyburg et al 2009), severe cases of urea cycle disorder require liver transplantation at a young age to improve outcomes (Campeau et al 2010). Accordingly, HTx has been attempted in the majority of the urea cycle disorders (Meyburg and Hoffmann 2010).
HTx in urea cycle disorders

Since the urea cycle is almost entirely localized to hepatic tissue, defects in this cycle are excellent candidates for HTx as well as OLT. The first attempted transplant of hepatocytes into a patient with a urea cycle disorder was performed by Strom and colleagues (1997) in a patient with OTC, although the patient died by day 43 of life in hyperammonemic coma. In 2003, this was followed by a further demonstration of HTx in a newborn male with severe OTC deficiency. HTx was begun on DOL 1, and by DOL 23 he had received 11 infusions totaling \(~5 \times 10^9\) hepatocytes derived from seven different donors. Protein intake was gradually increased (1.5 g/kg from formula, 0.5 g/kg from breastmilk) until DOL 31 when onset of hyperammonemia was noted and dietary protein intake was subsequently reduced (1 g/kg). Metabolic decompensation was attributed to inadequate immunosuppression, and further cell infusions beyond this time point were acutely rejected (Horslen et al 2003).

The efficacy of cryopreserved hepatocytes as a source of administered cells was first documented in OTC patients (Mitry et al 2004; Stéphenne et al 2005). Three hepatocyte isolations (80 % of segment IV, resected proximal to the middle hepatic vein, or segment IV with the caudate lobe) were cryopreserved and infused into three male OTC patients (age 18 to 33 years). The total hepatocyte infusion number was \(~10^8\) per patient with 80–95 % initial viability. Ammonia levels and serum urea improved, and provided proof-of-principle for the utility of segment IV as a viable source of hepatocytes (Mitry et al 2004). A 14-month-old OTC patient received ten hepatocyte infusions over 1.5 months with the goal of bridging to OLT (OLT performed 6 months after the first infusion). In total, the patient received 109 cryopreserved hepatocytes. Ammonia levels were modestly improved or static. Two hyperammonemic
episodes occurred during the infusion protocol, and liver enzymes (aspartate and alanine aminotransferase) increased after one infusion; nonetheless, blood urea levels and psychomotor development were improved (Stéphenne et al 2005).

Additional HTx studies reported successful outcomes in OTC deficiency that coupled HTx as a bridge to auxiliary partial orthotopic liver transplantation (APOLT) (Puppi et al 2008; Legido-Quigley et al 2009). In the latter, partial correction of plasma and urine metabolic disturbances was rapidly monitored employing NMR spectroscopy. Despite promising outcomes in this study, recurrent bouts of sepsis in the proband thwarted long-term correction and APOLT was required to achieve long-lasting metabolic control. In other urea cycle disorders, sequential HTx in a child with argininosuccinate lyase (ASL) deficiency led to long-term metabolic control, psychomotor catch-up, and 3% of control ASL activity in biopsied liver obtained 8 months post HTx (Stéphenne et al 2006). Meyburg and coworkers (2009) employed a single neonatal donor liver for cell isolation and HTx in four patients with urea cycle disorders, including CPS, OTC and ASS (argininosuccinate synthetase; type I citrullinemia) deficiencies. All patients showed metabolic improvements and were effectively bridged to OLT, although the patient with prenatally diagnosed OTC died within 6 months from severe metabolic crisis (Meyburg et al 2009). It is worth noting that Cytonet GmbH & Co. (www.cytonetllc.com) is currently pursuing an FDA-approved clinical trial of HTx in urea cycle disorders, although preliminary outcomes have not been presented at this time.

**Crigler-Najjar (CN) syndrome**

The Crigler-Najjar syndrome, first described in 1952, represents a heritable form of jaundice resulting from high levels of unconjugated bilirubin and, untreated, leading to severe
neurotoxicity in the central nervous system (Crigler and Najjar 1952). Bilirubin represents a by-product of heme metabolism generated during the normal recycling of red blood cells, and it is cleared in the endoplasmic reticulum of hepatocytes by bilirubin-UDP-glucuronosyl transferase (UGT1A1). Bilirubin accumulates in the normal turnover of hemoglobin, and increases in serum when production from heme exceeds 4 mg/kg/day. Two forms of the disease exist, type I and II, differing in age of onset (birth for type I, later in life for type II; also referred to as Arias syndrome) and autosomal-recessively inherited mutations in the UGT gene are known for both subtypes (Sampietro and Iolascon 1999).

Types I and II CN syndrome are characterized clinically by hepatic and CNS pathology including jaundice, hypotonia, oculomotor palsy, kernicterus and encephalopathy. Daily treatment of the type II disease with phenobarbital effectively lowers serum bilirubin via induction of UGT expression. Additionally, blood transfusion can lower plasma bilirubin, and calcium chelators can complex and lower bilirubin in the gut. CN type I, however, lacks appreciable UGT1A1 activity associating with very high levels of unconjugated bilirubin. CN I patients rely upon phototherapy, which involves exposure to high intensity blue light on a regular basis, depending upon patient age and the nature of the gene mutation (Strauss et al 2006b; Sampietro and Iolascon 1999). The only effective cure for CN I is liver transplantation, including APOLT (Rela et al 1999) and living donor liver transplantation (Ozçay et al 2009; Ribes-Koninckx et al 2012).

**The Gunn rat**

Gunn first reported on a Wistar rat strain that exhibited jaundice and a recessive inheritance pattern. Subsequently, deficiency of UGT1A1 was identified, representing a relevant
phenocopy of CN I syndrome (McCandless 2011). A number of preclinical HTx studies have shown efficacy in this model, including application of immortalized hepatocytes, bone marrow-derived cells, as well as intrasplenic administration of both fetal and adult hepatocytes (Kawashita et al 2008; Muraca et al 2007; Cubero et al 2007; Guha et al 2002). In these studies, engraftment advantage was achieved by irradiation, partial hepatectomy, or induction of ischemic/reperfusion injury to damage endogenous tissue. More recently, single liver lobe HTx in this model, employing preparative irradiation and concomitant application of hepatic growth factor (HGF), have shown correction of bilirubin excretion and associated jaundice (Zhou et al 2012). Studies in the Gunn rat have provided some of the most important proof-of-principle data to support human HTx trials. In this model, it has been estimated that repeated hepatocyte infusion could result in 5–10 % of hepatic mass replacement, without the requirement for irradiation or partial hepatectomy (Horslen and Fox 2004).

**Clinical application of HTx in CN I**

In a 10 year-old female with confirmed CN I disease, 7.5 × 10^9 hepatocytes were administered via portal vein access over 15 h. Immunosuppression consisted of corticosteroids, prednisone, and tacrolimus. Partial correction was observed 11 months post-transplant that required 6–7 h of phototherapy/day (reduced from 10 to 12 h/day) and an improved total serum bilirubin level (post-transplant, 14 mg/dL; pre-transplant, ~26) (Fox et al 1998). A 9-year-old male with confirmed CN type I boy received 7.5 × 10^9 hepatocytes from a split-liver cadaveric donor, without infusion complications. Two weeks post-transplant he was supplemented with phenobarbitone, and the serum bilirubin was decreased by 30 % in comparison to pre-transplant level. Parental noncompliance for prescribed phototherapy
coupled with inadequate immunosuppression ultimately led to OLT (Ambrosino et al 2005). Lysy et al (2008) reported HTx in two pediatric CN I patients. Patient 1 received $6.1 \times 10^9$ hepatocytes at age 9 years using a series of 18 infusions (delivered via catheter to the jejunal vein) over a 5 month timeframe. Serum bilirubin levels decreased approximately 30 % in comparison to pre-transplant levels, but spiked 6 months post-transplant and he progressed clinically to OLT. The second patient (a female) received 14 infusions of $2.6 \times 10^9$ hepatocytes at 1 year of age (Lysy et al 2008). The total hepatic mass transplanted was ~9 %; serum bilirubin fell approximately 25 % and skin jaundice rapidly improved. Unfortunately, viral infection led to rapid deterioration which required OLT.

**Familial hypercholesterolemia**

An autosomal dominant disorder, familial hypercholesterolemia (FH) results from mutations in either the low density lipoprotein receptor gene (LDLR) or apolipoprotein B-100 gene (APOB). The frequency of FH is ~1:500 in caucasians, but higher in certain populations due to founder effects. Homozygous mutations are particularly deleterious, leading to blood cholesterol levels that exceed 1000 mg/dL (Gautschi et al 2012). FH is characterized by significantly increased plasma total cholesterol and low density lipoprotein cholesterol, xanthomas (cholesterol-rich deposition in tendons and joints) and cardiovascular disease. Interventional targets include reduction of saturated fat intake and lowering of blood cholesterol. Cholesterol-lowering drugs (statins) and cholesterol-sequestering agents remain the mainstay of therapeutic intervention. Extracorporeal apheresis and hemodynamic filtration may be required in instances of hypercholesterolemia that is non-responsive to medications (Gautschi et al 2012).
Preclinical HTx in the Watanabe heritable hyperlipidemic (WHHL) rabbit

The WHHL rabbit was developed by Yoshio Watanabe (Watanabe 1980), and these animals demonstrate hypercholesterolemia due to a deficiency of LDL receptors, with lipoprotein metabolism quite comparable to humans. WHHL rabbits have undergone HTx with ex-vivo transfected hepatocytes. LDLR deficiency was corrected in harvested hepatocytes employing recombinant retrovirus administration. Subsequently, harvested cells were administered to WHHL rabbits via portal vein infusion at 1–2 × 10^8 cells per subject (up to 4 % of hepatic mass). Total serum cholesterol declined significantly within 5 days post-transplant (70 % reduction compared to pre-treatment level). Lipid abnormalities (LDL, VLDL) resolved within 1 week post-transplant and remained within normal levels for 6 months (Kakaei et al 2009; Okura et al 2011; Mohamadnejad et al 2010; Allian-Sauer and Falko 2012).

Clinical HTx in FH

HTx in FH was first achieved using ex vivo autologous hepatocytes transfected with recombinant retroviral LDLR (Grossman et al 1994). To improve transplant-cell engraftment and proliferation, and to obtain hepatocytes for transformation, the recipient underwent left lateral segment lobectomy prior to cell administration. The recipient (homozygous FH) demonstrated modest improvements in LDL/HDL (30 % improvement) levels 18 months post-surgery (Grossman et al 1994; Burlina 2004).

Refsum disease, a disorder of peroxisome biogenesis

Refsum disease results from defects in the α-oxidation of long chain fatty acids or in catabolic enzymes within the peroxisome. Inherited peroxisomal disorders group into either
single metabolic enzyme disorders or peroxisome biogenesis disorders (PBD) (Kim 2009), with a cumulative incidence of ~1:20,000 live births. Prior to transport to the mitochondria for subsequent β-oxidation, pristanic acid is generated from phytanic acid via α-oxidation. The enzyme deficiency normally associated with Refsum’s disease, phytanoyl-CoA 2-hydroxylase, catalyzes the conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA. Mutations in hydroxyphytanoyl-CoA lyase are also associated with Refsum’s disease. Clinical and biochemical manifestations of Refsum disease include marked elevation of plasma phytanic acid, retinitis pigmentosa, blindness, anosmia, deafness, sensory neuropathy, and ataxia. Treatment consists of elimination of phytanic acid from the diet, plasmapheresis (in conjunction with albumin replacement), and/or cascade filtration to reduce blood phytanic acid levels. Additional treatment options include administration of dermatological drugs that soften the skin and ultimately upregulate α-hydroxylation of phytanic acid to a limited extent. OLT decreases phytanic acid and very long chain fatty acids in serum and improves elimination of abnormal plasma bile acids (Van Maldergem et al 2005; Zolotov et al 2012).

**HTx in Refsum disease**

A 4 year-old female diagnosed with Refsum disease manifested advanced neurological morbidity, which obviated the potential therapeutic benefit of OLT. She was subsequently scheduled for HTx. Hepatocytes were derived from the unused left liver lobes of two compatible male donors. The patient received two transfusions of 109 fresh hepatocytes initially, followed by two additional administrations of 106 cryopreserved hepatocytes on days 3–5. Biochemical improvements included a 30 % decrease in blood very long chain fatty acids, ~1/3 decrease in plasma pipecolic acid (an additional biomarker of peroxisome biogenesis
deficiency), and improvements in blood dihydroxycoprostanic acid, the precursor to chenodeoxycholic acid. Visual and auditory evoked potentials showed no improvement, although somatic growth improved as did locomotor activity (Sokal et al 2003).

**Wilson’s disease**

Wilson’s disease results from mutations in the ATP7B-encoding copper transporting ATPase (P-type) 2, resulting in copper accumulation in hepatocytes and the central nervous system. Neuropsychiatric morbidity may develop in the 2nd and 3rd decades of life. Pathology may manifest as clinical liver disease, but timely intervention can retard disease progression and prevent selected pathological features, including corneal Kayser-Fleischer rings (copper deposition in the Descemet membrane of the cornea), elevated urinary copper excretion, reduced circulating ceruloplasmin, and elevated hepatic copper stores (Fenu et al 2012). Copper is an essential trace metal required for growth and development, and as a cofactor for numerous cuproproteins. Cytotoxicity results from excess copper levels associated with the production of reactive oxygen species (Peña et al 1999). More than 500 pathogenic mutations have been identified in Wilson’s disease, with an estimated incidence of ~1:30–50,000 live births (Olivarez et al 2001). Intervention includes lifelong adherence to a low copper diet (avoidance of high copper foods such as chocolate, dried fruit, liver, nuts, and mushrooms), and use of copper-chelators including penicillamine or trientine, and zinc acetate (which blocks copper absorption in the GI).
**HTx in Long Evans Cinnamon rats**

HTx has been extensively investigated in the Long Evans Cinnamon (LEC) rat model which harbors an atp7b point mutation and associated copper toxicosis of the liver (Yoshida et al 1996; Irani et al 2001; Malhi et al 2002; Park et al 2006; Joseph et al 2009). A variety of preconditioning approaches have been employed in these studies, including administration of retrorsine, partial hepatectomy, and the use of intraperitoneal tacrolimus administration to immunosuppress recipient animals. Outcome measures have included serum aminotransferases, liver, bile and copper output, ceruloplasmin oxidase activity, and quantitation of atp7b poly A+ RNA levels. Long-term analyses of metabolic correction ranged from 70 % to 90 % in most of these studies. In the most recent study, liver irradiation pretreatment associated with cholic acid (bile salt) administration prior to HTx resulted in 75 % of test animals achieving long-term metabolic improvements (Joseph et al 2009). In corrected animals, fulminant hepatitis, chronic inflammation, and cholangiofibrosis, often observed in untreated LEC animals, were absent and liver histology was improved (Irani et al 2001). Joseph and colleagues (2009) suggested that bile-salt-induced liver damage was very effective as a preparative HTx treatment for this model, in view of the pre-existing hepatic damage already present.

**Primary hyperoxaluria (PH)**

Autosomal-recessively inherited mutations in the alanine-glyoxylate aminotransferase (AGT) gene, catalyzing the transamination of glyoxylate to glycine, give rise to primary hyperoxaluria (PH). The inherited enzyme defect leads to excessive conversion of glycine to oxalate, the latter depositing (oxalosis) in bones, heart, retina, arteries, nerves, and
predominantly in the kidney. Accordingly, end-stage renal failure occurs in ~1/2 of patients by the age of 15 years. Partial liver transplant is insufficient to reduce oxalate, since remaining hepatocytes continue producing oxalate. OLT is metabolically corrective, but where systemic oxalosis has progressed to end-stage renal failure, combined liver-kidney transplantation is mandated (Ellis et al 2001; Hoppe 2012; Beck et al 2012).

Preclinical and clinical HTx in PH

Guha and colleagues (2005) developed preparative irradiation and hepatocyte mitotic stimulation procedures in order to evaluate the feasibility of HTx in PH. These approaches were applied in a murine PH model, the agxt −/− mouse, and were sufficient to correct urinary oxalate excretion via intrasplenic cell administration. Mitotic stimulation was achieved via application of adenoviral-based HGF (Guha et al 2005). Beck and colleagues (2012) extended these results by piloting HTx as a bridge to OLT in a 15 month old female patient with severe PH. Intraportal administration of 109 cryopreserved cells was undertaken over a 6 day period with tacrolimus as immunosuppressant. Plasma oxalate continuously decreased, with long-term values (8 weeks to 11 months) significantly lower than pre-HTx values. However, due to vascular complications and graft dysfunction the patient underwent OLT at 12 months. During the period of plasma oxalate correction, there was marked clinical improvement that effectively bridged the patient to combined liver-kidney transplantation (Beck et al 2012; Jiang et al 2008).

Progressive familial intrahepatic cholestasis (PFIC)

A heterogeneous class of autosomal-recessively inherited disorders, the subtypes of PFIC associate with different mutations in bile forming hepatocellular transport-systems. PFIC1
and PFIC2 are early onset, while PFIC3 may arise later in childhood or adulthood. PFIC presents with unrelenting hepatocellular cholestasis, pruritis and jaundice (Jacquemin 2012). Extensive fibrotic lesions often associate with end-stage liver disease in PFIC. Molecular defects in ATP8B1 (encoding FIC1 protein) and ABCB11 (encoding the bile salt export pump (BSEP) protein) represent the etiological underpinnings of PFIC 1 and 2. Bile salt secretion is severely impaired in both, as expected. PFIC 3 associates with defects in ABCB4, which encodes multidrug resistance 3 protein (MDR3) and impairs biliary phospholipid secretion. Bile salt intervention (e.g., ursodeoxycholic acid) represents a mainstay of therapy to mitigate liver damage, and biliary diversion may be beneficial to slow disease progression (Jankowska and Socha 2012). Nonetheless, most cases of PFIC progress to OLT.

Preclinical and clinical HTx in PFIC

Targeted ablation of the murine orthologue of MDR3, mdr2, yielded a representative model of PFIC with hepatic lesions indicative of primary sclerosing cholangitis (Popov et al 2005). Male mdr2 −/− mice were injected intrasplenically with 106 hepatocytes, and 1 week post-HTx both transplanted and control mice were placed on a diet of 20 % casein or the same supplemented with 0.3 % cholate. Mice were harvested for tissue collection (bile, liver) up to 50 weeks, as well as for assessment of pathology and engraftment efficiency (De Vree et al 2000). Engraftment efficiency of exogenous cells (mdr2 +/+) was enhanced by bile salt supplementation. Unfortunately, cholate supplementation induced development of multiple hepatic tumors within 1 year post-HTx. Conversely, transplanted animals on casein-only diet repopulated more slowly and improvement of liver pathology was significantly curtailed. These studies provided proof-of-principle for HTx in PFIC type 3 (De Vree et al 2000). Nonetheless,
there is still controversy as to whether these animal models are a faithful phenocopy of the human disease.

Bile salt export pump (BSEP) deficiency (PFIC-2) is the result of inherited mutations in the ABCB11 gene, coding for an ATP-binding cassette transporter. The BSEP protein is critical for bile transport and maintenance of liver function. In the Bsep −/− null mouse model, Chen and colleagues (2012) found that feeding of cholic/ursodeoxycholic acids to Bsep +/+ and Bsep −/− subjects resulted in increased proliferative capacity in wild-type subjects, but cholestasis and apoptosis in Bsep −/− subjects. The observed differential toxicity was employed as a preparative preconditioning technique for HTx in the same subjects. This approach resulted in biliary total bile acid levels in Bsep −/− mice that was 80 % of normal, providing valuable preclinical data for future trials of HTx in PFIC-2 patients (Chen et al 2012). Human HTx has been report in two PFIC-2 patients, with partial recovery of metabolic functions, but both patients rapidly progressed to OLT (Quaglia et al 2008).

**Glycogen storage diseases (GSD)**

Glycogen storage disease type I (GSDI) represents an autosomal-recessively inherited disorder resulting in the accumulation of hepatic glycogen and fat. The phenotype encompasses hypoglycemia, hepatomegaly, lactic acidosis, hyperuricemia, and hyperlipidemia. Mutations in the G6PC (glucose-6-phosphatase) gene at locus 17q21.31 give rise to GSDIa, whereas mutations in the SLC37A4 at locus 11q23.3 produce GSDIb, with an approximate 4:1 prevalence of GSDIa vs1b. GSDIb patients manifest hypoglycemia, growth retardation, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia. Chronic neutropenia and
functional deficiency of neutrophils and monocytes in GSD 1b often leads to transplant with granulocyte colony stimulating factor (Ribes-Koninckx et al 2012).

G6PC mutations result in loss of glucose-6-phosphatase activity, which curtails glycogenolysis and results in the typical hypoglycemia observed in GSD1a. Conversely, SLC37A4 mutations lead to impaired glucose-6-phosphate (G6P) translocase activity, preventing migration of G6P to the endoplasmic reticulum and resulting in hypoglycemia and hepatomegaly. The cumulative frequency of all GSDs is ~1 in 20,000–25,000 live births, with GSDI (a or b) occurring in ~1 in 100,000 live births. Treatment is usually dietary or occasionally pharmacologic; nonetheless, in severe cases, liver transplantation has been performed.

**HTx in GSD1a**

HTx was undertaken in a 47-year-old female with GSD1a who suffered severe fasting hypoglycemia. The clinical history included diagnosis at 3 years of age following presentation with hepatomegaly, hypoglycemia, hypertriglyceridaemia, hyperuricaemia, and lactic acidosis. G-6-Pase activity was undetectable in liver biopsy. Frequent meals, including nocturnal nasogastric feeds and uncooked corn starch meals every 3 h were employed to maintain blood glucose. Inability to comply with dietary requirements and an inability to maintain blood glucose >3.5 mmol/L (normal 3.7–6.1) eventually led to HTx intervention. Hepatocytes (109) were infused via an indwelling catheter inserted into the portal vein, which was followed by a cocktail of immunosuppressants including mycophenolate mofetil, tacrolimus, and steroids. Nine months post-HTx immunosuppression was limited to tacrolimus, and she was able to consume a normal diet and fast up to 7 h without overt evidence of hypoglycemia (Muraca et al 2002).
**HTx in GSD1b**

An 18-year-old male with GSD1b, referred for HTx, displayed severe growth retardation, epistaxis, hepatomegaly, hypoglycemia, and neutropenia. Hepatocytes were obtained from cadaveric whole livers derived from two donors, and were infused at days 1, 7, and 30 (each infusion, 109 cells). Post-transplant, the patient maintained normal blood glucose and neutrophil levels and discontinued cornstarch meals. After 1 year, the patient displayed pubertal development (significantly delayed pre-HTx) and a 12 cm gain in height. Nonetheless, hepatosplenomegaly persisted. Two liver biopsies were undertaken post-HTx in order to assess G-6 P translocase activity. Since G-6 P translocase defects in GSD1b impede transport of G-6 P into the endoplasmic reticulum and G6Pase is inactive in the cytoplasm, G-6 Pase activity is increased post-sonication with substrate accessibility (in GSD1, G-6 Pase activity is well below the control presonication range of 5.9–93.0 nmol/min). Translocase values (0, 20 and 250 days post-HTx) showed increases in G-6 Pase ER internalization from 0.18 (baseline) to 21 and 18 nmol/min/mg protein, pre- and post-sonication, respectively. These results provide evidence for engraftment and functional activity (Lee et al 2007).

**Conclusions from HTx in metabolic disorders**

Results for HTx in a number of metabolic (and non-metabolic liver diseases) have underscored the utility of this procedure to bridge the patient with life-threatening liver disease to orthotopic liver transplantation (Table 1). Two critical obstacles remain to be overcome to bring HTx to full clinical utilization: 1) transplanted cells, which are gene-replete, must have an engraftment advantage over endogenous gene-defective cells (if not an advantage, then minimally some type of selection opportunity); and 2) transplanted cells must have expansion
advantage, and this is particularly of concern with immunorejection of the transplanted cells (Puppi et al 2012). Distinctions between these terms (engraftment, expansion) may be small, and likely in the clinical setting the goals for both are overlapping. While HTx studies in animal models have shown utility, these promising results have not yet translated to the clinic, for the above reasons. Selected disorders in which the gene defect leads to cellular apoptosis in the absence of intervention (e.g., fah −/− mice) confer a distinct potential engraftment advantage to exogenous cells that is not universally available in other metabolic liver diseases. Also, endogenous liver damage, such as that found in the LEC rat model of Wilson’s disease provide a unique venue amenable to HTx, due to the nature of the pre-existing damage. Limited recent studies have begun to highlight the potential utility of human amniotic epithelial cells as a preclinical donor model for metabolic liver diseases (Skvorak et al 2013a; (Ordonez and Goldstein 2012). Many stem cells show strong immunosuppressive qualities and the pluripotent nature of these cells provides the opportunity for differentiation to mature hepatocytes, yet safety and efficacy remains to be demonstrated clinically. A more thorough understanding of stem cell signaling, differentiation and manipulation will be important for the full clinical potential of stem cells to be reached. Although further studies continue to underscore the potential for HTx to treat metabolic liver diseases, until such time as effective and non-invasive protocols are developed that can enhance engraftment and expansion of exogenous cells, HTx will remain in the short term primarily a “bridging” protocol to OLT and APOLT.
Table 1  
Heritable metabolic disorders for which hepatocyte transplantation has been attempted (preclinically or clinically)

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Affected enzyme</th>
<th>OMIM entry</th>
<th>Preclinical/clinical approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinemia type I</td>
<td>Fumarylacetoacetate hydrolase</td>
<td>276700</td>
<td>Preclinical/clinical</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>Phenylalanine hydroxylase</td>
<td>261600</td>
<td>Preclinical/clinical</td>
</tr>
<tr>
<td>Maple syrup urine disease</td>
<td>Branched chain ketoacid dehydrogenase</td>
<td>248600</td>
<td>Preclinical</td>
</tr>
<tr>
<td>NAGS</td>
<td>N-Acetylglutamate synthetase</td>
<td>237310</td>
<td>Clinical</td>
</tr>
<tr>
<td>CPS I</td>
<td>Carbamoyl phosphate synthetase</td>
<td>237300</td>
<td>Clinical</td>
</tr>
<tr>
<td>Argininosuccinic aciduria</td>
<td>Argininosuccinate lyase</td>
<td>207900</td>
<td>Clinical</td>
</tr>
<tr>
<td>Citrullinemia</td>
<td>Argininosuccinate synthetase</td>
<td>215700</td>
<td>Clinical</td>
</tr>
<tr>
<td>OTC</td>
<td>Ornithine Transcarbamylase</td>
<td>311250</td>
<td>Clinical</td>
</tr>
<tr>
<td>Crigler Najjar</td>
<td>Glucuronyl transferase type I</td>
<td>218800</td>
<td>Clinical</td>
</tr>
<tr>
<td>Familial hypercholesterolemia</td>
<td>LDL receptor</td>
<td>143890</td>
<td>Clinical</td>
</tr>
<tr>
<td>Refsum disease</td>
<td>Phytanic acid oxidase deficiency</td>
<td>266500</td>
<td>Clinical</td>
</tr>
<tr>
<td>Wilson’s disease</td>
<td>Copper transporter</td>
<td>ATP7B 277900</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Primary hyperoxaluria</td>
<td>Alanine glyoxylate aminotransferase</td>
<td>259900</td>
<td>Clinical</td>
</tr>
<tr>
<td>Familial intrahepatic cholestasis</td>
<td>Byler disease; bile transporters</td>
<td>211600</td>
<td>Preclinical/clinical</td>
</tr>
<tr>
<td>Glycogen storage disease</td>
<td>GSD 1a, 1b</td>
<td>232200, 602671</td>
<td>Clinical</td>
</tr>
</tbody>
</table>

LDL low-density lipoprotein; ATP adenosine-5′-triphosphate; OMIM online mendelian inheritance in man (www.ncbi.nlm.nih.gov); NAGS N-acetylglutamate synthetase; CPS 1 carbamoyl phosphate synthetase 1; OTC ornithine transcarbamylase; see text for additional abbreviations

Conflict of interest

None.
References


de Vries PM, de Jong BM, Bohning DE, Hinson VK, George MS, Leenders KL (2012) Reduced parietal activation in cervical dystonia after parietal TMS interleaved with fMRI. Clin Neurol Neurosurg Mar 1 [Epub ahead of print]


CHAPTER SIX
Conclusions

Within my field of research, inborn errors of metabolism and molecular genetics, my objective is to unveil new treatment approaches through advancing the understanding of underlying mechanisms in the pathophysiology of inherited diseases, or piloting new drugs in translational studies. I have contributed the following milestones within this body of work.

Chapter Two: Novel treatments in PKU

- My work has demonstrated for the first time the potential for non-physiological amino acids to inhibit blood-brain transport of phenylalanine in PKU mice (a potential novel approach to treat PKU)

Chapter Three: Protein restriction in MSUD

- My studies have demonstrated for the first time, conclusively, that evaluation of disease control in msud mice, via evaluation of blood amino acids, does not reflect brain homeostasis of amino acid levels, providing new insights into the long-term neurocognitive deficits in patients with MSUD

Chapter Four: GABA’s role in “self-eating” or autophagy

- My work has served to document a novel role for the inhibitory neurotransmitter GABA in the control of cellular autophagy, primarily focused on a role in mitophagy, in mammals, as initially documented in yeast
- My studies have documented novel pathophysiological processes in SSADH-deficient mice that are amenable to therapeutic intervention with FDA-approved autophagy-inducing drugs
Future Directions

I propose to extend the studies listed in this Dissertation to continue my goals of developing new approaches to therapeutics for the diseases that I have worked on herein, as well as others in the future. As any respectable scientist will confirm, today’s studies are no more than the springboard for tomorrow’s experiments and hypotheses. With that in mind, I have several goals for my future work.

Chapter Two: LAT1 inhibition in PKU

- Moving MAIB and AIB studies in PKU mice to a study of pharmacokinetics (PK) in collaboration with UW and Dr. Danny Shen. Supported by a grant from the ITHS (Ignition Award). The goal is to obtain data to develop a Phase 0 trial for MAIB.

Chapter Three: Pathophysiology in PKU and MSUD

- Beginning to explore the role of autophagy, oxidative stress and inhibition of mitophagy, and altered regulation of mTor as a mitigating pathophysiological process in MSUD and PKU mice, and the correlation with elevated levels of BCAAs and Phe. Collaboration with Dr. Mleczko Sanecke (EMBO Institute, Heidelberg, Germany) is planned.

Chapter Four: Autophagy-inducing drugs in SSADH

- Further explore the roles of mTor inhibitors (rapamycin, Torin, etc) in the treatment of SSADH-deficient mice, through evaluation of lifespan and EEG characteristics. This is supported by a new R21 grant on the topic (collaboration with Dr. J. Wisor).
- Developing the IRB paperwork and approaches to verify that findings related to autophagy and mTor in SSADH-deficient mice are reproduced in the blood of patients
with SSADH deficiency, thereby strengthening a potential role for autophagy-inducing drugs in SSADH-deficient patients.

Chapter Four: Other new treatments for SSADH

- Continue working on human clinical trials in SSADH deficiency (taurine, SGS-742). Assist with the clinical trials of NAC in SSADH-deficient patients if funding is obtained from NIH, especially as pertains to the analytical perspectives.

- Perform parallel studies on the survival characteristics and EEG changes in SSADH-deficient mice using autophagy-inducing drugs, NCS-382 and HOP-CA (the latter two high-affinity GHB receptor ligands). The objective is to garner the preclinical data to verify the potential utility of moving forward with clinical development of these drugs for SSADH deficiency, and other instances of chronic GHB intoxication.

- Garner PK and toxicity data on NCS-382 and HOP-CA (collaborations with Dr. E. Weerts and Dr. P. Wellendorph) to approach the FDA for an IND to perform a phase 0 on these species, whichever is most beneficial preclinically in our animal model.

Chapter Four: A new fruit fly model for SSADH

- Developing a Drosophila knockout model of SSADH in collaboration with Dr. Doris Kretschzmar of Oregon Health and Sciences University. Dr. Kretschzmar has developed several robust methods to assess “autistic behavior” in the fly, and interestingly SSADH-deficient patients are frequently misdiagnosed as autistic or “utism-spectrum disorder.

Chapter Five: A new mouse model for hepatocyte transplantation

- Developing a murine knockout model of TALDO (Transaldolase) deficiency, a rare polyl defect that manifests predominantly with hepatic dysfunction and hepatocarcinoma. A
major objective with this animal is to perform hepatocyte transplant with a goal of humanizing the liver, and moving to larger animal platforms.

Attribution

This Dissertation is compiled from six publications in the scientific literature. By definition, the Journals involved (J Inherited Metabolic Disease, Molecular Genetics and Metabolism, and EMBO Molecular Medicine) all possess different submission requirements, layouts, formatting for references, etc. Nonetheless, generally speaking, all have the same “guiding” landscape (abstract, introduction, results, discussion, references, supplementary material).

With regard to multiple authorship, I confirm that for the five manuscripts for which I was primary (lead) author, I performed the majority of the laboratory effort, including design of the study and oversight of protocols, animal breeding, dissection, and all other critical measures. I also contributed to drafting of the initial text, figures and schematics, statistics and data analysis, etc., of the manuscript, which was edited by my advisor, Dr. Gibson, and colleagues. In the case in which I am second author, I performed all mammalian (animal) work in the study, as well as all electron microscopy work, that was supplementary to yeast studies performed by our collaborators, collectively enabling its publication in a high-impact journal.

Efforts by co-authors

Arning E, Wasek BL, Bottiglieri T: These collaborators performed analytical procedures on tissue samples using techniques not available at WSU College of Pharmacy

Kennedy AA, Whitehouse LA: These collaborators assisted with literature review for the manuscript.
Pearl PL, Theodore WH, McCarter RC, Jakobs C: These are longstanding collaborators of Dr. Gibson, both clinicians and basic investigators. They have been actively involved in the clinical trials of SSADH (succinic semialdehyde dehydrogenase) deficiency for many years. They assisted through editing the manuscript and assisting with important suggestions to the text.

Lakhani R, Till A, Liu J, Burnett SF, Subramani S: These collaborators (all from UC San Diego) studied the components of autophagy, and its response to GABA, in yeast. These studies were supplemented by my mammalian studies in the murine model of SSADH deficiency.

McPherson S: Dr. McPherson is a biostatistician in the College of Nursing who provided oversight of our studies using statistical analyses.

Gibson KM: Dr. Gibson is my advisor and mentor, and worked in the procurement of funds for all studies, oversight of my investigations, guidance, drafting and editing, and other components of all of my studies over the previous four years.