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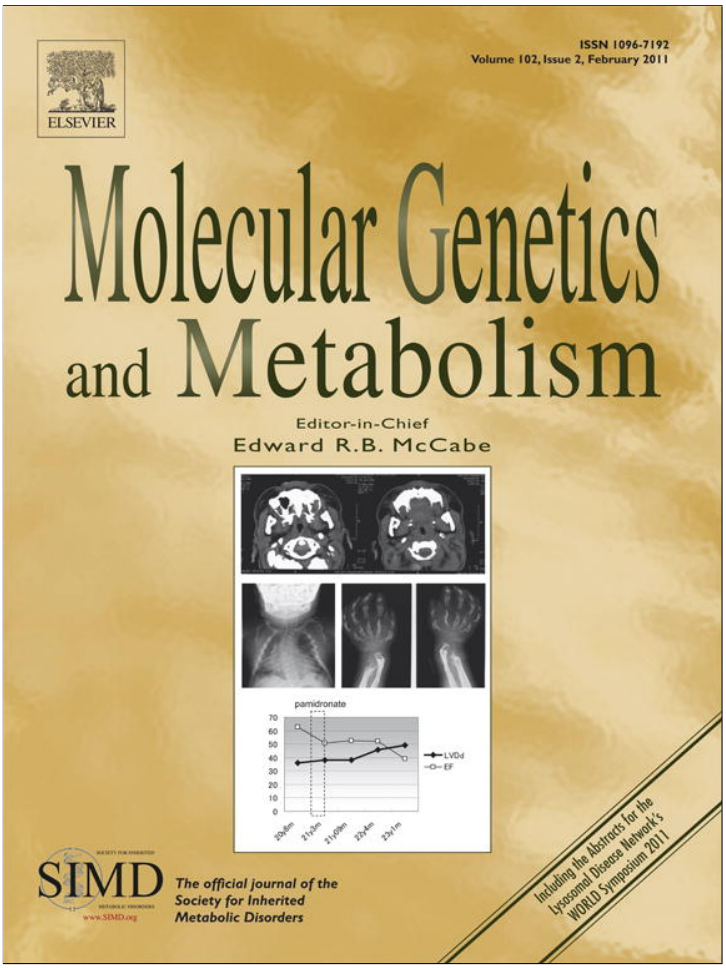

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Modification of aspartoacylase for potential use in enzyme replacement therapy for the treatment of Canavan disease

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ABSTRACT

Canavan disease is a fatal neurological disease without any effective treatments to slow the relentless progress of this disorder. Enzyme replacement therapy has been used effectively to treat a number of metabolic disorders, but the presence of the blood–brain-barrier presents an additional challenge in the treatment of neurological disorders. Studies have begun with the aim of establishing a treatment protocol that can effectively replace the defective enzyme in Canavan disease patients. The human enzyme, aspartoacylase, has been cloned, expressed and purified, and the surface lysyl groups modified through PEGylation. Fully active modified enzymes were administered to mice that are defective in this enzyme and that show many of the symptoms of Canavan disease. Statistically significant increases in brain enzyme activity levels have been achieved in this animal model, as well as decreases in the elevated substrate levels that mimic those found in Canavan disease patients. These results demonstrate that the modified enzyme is gaining access to the brain and functions to correct this metabolic defect. The stage is now set for a long term study to optimize this enzyme replacement approach for the development of a treatment protocol.

1. Introduction

Canavan disease (CD)[2] is a fatal, genetically transmitted neurodegenerative disorder that was first identified by brain pathology [1], and was subsequently shown to be a medical disorder that is distinct from the previously identified leukodystrophies and other demyelinating diseases [2]. Symptoms of CD appear in early infancy and typically progress very rapidly. The early symptoms include loss of motor skills, loss of muscle control, and megalocephaly. Progression of the disease leads to paralysis, blindness, and epileptic seizures, with death usually occurring within the first decade of life [3]. A deficiency in aspartoacylase (ASPA, EC3.5.1.15) activity caused by mutations in the *aspA* gene has been implicated as the cause of CD [4]. Analysis of DNA isolated from CD patients has identified numerous mutants resulting in low activity or the complete loss of aspartoacylase activity [5], with several common mutations prevalent in different ethnic groups [6,7]. This enzyme plays a critical role in brain metabolism, the deacetylation of *N*-acetyl-L-aspartic acid (NAA) to produce acetate and aspartate, and appears to be the only enzyme in brain that can effectively metabolize NAA.

NAA is one of the most abundant amino acid metabolites in the brain, but its precise biological function is still uncertain. Among the diverse roles suggested for NAA includes functioning as a storage form and source of acetyl groups in neurons [8] that could then either serve as precursors for the fatty acid biosynthesis necessary for myelin production [9,10], or could be transported for extramitochondrial fatty acid biosynthesis [11]. A myriad of other hypotheses have also been advanced that support the need for functionally viable aspartoacylase in the developing brain. While the precise role of NAA has not been defined, it has been conclusively demonstrated that the concentration of this metabolite must be controlled within a narrow range for optimal health. This control requires a balance between the activities of aspartate *N*-acetyltransferase and aspartoacylase, the enzymes responsible for the synthesis and degradation of NAA. Low brain NAA levels are a hallmark of neuronal injury and death. A decrease in neuronal NAA concentration has been observed in many neurodegenerative diseases, including epilepsy [12], multiple sclerosis [13], myotrophic lateral sclerosis [14], and Alzheimer's disease [15]. In contrast, it is the elevated levels of NAA found in CD patients that leads to the symptoms of this disorder.

Several gene replacement therapy trials have been approved for the treatment of infants with defective aspartoacylase [16,17], and some limited success has been seen in slowing the relentless progress of this disease. The primary barriers that must be overcome in gene therapy are the successful incorporation of the replacement gene into

Abbreviations: ASPA, aspartoacylase; CD, Canavan disease; ERT, enzyme replacement therapy; NAA, *N*-acetyl-L-aspartate; PEG, polyethylene glycol.

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the patient's genomic DNA and the expression and control of the protein product. A potential alternative approach that would bypass these issues is direct administration of the properly functioning protein. However, for enzyme replacement therapy (ERT) to succeed a different set of barriers must be surmounted. Injection of foreign proteins into a patient will cause an immune response, the half-life of injected proteins can be quite short, and to be most effective the protein must become localized in the cells where the deficiency is manifest.

Surface modifications of proteins can decrease the immunogenicity of foreign proteins. The introduction of polyethylene glycol (PEG) polymers through covalent modification of exposed protein functional groups has been shown to decrease the immune response to these modified proteins and increase their circulation half-life [18]. Recent studies have demonstrated the efficacy of ERT in an animal model of the metabolic disorder phenylketonuria in which mutations in the gene that encodes for phenylalanine hydroxylase result in the inability to metabolize phenylalanine. Treatment of recombinant phenylalanine hydroxylases with several PEGs that are terminated in reactive functional groups lead to modified forms of these enzymes that retain full catalytic activity and achieve increased stability [19]. Injection of these PEGylated enzymes into a mouse model with defective phenylalanine hydroxylase results in enhanced *in vivo* catalytic activity, decreased serum phenylalanine levels, and significantly reduced immunogenicity when compared to control studies with the untreated enzyme [20]. Administration of a PEGylated form of adenosine deaminase is a well established treatment therapy for children with severe combined immune deficiency caused by a defect in purine metabolism [21]. Achieving similar success with aspartoacylase will be needed to consider ERT as a viable approach for the treatment of CD.

An additional potential complication in the use of ERT for the treatment of neurological disorders is the presence of a blood–brain barrier which serves as the gatekeeper controlling access to the neurological system in higher organisms. This structure provides a physical barrier, by means of tight junctions composed of membrane proteins and lipids that seal the gaps between endothelial cells, a chemical barrier that regulate the transport of material through these cells and pump foreign substances away from the brain, and a metabolic barrier that hydrolyzes and inactivates toxic compounds. Thus the blood–brain barrier effectively protects that brain against foreign substances, but also limits access to many therapeutic agents designed to treat neurological disorders. The modified replacement enzyme must be able to traverse this barrier if it is to function to overcome the metabolic defect that is responsible for CD.

In this study we have examined the effect of PEGylation on the catalytic activity and the protein stability of aspartoacylase. Several PEGylated forms of this enzyme have been injected into mice that are deficient in aspartoacylase. Statistically significant increases in brain aspartoacylase activity and decreases in NAA levels have now been achieved with this approach, demonstrating the capability of modified aspartoacylase to cross the blood–brain barrier.

2. Materials and methods

2.1. Materials

The plasmid containing the *acy2* gene was transformed into KM71H *Pichia pastoris* cells following the directions in the Easy Select Pichia Transformation kit (Invitrogen). Reactive PEG reagents were purchased from NOF (Japan) and the protease inhibitor cocktail was obtained from Sigma. Mice were from the Canavan original black strain [22].

2.2. Purification of aspartoacylase

Aspartoacylase was expressed in a *P. pastoris* cell line using standard protocols, with methanol induction of the *acy2* gene under control of an alcohol oxidase promoter [12,13]. Fifteen grams of cells obtained from 4 l of growth media were resuspended in 50 ml of buffer containing 20 mM potassium phosphate, pH 7.4, 20 mM imidazole, 5% glycerol and 0.5 M NaCl. Fifty microliters of protease inhibitor cocktail was added to the lysate to inhibit proteases and the cells were mechanically disrupted by using a bead beater. The crude supernatant was loaded on a 10 ml Fast flow Nickel Sepharose column (Amersham Biosciences) using an Akta Explorer 100 chromatography system. After washing the column with buffer containing a low level of imidazole (20 mM) to remove any non-specifically adsorbed proteins the enzyme was eluted with a linear imidazole gradient (from 20 to 400 mM). The active fractions were pooled and dialyzed into 50 mM Hepes, pH 7.5, with 1 mM dithiothreitol (DTT) and loaded onto an anion-exchange column (Source 15Q). The enzyme was eluted from this column using a linear salt gradient (from 0 to 0.5 M NaCl), yielding from 5 to 10 mg of highly purified enzyme. The purified enzyme was dialyzed into 50 mM Hepes, pH 7.5, 1 mM DTT and 0.1 M NaCl for immediate use or storage at −80 °C.

2.3. Activity measurements

Aspartoacylase activity was measured by a coupled assay previously developed for this enzyme [23]. In this assay, carried out in the presence of 0.75 mM of *N*-acetyl-L-aspartate in a reaction buffer containing 50 mM Hepes, pH 7.5, and 10 mM $Mg(OAc)_2$, the aspartic acid produced by the deacetylation reaction of aspartoacylase is deaminated by using an excess of L-aspartase as the coupling enzyme. Production of the resulting fumarate product was followed at 240 nm ($\varepsilon = 2.53\ mM^{-1}\ cm^{-1}$) using a Cary 50 UV-visible spectrophotometer.

2.4. PEGylation of aspartoacylase

Aspartoacylase samples (0.8 mg per reaction) were treated with different PEG reagents containing terminal activating aldehyde or succinimidyl groups attached with either carboxymethyl or succinyl linkers. Linear 2 kDa, 5 kDa, 10 kDa, 20 kDa and branched 40 kDa PEG molecules were added to the reaction mixtures in varying enzyme to polymer ratios from 1:2 to 1:64 and incubated pH 7.5 or 8.3 and at temperatures ranging from 4 to 37 °C. Aliquots were removed from the reactions at different time points, quenched by the addition of excess lysine and then frozen for subsequent analysis. For the enzyme distribution studies aspartoacylase was treated with fluorescein-labeled 5 kDa PEG. The enzyme samples were each concentrated by using a 30 kDa spin concentrator to remove the excess PEG. After completion of the reaction, followed by several buffer exchanges, the sample was concentrated to 1 mg/ml in a buffer containing 50 mM Hepes, pH 7.5, 1 mM DTT, 0.1 M NaCl. PEGylated enzyme samples that were used in the animal studies were further purified by binding and elution from an anion-exchange column (Source 15Q) with a linear NaCl gradient to remove any residual unmodified enzyme.

Aspartoacylase was also directed labeled by treatment with fluorescein dye dissolved in DMSO at a final dye to protein ratio of 1:10 (w/w) with the reaction run at 20 °C for 1 h. After spin concentration to remove any unreacted dye the extent of modification was determined by the A_{495}/A_{280} ratio.

2.5. Animal treatment protocol

Canavan, black original strain mice [22] were used for the animal studies, and the studies were conducted with five groups of four mice each, two males and two females per groups. All mice were humanely handled in accord with IACUC protocol and policies. All KO/KO genotypes were examined twice, with mice genotyped before weaning and again at harvest time. Treatment of all mice began at 23 days of age and consisted of two doses of enzyme per week from age 3 to 5 weeks and then one dose per week from age 6 to 10 weeks.

The unmodified, 2 kDa- and 5 kDa-modified enzyme samples were each administered by i.p. injection at two dosing levels, a low dose of 30 μg/mouse/dose and a high dose of 60 μg/mouse/dose. All mice were harvested at 11 weeks of age, at least 3 days after the final dosing, and were anesthetized prior to sacrificing. The brain, liver and kidneys from each mouse were removed and immediately placed into pre-labeled containers, then onto dry ice, and stored at −80°.

For the enzyme distribution studies mice were treated with two injections of 120 μg of fluorescein-labeled aspartoacylase at 0 and 18 h, and then sacrificed at 24 h. Brains were perfused with saline solution, then removed and sectioned into four regions: (1) olfactory/anterior frontal region, (2) posterior frontal region, anterior basal ganglia and hippocampus, (3) midbrain, thalamus and occipito-parietal region, and (4) brain stem/cerebellar region. These tissue sections were flash frozen and stored at −80°.

2.6. Measurement of tissue samples

The mice tissue samples were cut and homogenized in 50 mM Tris, pH 8.0 using a tissue grinder (Kontes Glass). The homogenate was sonicated and centrifuged to obtain a clear supernatant. Analysis of the brain tissues from the mice was performed by making an acetone powder after sonication of the tissues. Eight volumes of cold acetone on dry ice (with alcohol) was mixed with the homogenate and allowed to stand for 30 min. The supernatant was removed and the pellet was dried to remove traces of acetone. The pellet was resuspended in water, the pH of the solution was adjusted to 8.0, and the solution was centrifuged at 4000 rpm for 15 min to remove any insoluble material. Enzymatic activity was measured by using 10 μg of total protein in the assay described above.

For the brain distribution studies each brain section was homogenized by sonication, centrifuged, and the supernatants were analyzed for protein concentration (A_{280}) and for fluorescence intensity by excitation at 495 nm and measurement of emission at 515 nm. Fluorescence intensities for each brain section were corrected by subtracting any background intensity from saline injected controls for that region and then normalized per mg of tissue.

3. Results and discussion

3.1. Modification of aspartoacylase by PEGylation

The charged and polar amino acids found on the surface of all soluble proteins provide many of the binding epitopes that are recognized during an immune response to a foreign protein. These functional groups also provide reactive sites that can be selectively modified to alter protein surfaces. Human aspartoacylase is a functional dimer containing 23 lysines per monomer. Recent structures of this enzyme [24,25] reveal that all of these lysyl groups are solvent exposed to varying degrees (Fig. 1), and modification of any of these lysines would be expected to alter the surface properties of aspartoacylase. These modifications will produce altered forms of the enzyme that could have decreased immunogenicity and increased *in vivo* stability.

To test the validity of this approach ASPA has been treated with different PEG reagents that can react through nucleophilic attack of the side chain lysyl amino groups on the reactive terminal functional groups of these PEGs. Activated PEGs containing different terminal functional groups and different linkers have been employed for these modifications, using both linear, lower molecular weight as well as branched, higher molecular weight polymers ranging from 2 kDa to 40 kDa PEG. In order to achieve a reasonable degree of lysine modifications for the PEGylation reactions different ratios of enzyme to PEG have been investigated along with different reaction conditions (pH, reaction times and temperatures). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated the successful PEGylation of ASPA, with the different ASPA to PEG ratios generating different distributions of the PEGylated species. In each cases reactions conducted for sufficient times with these PEG reagents has been shown to modify multiple groups of surface lysine residues of ASPA.

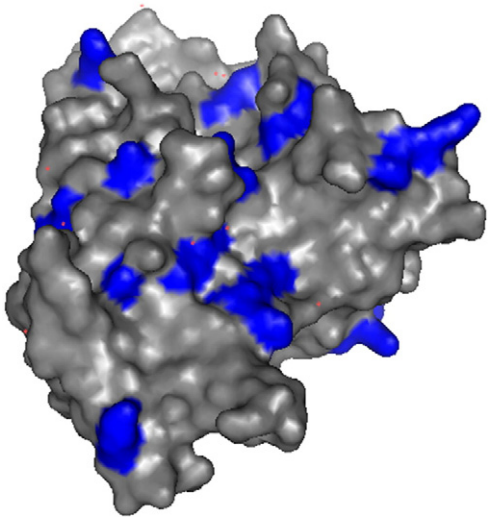

Fig. 1. A surface representation of the monomer structure of human aspartoacylase (PDB ID 204H) with the positively-charged lysyl side chains shown in blue. All 23 lysines in each monomer are solvent exposed to varying degrees.

The reactions run at the lowest temperature (4 °C) did not yield an appreciable amount of modified enzyme except when run at the highest ratios of PEG to enzyme, while the reactions run at the highest temperature (37 °C) caused considerable enzyme denaturation. The best yields of modified enzyme were obtained from the reactions run at 20 °C. Under these conditions a mixture of modified enzyme forms were produced even at the shorter time points, with the distribution shifting towards greater numbers of modified lysines as the reaction was allowed to continue. When the reaction was run at higher ratios of PEG to enzyme and for longer times the residual amount of unmodified enzyme decreased, but some enzyme denaturation was observed at the longer reaction times. Increasing the pH of the reaction from 7.5 to 8.3 causes a significant increase in the rate of the modification reaction, as a consequence of the higher fraction of lysyl amino groups in the neutral ionized form. At the shortest times two highly reactive lysines are rapidly modified, followed by modification of an additional set of three lysines (Fig. 2). Continued reaction results in the modification of two more groups of lysines, with almost no unmodified enzyme remaining after 36 h. At this point the enzyme is a mixture of modified forms, with from 2 to 9 lysines per monomer containing a covalently attached PEG (Fig. 2).

3.2. Assessment of PEGylation effects on activity and stability

To determine the effects of modification on aspartoacylase the catalytic activity of the PEGylated enzyme forms were measured by using our coupled assay [23] as described in Materials and methods, and the values obtained were compared with the activity of untreated native enzyme. ASPA is quite sensitive to oxidation and some variability in specific activity has been observed depending on the age of the purified enzyme and the storage conditions. Despite this variability all of the PEGylated forms of ASPA showed similar activities to that of the native enzyme when examined at the same protein levels in the assay (Table 1). Hence, the modification of these surface lysyl residues does not have a significant effect on the catalytic activity of the enzyme regardless of the polymer size, the nature of the activating group, or the extent of modification within the limits

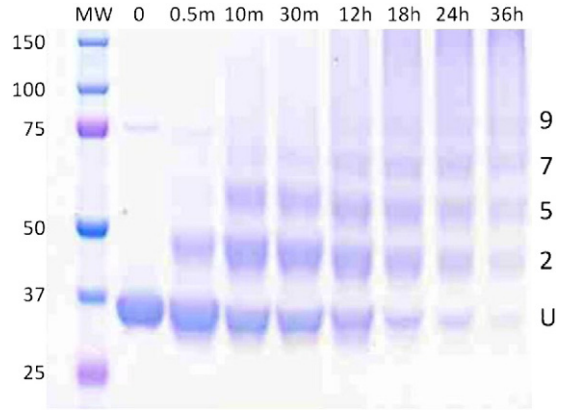

Fig. 2. SDS gel profile of a time course for the PEGylation of aspartoacylase with 5 kDa PEG. Lane 1: molecular weight markers; Lanes 2–9: formation of PEGylated enzyme, from time = 0 to 36 h. Protein bands show the unmodified enzyme (U) and a molecular weight ladder of modified enzyme forms with an increasing number of modified lysines from 2 to approximately 9 per monomer.

examined. The activity of each of the PEGylated ASPA samples was also monitored for 72 hours without observing a substantial loss in activity, showing that the longer term stability of the enzyme is not affected by these modifications.

Because lysine modifications do not alter the catalytic activity of ASPA to any great extent it is not absolutely essential to obtain a completely homogeneous sample of modified enzyme for the planned *in vivo* studies in an animal model of CD. However, it is important to remove any unmodified enzyme from these samples to allow comparison of the effects of treatment with either modified or unmodified enzyme on brain activity levels. Since modification of these lysines alters the surface charge of the enzyme any remaining unmodified enzyme would have a greater number of positively charged surface residues and therefore a higher isoelectric point than the modified enzyme forms. This charge difference was used in an ion-exchange chromatography step to remove any remaining unmodified enzyme after the PEGylation reaction. These purified samples were then used for the subsequent animal treatment studies.

3.3. In vivo investigation of PEGylated aspartoacylase in a mouse model of Canavan disease

Now that modified forms of ASPA have been produced that are stable and active, a pilot study was conducted to determine if these enzyme forms can penetrate the blood–brain barrier and lead to an increase in enzyme activity in the brain of an animal model of CD. The mouse model of CD was produced by deletion of the *aspA* gene that encodes for aspartoacylase [22]. These Canavan mice have somewhat elevated brain NAA levels, show a loss of coordination and have a significantly shortened lifetime. After breeding and genetic typing these animals were treated with a dosing protocol of ASPA by i.p. injections as described in Materials and methods. Treatment consisted of either lower or higher levels of unmodified enzyme or enzyme covalently modified with either 2 kDa or 5 kDa PEG. After conclusion of the protocol the animals were sacrificed and the tissues analyzed for enzyme activity levels. The untreated mice had the expected low levels of brain ASPA activity (~0.06 units/mg). The animals treated with the unmodified enzyme showed over a 2-fold increase in brain enzyme activity, and comparable increases were seen upon treatment with the 2 kDa-modified enzyme (Table 2). A 2-fold increase was also observed for the lower dosage of 5 kDa-modified enzyme, however treatment with higher levels of this modified form of ASPA causes a greater than 5-fold increase in brain enzyme activity. In contrast, enzyme activity in the kidney samples from each animal remained low (0.05–0.07 units/mg) regardless of the treatment protocol. It is not clear if these higher brain enzyme levels that are observed are a consequence enhanced of diffusion of this PEGylated enzyme across the blood–brain barrier or if an existing protein transport mechanism is being accessed by this modified enzyme. Additional studies will be needed to determine the mechanism by which this modified enzyme gains entry to the brain.

Because the animals in this study were not sacrificed until at least several days after the final injection of modified enzyme, and the brain tissues were then extensively perfused with buffer before homogenization, it is clear that the enhanced enzyme levels demonstrate that aspartoacylase has been able to cross the blood–brain barrier. This observation was confirmed by repeating this treatment experiment using both fluorescein-labelled and fluorescein-PEGylated enzyme. Increased fluorescence signals above the saline-injected controls were observed in several regions of the brain, with the highest fluorescence above background seen in the frontal cortex and in the midbrain and parietal regions. The midbrain and thalamus are also the regions of the brain that show the greatest vacuolation in the animal model of CD. Introduction of functional aspartoacylase into these regions would likely provide the greatest potential to slow brain deterioration.

The cellular distribution of these introduced enzyme forms has not yet been determined and will require either detailed fluorescence microscopy or labelled antibody studies to assess enzyme distribution at the cellular level [26]. However, if some of the increased brain enzyme activity is localized in the oligodendrocytes that are responsible for the metabolism of NAA then we would expect to see a corresponding decrease in the elevated levels of this metabolite for these treated animals. Enhanced levels of NAA (nearly 2 mM) are seen in the untreated Canavan mice as a consequence of the diminished catalytic activity of aspartoacylase (Table 2). Treatment with native aspartoacylase or with the 2 kDa-PEGylated enzyme forms causes a 1.3- to 2-fold decrease in brain NAA levels. The greatest decrease

Table 1
Effect of PEGylation on the activity of human aspartoacylase.

Enzyme form	Activating group	Ratio (ASPA:PEG)	Specific activity (U/mg)
Native ASPA	–	–	5–10[a]
ASPA-2 kDa PEG	succinimide	1:8	5.1
ASPA-2 kDa PEG	succinimide	1:24	3.2
ASPA-2 kDa PEG	succinimide	1:64	5.7
ASPA-5 kDa PEG	aldehyde	1:2	8.2
ASPA-5 kDa PEG	aldehyde	1:8	5.0
ASPA-5 kDa PEG	aldehyde	1:24	6.2
ASPA-10 kDa PEG	aldehyde	1:2	7.5
ASPA-20 kDa PEG	succinimide	1:2	8.5
ASPA-20 kDa PEG	succinimide	1:4	3.8
ASPA-40 kDa PEG	succinimide	1:2	6.0
ASPA-40 kDa PEG	succinimide	1:8	5.2

[a] Typical values for freshly purified enzyme [28].

Table 2
Treatment to increase brain enzyme activity and lower substrate levels in Canavan mice.

Treatment protocol	Enzyme dosage[a] (μg/dose)	Number of animals	Specific activity (U/mg)	Fold increase	[NAA] mM	Fold decrease
None	–	4	0.059 ± .007	–	1.89 ± .23	–
Unmodified enzyme	30–60	4	0.140 ± .045	2.4	0.96 ± .15	2.0
2 kDa modified	30	2	0.183 ± .003	3.1	1.21 ± .01	1.6
2 kDa modified	60	2	0.134 ± .017	2.3	1.48 ± .19	1.3
5 kDa modified	30	2	0.112 ± .003	1.9	1.28 ± .06	1.5
5 kDa modified	60	2	0.325 ± .017	5.5	0.71 ± .10	2.7

[a] Dosage protocol: age 3–5 weeks, 2 doses/week; age 6–10 weeks, 1 dose/week.

(nearly 3-fold) is seen in the brains of animals treated with the higher level of 5 kDa-PEGylated enzyme, the same brain tissues that show the greatest increase in enzyme activity (Table 2). These decreases in NAA levels clearly reflect the ability of the introduced enzyme to gain access to the accumulated substrate levels produced in the oligodendrocytes of these test animals.

Recent studies have tracked the positive effects on brain development and motor function resulting from the treatment of a rat model of CD with exogenous acetate to overcome this metabolic defect [27]. This result supports the hypothesis that the demyelination observed in the brain of CD patients is caused by the failure to produce sufficient acetate to support fatty acid biosynthesis. Our introduction of functional enzyme that is capable of metabolizing the accumulating NAA in the brain results in endogenous acetate production that should be effective in overcoming this defect and at least slowing, if not reversing the progress of CD.

4. Conclusions

The results of these studies show that PEGylated forms of aspartoacylase are a viable approach to use in ERT trials for the treatment of Canavan disease. Introduction of active enzyme into the brain of Canavan mice leads to a correction of the metabolic defect and metabolism of the accumulating NAA. The stage is now set for a larger scale animal study to optimize the treatment protocol and to examine the long term impact of ERT on brain development in this animal model.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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