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Enhanced brain distribution of modified aspartoacylase

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Abstract

Canavan disease is a fatal neurological disorder caused by defects in the gene that produces the enzyme aspartoacylase. Enzyme replacement therapy can potentially be used to overcome these defects if a stable enzyme form can be produced that can gain access to the appropriate neural cells. Achieving the proper cellular targeting requires a modified form of aspartoacylase that can traverse the blood-brain barrier. A PEGylated form of aspartoacylase has been produced that shows dramatic enhancement in brain tissue access and distribution. While the mechanism of transport has not yet been established, this modified enzyme is significantly less immunogenic than unmodified aspartoacylase. These improved properties set the stage for more extensive enzyme replacement trials as a possible treatment strategy.

Keywords

blood-brain barrier; Canavan disease; aspartoacylase; enzyme replacement therapy; PEGylation

1. Introduction

A defect in the human *acy2* gene has been identified as the cause of Canavan disease (CD)¹ [1], a neurodegenerative disorder for which there is currently no effective treatment or cure. This defective enzyme, aspartoacylase (ASP), plays a critical role in brain metabolism, the deacetylation of N-acetylaspartate (NAA) to produce acetate and aspartate. ASP is the only brain enzyme that has been shown to be capable of metabolizing NAA [1]. Over 50 different mutations including numerous deletions, missense mutations, and terminations,

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

¹*Abbreviations used:* ASP, aspartoacylase; BBB, blood-brain barrier; CD, Canavan disease; CNS, central nervous system; ERT, enzyme replacement therapy; GFAP, glial fibrillary acidic protein; IBA, ionized calcium binding adaptor protein-1; NAA, N-acetyl-L-aspartate; NECA, 1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide; PEG, polyethylene glycol

have been reported in the enzyme from Canavan patients. In most cases these mutations result in an altered enzyme that is either not expressed or is expressed but retains little catalytic activity [2]. Recent studies have shown correlations between aspartoacylase activity, protein stability and disease severity [3]. Our functional studies have shown that ASPA is a glycoprotein in which the glycan stabilizes the protein structure [4]. A bound zinc atom was identified and showed to play an essential catalytic role [5], and the function of many of the substrate binding and catalytic groups in the enzyme active site have also been determined [6]. Our detailed understanding of the mechanism and function of this enzyme is the basis for our studies to prepare stable and non-immunogenic forms of this enzyme for replacement therapy.

In addition to its role in acetate production, it has been proposed that NAA can be converted to glutamate in the brain and that this conversion is energetically favorable [7]. This possibility has important consequences given the well documented findings that high levels of glutamate are excitotoxic to cells [8]. In fact, we have shown that high levels of glutamate contribute to the neurotoxic effects of the drug of abuse, methamphetamine, through oxidative and metabolic stress [9–11]. NAA and glutamate are inherently linked through a series of metabolic reactions, mainly the tricarboxylic acid (TCA) and the glutamate–glutamine cycle [12]. NAA is proposed to be a reservoir for regulating the concentration of glutamate, maintaining low levels of glutamate yet having the capability to rapidly produce it when needed [7]. Several additional studies also support this proposed connection between NAA and glutamatergic neurotransmission, showing a strong correlation in different brain regions between NAA and Glx synthesis [13;14].

The introduction of ASPA into the brain may serve not only to supply much needed acetate to the brain for myelination, but could also buffer the brain against extremely high levels of NAA and consequently, excitotoxic levels of glutamate. Therefore, enzyme replacement therapy (ERT) may be effective for the treatment of diseases associated with deficiencies in fatty acid biosynthesis such as CD, and may also provide a therapeutic approach for the mitigation of brain injury produced by stimulant drugs of abuse.

ERT has shown success for a number of different metabolic disorders, including phenylketonuria [15] and a glycogen storage disease called Pompe disease [16] where lost catalytic function was restored in animal models of these disorders. ERT trials have been approved for the treatment of a number of metabolic disorders, including some that lead to neurological defects. ERT has shown varying levels of success in the treatment of these disorders, including patients with lysosomal storage diseases [17] such as Gaucher and Fabry diseases as well as Hunter and Hurler syndromes. Treatment with recombinant human enzymes has been used to minimize adverse immune responses in these patients thereby leading to enhanced *in vivo* stability. While these disorders each cause significant neurological symptoms, the underlying causes are genetic defects that are manifest both in neural and in non-neural cells. For disorders such as CD in which the defects are found exclusively in neural cells there is an additional hurdle that must be overcome in order for ERT to succeed, the blood-brain barrier.

The presence of a blood-brain barrier (BBB) was first identified because injected dyes failed to penetrate into cells in the central nervous system. The BBB 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 BBB effectively protects the brain against foreign substances, but it also limits access to many therapeutic agents designed to treat neurological disorders. This barrier can potentially be overcome either by delivering drugs behind the barrier through intracerebral injections or implants, or by increasing the flux across the barrier. While these approaches have led to the successful delivery of certain drugs and therapeutic agents, the existence of a BBB makes ERT significantly more difficult to achieve for the treatment of neurological disorders. The potential importance of effective approaches for the delivery of materials to the CNS has led to several patents for different proposed therapies [18–20]. However the relative merits and potential effectiveness of these approaches have not yet been fully examined.

Since replacement enzymes do not typically cross the BBB, severely affected patients with central nervous system symptoms would not be expected to show significant improvements through this approach unless this issue of limited brain access can be overcome. So, while ERT is proving to be a viable approach for the treatment of certain genetic disorders, this promising new therapeutic approach will have only limited applications and modest successes for most neurological disorders until the issues of enzyme stability, protein immunogenicity, and bioavailability are addressed. We have begun to explore approaches that address each of these issues for the application of ERT in the treatment of CD. Preliminary studies have shown that administration of PEGylated forms of ASPA causes increased enzyme activity and decreased substrate accumulation in brain homogenates from an animal model of CD [21]. However, it has not been established if this treatment leads to uptake and transport of the modified enzyme into brain tissue. These issues are the focus of this study.

2. Materials and methods

2.1. Materials

The plasmid containing the *acy2* gene was transformed into *P. pastoris* KM71H cells following the directions in the Easy Select Pichia Transformation kit (Invitrogen). Aspartoacylase (ASPA) was purified by a previously published protocol [21]. Reactive polyethylene glycol (PEG) reagents were purchased from NOF (Japan), amine-reactive AlexaFluor® 594 carboxylic acid, succinimidyl ester was from Life Technologies, polyclonal rabbit anti GFAP antibodies were from Millipore (catalog no. AB5804) and anti-Rb-IBA antibody was purchased from Wako (catalog no. 019-19741). Fluoromount-G mounting media were purchased from Southern Biotech (catalog no. 0100-01). NECA was purchased from Tocris (catalog no. 1691) and Hyaluronidase was purchased from Sigma (catalog no. H3884).

2.2. Production of modified aspartoacylase

ASPA samples were treated with a methoxy-PEG reagent containing terminal activating aldehyde or ester groups attached with a carboxymethyl linker. Linear 5 kDa PEG molecules were added to the reaction mixture in varying enzyme to polymer ratios and incubated at 25 °C. Aliquots were removed from the reactions at different time points and quenched by the addition of excess lysine. The samples were then treated by using a spin concentrator with a 30 kDa molecular weight cut-off to remove the excess PEG, and concentrated to 0.8 to 3.0 mg/ml in a buffer containing 50 mM Hepes, pH 8.3, 1 mM DTT, 0.1 M NaCl. PEGylated enzyme samples for the animal studies were analyzed by SDS-PAGE by using with Coomassie dye or bariumiodide staining to determine the extent of PEGylation and to detect the presence of any unmodified enzyme. For protein staining gels were soaked in Coomassie brilliant blue R-250 solution (0.1 % v/v in 50% v/v methanol and 10% v/v acetic acid) for 10 min at ambient temperature with gentle shaking, followed by de-stained using a solution containing 50% v/v methanol and 10% v/v acetic acid. For PEG staining gels were soaked in 5% glutaraldehyde solution for 15 min, followed by a 0.1 M perchloric acid treatment for an additional 15 min. Treatment with a 5% barium chloride solution and 0.1 M iodine solution was used to detect the protein-PEG conjugates [22]. If necessary, the enzyme samples were purified by elution from an anion-exchange column (Source 15Q) with a linear NaCl gradient to remove any residual unmodified enzyme.

The ASPA samples were pre-treated with AlexaFluor® 594 to provide a covalently attached fluorescent probe. This labeling dye was dissolved in anhydrous DMSO and reacted with the enzyme (1:10 w/w ratio) at ambient temperature for 2 to 3 min. The reaction was quenched by adding excess lysine, and the excess dye and lysine were subsequently removed using a spin concentrator of the appropriate molecular weight cut-off. The degree of labeling was determined following the equation in the amine-reactive probes manual by Life Technologies. The labeled samples were concentrated as described above and stored at -80°C until used for the animal studies.

2.3. Brain distribution of aspartoacylase

To examine the brain distribution of the modified enzyme, 0.8, 1.3, 2.0 or 3.0 mg/Kg of fluorescently-labeled modified (PEGylated) enzyme was injected i.p into adult male Sprague- Dawley rats (200-250 g), with labeled unmodified enzyme and saline serving as controls. After 4 hours, the rats were sacrificed, brains were extracted, frozen in dry ice and sliced at a thickness of 25 µm through the striatum and hippocampus. The sections were then mounted onto subbed slides, coverslipped using Fluoromount-G mounting media and air dried overnight in the dark and imaged the next day. To determine if the enzyme was localized to the capillaries, and whether PEGylation increased extravasation of the enzyme from the capillaries, fresh brain sections rather than perfused and fixed sections were used for imaging, since perfusing the brain would remove the enzyme in the capillaries. The dentate region of the hippocampus and the region adjacent to the lateral ventricle in the striatum were imaged using the Olympus Fluoview FV1000 confocal scanning laser microscope system. The fluorophores were excited using the argon laser at 561 nm and images were obtained using the 20X objective. The gain, offset, voltage, aperture size and

laser power were each kept constant between treatment groups in order to allow for unbiased comparisons.

Vehicle saline or 0.08 mg/Kg NECA was injected i.p. into a separate group of rats 5 h prior to PEGylated enzyme treatment, or 20 mg/Kg hyaluronidase was injected 20 min prior to PEGylated enzyme injections. Four hours after enzyme treatment, brains were extracted and sectioned as described above.

2.4. Immunofluorescence Protocol

Slide mounted sections were post-fixed and cryoprotected by serial overnight immersion in 4% paraformaldehyde, 10% and 20% glycerol. The following day sections were washed with 0.1 M PBS for 5 min, followed by 1 h incubation in 10% normal goat serum in 0.1 M phosphate buffered saline (PBS) containing 0.1% Triton at room temperature. After blocking, the sections were incubated in primary antibody - rabbit polyclonal anti GFAP (Glial fibrillary acidic protein, 1:1000) or rabbit polyclonal anti IBA1 (ionized calcium binding adaptor protein-1, 1:1000) for 1 h, washed in 0.1 M PBS and incubated in secondary antibody - Alexa Fluor 488 conjugated goat anti rabbit at a 1:2000 dilution in 0.1 M PBS containing 0.01% tween20 for 1 hr at room temperature. Any unbound secondary antibodies were then rinsed off the sections with 0.1 M PBS, and the slides were cover slipped using Fluoromount-G mounting media and allowed to set at room temperature overnight.

2.5 Data Analysis

The sections were imaged using confocal microscopy and images were quantified using Image J software (<http://rsb.info.nih.gov/ij>). Because of the high background fluorescence in the striatal images, the threshold levels were adjusted to eliminate basal and outlier fluorescence signals prior to analysis. Background intensities were subtracted from each image and a maximum threshold was set to moderate the impact of bright outlier regions from dominating the image intensities. Before normalizing the images, it is necessary to remove pixels that are not part of the image and to eliminate high intensity pixels that are localized within the capillaries of the brain. Images were normalized by setting it between the minimum and maximum level of intensity. In order to suppress the unwanted high intensity pixels, the same image was duplicated, then the global maximum threshold was manually set to identify the highest intensity regions of each image. Next, each duplicate image was despeckled and then converted to a binary image, followed by a dilation and smoothing process on the images. The processed image was subtracted from the original image to remove the high intensity punctuated pixels (Figure S1). Finally, the background intensities were corrected from each final image. The mean fluorescence intensities of the pixels within each image were summed to determine the overall fluorescent signal.

For the immunofluorescence images, the background intensities were subtracted using the rolling ball algorithm method, followed by the same smoothing and despeckle process on the images. The outlier radius was set to 20 and the threshold value to 50 to remove the unwanted bright intensity outliers. Finally, the mean value of overall fluorescent images was measured to obtain the mean fluorescence intensity.

3. Results

3.1. Optimizing the production of modified aspartoacylase

Aspartoacylase, the defective enzyme that causes CD, was treated with activated forms of polyethylene glycol (PEG), leading to modification of some of the surface-exposed lysine residues. The goal of this treatment is to produce stable forms of ASPA that have the potential to traverse the blood-brain barrier. Changes in the reaction conditions (type of PEG, pH, polymer to enzyme ratio) and in reactions times have led to the efficient modification of an increasing number of lysines out of the total of 23 lysines per enzyme monomer. Alterations in the reaction conditions can lead to the production of either lightly or heavily modified enzyme forms, with the products obtained from these reactions consisting of mixtures of aspartoacylases with differing numbers of PEG-modified lysines. In all cases the enzyme retains a high level of catalytic activity.

Optimization of the reaction pH and reaction time, as well as the use of PEGs with more reactive activating groups, leads to a more homogeneously PEGylated enzyme (Fig. 1). Analysis by SDS-PAGE indicates the modification of 11 lysines per subunit based on protein molecular weight standards (Fig. 1A), while analysis based on PEG molecular weight standards yielded a total of 9 lysines per subunit. (Fig.1B). These homogeneously-PEGylated aspartoacylase samples were used for the brain distribution studies.

3.2. Brain cellular distribution of aspartoacylase

Both PEG-modified and unmodified forms of ASPA were pre-labeled with a fluorescent dye to follow the uptake and distribution of the enzyme after injection into animals. As expected, the injection of saline controls showed no enhanced signal over the background autofluorescence in imaged brain sections. The brain sections of the animals that were administered the unmodified enzyme showed an increased level of fluorescence that appear primarily as punctate signals, suggesting that the enzyme is localized within the capillaries that provide blood supply to those regions of the brain (Fig. 2A). In contrast, the brain sections of animals that were administered increasing amounts of the PEGylated form of ASPA showed more diffuse fluorescence that is evidence of enzyme being distributed throughout the brain tissue (Fig. 2B to 2E). After elimination of the basal and outlier fluorescence in the hippocampus region, a 2-fold increase was evident in enzyme fluorescence relative to the non-PEGylated control at the lower dosing level (Fig 3). As the concentration of administered PEGylated enzyme was increased, further enhancements were observed in the levels of aspartoacylase present in the brain tissue. Enzyme uptake shows the expected saturation behavior, with the maximum enzyme levels seen at doses of 1.3 mg/Kg and above (Fig. 3). A similar enhanced distribution of PEGylated ASPA was observed in sections taken from the striatum regions of the brain (data not shown).

3.3. Exploring possible mechanisms of brain uptake

Now that enhanced brain uptake of PEG-modified aspartoacylase has been established, several potential mechanisms were investigated by which this enzyme could traverse the BBB. Recent work has shown that activation of the adenosine family of receptors can increase the permeability of the BBB to facilitate the entry of macromolecules into the CNS

[23]. Intravenous administration of the agonist NECA was shown in this study to activate all adenosine receptor subtypes and led to the enhanced uptake of high molecular weight dextrans and antibodies. To test whether this mode of access is responsible for the observed increase in PEGylated aspartoacylase distribution, NECA was injected i.p. 5 hours prior to treatment with the enzyme samples, the optimal time established for enhanced permeability [23]. No significant increase in fluorescence was observed for the non-PEGylated enzyme following NECA treatment, nor was an increase observed for the PEGylated enzyme at either the lower or higher dosing levels.

Treatment with certain glycosidases had previously been found to alter the permeability of the BBB towards selected drugs [24]. In particular, hyaluronidase treatment was found to cause a short term degradation of the extracellular matrix, and allowed increased penetration of polymeric materials [25] and increased biodistribution in enzyme replacement therapy [16]. Administration of hyaluronidase for 20 minutes prior to enzyme treatment showed a slight enhancement in the already elevated levels of PEGylated ASPA at the lower dosing levels with hyaluronidase treatment, but no further enhancement was observed at the higher dosing.

3.4. Immune response to aspartoacylase treatment

PEGylation of aspartoacylase will alter the surface properties of the enzyme and, based on the results of PEGylation studies with other proteins, would be expected to decrease protein immunogenicity. Several different biomarkers were selected to examine the effect of ASPA administration on the immunological response. Glial fibrillary acidic protein (GFAP) is a marker of reactive astrocytes and will show an increase after tissue exposure to pro-inflammatory agents [25]. IBA (ionized calcium binding adaptor protein-1) binds to brain microglia/macrophages, the resident brain cells involved in immune and inflammatory response [26;27]. The fluorescence intensity of the GFAP and IBA responses were measured after treatment with either non-PEGylated or PEGylated ASPA. A fairly intense signal was observed in response to administration of non-PEGylated ASPA for both GFAP (Fig. 4A) and IBA (Fig. 4B). Treatment with low levels of the PEGylated form of ASPA resulted in a significant decrease in GFAP biomarker response, to half that of the non-PEGylated values when standardized for the differences in brain ASPA levels (Fig. 4A). These decreases were further enhanced at the higher PEGylated enzyme dosing, with the immune response at the highest enzyme doses only about 10% of the response observed with the non-PEGylated enzyme (Fig. 4A). Treatment with low levels of PEGylated ASPA also decreased IBA response, with only about one-third the response seen upon treatment with the non-PEGylated enzyme and a similar decrease observed at the higher dosing levels (Fig. 4B). However, there were no significant changes in these responses in the animals that were pretreated with NECA, or after hyaluronidase treatment (data not shown).

4. Discussion

4.1. Enhanced brain distribution of aspartoacylase

The localization of unmodified aspartoacylase suggests that this enzyme is found primarily confined to the capillaries in the hippocampus region of the brain (Fig. 2A), consistent with

the capability of the BBB to restrict access of macromolecules to the brain. In contrast, the much more diffuse fluorescent signal observed upon treatment with PEGylated enzyme (Fig. 2B to 2E) shows the significant alteration in the permeability properties of aspartoacylase upon modification. This observation establishes the newly acquired capability of PEGylated ASPA to traverse the BBB, reflected in the increased fluorescence in the hippocampus region. A similar outcome was observed in the striatum, where treatment with PEGylated ASPA also showed a dose-dependent increase in the fluorescence signal.

The enhanced capability of aspartoacylase to traverse the BBB upon PEGylation was unexpected, and is counterintuitive based on what is known about the properties of the types of molecules that can gain access [28]. Modification of this enzyme through the covalent attachment of multiple PEG molecules further increases the size of an already large protein. Also, while the ethylene repeating units in PEG are hydrophobic, the strong interactions of this polymer with water tend to increase the solubility of proteins and make them less lipophilic. Each of these changes in properties should make passage of aspartoacylase across the BBB less likely.

Despite the physical, chemical and metabolic barriers that are imposed, there are diverse routes for potential transport across the BBB. More lipophilic molecules can gain access by passive diffusion, while polar molecules must be carried by selective transporters. Some macromolecules can be transported by receptor-mediated transcytosis, while others can gain access through adsorptive-mediated mechanisms [29]. It is not yet clear how PEGylated ASPA traverses the BBB and whether it is diffusely distributed in the brain parenchyma or localized to specific cell types such as oligodendrocytes. Studies have shown that endogenous ASPA is restricted to the cytosol and nucleus of oligodendrocytes, and is not found in the membrane fraction or in neurons [30;31]. While it is possible that PEGylated ASPA is also primarily in oligodendrocytes, PEGylation may alter the distribution and localization of the enzyme.

In order to provide some insights on the possible mechanisms underlying the distribution of PEGylated ASPA in the brain, several experiments were conducted that focused on factors that can affect BBB permeability. The failure to observe a significant enhancement in enzyme uptake upon addition of NECA, a non-selective adenosine receptor agonist, indicates that the adenosine receptor system is unlikely to be involved in transporting the PEGylated ASPA. Furthermore, pre-treatment with hyaluronidase has been shown to alter the viscosity and permeability of the extracellular matrix and allow increased access across the BBB. However, our pre-treatment conditions neither enhanced uptake of the non-PEGylated ASPA, nor was there a significant increase in the already elevated levels of PEGylated ASPA upon hyaluronidase treatment. There is a threshold in macromolecule uptake since there is no further increase in brain enzyme concentration at the highest dosing levels of PEGylated ASPA. It is possible that changes in the pre-treatment conditions or examination under sub-saturating enzyme concentrations could identify glycosidase treatment conditions that could lead to enhanced enzyme uptake. However, the present results do not support a diffusion mechanism for aspartoacylase brain transport.

4.2. Decreased immunogenicity of modified aspartoacylase

While there is little precedent for increased BBB penetration of PEGylated proteins, there are numerous examples of decreased protein immunogenicity upon PEGylation [32]. Because the production of antibodies requires access to potential antigens, the interactions with “foreign” proteins involves the recognition of surface structural features that differ from those present in naturally occurring proteins. Altering the surface properties through covalent modifications with polymers would be expected to have a significant effect on antibody recognition. The expected improvement in immunogenicity and increase in circulating lifetime has been realized for numerous PEGylated proteins, including catalase [33], albumin [34] and adenosine deaminase [35]. As a consequence, an increasing number of PEGylated proteins have now been approved for therapeutic uses [36;37].

In the case of aspartoacylase, the expected enhancement in immunological biomarker response was seen following administration of unmodified enzyme. However, the immune response to the PEGylated enzyme was significantly decreased, to as little as 10% that of the non-PEGylated control. Lower GFAP and IBA immunoreactivity in the PEGylated enzyme groups suggest decreased microglial and astrocytic activation in the brain, indicating that PEGylation suppresses neuroinflammatory responses to the enzyme. Under resting conditions, microglia and astrocytes serve a house keeping function by clearing brain parenchyma of metabolic wastes and maintaining homeostasis [38;39]. Upon activation by foreign antigens, microglia can proliferate and undergo morphological and functional transformation that includes astrocytic activation and phagocytosis and degradation of the antigen [40;41]. Furthermore, overactivation of microglia and astrocytes has been shown to contribute to neural damage and underlie multiple neurodegenerative diseases [42–44]. Therefore, the lower levels of microglial activation attributed to PEGylation appear to reflect increased bioavailability of the enzyme without inciting an inflammatory response in the brain. Changes in the striatal region have been associated with neurodegenerative disorders such as Parkinson’s and Huntington’s diseases. An understanding of the mechanism of enzyme transport could make ERT available as potential therapies for these types of disorders.

5. Conclusions

An unexpected improvement in the transport properties of aspartoacylase was observed upon PEGylation, with a several-fold increase in total enzyme delivered to the hippocampus and striatum regions of the brain. This increased brain distribution appears to be unprecedented among PEGylated proteins. The only reported evidence of PEGylation having a positive effect on BBB penetration by macromolecules involved increases access of radiolabeled nanoparticles upon modification with PEG [45]. Further studies will be needed to determine the mechanism of transport and the specific properties of PEGylated aspartoacylase that allows for enhanced brain uptake and distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. SDS-PAGE of PEGylated aspartoacylase. The gels were run under identical conditions, and were stained by using: (A) Coomassie blue for proteins or (B) Barium-iodine for PEGs. Lanes 1-2 are duplicates of the reaction mixture of ASPA with methoxy-PEG 5 kDa. Lane 3 is the protein molecular weight standards in (A & B) and Lane 4 is the PEG molecular weight standards in (B).

Fig. 2.

Brain imaging (hippocampus) after treatment with fluorescently-labeled aspartoacylase.

Panel A: treatment with unmodified enzyme showing confinement primarily in the capillary regions, *Panels B through E:* treatment with PEGylated enzyme at increasing dosing levels, 0.8 mg/kg; 1.3 mg/kg; 2.0 mg/kg and 3.0 mg/kg, respectively, showing diffusion into the surrounding tissues.

Fig. 3. Fluorescence quantification of brain hippocampus enzyme levels after eliminating basal and outlier fluorescence as described in methods. These results are the averages of three brain slices from each of two animals, with the standard deviations shown in the error bars.

Fig. 4. Immune response in the hippocampus region of the brain after treatment with fluorescently-labeled aspartoacylase (ASPA), measured with two different biomarkers normalized to enzyme fluorescence intensity illustrated in Fig 3. *Panel A:* GFAP marker, *Panel B:* IBA marker. Each treatment condition was assessed from two animals per group with three brain sections examined per animal.