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Quantifying CNS protein production and clearance rates in humans using in vivo stable isotope labeling, immunoprecipitation, and tandem mass spectrometry

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Abstract

Certain disease states are characterized by disturbances in protein production, accumulation, or clearance. In the central nervous system (CNS), alterations in metabolism of proteins such as amyloid-beta ($A\beta$), alpha-synuclein, or tau may cause degenerative diseases such as Alzheimer's disease (AD), Parkinson's disease or fronto-temporal dementias respectively. In AD, dysregulation of $A\beta$ metabolism is indicated by a massive buildup of this protein in the brains of those with AD. In rare, autosomal dominant forms of AD, mutations in the amyloid precursor protein or in components of the enzymes which produce $A\beta$ (presenilin-1 and presenilin-2) appear to result in $A\beta$ overproduction. However, whether dysregulation of $A\beta$ metabolism (increased synthesis or clearance) causes the most common form of AD (sporadic >99%) is not known. Furthermore, there has not been a method available which could determine the synthesis or clearance rate of $A\beta$ or any other protein produced in the CNS. This report describes a method to determine the production rate and clearance rate of proteins produced by the CNS in vivo in humans. We report the first measurements of the fractional production and clearance rates of $A\beta$ in vivo in the human CNS to be 7.6%/hr and 8.2%/hr respectively. This method may be used to search for novel biomarkers of disease, assess underlying differences in protein metabolism that contribute to disease, and to evaluate treatments in terms of their pharmacodynamic properties on proposed disease causing pathways.

INTRODUCTION

Protein production and clearance

Protein production and clearance are important parameters that are tightly regulated and reflect normal physiology as well as disease states^{1–6}. Previous studies of protein metabolism in humans have focused on whole body or peripheral body proteins, but not on proteins produced in the central nervous system (CNS). A technique to measure specific protein metabolism in the CNS could provide important insights in CNS protein physiology in health and disease. Certain disease states are characterized by disturbances in protein production, accumulation, or clearance. In the CNS, disturbances in metabolism of proteins such as the prion protein⁷, alpha-synuclein⁸, tau⁹, or amyloid-beta¹⁰ (A β), can contribute to, and in some cases, cause neurodegenerative diseases such as Creutzfeldt–Jakob disease, Parkinson’s disease, frontotemporal dementia, or Alzheimer’s disease (AD) respectively.

A β in AD

Biochemical, genetic, and animal model evidence implicates A β as a pathogenic peptide in AD. The neuropathologic and neurochemical hallmarks of AD include synaptic loss and selective neuronal death, a decrease in certain neurotransmitters, and the presence of abnormal proteinaceous deposits in neurons (neurofibrillary tangles), in the cerebral vasculature (amyloid angiopathy), and in the extracellular space (diffuse and neuritic plaques). The main constituent of plaques is A β , a 38–43 amino acid peptide cleaved from the amyloid precursor protein (APP)^{11,12} (fig. 1). Throughout life, soluble A β is secreted mostly by neurons, but also other cell types. In late-onset AD, the total amount of A β that accumulates in brain is ~100–200-fold higher in AD brain homogenates versus control brains^{13,14}. Disturbance of A β production can lead to rare forms of AD in humans. Mutations in 3 different genes (*APP*, *PS-1* and *PS-2*) which cause early-onset autosomal dominant AD, all result in overproduction of total A β or A β ₄₂¹¹. In Down’s syndrome, three copies of APP result in increased A β production and 100% of these individuals develop AD pathology by age 35¹⁵. However, in late-onset AD (~99% of AD cases), there is not strong evidence for A β over-production. Therefore, the underlying cause for A β deposition (increased production vs. decreased clearance) is not known in the vast majority of AD.

Measurement of A β synthesis and clearance in humans

No methods were previously available to quantify protein synthesis or clearance rates in the CNS of humans. Such a method would be valuable to assess not only A β synthesis or clearance rates in humans but also the metabolism of a variety other proteins relevant to diseases of the CNS. In order to address critical questions about underlying AD pathogenesis and A β metabolism, we developed a method for quantifying A β fractional synthesis rate (FSR) and fractional clearance rate (FCR) *in vivo* in the CNS of humans. The following results indicate that by administering a stable isotope labeled amino acid (¹³C₆-leucine), sampling cerebrospinal fluid (CSF), and using high-resolution tandem mass spectrometry to quantify labeled A β , reproducible rates of A β synthesis and clearance can be quantified in humans.

METHODS

Participants and Sampling

All human studies were approved by the Washington University Human Studies Committee and the General Clinical Research Center (GCRC) Advisory Committee. Informed consent was obtained from all participants. All participants were screened to be in good general health and without neurologic disease. Seven men and three women (23–45 yrs old) participated. Each research participant was admitted to the GCRC at 7:00 AM after an overnight fast from

8PM the preceding evening. The GCRC Research Kitchen provided meals (60% carbohydrate, 20% fat, 20% protein, low leucine diet during labeled leucine infusion) at 9AM, 1PM, and 6PM and the participant had free access to water. All food and water consumption was recorded during the admission by nursing staff and the GCRC kitchen. One intravenous catheter was placed in an antecubital vein and used to administer the stable isotope labeled leucine solution. A second intravenous catheter was placed in the contra-lateral antecubital vein to obtain blood samples. A subarachnoid catheter was inserted at the L3–L4 interspace via a Touhy needle, so that CSF could be sampled without performing multiple lumbar punctures¹⁶. The intravenous catheters were placed by trained registered nurses and the lumbar catheter was placed by trained physicians with extensive experience in lumbar puncture. Blood samples were obtained hourly except from 11PM to 7AM, when they were obtained every other hour. CSF samples were obtained hourly throughout the study. The participants were encouraged to stay in bed except to use the restroom.

In Vivo labeling

¹³C₆-leucine was dissolved in medical grade normal saline and then filtered through a 0.22 micron filter the day before each study. The labeled leucine was bolused and infused intravenously using a medical IV pump at a rate of 1.8, 2.0, or 2.5 mg/kg/hr.

Materials

¹³C₆-leucine was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). M266 antibody was generously provided by Eli Lilly¹⁷. CNBr-activated sepharose 4B beads were obtained from Amersham Biosciences. Formic acid (98%) and ammonium bicarbonate (ultra >99.5%) were obtained from Fluka. Sequence Grade Modified Trypsin was obtained from Promega (Madison, WI).

Amyloid-β immunoprecipitation

Antibody beads were prepared by covalently binding m266 antibody to CNBr sepharose beads per the manufacturers protocol at a concentration of 10 mg/ml m266 antibody. The antibody beads were stored at 4°C in a 50% slurry of PBS 0.02% azide. The immunoprecipitation mixture was 250 μl of 5x RIPA, 12.5 μl of 100x protease inhibitors, and 30 μl of antibody-bead slurry added to 1 ml of sample in an eppendorf tube which was rotated overnight at 4°C. The beads were rinsed once with 1x RIPA and twice with 25 mM ammonium bicarbonate. They were aspirated dry after the final rinse and Aβ was eluted off the antibody-bead complex using 30 μl of pure formic acid. After centrifuging the beads again, the formic acid supernatant was transferred to a new eppendorf tube and speed-vacuum dried. The formic acid was evaporated in a Savant speed-vac (model AES2010) for 15 minutes at low rate (ambient temperature) with radiant cover and full vacuum, followed by 30 minutes at medium rate (43°C) with radiant cover and full vacuum. The sample was reconstituted in 5 μl of acetonitrile and 20 μl of 25 mM ammonium bicarbonate, pH at 8.0. The sample was digested with 400 ng sequence grade trypsin and incubated at 37 °C for 16 hours.

Liquid Chromatography/Mass Spectrometry

A Waters (Milford, MA) capillary liquid chromatography system with auto injector was interfaced to a Thermo-Finnigan (San Jose, CA) LCQ-DECA equipped with an electrospray ionization source (LC-ESI-tandem MS). A 5 μl aliquot of each sample was injected onto a Vydac C-18 capillary column (0.3 × 150mm MS 5 μm). In positive-ion scanning mode, LC-ESI-MS analysis of trypsin-digested synthetic and immunoprecipitated Aβ yielded the expected parent ions at masses 1325.2 for Aβ_{17–28} and 1331.2 for ¹³C₆-leucine labeled Aβ_{17–28}. To obtain amino acid sequence and abundance data, these parent ions were subjected to collision induced dissociation (CID 28%), and tandem MS analysis of their doubly-charged

species ($[M+2H]^{+2}$; m/z 663.6 and 666.6) were scanned in selected reaction monitoring mode (SRM), so that the y- and b-series ions generated were used for isotope ratio quantitation.

Calculation of labeled ratio

Percent labeled A β was calculated as the ratio of all labeled MS/MS ions from A β_{17-28} divided by all unlabeled MS/MS ions from A β_{17-28} . A custom Microsoft Excel spreadsheet with macros was used to calculate the ratio. A detailed description of the MS/MS quantification of labeled versus unlabeled peptides is under preparation (R. Bateman, D.M. Holtzman, K.E. Yarasheski).

RESULTS

In Vivo labeling and quantitation of A β

To determine if labeled A β could be produced and detected *in vivo* in a human, a single participant underwent a 24 hour infusion of labeled leucine followed by a lumbar puncture to obtain CSF. A β was immunoprecipitated from the CSF sample with the anti-A β antibody m266, trypsin digested and analyzed by liquid chromatography mass spectrometry (LC-MS). The m266 antibody is directed against the central domain of A β and binds to all A β species containing amino acids 13–28. The results demonstrated that unlabeled and labeled A β could be detected and measured in human CSF (fig. 2). A pharmacokinetic study was conducted to optimize the labeling and sampling times, so that detectable $^{13}\text{C}_6$ -leucine labeling of A β was achieved and maintained for an adequate period of time that permitted us to use steady-state equations to calculate A β synthesis and clearance rates. We tested a range of $^{13}\text{C}_6$ -leucine intravenous infusion dosages (1.8 to 2.5 mg/kg/hr), durations (6, 9, or 12 hours) and CSF/blood sampling times (from 12 to 36 hours duration). We found that labeled A β could be reliably quantified after 9 or 12 hours of label infusion, but not after 6 hours of label infusion. The synthesis portion of the labeling curve could be determined in the first 12 hours of sampling; however, the clearance portion of the labeling curve could only be determined with 36 hours of sampling. Based on these results, optimal labeling parameters for A β were defined to be 9 hours of IV infusion of the label and 36 hours of sample collection. These parameters allowed for assessment of both the fractional synthesis rate and fractional clearance rate portions of the labeling curve.

In Vivo labeling protocol—In the last three participants, $^{13}\text{C}_6$ -labeled leucine was administered with an initial bolus of 2 mg/kg over 10 minutes to reach a steady state of labeled leucine, followed by 9 hours of continuous intravenous infusion at a rate of 2 mg/kg/hr. Blood and CSF were sampled for 36 hours in the last 3 participants. Serial samples of 12 ml blood and 6 ml CSF were taken at one or two hour time intervals (fig. 3a). CSF has a production rate of ~20 ml per hour¹⁸ in a normal sized adult and replenishes itself throughout the procedure. The total amount of blood collected over the 36 hour study is 240 ml.

There were a total of 10 participants enrolled in the study, with 8 completing the pre-defined protocols and 2 studies stopped before completion due to post-lumbar puncture headache associated with the study. Two of the 8 completed studies had a 6 hour labeled leucine infusion, and labeled A β levels in these 2 participants were too low to accurately measure and were not used for analysis. The findings from the remaining 6 studies are reported here.

Labeled leucine quantitation

Plasma and CSF samples were analyzed to determine the amount of labeled leucine present in each fluid (fig. 3b). The tracer to tracee ratios (TTR) for plasma and CSF $^{13}\text{C}_6$ -leucine were quantified using capillary gas chromatography-mass spectrometry (GC-MS)^{19,20}. $^{13}\text{C}_6$ -leucine reached steady state levels in both plasma and CSF within an hour of 14% and 10%

respectively. This confirmed that leucine is rapidly transported across the blood-brain-barrier via known neutral amino acid transporter systems²¹.

Labeled A β dynamics

For each hourly sample of CSF collected, the percent of labeled A β was determined by immunoprecipitation-MS/MS as described above (fig. 2). The MS/MS ions from labeled A β_{17-28} and unlabeled A β_{17-28} were summed and divided to produce a ratio of labeled A β to unlabeled A β . The mean labeled A β ratio and standard error (n=6) of each time point are shown in figure 3c. There was no measurable labeled A β for the first 4 hours, followed by an increase from 5 to 13 hours. There was no significant change from 13 to 24 hours. The labeled A β decreased from 24 to 36 hours.

Calculation of FSR and FCR

Fractional Synthesis Rate (FSR) was calculated using the standard formula: the slope of increase of labeled A β divided by the percentage of labeled leucine²² and reported in units of percent per hour. FSR, in percent per hour, was operationally defined as the slope of the linear regression from 5 to 13 hours divided by the average of CSF ¹³C₆-labeled leucine level during infusion (fig. 4a–c).

Fractional Clearance Rate (FCR) was calculated by fitting the slope of the natural logarithm of the clearance portion of the labeled A β curve. This was operationally defined as the natural log of the labeled A β from 24 to 36 hours (fig 4d–f). The average FSR of A β for these 6 healthy young participants was 7.6%/hr and the average FCR was 8.3%/hr (fig. 4g). These values were not statistically different from each other.

DISCUSSION

First results of A β synthesis and clearance rates in humans

Summary of technique—We report a novel method to determine the production and clearance rates of CNS proteins. The technique offers an accurate and reproducible way to determine the labeled amount of a protein that is in low picomolar abundance in human CSF. This technique may be adapted to measure proteins that are made in the CNS, present in the CSF or blood, and can be collected and measured by mass spectrometry, since all proteins are labeled simultaneously. The method may be used to determine changes that may be present in a disease state (e.g. AD vs. control), to find possible biomarkers, and to test proposed disease modifying therapies. This general approach may also be applied to other macromolecules produced in the CNS, including lipids, carbohydrates, and inflammatory markers by infusing a labeled precursor that crosses the blood-brain-barrier and isolating the labeled and unlabeled products. In addition, the method may offer information on the compartmentalization of proteins if labeled species are measured in separate compartments such as blood and CSF.

There exist established stable isotope tracer methods for quantifying synthesis and clearance rates of abundant (μ g–mg) quantities of peripheral (liver, muscle, lung) human proteins, e.g., albumin, apolipoprotein B, myosin, and surfactant^{6,23–27}. In general, these methods measure the abundance of the labeled and unlabeled proteins by using gas chromatography mass spectrometry of the derivitized amino acids. This approach is sensitive and accurate, but requires relatively large amounts of purified protein, and as such, is not ideal for low abundance proteins like A β (low picomolar quantities). Recent advances in electrospray ionization mass spectrometers and MS/MS quantitation provided the opportunity to further refine this approach so that sensitive, accurate, and reproducible quantitation of stable isotope labeling ($\pm 1\%$) in very low abundance proteins/peptides (pM) can be achieved. Our method exploits this technology to quantify *in vivo* ¹³C-incorporation into A β that has been immunoprecipitated

from human CSF, digested with trypsin, and used to quantify *in vivo* A β protein synthesis and clearance rates.

Labeling—During labeling, all proteins that are being produced are labeled simultaneously, which offers the possibility of measuring multiple protein production and clearances rates on the same sample. In addition other isotopomer labels may be used, including other amino acids, simple carbohydrates, or acetate to determine kinetics of proteins, carbohydrates, or fatty acids respectively.

We chose to use a steady-state labeling infusion; however, others have used a bolus/chase design peripherally²⁸. As demonstrated in figure 3b, plasma and CSF labeled leucine were similar in shape, with both having a steady state of labeling for 9 hours and rapid clearance of the label. However, the plasma leucine was labeled at 14% while CSF was labeled at 10% during label infusion. We chose to use the plateau of labeled leucine in the CSF in our estimates of FSR. Brain labeled leucine content is likely between the measured plasma levels and the CSF levels at each time point. However, CSF labeled leucine may better reflect the precursor enrichment in the brain.

A β production and clearance—Our results indicate A β is rapidly produced and cleared in the CNS in humans. To our knowledge, this represents the first estimate of the synthesis and clearance rate of a protein produced in the CNS in humans. The clearance rate of A β in humans measured by this technique is similar to, but slower than that measured in mouse brain by *in vivo* microdialysis²⁹. Possible reasons for the differences include species difference in metabolism rate or measurement technique. In our current experiments, we have determined the synthesis and clearance of all A β species as we immunoprecipitated A β with an antibody to the central domain of the molecule. To measure synthesis and clearance of specific A β species such as A β ₄₀ or A β ₄₂, immunoprecipitation of these species with C-terminal specific antibodies would allow such assessment. This method may be used to determine changes in underlying pathophysiology associated with AD, as a biomarker for diagnosis, or as a way to test new therapeutics (e.g. beta or gamma-secretase inhibitors) in terms of the pharmacodynamics on the proposed actions of drugs or other treatments.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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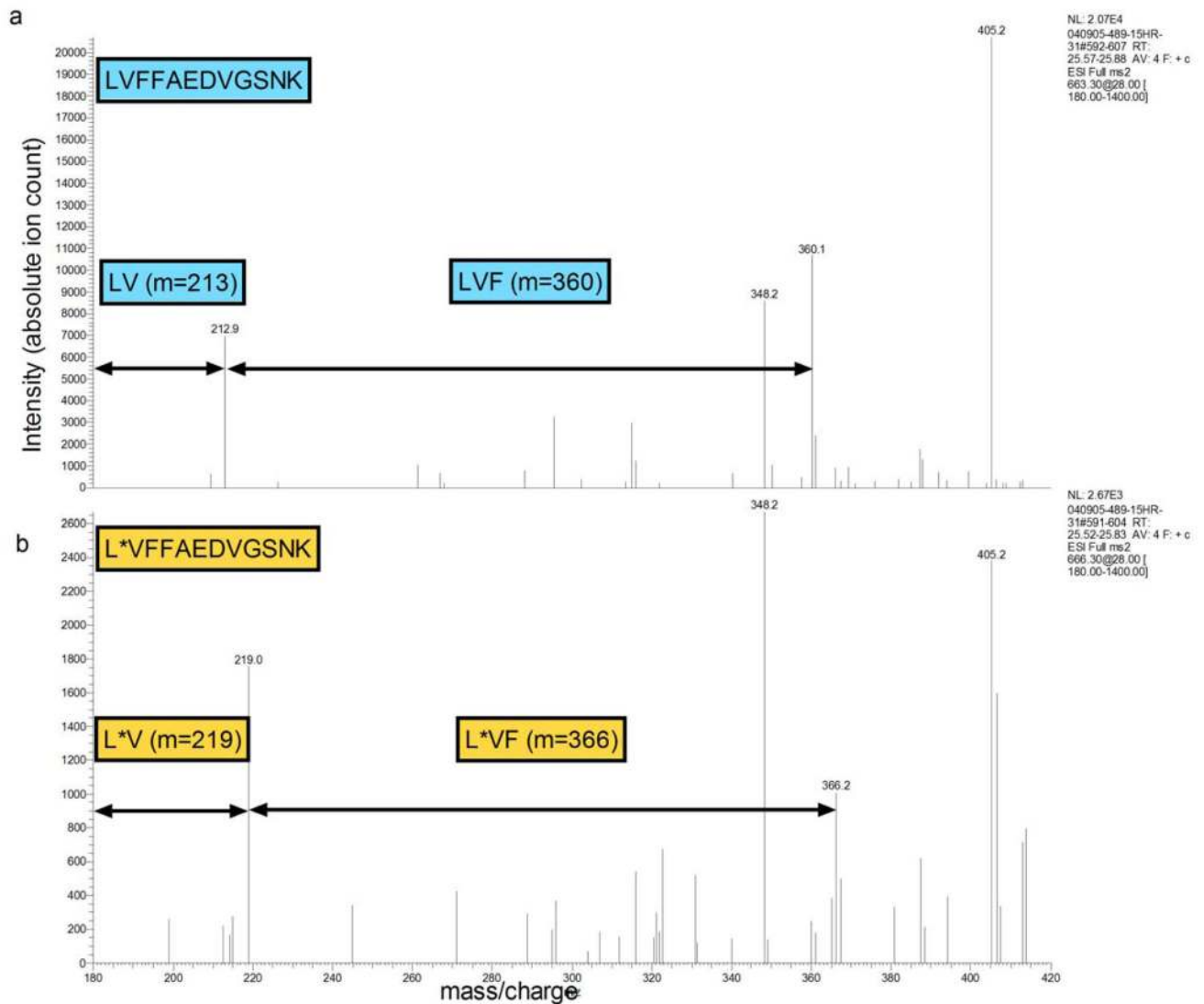
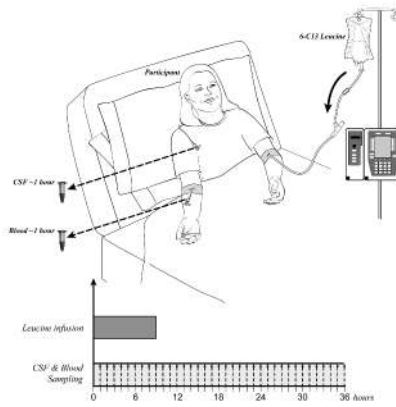
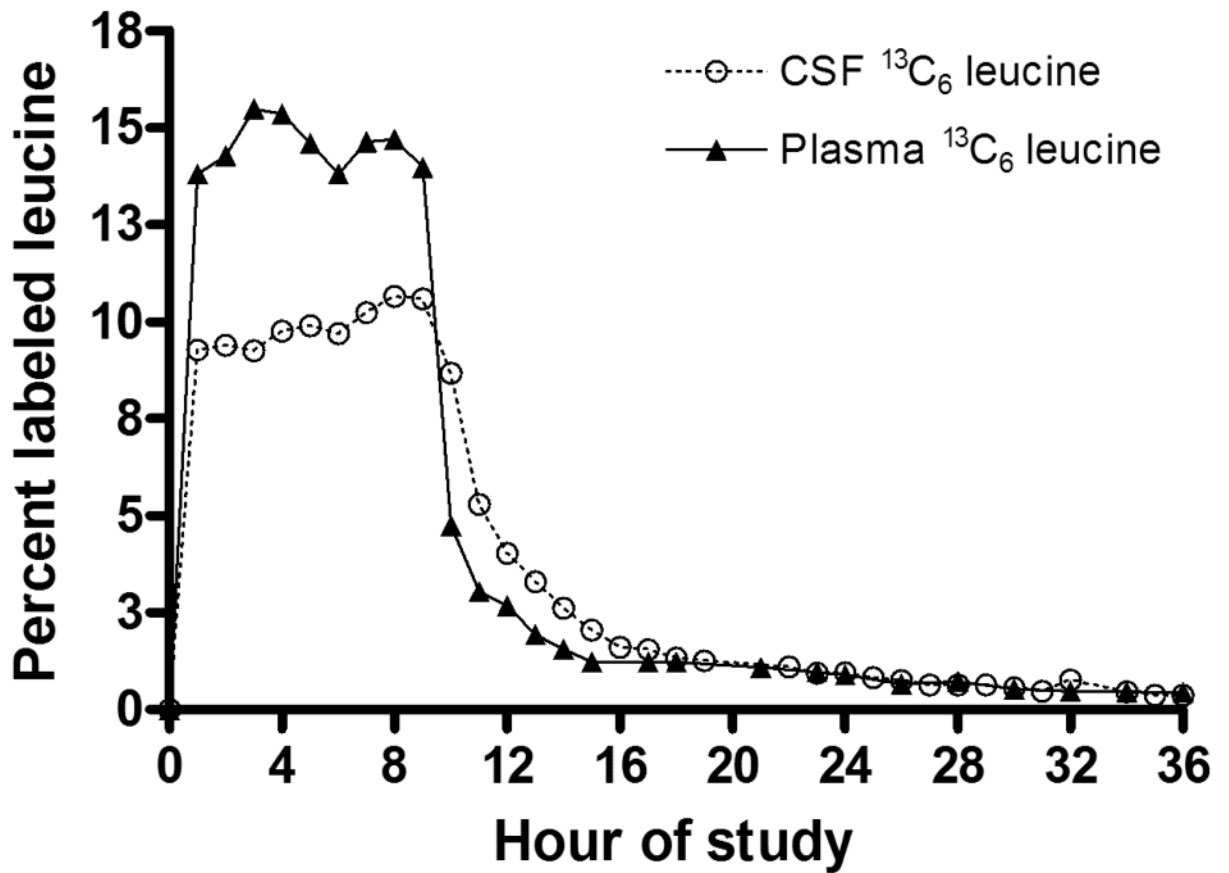


Figure 2. Human CSF was collected after intravenous infusion of $^{13}\text{C}_6$ -leucine. Representative spectra of unlabeled (**a**) and labeled (**b**) $\text{A}\beta_{17-28}$ are shown. The spectra were obtained using MS/MS analysis of unlabeled parent ion $\text{A}\beta_{17-28}$ at m/z 663.3 or labeled parent ion $\text{A}\beta_{17-28}$ at m/z 666.3. Note the MS/MS ions containing leucine ($\text{A}\beta_{17}$) are mass shifted by 6 Daltons demonstrating the labeled leucine. The $\text{A}\beta$ ions without leucine at position 17 are not labeled and are not mass shifted by 6 Daltons.



3b



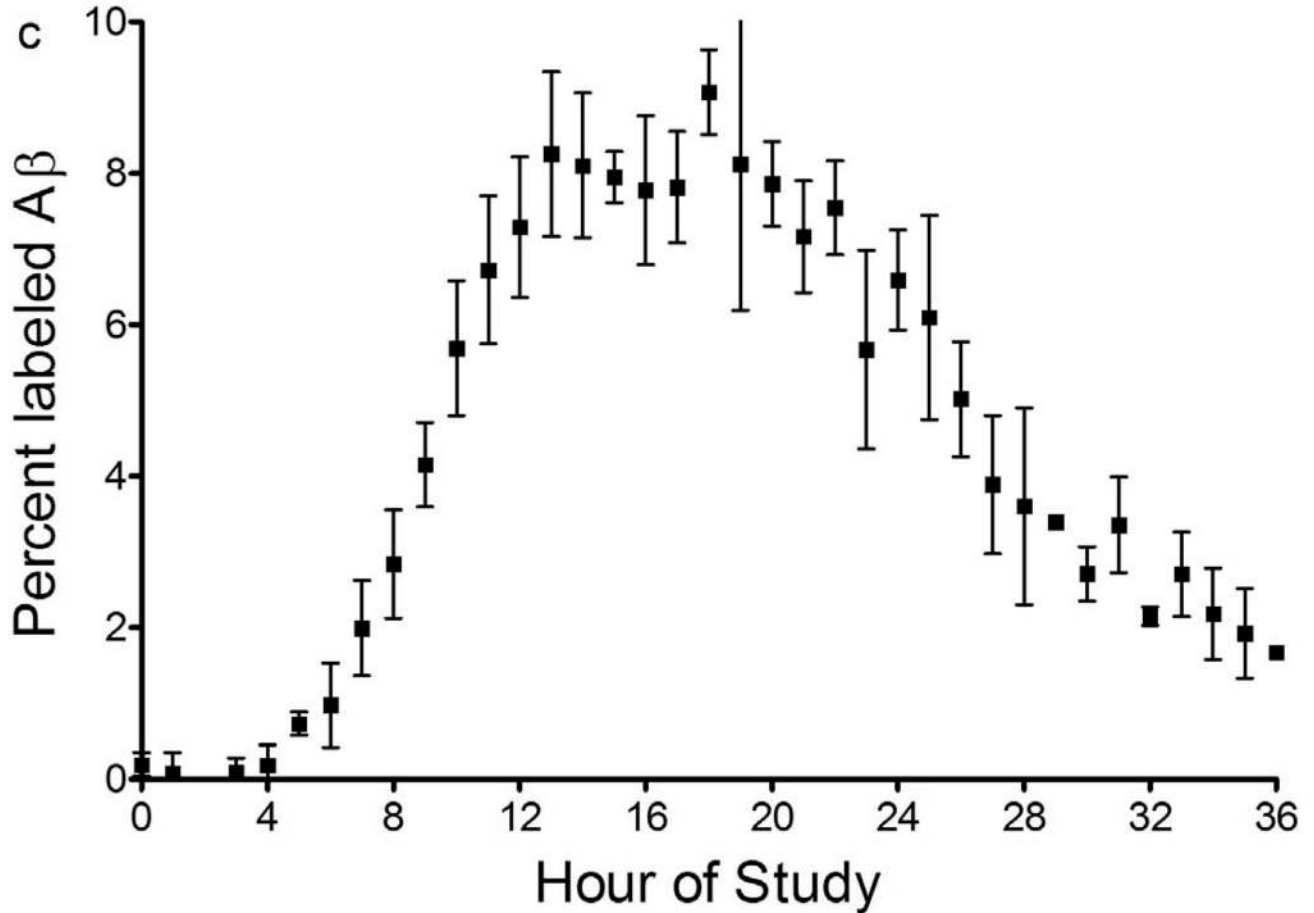
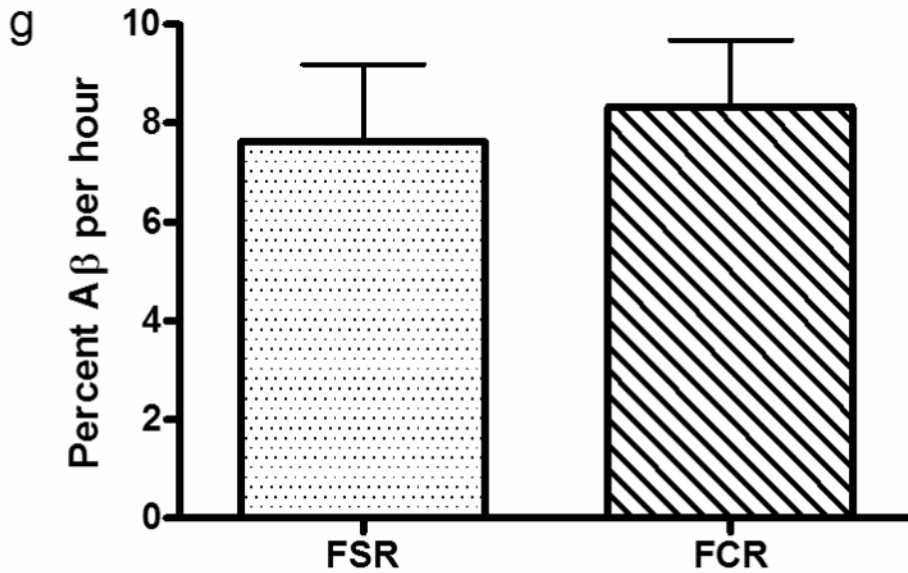
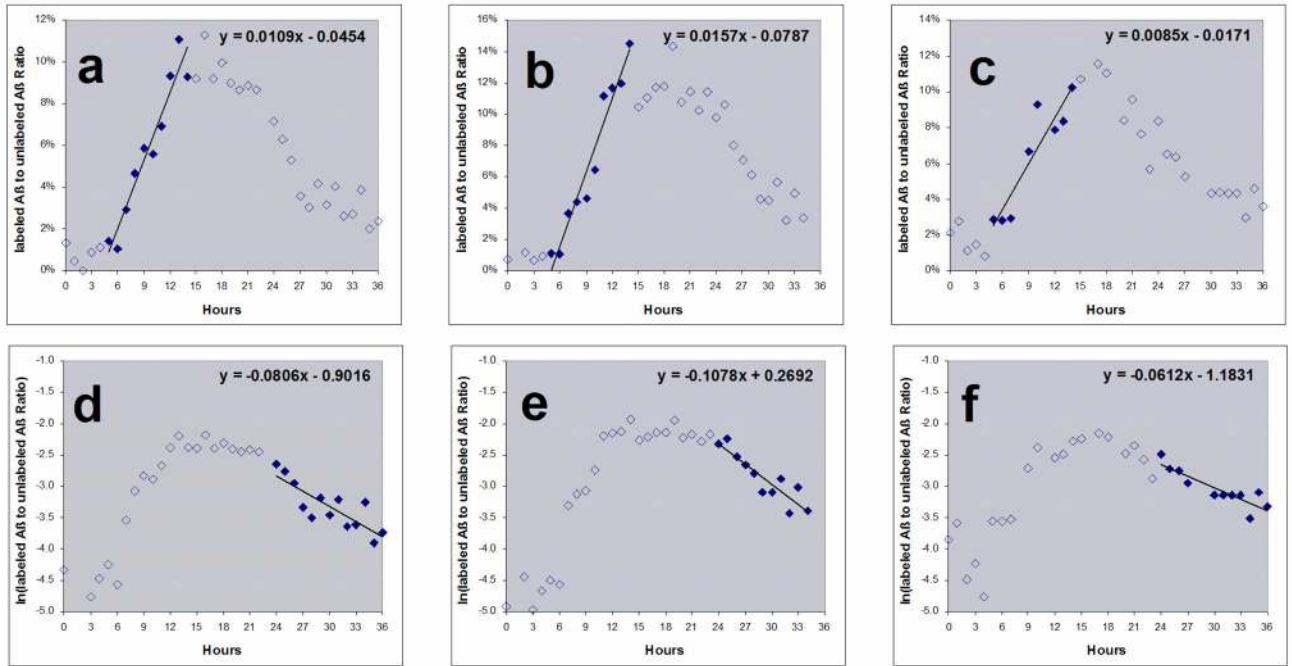


Figure 3.

(a) Diagram of participant with an intravenous catheter in either antecubital vein and a lumbar catheter in the L3-4 intrathecal space. Participants were admitted to the Washington University GCRC at 7 AM and 2 IVs and one lumbar catheter were placed. In one IV, $^{13}\text{C}_6$ -labeled leucine was infused at a rate of 1.8 to 2.5 mg/kg/hr for 9 or 12 hours, after an initial bolus of 2 mg/kg. Each hour, 12 ml of plasma and 6 ml of CSF were obtained through the other IV and the lumbar catheter respectively. Each sample was then processed as described above with immunoprecipitation of A β , trypsin digest, and LC-ESI-MS analysis to determine the percent labeled A β at each hour time point.

(b) Labeled leucine in CSF and blood from a participant during a 36 hour study. Note that CSF and plasma labeled leucine reaches near steady state within an hour after the initial bolus of 2mg/kg. Also note the exponential decay in labeled leucine clearance after the infusion of leucine into the bloodstream is stopped at 9 hours. The plasma labeled leucine is ~4% higher than the CSF labeled leucine during infusion.

(c) Average labeled CSF A β over 36 hours from 6 participants. The labeled A β curves were averaged and the mean for each time point shown \pm SEM. Each participant was labeled for 9 or 12 hours, while sampling occurred hourly from 0 to 12, 24, or 36 hours. There is no detectable incorporation of label in the first 4 hours, followed by an increase in percent labeled A β which plateaus near steady state labeled leucine levels (~10%), before decreasing over the last 12 hours of the study.



FSR=Fractional Synthetic Rate 7.6 %/hour
FCR=Fractional Clearance Rate 8.3 %/hour

Figure 4. FSR and FCR of labeled Aβ curves from 3 participants with 9 hour label infusion and 36 hour sampling. FSR (a–c) is calculated by the slope of increasing labeled Aβ divided by the predicted steady state value. The predicted steady state value was estimated as the average CSF labeled leucine measured during labeling. The slope was defined to start after 4 to 6 hours lag time when there was no increase in labeled Aβ and ending 9 hours later (solid diamonds). The slope of FCR (d–f) is calculated by the slope of the natural logarithm of percent labeled Aβ from

hours 24 to 36. (g) Average A β FSR & FCR: The average A β FSR of 6 participants and the average A β FCR of 3 participants is shown with standard deviation.