nature medicine

Human amyloid-β synthesis and clearance rates as measured in cerebrospinal fluid *in vivo*

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Certain disease states are characterized by disturbances in production, accumulation or clearance of protein. In Alzheimer disease, accumulation of amyloid-β (Aβ) in the brain and disease-causing mutations in amyloid precursor protein or in enzymes that produce Aβ indicate dysregulation of production or clearance of Aβ. Whether dysregulation of Aβ synthesis or clearance causes the most common form of Alzheimer disease (sporadic, >99% of cases), however, is not known. Here, we describe a method to determine the production and clearance rates of proteins within the human central nervous system (CNS). We report the first measurements of the fractional production and clearance rates of Aβ *in vivo* in the human CNS to be 7.6% per hour and 8.3% per hour, respectively. This method may be used to search for novel biomarkers of disease, to assess underlying differences in protein metabolism that contribute to disease and to evaluate treatments in terms of their pharmacodynamic effects on proposed disease-causing pathways.

Protein production and clearance are important parameters that are tightly regulated and reflect normal physiology as well as disease states[1–4]. Previous studies of protein metabolism in humans have focused on whole-body or peripheral-body proteins, but not on proteins produced in the CNS. A technique to measure specific protein metabolism in the CNS could provide important insights into 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 protein[5], alpha-synuclein[6], tau[7] or Aβ[8] can contribute to and, in some cases, cause neurodegenerative diseases such as Creuzfeldt-Jakob disease, Parkinson disease, frontotemporal dementia or Alzheimer disease, respectively.

Biochemical, genetic and animal model evidence implicates Aβ as a pathogenic peptide in Alzheimer disease. The neuropathologic and neurochemical hallmarks of Alzheimer disease 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 Aβ, a peptide of 38–43 amino acids cleaved from the amyloid precursor protein (APP)[9,10]. Throughout life, soluble Aβ is secreted mostly by neurons but also other cell types. In late-onset Alzheimer disease, the total amount of Aβ that accumulates in brain is ~100–200-fold higher in homogenates from Alzheimer disease brains than from control brains[11]. Disturbance of Aβ production can lead to rare forms of Alzheimer disease in humans. Mutations in three different genes (*APP, PSEN1* and *PSEN2*), which cause early-onset autosomal dominant Alzheimer disease, all result in overproduction of total Aβ or $Aβ_{42}$ (ref. 9). In Down syndrome, three copies of *APP* result in increased production of Aβ, and 100% of individuals with Down syndrome develop Alzheimer disease pathology by age 35 (ref. 12). In late-onset Alzheimer disease (~99% of cases), however, there is not strong evidence for overproduction of Aβ. Therefore, the underlying cause of deposition of Aβ (increased production versus decreased clearance) is not known for most cases of Alzheimer disease.

No methods were previously available to quantify protein synthesis or clearance rates in the human CNS. Such a method would be valuable to assess not only Aβ synthesis and clearance rates in humans but also the metabolism of a variety other proteins relevant to diseases of the CNS. To address crucial questions about underlying pathogenesis of Alzheimer disease and Aβ metabolism, we developed a method for quantifying the fractional synthesis rate (FSR) and fractional clearance rate (FCR) of Aβ *in vivo* in the human CNS. Our results indicate that by administering a stable isotope-labeled amino acid ($^{13}C_6$-leucine), sampling cerebrospinal fluid (CSF) and using high-resolution tandem mass spectrometry to quantify labeled Aβ, reproducible rates of Aβ synthesis and clearance can be quantified in humans.

RESULTS

In vivo labeling and quantification of Aβ

To determine whether labeled Aβ (**Fig. 1**) could be produced and detected *in vivo* in a human, one individual underwent a 24-h infusion of labeled leucine followed by a lumbar puncture to obtain CSF. We immunoprecipitated Aβ from the CSF sample with the Aβ-specific

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Received 2 November 2005; accepted 24 January 2006; published online 25 June 2006; doi:10.1038/nm1438

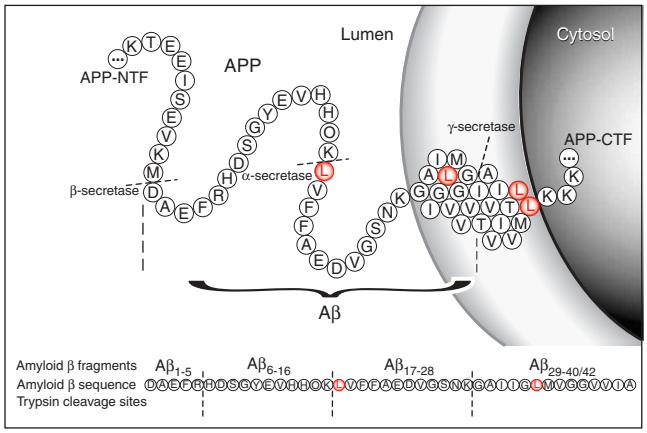

Figure 1 The amino acid sequence of Aβ is depicted in the amyloid precursor protein (APP) in the cell membrane with the leucines (L) labeled in red to indicate possible labeling sites. The sequence of Aβ is shown below with the trypsin digest sites indicated to show the fragments that were analyzed by mass spectrometry.

antibody m266, digested it with trypsin and analyzed it using liquid chromatography–mass spectrometry (LC-MS). The m266 antibody is directed against the central domain of Aβ and binds to all Aβ species containing amino acids 13–28. The results showed that unlabeled and labeled Aβ could be detected and measured in human CSF (**Fig. 2**).

We conducted a pharmacokinetic study to optimize the labeling and sampling times, so that detectable $^{13}C_6$-leucine labeling of Aβ was achieved and maintained for an adequate period of time that permitted us to use steady-state equations to calculate Aβ synthesis and clearance rates. We tested a range of $^{13}C_6$-leucine intravenous infusion dosages (1.8–2.5 mg/kg/h), durations (6, 9 or 12 h) and CSF or blood sampling times (sampling at 12–36 h; **Table 1**). We found that labeled Aβ could be reliably quantified after 9 or 12 h of infusion of the label but not after 6 h of infusion of the label. The synthesis portion of the labeling curve could be determined in the first 12 h of sampling; however, the clearance portion of the labeling curve could only be determined with 36 h of sampling. Based on these results, we defined optimal labeling parameters for Aβ to be 9 h of intravenous infusion of the label and 36 h of sample collection. These parameters allowed for assessment of both the FSR and FCR portions of the labeling curve.

In vivo labeling protocol

For the last three individuals, we administered $^{13}C_6$-labeled leucine with an initial bolus of 2 mg/kg over 10 min to reach a steady state of labeled leucine, followed by 9 h of continuous intravenous infusion at a rate of 2 mg/kg/h. We sampled blood and CSF for 36 h in the last three individuals. We took serial 12-ml blood samples and 6-ml CSF samples at 1- or 2-h time intervals (**Fig. 3a**). CSF has a production rate of ~20 ml/h[13] in a normal-sized adult and replenishes itself throughout the procedure. Over a 36-h study, the total amounts of blood and CSF collected were 312 ml and 216 ml, respectively.

There were a total of ten individuals enrolled in the study, with eight completing the predefined protocols; we stopped two studies before completion because of postlumbar puncture headache associated with the study. Two of the eight completed studies had a 6-h labeled leucine infusion, and labeled Aβ levels in these two individuals were too low to accurately measure and were not used for analysis. The findings from the remaining six studies are reported here.

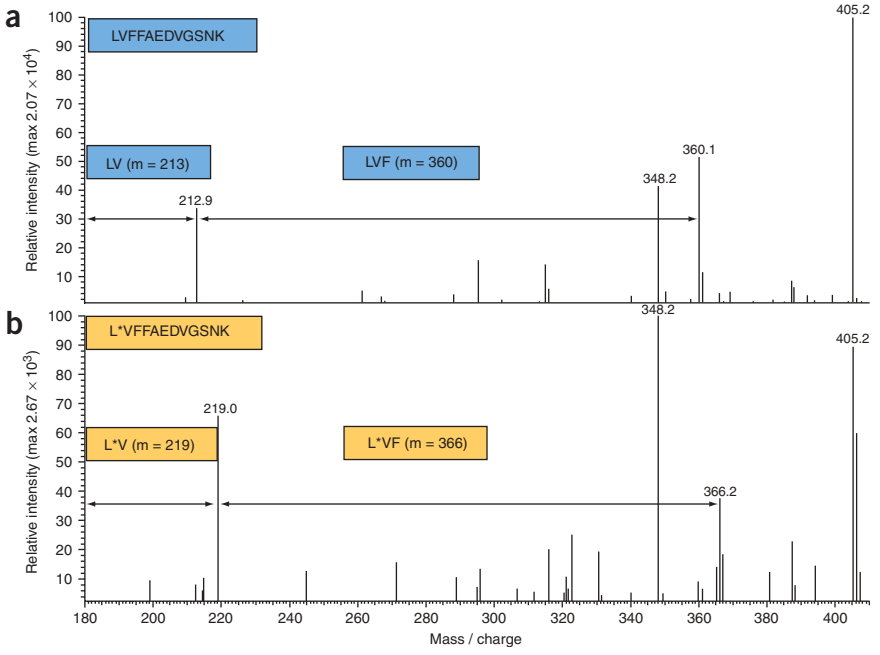

Figure 2 Spectra of unlabeled and labeled $A\beta_{17-28}$. We collected human CSF after intravenous infusion of $^{13}C_6$-leucine. Representative spectra of unlabeled (**a**) and labeled (**b**) $A\beta_{17-28}$ are shown. We obtained the spectra using MS/MS analysis of unlabeled parent ion $A\beta_{17-28}$ at *m/z* 663.3 or labeled parent ion $A\beta_{17-28}$ at *m/z* 666.3. The MS/MS ions containing leucine ($A\beta_{17}$) are mass shifted by 6 Da, indicating the labeled leucine. The MS/MS ions without leucine are not labeled and are not mass shifted by 6 Da (348 and 405).

Quantification of labeled leucine

We analyzed plasma and CSF samples to determine the amount of labeled leucine present in each fluid (**Fig. 3b**). We quantified the labeled-to-unlabeled leucine ratios for plasma and CSF $^{13}C_6$-leucine using capillary gas chromatography–mass spectrometry (GC-MS)[14], which is more suitable than LC-MS for analysis of low-mass amino acids. Within 1 h, the $^{13}C_6$-leucine reached steady-state levels in both plasma and CSF of 14% and 10%, respectively. This confirmed that leucine is rapidly transported across the blood-brain barrier through known neutral amino acid–transporter systems[15].

Dynamics of labeled Aβ

For each sample of CSF collected, we determined the labeled-to-unlabeled ratio of Aβ by immunoprecipitation–tandem mass spectrometry (MS/MS) as described above. The number of MS/MS ions from labeled $A\beta^*_{17-28}$ was divided by the number of MS/MS ions from unlabeled $A\beta_{17-28}$ to produce a ratio of labeled Aβ to unlabeled Aβ. The mean labeled Aβ ratio and standard error ($n = 6$) of each

Table 1 Participant labeling and sampling parameters

Participant number	Infusion dosage (mg/kg/h)	Infusion duration (hours)	CSF or blood sampling (hours)
1	1.8	24	1 time at 24 h
2	1.9	12	24
3	2.5	12	13
4	2.5	9	24
5	2.4	6	6
6	2	6	36
7	2	6	36
8	2	9	36
9	2	9	36
10	2	9	36

We tested a range of labeling dosages, durations and sampling times. A labeling duration of 9 h and a sampling duration of 36 h allowed for calculation of FSR and FCR.

time point are shown in **Figure 3c**. There was no measurable labeled Aβ for the first 4 h, followed by an increase from 5 to 13 h. There was no significant change from 13 to 24 h. The labeled Aβ decreased from 24 to 36 h.

Calculation of FSR and FCR

We calculated FSR using the standard formula[16]:

$$FSR = \frac{(E_{t2} - E_{t1})_{A\beta}}{(t_2 - t_1)} \div Precursor\ E$$

Where $(E_{t2} - E_{t1})_{A\beta}/(t_2 - t_1)$ is defined as the slope of the line during labeling, and Precursor E is the ratio of labeled leucine to unlabeled. FSR, in percent per hour, was operationally defined as the slope of the linear regression from 5 to 14 h divided by the average of CSF $^{13}C_6$-labeled leucine level during infusion (**Fig. 4a–c**). For example, a FSR of 7.6% per hour means that 7.6% of total Aβ is produced each hour.

We calculated FCR by fitting the slope of the natural logarithm of the clearance portion of the labeled Aβ curve.

$$FCR = \ln\left(\frac{\text{labeled A}\beta/\text{unlabeled A}\beta}{\Delta time(hours)_{24-36}}\right)$$

We operationally defined this as the natural log of the labeled Aβ from 24 to 36 h (**Fig. 4d–f**). For example, a FCR of 8.3% per hour means that 8.3% of total Aβ is cleared each hour. The average FSR of Aβ for these six healthy young individuals was 7.6% per hour, and the average FCR was 8.3% per hour (**Fig. 4g**). These values were not statistically different from each other.

DISCUSSION

We report a new method to determine the production and clearance rates of proteins present in the human CNS. 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 as all proteins are labeled simultaneously. The method may be used to determine changes that may be present during a disease state (for example, Alzheimer disease), 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.

Several earlier studies have shown that some plasma Aβ can enter the CNS[17–19], although the percentage of CSF Aβ that is derived from

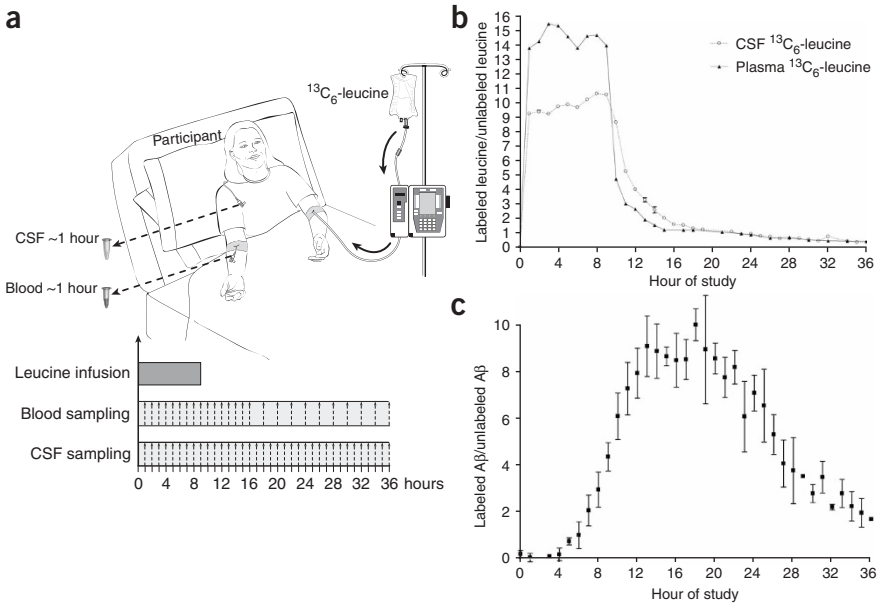

Figure 3 Diagram of *in vivo* human CNS protein labeling. Sample collection and measurements of labeled amino acid and Aβ. (**a**) Diagram of individual with an intravenous catheter in either antecubital vein and a lumbar catheter in the L3–L4 intrathecal space. In one intravenous line, we infused $^{13}C_6$-labeled leucine at a rate of 1.8–2.5 mg/kg/h for 9 or 12 h, after an initial bolus of 2 mg/kg. We obtained 12 ml of plasma through the other intravenous line every hour for the first 16 h and every other hour thereafter, as depicted. We obtained 6 ml of CSF through the lumbar catheter every hour. (**b**) Labeled leucine in CSF and blood from an individual during a 36-h study. Labeled leucine in the CSF and plasma reaches a near steady-state level within an hour after we gave the initial bolus of 2 mg/kg. There was an exponential decay in labeled leucine levels after the infusion of leucine into the bloodstream was stopped at 9 h. The plasma labeled leucine is ~4% higher than the CSF labeled leucine during infusion. (**c**) Average labeled CSF Aβ over 36 h from six individuals. We averaged the labeled Aβ curves and the mean for each time point is shown ± s.e.m. Each participant underwent labeling for 9 or 12 h, whereas sampling occurred hourly from 0 to 12, 24 or 36 h. There is no detectable incorporation of label in the first 4 h. This is followed by an increase in percent labeled Aβ, which plateaus near steady-state levels of labeled leucine (~10%), before decreasing over the last 12 h of the study.

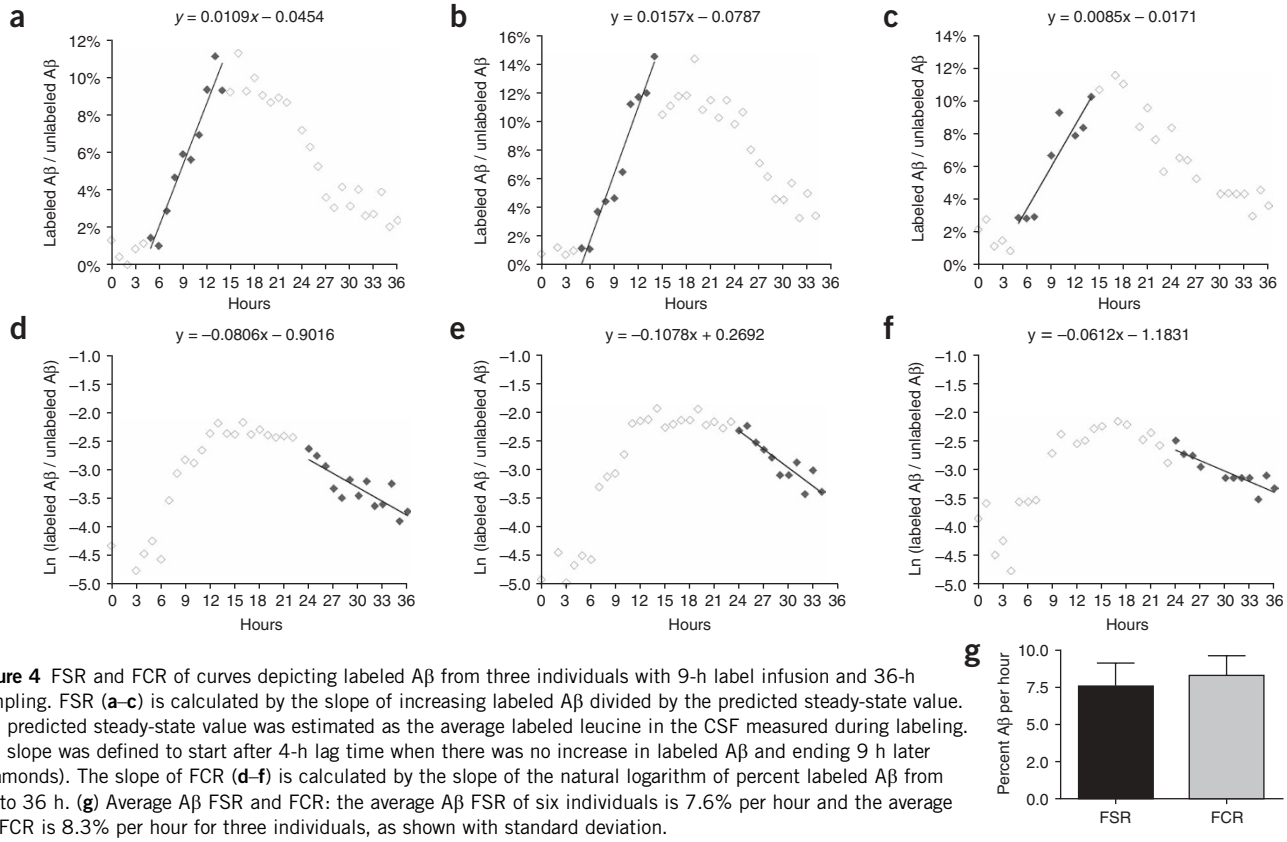

Figure 4 FSR and FCR of curves depicting labeled Aβ from three individuals with 9-h label infusion and 36-h sampling. FSR (**a**–**c**) is calculated by the slope of increasing labeled Aβ divided by the predicted steady-state value. The predicted steady-state value was estimated as the average labeled leucine in the CSF measured during labeling. The slope was defined to start after 4-h lag time when there was no increase in labeled Aβ and ending 9 h later (diamonds). The slope of FCR (**d**–**f**) is calculated by the slope of the natural logarithm of percent labeled Aβ from 24 to 36 h. (**g**) Average Aβ FSR and FCR: the average Aβ FSR of six individuals is 7.6% per hour and the average Aβ FCR is 8.3% per hour for three individuals, as shown with standard deviation.

peripheral versus CNS sources has not been directly measured. The concentration of Aβ in human brain and CSF is 50–100 times higher than in plasma[20], and from γ-secretase inhibitor studies, it seems that Aβ generated in the CNS contributes to most Aβ found in the CSF. For example, inhibition of Aβ production outside the CNS can decrease plasma Aβ by as much as 50% with no detectable change in brain or CSF Aβ[21,22]. Measurements of the FSR and FCR of Aβ in the CSF are likely to be measurements of Aβ produced largely in the CNS, but may also reflect a small amount of Aβ synthesized in the periphery.

There exist established stable isotope–tracer methods for quantifying synthesis and clearance rates of abundant (microgram to milligram) quantities of peripheral (liver, muscle lung) human proteins, for example, albumin, apolipoprotein B, myosin and surfactant[4,23,24]. In general, these methods measure the abundance of the labeled and unlabeled proteins by using GC-MS 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 Aβ (present in low picomolar quantities). Recent advances in electrospray ionization mass spectrometers and MS/MS quantification provided the opportunity to further refine this approach so that sensitive, accurate and reproducible quantification of stable isotope labeling (±1%) in very-low-abundance proteins and peptides (picomolar) can be achieved. Our method exploits this technology to quantify *in vivo* incorporation of ^{13}C into Aβ that has been immunoprecipitated from human CSF, digested with trypsin and used to quantify *in vivo* Aβ protein synthesis and clearance rates.

During labeling, all proteins that are being produced are labeled simultaneously, which offers the possibility of measuring multiple protein production and clearance 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 peripherally[25]. As shown, curves depicting plasma and CSF labeled leucine were similar in shape, with both having a steady state of labeling for 9 h and rapid clearance of the label. Plasma leucine was labeled at 14% whereas CSF leucine was labeled at 10% during infusion of the label. In individuals in our study, levels of labeled Aβ in the CSF approached levels of labeled leucine in the CSF (average, 8.9% and 11.2%, respectively), suggesting that labeled leucine in the CSF more closely reflects the labeled leucine precursor in the brain compared to plasma. We chose to use the plateau of labeled leucine in the CSF in our estimates of FSR, as levels of labeled leucine in the brain are likely to be closer to levels in CSF than plasma.

Our results indicate Aβ is rapidly produced and cleared from the CNS in humans. To our knowledge, this represents the first estimate of the synthesis and clearance rate of a protein produced in the human CNS. The clearance rate of Aβ in humans measured by this technique is similar to, but slower than, that measured in mouse brain by *in vivo* microdialysis[26]. Possible reasons for the differences include species difference in metabolism rate or the measurement technique.

In our current experiments, we have determined the synthesis and clearance of all Aβ species, as we immunoprecipitated Aβ with an antibody to the central domain of the molecule. To measure synthesis and clearance of specific Aβ species, such as $Aβ_{40}$ or $Aβ_{42}$, we would need to perform an immunoprecipitation of these species with C-terminal–specific antibodies. This method may be used to determine changes in the underlying pathophysiology of Alzheimer disease

or as a way to test new therapeutics (for example, β- or γ-secretase inhibitors) by measuring the pharmacodynamic effect of the therapy on Aβ synthesis or clearance in humans.

METHODS

Human studies. 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 (between 23 and 45 years) participated. Each individual was admitted to the GCRC at 07:00 after an overnight fast from 20:00 the preceding evening. The GCRC Research Kitchen provided meals (60% carbohydrate, 20% fat, 20% protein, low-leucine diet during labeled leucine infusion) at 09:00, 13:00 and 18:00. Individuals had free access to water. All food and water consumption was recorded during the admission by nursing staff and the GCRC kitchen. We placed one intravenous catheter in an antecubital vein and used it to administer the stable isotope–labeled leucine solution. We placed a second intravenous catheter in the contralateral antecubital vein to obtain blood samples. We inserted a subarachnoid catheter at the L3–L4 interspace using a Touhy needle, so that CSF could be sampled without performing multiple lumbar punctures[27]. The intravenous catheters were put in place by trained registered nurses and the lumbar catheter by trained physicians with experience in lumbar puncture. We obtained blood samples hourly, unless the study was 36 h, in which case blood was obtained hourly for the first 16 h and every other hour thereafter. We obtained CSF samples hourly throughout the study. We encouraged the participants to stay in bed except to use the restroom.

We dissolved $^{13}C_6$-leucine in medical-grade normal saline and then filtered it through a 0.22-μm filter the day before each study. We infused the labeled leucine intravenously using a medical intravenous pump at a rate of 1.8–2.5 mg/kg/h.

We obtained $^{13}C_6$-leucine (CLM-2262) from Cambridge Isotope Laboratories. M266 antibody was generously provided by Eli Lilly[28]. We obtained CNBr-activated Sepharose 4B beads from Amersham Biosciences. We obtained formic acid (98%) and ammonium bicarbonate (ultra >99.5%) from Fluka and sequence grade–modified trypsin from Promega.

Aβ immunoprecipitation. We prepared antibody beads by covalently binding m266 antibody to CNBr Sepharose beads per the manufacturer's protocol at a concentration of 10 mg/ml m266 antibody. We stored the antibody beads at 4 °C in a 50% slurry of PBS 0.02% azide.

The immunoprecipitation mixture consisted of 250 μl 5× RIPA, 12.5 μl 100× protease inhibitors and 30 μl antibody-bead slurry added to 1 ml of sample in an Eppendorf tube, which we rotated overnight at 4 °C. We rinsed the beads once with 1× RIPA and twice with 25 mM ammonium bicarbonate. They were aspirated dry after the final rinse and we eluted Aβ off the antibody-bead complex using 30 μl pure formic acid. After centrifuging the beads again, we transferred the formic acid supernatant to a new Eppendorf tube for speed-vacuum drying. We evaporated the formic acid in a Savant speed-vac (model AES2010) for 15 min at low rate (ambient temperature, 20 °C) temperature with radiant cover and full vacuum, followed by 30 min at medium rate (43 °C) with radiant cover and full vacuum. We reconstituted the sample in 5 μl acetonitrile and 20 μl 25 mM ammonium bicarbonate, pH 8.0. We digested the sample with 400 ng sequence-grade trypsin and incubated it at 37 °C for 16 h.

Liquid chromatography–mass spectrometry. We interfaced a Waters capillary liquid chromatography system with autoinjector to a Thermo-Finnigan LCQ-DECA equipped with an electrospray ionization source (LC-ESI-MS/MS). We injected a 5-μl aliquot of each sample onto a Vydac C-18 capillary column (0.3 × 150 mm). In positive-ion scanning mode, LC-ESI-MS/MS analysis of trypsin-digested synthetic and immunoprecipitated Aβ yielded the expected parent ions at a mass of 1,325.2 for $Aβ_{17–28}$ and 1,331.2 for $^{13}C_6$-leucine labeled $Aβ_{17–28}$. To obtain amino acid sequence and abundance data, we subjected these parent ions to collision-induced dissociation (28%), and MS/MS analysis of their doubly charged species ($[M+2H]^{+2}$; m/z 663.6 and 666.6) were scanned in selected reaction monitoring mode, so that the y- and b-series ions generated were used for isotope ratio quantification.

Calculation of labeled ratio. We calculated percent labeled Aβ as the ratio of the number of labeled MS/MS ions from $Aβ^*_{17-28}$ divided by the number of unlabeled MS/MS ions from $Aβ_{17–28}$. We used a custom Microsoft Excel spreadsheet with macros to calculate the Aβ ratio as the tracer-to-tracee ratio (TTR) of $Aβ_{17–28}$ using the formula:

$$TTR_{A\beta} = \frac{\sum MS/MS\ ions\ A\beta^*_{17-28}}{\sum MS/MS\ ions\ A\beta_{17-28}}$$

A detailed description of the mass spectrometry methods and quantification of labeled versus unlabeled peptides is in preparation (R.B., D.M.H. & K.E.Y., unpublished data).

ACKNOWLEDGMENTS

This work was supported by grants from the American Academy of Neurology Foundation, US National Institutes of Health (NIH) grants ADRC (P50 AG05681), GCRC (MO1 RR00036), Mass Spectrometry Resource (NIH RR000954), the Clinical Nutrition Research Unit (NIH DK056341) and The Alan A. and Edith L. Wolff Charitable Trust. We are grateful to the participants for their time and to Eli Lilly for providing m266 antibody.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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