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Cerebrospinal fluid concentrations of large neutral and basic amino acids in *Macaca mulatta*: diurnal variations and responses to chronic changes in dietary protein intake

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Abstract

In rats, dietary protein intake influences brain concentrations of tryptophan, tyrosine, and other large neutral amino acids (LNAAs) and the neurotransmitters to which they are linked. Few experiments have examined these dietary protein–amino acid relationships in nonhuman primates, in relation to time of day or dietary protein content. We therefore examined the effect in monkeys of changes in chronic protein intake on 24-hour plasma and cerebrospinal fluid (CSF) concentrations of LNAAs (tyrosine, phenylalanine, branched-chain amino acids) and basic amino acids. Juvenile male monkeys (*Macaca mulatta*) consumed for sequential 4-week periods diets differing in protein content (~23% → ~16% → ~10% → ~6% protein [percentage of energy]). The daily ration was presented as a morning meal of fruit and an afternoon meal of fruit and a commercial diet to mimic feeding patterns *in the wild*. During week 4 on each diet, blood and CSF were sampled repeatedly over a 48-hour period via indwelling catheters. Plasma and CSF LNAA concentrations varied markedly with time of day and dietary protein content, showing up to 4-fold variations. Diurnal variations in plasma and CSF basic amino acids were smaller in magnitude and generally not strongly linked to dietary protein content. A measure of the competitive transport of LNAAs across the blood-brain barrier, calculated using plasma concentrations of the LNAAs and their blood-brain barrier kinetic constants, predicted the observed CSF concentration of each LNAA examined remarkably well, except for phenylalanine. Based on observations in rats, the variations in the CSF concentrations of the LNAAs in monkeys may be large enough to influence metabolic and signaling pathways in brain to which they have been linked.

1. Introduction

The plasma concentrations of some amino acids show notable changes after the ingestion of single meals. These changes have been linked to rapid alterations in their brain concentrations and, in some cases, to neurotransmitter products derived from them. Such relationships have been described for tryptophan (TRP) and its transmitter product, serotonin (5HT), tyrosine (TYR) and the catecholamine transmitters that derive from it, and histidine (HIS) and histamine [1-5]. Such neurotransmitter modifications are of particular physiologic interest when they can be linked to the control of food intake, as they suggest possible pathways by which the brain might sense and modify the appetite for energy and specific macronutrients, such as protein (eg, Fernstrom and Fernstrom [2]).

In contrast, the impact of chronic changes in the ingestion of particular macronutrients, notably protein, on these amino acids and the transmitters into which they are transformed has been studied much less. From limited data in rats, chronic differences in protein intake between 0% and 70% (percentage energy) produce changes in the plasma and brain concentrations of several amino acids

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sufficient to influence transmitter production. For example, plasma and brain TYR concentrations increase as dietary protein content increases and produce like modifications in catecholamine production in the hypothalamus, an important brain center of appetite control [6-8]. The plasma and brain levels of the branched-chain amino acids (BCAAs) also rise as dietary protein content increases [6,7]. This effect is of interest, in part because the BCAAs share with TRP and TYR a competitive transport carrier across the blood-brain barrier (BBB); variations in blood BCAA levels can thereby indirectly influence TRP and TYR levels in brain and thus the rates of 5HT and catecholamine synthesis [9-12]. In addition, leucine (LEU) directly stimulates the mammalian target of rapamycin (mTOR) signaling pathway in brain (hypothalamus) appetite neurons and as a result modifies food intake in rats [13].

However, there are limitations to existing chronic studies in rats, namely, that sampling occurred at only a single time point over the 24-hour period, the time of day at which animals were killed differed among studies, and the feeding conditions were quite different [6-8]. Hence, one gains only a narrow view of the potential relationship of chronic dietary protein intake to brain amino acid levels. Most notably, one cannot infer from such studies how the timing of meals impacts on such relationships, particularly in relation to eating patterns over the 24-hour period. In this latter regard, the temporal pattern of the nutrient intake of rats (eg, wild rats foraging in their natural habitat) is not known; studies of the eating habits of wild rats are restricted to the analysis of stomach contents of captured animals [14,15]. Thus, one cannot design a study that reasonably approximates the animal's natural eating patterns to ascertain how such natural dietary variations impact chemically on the brain. However, such a laboratory study can be conducted in nonhuman primates. Primates can bear chronic intravenous and intraventricular cannulas, allowing blood and cerebrospinal fluid (CSF) (a reflection of brain extracellular fluid [16]) to be sampled repeatedly over the 24-hour period. Such a model also allows the animal to be used for repeated measures under a variety of experimental conditions (here, multiple changes in chronic protein intake). Furthermore, unlike the rat, the primate diet in the wild has been extensively studied both diurnally and seasonally [17-19]. The findings permit the construction of a laboratory primate diet similar to that in the wild with which to examine the impact of chronic dietary protein intake on blood and CSF amino acid levels. We used such an approach in young macaques, initially focusing on TRP and the 5HT metabolite 5-hydroxyindoleacetic acid in plasma and CSF. We observed that CSF TRP concentrations increased chronically with increasing dietary protein intake and were accompanied by like changes in CSF concentrations of 5-hydroxyindoleacetic acid, an index of 5HT synthesis and/or turnover [20]. Because of recent interest in the relationship of TYR, LEU, and also HIS to food intake regulation, we now report results for plasma and CSF concentrations of aromatic, branched-chain, and basic amino acids. Although signaling molecules derived from or linked to these amino acids have not been measured in this study (such would have required sacrificing the animals), one object was to assess if variations in the CSF levels of these amino acids were large enough to stimulate interest in examining downstream signaling molecules (eg, catecholamines, histamine, mTOR). In addition, a second objective was to evaluate if CSF concentrations of the large neutral amino acids (LNAAs) were predicted by the effect of the diet on plasma LNAAs, as represented by a particular expression of their competitive transport across the BBB, the brain influx parameter of Pardridge [21]. All measurements were made at the time of the original study, but have not been reported previously (except for plasma valine concentrations [20]).

2. Materials and methods

2.1. Animals

Juvenile male rhesus monkeys (*Macaca mulatta*, N = 10) were studied. They were bred at the University of Pittsburgh Primate Research Laboratory and, at the time experiments commenced, were 21.5 ± 2.8 months of age and weighed 2.9 ± 0.4 kg (mean ± SE). Before study, they were housed individually in a controlled environment; lighting was provided 12 hours daily (7:00 AM-7:00 PM), and the ambient temperature was maintained at 24°C. Food (Monkey Chow 5045, 25% protein; PMI Ralston Purina, St Louis, MO) and water were available ad libitum; fresh food was provided daily at 11:00 AM, when the old food was removed. The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all experiments.

2.2. Catheterization

Subclavian venous catheters (0.03 in internal diameter × 0.065 in outside diameter Silastic tubing; Dow-Corning, Midland, MI) were implanted under anesthesia as previously described [20]. The free end of the catheter was passed through a flexible tether and connected to a swivel (Alice King Chatham, Tarzana, CA) at the top of each animal's cage, from which it passed a short distance into an adjacent room in which sampling and drug administration occurred. Patency was maintained via constant infusion (100 mL/d) of sterile saline containing 4U/mL sodium heparin. The monkeys were maintained in nylon jackets to prevent access to the catheters. Experiments began 3 weeks after catheterization.

Cerebrospinal fluid cannulas were inserted into the subarachnoid space of the spinal canal under ketamine anesthesia as previously described [20]. Silastic tubing (0.03 in internal diameter × 0.065 in outside diameter) was attached to the end of the catheter and brought subcutaneously to an interscapular exit site, where it passed through

the tether and swivel into the adjoining room. A small volume of CSF was removed daily to maintain catheter patency. After catheter insertion, monkeys recovered for 2 days before sampling. After the sampling period, which lasted 48 hours, the CSF catheters were removed under ketamine sedation. Cerebrospinal fluid catheters were implanted 4 times in each animal during the last week on each of the 4 experimental diets.

2.3. Sampling

During collection periods, blood samples (0.5 mL each) were collected hourly (except as noted) over 2 consecutive days; and the plasmas were separated and stored as previously described [20]. The red blood cells were resuspended in sterile saline and reinfused through the catheters every 2 hours. Using this procedure, the change in hematocrit over a 24-hour period rarely exceeded 5%. Cerebrospinal fluid samples (0.5 mL each) were collected, aliquoted, and stored at −80°C until assayed. The dead space in the CSF catheter and line was about 1 mL; this amount of CSF was drawn and discarded at the onset of each sampling day.

2.4. Experimental diets

Each diet provided monkeys with an energy intake of 180 kcal/kg/d, which is higher than that previously associated with normal health and growth of juvenile *M mulatta* in captivity [22] and approximately equal to that which macaques select in the wild [19,23].

The acclimatization diet consisted of 2 identical meals per day, provided at 9:00 AM and 3:00 PM daily. These meals each consisted of 42 g of Monkey Chow 5045 (25% protein) and 225 g pears (for a 3-kg monkey). The timing of meals approximates the typical diurnal pattern of feeding for monkeys in the wild [17,18]. Pears were chosen because the fiber content is high and similar to that of fruit consumed by monkeys in the wild [24].

All 4 experimental diets used the same morning meal, consisting of 450 g pears (266 kcal, 1.76 g protein). The first experimental diet (22.6% protein [percentage of energy], calculated here for a 3-kg monkey, the typical size) used an afternoon meal of 84 g of monkey chow (35% protein; catalog 5739C-E, PMI, Test Diet Laboratory, Ralston Purina). Each day, this diet provided 31.2 g/d protein, 99.5 g/d carbohydrate (CHO), and 4.8 g/d fat. The afternoon meal for the second experimental diet (16.4% protein) used Purina Monkey Chow 5045 (25% protein). Each day, the second diet provided 22.8 g/d protein, 109.8 g/d CHO, and 4.2 g/d fat. The afternoon meal for the third diet (9.7% protein) consisted of an afternoon meal of 42 g Purina Monkey Chow 5045 and 225 g pears. Overall, this diet provided 13.1 g/d protein, 122.9 g/d CHO, and 2.1 g/d fat. The afternoon meal for the fourth diet (6.2% protein) consisted of 21 g Purina Monkey Chow 5045 and 340 g pears. This diet provided 8.3 g/d protein, 129.8 g/d CHO, and 1.1 g/d fat. On each diet, the monkeys were at or above their known requirements for vitamins and minerals [22].

2.5. Experimental design

Food intake was monitored daily, and body weight was recorded weekly. Water was available at all times, ad libitum. Initially, monkeys were acclimated for 4 weeks to the regimen of receiving 2 meals a day; during this period, they ingested the acclimatization diet. Thereafter, they began 4 sequential 4-week periods during which they ingested each of the experimental diets, beginning with the highest-protein diet and proceeding to the lowest (22.6% → 16.4% → 9.7% → 6.2%). During the fourth week on each diet, the CSF catheters were implanted, the monkeys recovered for 2 days, and then blood and CSF samples were collected over 2 sequential 24-hour periods, as described above. Blood and CSF samples were collected at 8:00 AM, 9:00 AM, 10:00 AM, 11:00 AM, 12:00 PM, 2:00 PM, 3:00 PM, 4:00 PM, 5:00 PM, 6:00 PM, and 12:00 AM; CSF samples were also collected at 10:30 PM.

2.6. Analytical methods

Plasma and CSF samples were assayed for TYR, phenylalanine (PHE), LEU, isoleucine (ILE), valine (VAL), lysine (LYS), HIS, and arginine (ARG) using a Beckman 6300 amino acid analyzer (Beckman Instruments, Palo Alto, CA). Glucosaminic acid was used as the internal standard; samples were prepared as described previously [20]. Separate aliquots of serum were assayed fluorometrically for TRP [25-27]. Measurements of TRP, TYR, PHE, and the BCAAs were made at the time of the study; measurements of the basic amino acids (LYS, HIS, ARG) were made 1 year later from samples that had been continuously frozen at −80°C. Values for each amino acid examined are very similar to those obtained previously for human CSF (such measurements appear not to have been made previously in monkeys) [28]. For each LNAA, the brain influx value (in nanomoles per gram per minute), a measure of competitive transport across the BBB, was calculated using the equation described originally by Pardridge [21,29,30], and kinetic parameters for neutral amino acid transport obtained in human blood vessels [31].

2.7. Statistics

The choice of sample size (N = 10) was based on a power analysis using 24-hour data for plasma amino acids from humans subjected chronically to diets varying in protein content [32]. For each variable quantitated, the data obtained on each diet were pooled for each animal to obtain a mean concentration for each of 5 time bins (represented as boxes in the figures): bin 1—8:00 AM and 9:00 AM values (pre–AM-meal bin); bin 2—10:00 AM, 11:00 AM, and 12:00 PM values (post–AM-meal bin); bin 3—2:00 PM and

3:00 PM values (pre–PM-meal bin); bin 4—4:00 PM, 5:00 PM, and 6:00 PM values (post–PM-meal bin); and bin 5—12:00 AM (for plasma), or 10:30 PM and 12:00 AM (for CSF) values (late-night bin). Comparisons among dietary treatments and time of day were then made on the reduced data using a 2-way analysis of variance for repeated measures. Selected treatment and time comparisons were then made using the least squared means test with the Bonferroni correction. Results were considered significant when P was less than .05. Because no day effect was present, the data are pooled from the 2 sampling days and represented in the figures as a single 24-hour period. All data are reported as the mean ± SE.

3. Results

As reported previously, the monkeys received 180 kcal/kg/d but, after acclimation to the diets, elected to consume about 150 kcal/kg/d [20]. They showed a small, progressive weight gain throughout the study, gaining about 10% over their initial body weight by the end of the 16-week period [20]. All appeared healthy and active.

3.1. Tyrosine

Statistically significant effects for both time of day and dietary protein content were noted for plasma TYR, TYR brain influx (a predictor of competitive TYR uptake into brain [21]), and CSF TYR (see legend to Fig. 1 for F values and levels of significance). All diet-by-time interaction terms were also statistically significant. Plasma TYR concentrations declined significantly after the morning meal of fruit on each diet (comparing for each diet time bin 1 [prebreakfast time points; box 1 in Fig. 1] and time bin 2 [time points after breakfast presentation; box 2 in Fig. 1]). After the afternoon, protein-containing meal, plasma TYR concentrations rose significantly soon after meal presentation for each diet (comparing for each diet time bin 3, which preceded the meal [box 3 in Fig. 1, top panel], and time bin 4 [box 4 in Fig. 1]). Plasma TYR remained significantly elevated well into the evening (comparing for each diet time bin 3 and time bin 5 [box 3 and box 5 in Fig. 1, top panel]). Tyrosine brain influx declined significantly in response to the morning meal of fruit under all but the lowest-protein diet regimen and increased in response to the afternoon (protein-containing) meal in these same dietary groups (Fig. 1, middle panel). Cerebrospinal fluid TYR concentrations revealed no significant change in response to the morning meal under all but the 16.4%-protein diet regimens (Fig. 1, bottom panel, white circles, box 1 vs box 2). Soon after the afternoon meal, CSF TYR increased significantly only when the highest level of protein was consumed (Fig. 1, bottom panel, black circles, box 3 vs box 4); however, several hours later, values were significantly higher than those before the meal at all but the lowest dietary protein level (Fig. 1, bottom panel, box 3 vs box 5).

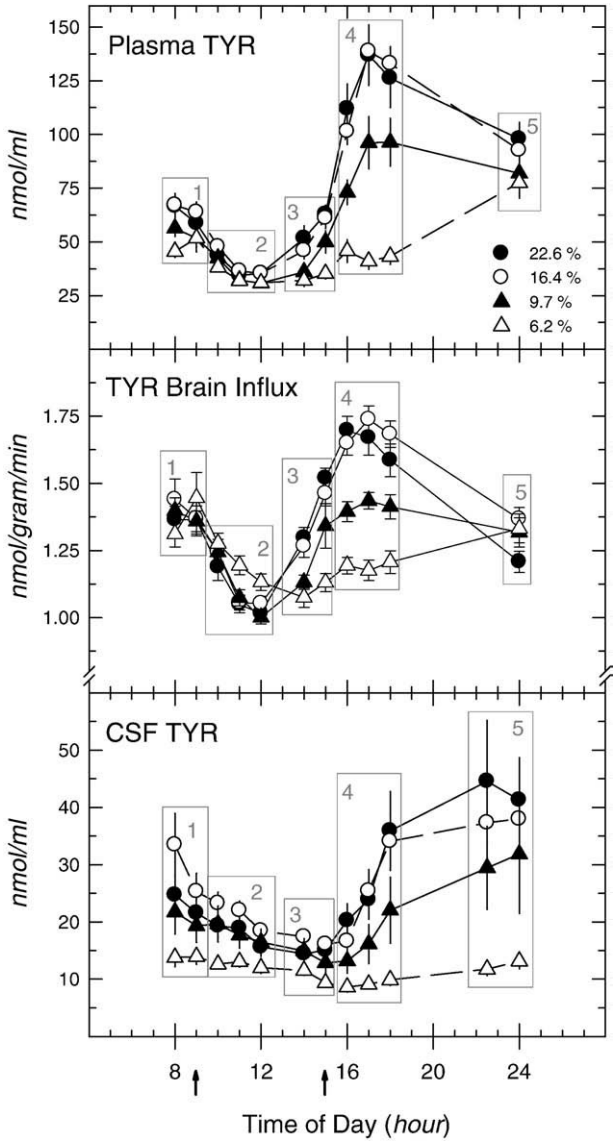

Fig. 1. Effect of chronic dietary protein intake on plasma and CSF TYR levels, and TYR brain influx. Data are means ± SE (N = 10). By analysis of variance, significant effects of both diet (F = 26.91, $P < .01$) and time (F = 91.39, $P < .01$) were noted on plasma TYR; the interaction was also significant (F = 9.35, $P < .01$). Significant effects of both diet (F = 5.62, $P < .02$) and time (F = 23.82, $P < .01$) were noted on TYR brain influx; the interaction was also significant (F = 10.44, $P < .01$). Significant effects of both diet (F = 5.95, $P < .01$) and time (F = 32.37, $P < .01$) were noted on CSF TYR; the interaction was also significant (F = 3.29, $P < .05$). The symbols are as follows: 22.6% protein, black circles; 16.4% protein, white circles; 9.7% protein, black triangles; 6.2% protein, white triangles. The boxes in the panels indicate the data groupings in time bins used for statistical comparisons (see "Statistics" section in "Materials and Methods"). The arrows indicate the times of day when food was presented: 9:00 AM and 3:00 PM. See "Materials and Methods" for additional details.

3.2. Phenylalanine

Statistically significant effects for both time of day and dietary protein content were noted for plasma PHE, PHE brain influx, and CSF PHE (see legend to Fig. 2 for

F values and levels of significance). Except for CSF PHE, all of the diet-by-time interaction terms were statistically significant (legend to Fig. 2). Plasma PHE concentrations declined significantly after the morning meal of fruit when monkeys ingested the 2 highest, but not the 2 lowest, levels of protein intake (box 1 vs box 2 in Fig. 2). After the afternoon meal, plasma PHE concentrations rose significantly, regardless of meal protein content, comparing premeal values (box 3) with those soon after (box 4) or several hours after (box 5) presentation of the meal. On each diet, PHE brain influx rose significantly after the morning meal; it declined significantly in the 3 hours immediately after presentation of the afternoon meal (for all but the lowest-protein diet). The late-night values differed significantly from those immediately preceding presentation of the afternoon meal (pre-PM bin) in all dietary protein groups. Cerebrospinal fluid PHE concentrations declined significantly after the morning meal of fruit

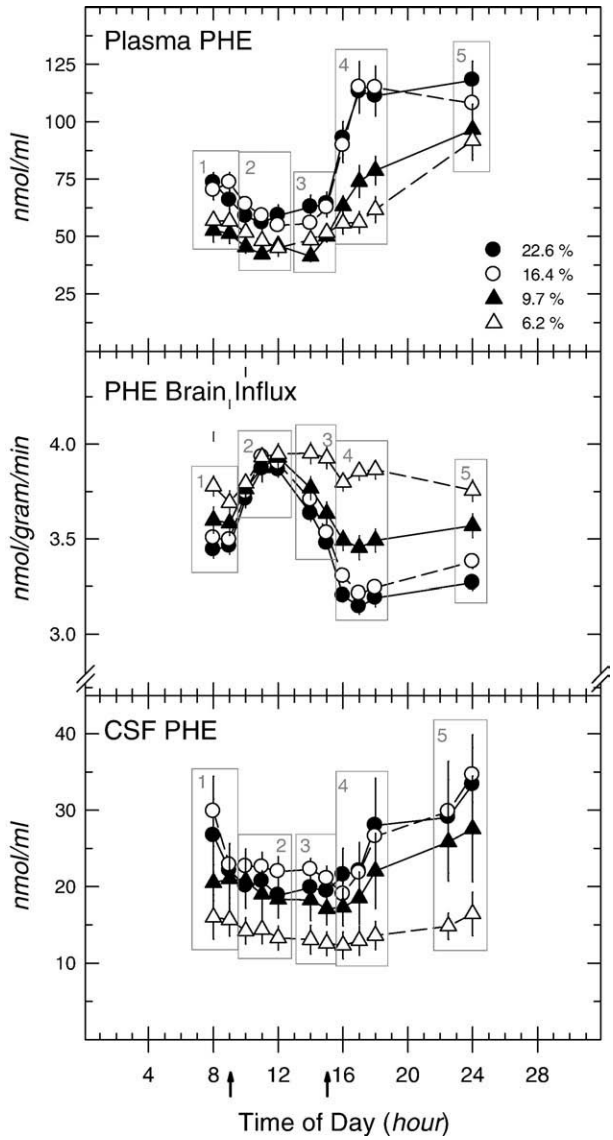

Fig. 2. Effect of chronic dietary protein intake on plasma and CSF PHE levels, and PHE brain influx. Data are means ± SE (N = 10). By analysis of variance, significant effects of both diet (F = 12.76, $P < .01$) and time (F = 83.42, $P < .01$) were noted on plasma PHE; the interaction was also significant (F = 5.80, $P < .01$). Significant effects of both diet (F = 16.10, $P < .01$) and time (F = 45.37, $P < .01$) were noted on PHE brain influx; the interaction was also significant (F = 10.33, $P < .01$). Significant effects of both diet (F = 3.08, $P < .05$) and time (F = 21.35, $P < .01$) were noted on CSF PHE, although the interaction was not significant (F = 1.73, $P > .05$). See legend to Fig. 1 for additional details.

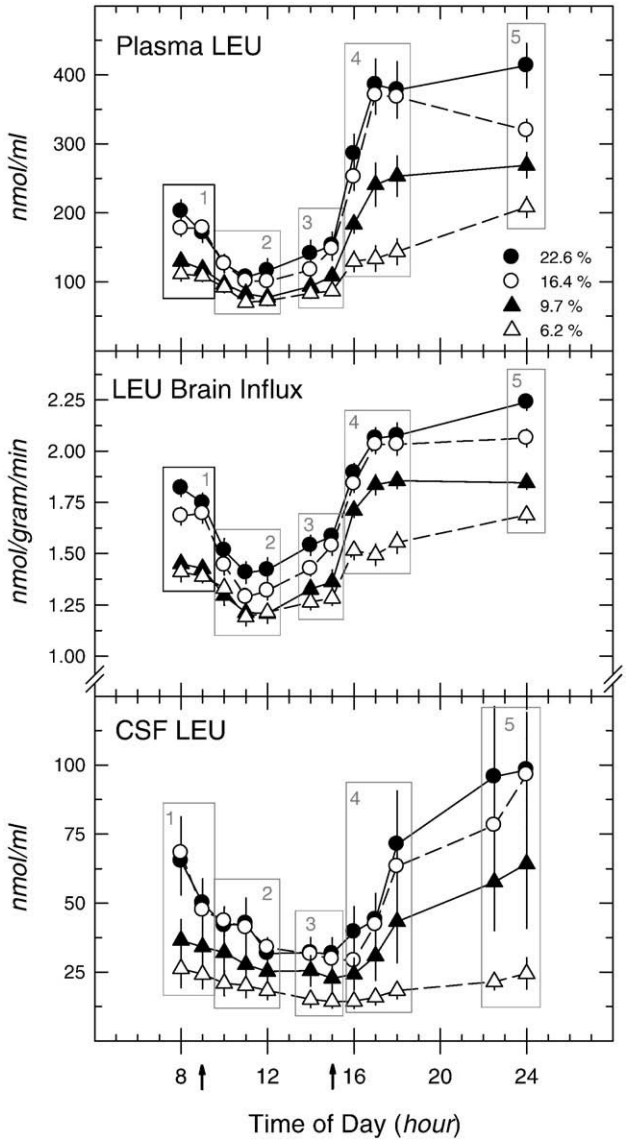

Fig. 3. Effect of chronic dietary protein intake on plasma and CSF LEU levels, and LEU brain influx. Data are means ± SE (N = 10). By analysis of variance, significant effects of both diet (F = 29.57, $P < .01$) and time (F = 93.33, $P < .01$) were noted on plasma LEU; the interaction was also significant (F = 9.85, $P < .01$). Significant effects of both diet (F = 18.44, $P < .01$) and time (F = 90.59, $P < .01$) were noted on LEU brain influx; the interaction was also significant (F = 4.26, $P < .01$).Significant effects of both diet (F = 5.07, $P < .01$) and time (F = 35.87, $P < .01$) were noted on CSF LEU; the interaction was also significant (F = 3.84, $P < .01$). See legend to Fig. 1 for additional details.

only when monkeys ingested the highest-protein diet (box 1 vs box 2, black circles in Fig. 2). In the afternoon, CSF PHE concentrations did not rise significantly, at any level of protein intake, soon after meal presentation (box 3 vs box 4); but later in the evening, values were significantly higher than premeal values at each level of dietary protein (box 3 vs box 5).

3.3. Leucine

Statistically significant effects for both time of day and dietary protein content were noted for plasma LEU, LEU brain influx, and CSF LEU (see legend to Fig. 3 for F values and levels of significance). The diet-by-time interaction terms were also statistically significant. Plasma LEU concentrations declined significantly after the morning meal when monkeys ingested the 2 highest, but not the 2 lowest, levels of protein intake (box 1 vs box 2 in Fig. 3, top panel). After the afternoon meal, plasma LEU concentrations rose significantly, regardless of meal protein content, comparing premeal values (box 3) with those soon after (box 4) or several hours after (box 5) presentation of the meal. On each diet, LEU brain influx declined significantly after the morning meal and was increased significantly above pre–afternoon-meal values during the 3 hours immediately after presentation of the afternoon meal and late at night (Fig. 3, middle panel). Cerebrospinal fluid LEU concentrations declined significantly after the morning meal only when monkeys ingested the 2 highest-protein diets (box 1 vs box 2, white and black circles in bottom panel of Fig. 3). In the afternoon, CSF LEU concentrations did not rise significantly, except at the highest level of protein intake, soon after meal presentation (box 3 vs box 4, black circles); but later in the evening, values were significantly higher than premeal values at all but the lowest level of dietary protein (box 3 vs box 5).

3.4. Isoleucine

Statistically significant effects for both time of day and dietary protein content were noted for plasma ILE, ILE brain influx, and CSF ILE (see legend to Fig. 4 for F values and levels of significance). The diet-by-time interaction terms were also statistically significant. Plasma ILE concentrations declined significantly after the morning meal when monkeys ingested the 2 highest, but not the 2 lowest, levels of protein intake (box 1 vs box 2 in Fig. 4, top panel). After the afternoon meal, plasma ILE concentrations rose significantly when all but the lowest level of protein were ingested, comparing premeal values (Fig. 4, top panel, box 3) with those soon after (box 4) meal presentation, and rose significantly for all diets, comparing premeal values (box 3) with those several hours after (box 5) presentation of the afternoon meal. Isoleucine brain influx declined significantly after the morning meal only when the highest–protein-content diet was consumed. After the afternoon meal, the change in the ILE brain influx was significant only for the 2 highest–protein-content diets; a significant late-night effect was present only when monkeys were consuming the 22.6%- or the 9.7%-protein diets (Fig. 4, middle panel). Cerebrospinal fluid ILE concentrations declined significantly after the morning meal only when monkeys ingested the highest-protein diet (box 1 vs box 2, black circles in bottom panel of Fig. 4). In the afternoon, CSF ILE concentrations did not rise significantly soon after meal presentation, except at the

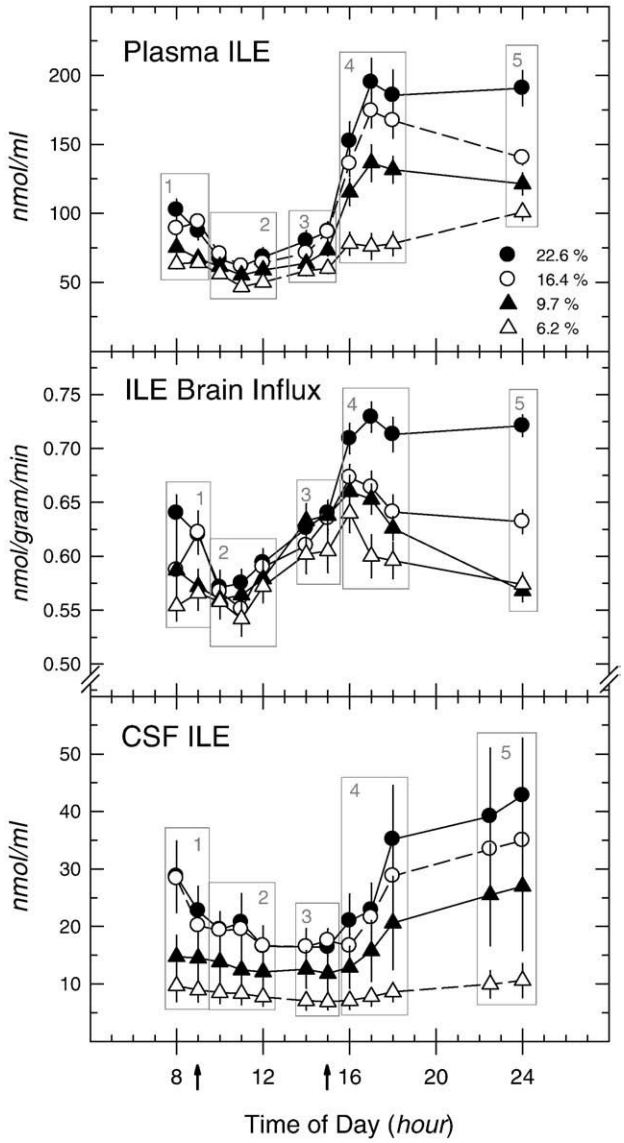

Fig. 4. Effect of chronic dietary protein intake on plasma and CSF ILE levels, and ILE brain influx. Data are means ± SE (N = 10). By analysis of variance, significant effects of both diet ($F = 32.25$, $P < .01$) and time ($F = 78.08$, $P < .01$) were noted on plasma ILE; the interaction was also significant ($F = 11.54$, $P < .01$). Significant effects of both diet ($F = 8.12$, $P < .01$) and time ($F = 21.48$, $P < .01$) were noted on ILE brain influx; the interaction was also significant ($F = 6.09$, $P < .01$). Significant effects of both diet ($F = 4.81$, $P < .01$) and time ($F = 29.17$, $P < .01$) were noted on CSF ILE; the interaction was also significant ($F = 4.78$, $P < .01$). See legend to Fig. 1 for additional details.

highest level of protein intake (box 3 vs box 4, black circles). Later in the evening, however, CSF ILE values were significantly higher than premeal values at all but the lowest level of dietary protein (box 3 vs box 5).

3.5. Valine

Statistically significant effects for both time of day and dietary protein content were noted for plasma VAL, VAL brain influx, and CSF VAL (see legend to Fig. 5 for F values and levels of significance). The diet-by-time interaction terms were also statistically significant. Plasma VAL concentrations declined significantly after the morning meal when monkeys ingested the 2 highest, but not the 2 lowest, levels of protein intake (box 1 vs box 2 in Fig. 5, top panel). After the afternoon meal, plasma ILE concentrations rose significantly when all but the lowest level of protein were ingested, comparing premeal values (Fig. 5, top panel, box 3) with those soon after (box 4) meal presentation, and rose significantly for all diets, comparing premeal values (box 3) with those several hours after (box 5) meal presentation. Valine brain influx showed no significant variation after the morning meal, regardless of dietary protein content (Fig. 5, middle panel). In the first 3 hours after presentation of the afternoon meal, the VAL brain influx declined significantly (vs values immediately preceding the afternoon meal) when monkeys ingested the 2 lowest–protein-content diets, but did not change significantly when they ingested the 2 highest–protein-content diets. Late-night values differed significantly from values just before the afternoon meal in all diet groups except the 9.7%-protein diet. Cerebrospinal fluid VAL concentrations declined significantly after the morning meal when monkeys ingested the 2 highest–protein-

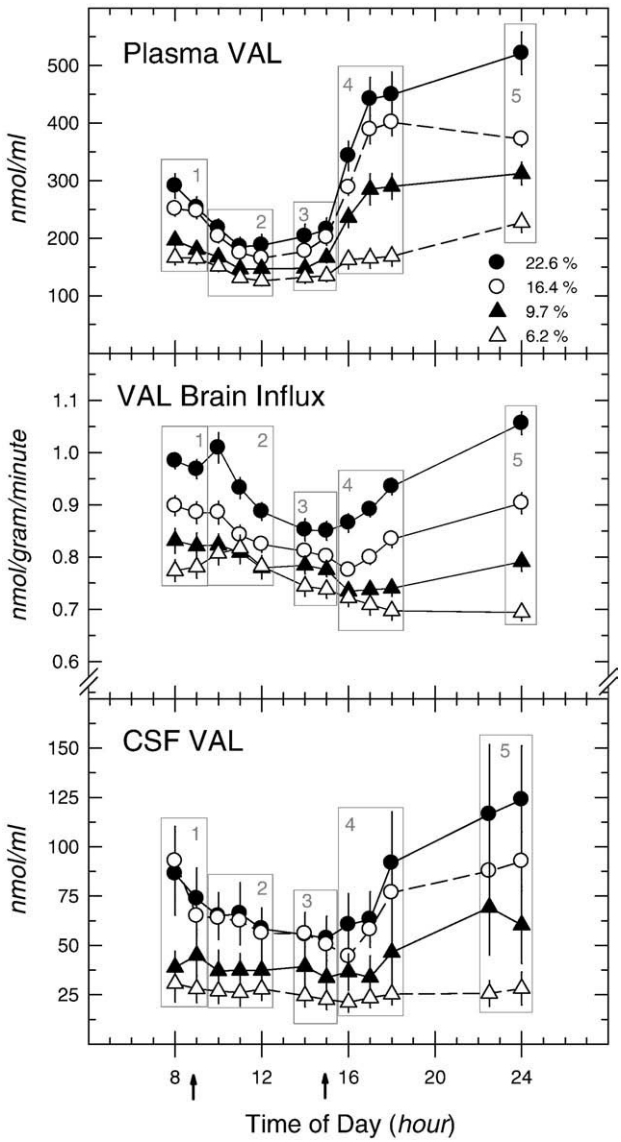

Fig. 5. Effect of chronic dietary protein intake on plasma and CSF VAL levels, and VAL brain influx. Data are means ± SE (N = 10). By analysis of variance, significant effects of both diet (F = 35.32, $P < .01$) and time (F = 109.06, $P < .01$) were noted on plasma VAL; the interaction was also significant (F = 15.90, $P < .01$). Significant effects of both diet (F = 28.44, $P < .01$) and time (F = 28.11, $P < .01$) were noted on *VAL* brain influx; the interaction was also significant (F = 11.34, $P < .01$). In addition, significant effects of both diet (F = 5.14, $P < .01$) and time (F = 20.26, $P < .01$) were noted on CSF VAL; the interaction was also significant (F = 4.60, $P < .01$). See legend to Fig. 1 for additional details.

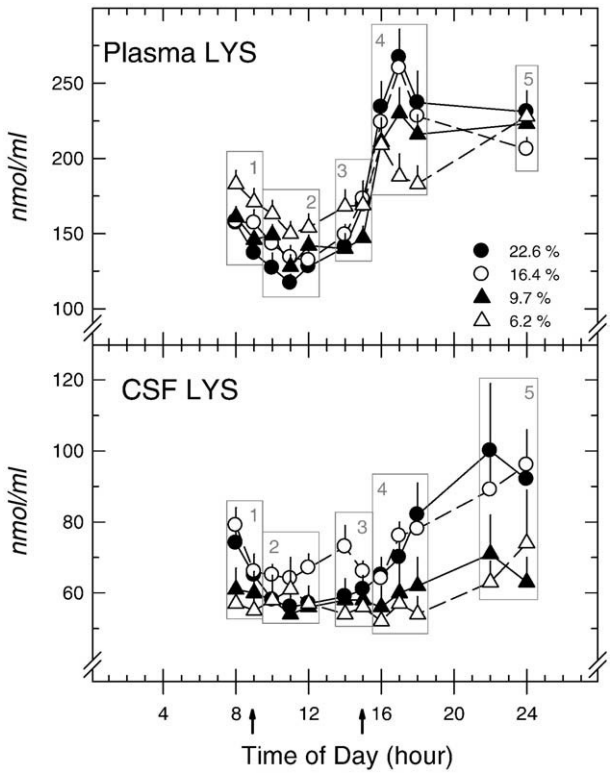

Fig. 6. Effect of chronic dietary protein intake on plasma and CSF LYS levels. Data are means ± SE (N = 10). By analysis of variance, a significant effect of time (F = 21.29, $P < .01$), but not diet, was noted on plasma LYS; the interaction was also significant (F = 3.48, $P < .05$). A significant effect of time (F = 13.55, $P < .01$), but not diet, was noted for CSF LYS; the interaction was not significant. See legend to Fig. 1 for additional details.

content diets (box 1 vs box 2, black and white circles in bottom panel, Fig. 5). In the afternoon, CSF VAL concentrations did not rise significantly soon after meal presentation, except at the highest level of protein intake (box 3 vs box 4, black circles). Later in the evening, CSF VAL values were significantly higher than premeal values when animals ingested the 2 highest–protein-content diets (black and white circles, box 3 vs box 5).

3.6. Lysine

A statistically significant effect of time of day, but not dietary protein content, was noted for plasma and CSF LYS concentrations (see legend to Fig. 6 for F values and levels of significance). The diet-by-time interaction term was statistically significant for plasma, but not CSF. Plasma LYS concentrations declined significantly after the morning meal only when monkeys consumed the lowest–protein-content diet (box 1 vs box 2 in Fig. 6, top panel, white triangles) and increased significantly in the first 3 hours after presentation of the afternoon meal only when animals ingested either of the 2 intermediate–protein-content diets (box 3 vs box 4, white circles and black triangles). The late-night plasma LYS values differed significantly from those preceding the afternoon meal for all except the lowest–protein-content diet (box 3 vs box 5). Cerebrospinal fluid LYS concentrations did not change significantly after the morning meal for any of the diets. In the afternoon, CSF LYS rose significantly soon after meal presentation only at the highest level of protein intake (box 3 vs box 4, black circles). In the evening, CSF LYS values were significantly higher than premeal values only when the highest–protein-content diet was ingested (box 3 vs box 5).

3.7. Histidine

Statistically significant effects of diet and time of day were noted for plasma HIS concentrations, and the diet-by-time interaction was also significant (see legend to Fig. 7 for F values and levels of significance). For CSF HIS, a statistically significant effect of time of day, but not diet, was present; and the interaction was not significant. Plasma HIS concentrations changed significantly after the morning meal only in the 22.6%-protein group (black circles, Fig. 7, top panel, box 1 vs box 2). Soon after presentation of the afternoon meal, plasma HIS rose significantly at all but the highest level of dietary protein intake (box 3 vs box 4). Late-night values differed significantly from pre–afternoon-meal values for all but the lowest–protein-content diet (box 3 vs box 5). Cerebrospinal fluid HIS concentrations showed no significant change after the morning meal, except on the highest–protein-content diet (black circles, Fig. 7, bottom panel, box 1 vs box 2). After presentation of the afternoon meal, a significant change occurred during the first 3 hours only when monkeys were on the 9.7%-protein diet (a decline: black triangles, box 3 vs box 4); no significant changes occurred in the late-night values

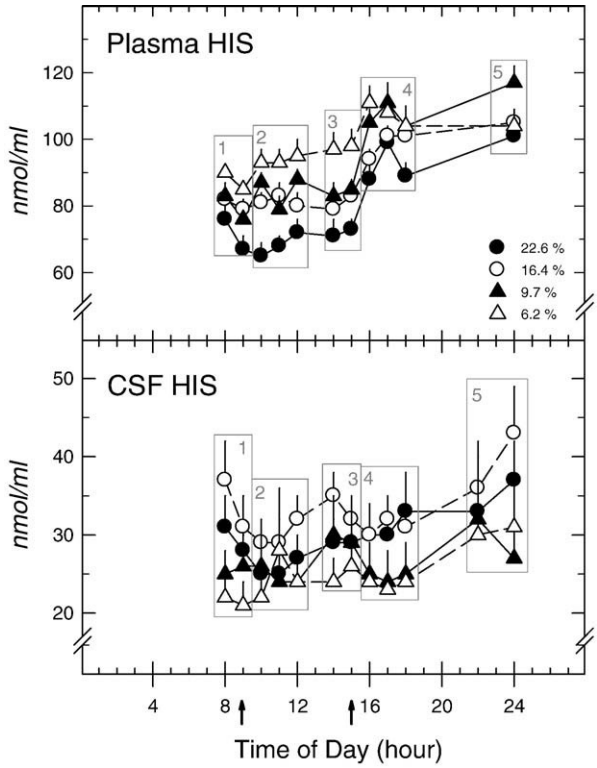

Fig. 7. Effect of chronic dietary protein intake on plasma and CSF HIS levels. Data are means ± SE (N = 10). By analysis of variance, significant effects of diet ($F = 26.79$, $P < .01$) and time ($F = 4.10$, $P < .05$) were noted on plasma HIS; the interaction was also significant ($F = 3.48$, $P < .05$). A significant effect of time ($F = 5.40$, $P < .05$), but not diet, was noted on CSF HIS. The interaction term was not significant. See legend to Fig. 1 for additional details.

(relative to pre–afternoon-meal values) on any of the diets (box 3 vs box 5).

3.8. Arginine

A statistically significant effect of time of day, but not dietary protein content, was noted for plasma and CSF ARG concentrations (see legend to Fig. 8 for F values and levels of significance). The diet-by-time interaction term was statistically significant for plasma, but not CSF. Plasma ARG concentrations declined significantly after the morning meal when monkeys ingested the 2 highest, but not the 2 lowest, levels of protein intake (black and white circles in Fig. 8, top panel, box 1 vs box 2). In the afternoon, plasma ARG concentrations rose significantly at each level of dietary protein intake when measured soon after meal presentation (box 3 vs box 4) or several hours later (box 3 vs box 5). Cerebrospinal fluid ARG concentrations changed (declined) significantly after the morning meal only when monkeys consumed the highest–protein-content diet (black circles in Fig. 8, bottom panel, box 1 vs box 2). After presentation of the afternoon meal, no significant change was observed during the first 3 hours on any of the diets (box 3 vs box 4). A significant increase (relative to pre–afternoon-meal values)

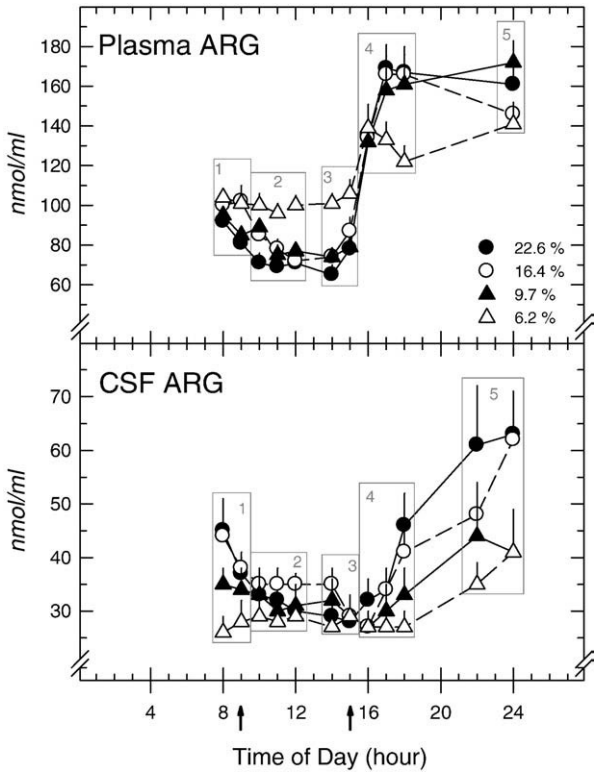

Fig. 8. Effect of chronic dietary protein intake on plasma and CSF ARG levels. Data are means ± SE (N = 10). By analysis of variance, a significant effect of time ($F = 48.54$, $P < .01$), but not diet, was noted on plasma ARG; the interaction was also significant ($F = 3.86$, $P < .05$). A significant effect of time ($F = 40.03$, $P < .01$), but not diet, was noted on CSF ARG. The interaction term was not significant. See legend to Fig. 1 for additional details.

in late-night values occurred when monkeys consumed the 2 highest–protein-content diets (box 3 vs box 5, black and white circles).

4. Discussion

These results reveal marked variations in the plasma and CSF concentrations of the LNAA as a function of time of day and chronic dietary protein content. Smaller effects were observed for the basic amino acids. The study design involved varying dietary protein content chronically in the context of a diurnal pattern of food consumption similar to that experienced by monkeys in the wild: ingestion of CHO foods in the morning and protein-containing foods in the afternoon/evening [17,18]. Generally speaking, LNAA values in plasma and CSF tended to fall over the course of the morning hours (8:00 AM-noon), when fresh pears (largely CHOs) were present. This effect was most evident when monkeys consumed the higher levels of dietary protein and least evident when they ingested the lowest level of dietary protein. In many cases, marked LNAA elevations occurred later in the afternoon and evening, after presentation (3:00 PM) of the protein-containing diet. These effects were also most evident when the dietary protein content was highest and least evident when it was low. Dietary protein content thus clearly had an impact on the plasma and CSF levels of the LNAAs. Because effects were most notable during the evening hours, when protein-containing food was being ingested, time of day and the temporal pattern of protein ingestion were clearly important factors in observing them.

The findings show similarities with those conducted previously in rats. Of note, in studies examining chronic protein ingestion in which rats were fed a single diet ad libitum and killed at the end of the daily dark period (ie, the end of the daily feeding period), serum and brain levels of TYR and PHE rose as dietary protein content was increased from very low levels to levels modestly above requirement and then plateaued even as protein content was stepped to high levels [6,7]. This was also evident in this study of plasma and CSF and is most clearly observed between the hours of 4:00 PM and 12:00 AM, which would correspond most closely to the diurnal feeding times examined in rats. Note in Figs. 1 and 2, for example, that there is little difference in plasma and CSF TYR and PHE values during this time interval between values obtained during ingestion of the highest (22.6%, black circles) and next highest (16.4%, white circles) levels of protein intake, whereas values drop notably as protein content steps to the next lower (9.7%, black triangles) and then the lowest (6.2%, white triangles) levels. In contrast, the rat studies observed that serum and brain values of the BCAA continued to increase as protein content rose beyond values at which TYR and PHE levels plateaued. This relationship is also evident for plasma and CSF in the present study, most notably during the afternoon and evening hours (Figs. 3-5; note that BCAA values at the highest [black circles] and next highest [white circles] protein intake levels are separated). In contrast, in rat studies, the basic amino acids (LYS, HIS, ARG) showed relatively unremarkable changes in serum as a function of chronic dietary protein intake (when studied at the indicated time of day) [6,7], an effect also evident in monkeys during afternoon and evening hours (Figs. 6-8). Of interest, HIS values in rat serum tended to fall with increasing levels of protein intake [6,7], an effect also observed in the primates at almost any time of day or night (Fig. 7). Of course, plasma levels of each basic amino acid rose during the afternoon and evening in the present study, in association with protein ingestion; but this effect would not be evident in single–time-point rat studies. The one distinction between rat brain levels and primate CSF levels appears to be for the basic amino acids: in rats, brain levels of LYS rose with increasing protein content, ARG values varied inconsistently, and HIS concentrations declined (measured at the end of the eating period) [6,7]. However, in primate CSF, in the late evening (end of the eating period), levels of LYS, HIS, and ARG all tended to *rise* with dietary protein content, although the main effect of diet was not statistically significant (Figs. 6-8).

Amino acids are transported from blood into brain across the BBB [16]. The LNAAs share a common carrier that is saturable and competitive [16]. The uptake into brain (and concentrations) of each therefore depends on the blood concentrations of all the LNAAs, expressed in a manner that captures the competitive effects. Reliable expressions have included simply the ratio of the plasma or serum concentration of any one LNAA to the sum of the concentrations of its LNAA competitors (eg, for TYR, TYR/ΣLNAA) or a ratio that accounts for the different affinity of each LNAA for the transport carrier (termed the *brain influx*) [29,30,33,34]. These expressions of LNAA transport at the BBB have been used repeatedly to examine the relationship of transport at the BBB on free LNAA concentrations in the brains of rats (eg, Fernstrom and Faller [30]). The present study afforded the opportunity to evaluate if CSF LNAA concentrations reflect an index of competitive BBB uptake, and thus possibly brain free LNAA concentrations, in a species in which frequent, direct biopsy of brain tissue is not permissible. How well do the data support this possibility? Figs. 1 to 5 include data for the brain influx for each LNAA; and in almost all cases, these predictors of BBB LNAA transport mirror quite well the CSF concentrations obtained, both acutely and chronically. Thus, brain influxes generally decline during the morning hours, after presentation of the morning meal, in parallel with CSF changes, and rise in the late afternoon and evening, again in parallel with CSF changes. Moreover, brain influx values also change chronically with dietary protein content in parallel with CSF concentrations. The lone exception is PHE, where influx values failed to follow CSF PHE concentrations (Fig. 2). The effect is most observable during the afternoon/evening, after ingestion of protein-containing meals. It is interesting that afternoon/evening values for plasma PHE and PHE brain influx behaved very similarly to that observed in rats after ingestion of a protein meal [30]: in both species, plasma PHE rises with the protein content of the meal, whereas the ratio and the brain influx decline. However, rat brain PHE levels follow these transport predictors [35], whereas in this study, CSF PHE concentrations do not (Fig. 2). There is no reason to suspect that BBB LNAA transport properties for PHE differ between rats and primates [36]. Indeed, PHE clearly shows competitive LNAA transport across the primate BBB [31,37,38]. Hence, the brain influx parameter is presumably predicting BBB PHE transport correctly in macaques. In addition, PHE flux across the blood-CSF barrier (studied in sheep) also occurs via a competitive, saturable LNAA transporter [39]. Phenylalanine brain influx, a reflection of competitive transport, should thus apply to the CSF as well as the brain PHE pool, even if CSF PHE derives from both blood-CSF uptake and brain extracellular fluid (and, indirectly, the BBB). Some data in monkeys, however, suggest that, under circumstances in which competition between PHE and another LNAA (fluoro-dihydroxyphenylalanine) is clearly seen in brain, no such competition is seen in CSF [38]. The issue is not further clarified by available data from subjects with phenylketonuria (PKU), in whom blood PHE concentrations are markedly elevated because of an enzyme deficiency [40]. Although subjects with PKU have very high CSF concentrations of PHE, CSF levels of LEU, ILE, and VAL are not reliably below normal [41,42]. Hence, a clear demonstration in CSF of LNAA transport inhibition by chronically high circulating PHE concentrations is not seen; and yet, CSF PHE concentrations can be reduced in subjects with PKU by dosing them with repeated, large doses of BCAA [42]. Such apparent paradoxes, together with the paucity of CSF studies of amino acids, suggest that further work is needed to unravel this transport puzzle.

For those amino acids where variations in brain level in rats have been reported to be associated with chemical or functional consequences in brain, does the magnitude of variation found here in CSF suggest that such functional differences might be expected in primates? For TYR, which influences catecholamine synthesis in rat retina and hypothalamus, clear effects on catecholamine production are observed when tissue TYR values vary by 2-(hypothalamus) to 4-fold (retina) [8]. Such large differences occurred in monkey CSF, where TYR values differed by 4-fold between the lowest and highest levels of protein intake. Hence, the magnitude of change in CSF TYR levels, if reflective of brain levels, should influence catecholamine production because changes in TYR supply to brain, induced by oral amino acid mixtures, are known to modify catecholamine synthesis in humans [43,44].

For LEU, plasma concentrations varied up to 4-fold (Fig. 3). Cerebrospinal fluid LEU concentrations showed variations of similar magnitude. Leucine promotes muscle protein synthesis, an effect mediated at least partly through the mTOR signaling pathway [45-47]; effects are seen when plasma LEU concentrations are raised only 40% to 50% over control values [47]. Recently, LEU has been reported to stimulate mTOR signaling in hypothalamic appetite neurons and to reduce food intake when administered into the third ventricle [13,48]. Unfortunately, hypothalamic LEU concentrations were not measured. However, if the sensitivity of the mTOR pathway in hypothalamic neurons is similar to that in muscle, perhaps the 4-fold changes in CSF LEU observed in the present study are sufficient to influence mTOR signaling in hypothalamus.

In rats, large doses of HIS reduce food intake and increase brain HIS and histamine pools [49-51]. The rise in brain HIS necessary to elicit a decline in food intake is reported to be 4-fold or more [49]. Serum and brain HIS levels are also observed to increase markedly in rats as chronic dietary protein content falls from 15% to 0%; this effect has been suggested to be linked to the decline in food intake associated with the ingestion of low-protein diets [5,7]. In dietary studies, the observed rise in brain HIS was about 4-fold [5]. Whereas a rise in serum HIS was observed in the present study as dietary protein content declined, CSF HIS concentrations showed no main effect of diet and, if

anything, tended to decline as dietary protein content was reduced (Fig. 7). Hence, CSF levels in macaques do not respond to dietary protein content in a manner like that seen for free brain HIS levels in rats. As discussed above, the concentrations of each LNAA in monkey CSF change markedly in response to dietary protein intake in a manner like that seen for brain free LNAA concentrations in rats [6,7]. It is thus notable that the species comparison fails for HIS. At the very least, it suggests that the diet-related changes in CSF HIS seen here in monkeys are probably insufficient to elicit changes in food intake like those reported in rats.

Tyrosine concentrations in brain and the rate of catecholamine synthesis in hypothalamus increase in adult rats as chronic dietary protein content is raised from 2% to 10% and plateaus at 10% and higher levels of protein intake [8]. The range of 2% to 10% protein intake brackets the adult rat's protein requirement (5%-6%) [52], suggesting that this dietary–amino acid–neurochemical relationship might function as a signal to brain regarding the adequacy of protein intake. This hypothesis seems compatible with the observation that rats in the wild (ie, freely selecting a natural diet) ingest a level of protein modestly above their requirement [2], similar to humans [53]. A similar suggestion might also be made for monkeys. These animals are observed in the wild, their eating habits are recorded, and samples of the foods selected are analyzed for nutritional content. Such analysis shows that they undergo diurnal and circannual variations in protein and caloric intake [17,19]; the circannual variation places them above and below their requirement levels, depending on the time of year [19]. Because the daily and annual pattern of food ingestion in wild monkeys had been defined, we modeled a primate diet according to these eating habits to examine whether an amino acid signal (our primary interest was TYR) observed to vary markedly with chronic protein ingestion in rats would show a similar variation in monkeys under more natural dietary conditions. Not only did CSF TYR show a relationship to chronic dietary protein intake like that seen in the rat, CSF TYR levels were directly related to dietary protein content around the range of the animal's requirement (4.6%-7.5% energy in monkeys and possibly somewhat higher in *M mulatta*) [22]. It is also of interest, however, that CSF LEU values in monkeys were observed to rise with dietary protein content. Leucine administration stimulates hypothalamic mTOR expression and influences food intake in rats [13]. Hence, the possibility emerges that the brain may receive more than a single amino acid signal that reflects the level of protein intake. Such a possibility seems sensible, particularly in monkeys, who obtain their dietary protein almost exclusively from plant sources [54] and thus must select from a variety of plants to obtain sufficient quantities of all essential amino acids. Future studies will hopefully evaluate these possibilities further.

In summary, this study demonstrates that growing macaques undergo marked CSF variations in the concentrations of amino acids, as a function of dietary protein content, that are known to influence neurochemical pathways in brain involved in food intake control. The diurnal variations are most pronounced during the time of day when protein is actively consumed, but generally persist throughout the 24-hour period. Because these variations occur in the range of the animal's protein requirement, one interesting possibility is that they may provide signals to the brain regarding dietary protein adequacy.

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