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Free amino acid and dipeptide changes in the body fluids from Alzheimer's disease subjects

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Summary. Our aim was to determine changes in free amino acid (FAA) and dipeptide (DP) concentrations in probable Alzheimer's disease (pAD) subjects compared with control (CT) subjects using liquid chromatography and electrospray ionization tandem mass spectrometry ($LCMS^2$). We recruited gender- and age-matched study participants based on neurological and neuropsychological assessments. We measured FAAs and DPs in cerebrospinal fluid (CSF), plasma and urine using $LCMS^2$ with selected reaction monitoring (SRM). Imidazole-containing FAAs (histidine, methylhistidine), catecholamines (L-DOPA and dopamine), citrulline, ornithine, glycine and antioxidant DPs (carnosine and anserine) accounted for the major changes between CT and pAD. Carnosine levels were significantly lower in pAD (328.4 ± 91.31 nmol/dl) than in CT plasma (654.23 ± 100.61 nmol/dl). In contrast, L-DOPA levels were higher in pAD (1400.84 ± 253.68) than CT (513.10 ± 121.61 nmol/dl) plasma. These data underscore the importance of FAA and DP metabolism in the pathogenesis of AD. Since our data show changes in antioxidants, neurotransmitters and their precursors, or FAA associated with urea metabolism in pAD compared with CT, we propose that manipulation of these metabolic pathways may be important in preventing AD progression.

Keywords: Amino acids – Dipeptides – Tandem mass spectrometry – Cerebrospinal fluid – Plasma – Alzheimer's disease

Abbreviations: CT, control study participants; pAD, probable Alzheimer's disease study participants; CT_M, control male; CT_F, control female; pAD_M, pAD male; pAD_F, pAD female; MMSE, mini-mental state examination; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; $LCMS^2$, liquid chromatography electrospray ionization tandem mass spectrometry; SRM, selected reaction monitoring; FAA, free amino acid; DP, dipeptide; GABA, γ-aminobutyric acid; βABA, β-aminobutyric acid; ABA, α-aminobutyric acid; DABA, 2,4-diaminobutyric acid; DOPA, 3,4-dihydroxyphenyl-L-alanine

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder with an estimated worldwide prevalence of over eighteen million people, and is predicted to increase with an increasing elderly population (Cummings and Jeste, 1999). AD is characterized by cognitive deficits and memory impairment, and there is currently no cure for this disease. Several genetic, age, demographic and environmental factors are linked to AD pathophysiology (Chapman et al., 2001; Cummings et al., 1998; Doraiswamy et al., 2002; Markesbery and Carney, 1999; Sandbrink et al., 1996). Biochemical processes accounting for neurodegeneration are not known but are likely to include the metabolism of amino acids and dipeptides (Advokat and Pellegrin, 1992; Bowen, 1990; Greenamyre and Young, 1989; Molina et al., 1998). Since FAA are important in neurotransmission, receptor function and are implicated in neurotoxicity, changes in FAA metabolism can be an early indicator of neurodegeneration in AD.

Of all neurotransmitter pathways known in man, the cholinergic pathway has received the most attention in AD research because it is extensively impaired and drugs that increase available acetylcholine improve memory (Cummings and Knopman, 1999). Studies have shown age-dependent decreases in dopamine receptor levels in blood lymphocytes from AD and Parkinson's disease (PD) subjects (Barbanti et al., 2000; Barili et al., 1996; Caronti et al., 1999). However, a comprehensive study of FAA and DP levels has not been undertaken in biological fluids from CT or pAD subjects. Thus, it is not known whether there are changes in other amino acids in pAD. Moreover, various dietary supplements containing amino acids are proposed to influence aging, memory loss and cognitive function, despite sparse scientific data for the efficacy of these supplements.

The aim of these studies was to use $LCMS^2$ to measure the concentrations of FAAs and DPs in the cerebrospinal fluid (CSF), plasma and urine from CT and pAD subjects.

Our data show a decrease in imidazole-containing antioxidants in pAD compared to CT plasma. In pAD plasma, there is a significant increase in L-DOPA, accompanying a decrease in dopamine concentrations when compared with CT levels. There are changes in FAAs associated with urea cycle or detoxification and an increase in urinary glycine in pAD compared with CT. Together, our data show changes in the metabolism of FAAs and DPs linked to anti-oxidation, neurotransmission, and urea metabolism. These changes are not directly related to their medications. These studies also underscore the use of $LCMS^2$ methods to discover metabolites that may be useful biomarkers for disease progression or to elucidate biochemical pathways for therapeutic or nutraceutical intervention in AD.

Materials and methods

Materials

All reagents, solvents, amino acid standards, dipeptide standards, deuterated internal standards, protein dye reagent concentrate and the C18 liquid chromatography column were obtained from previously described sources (Fonteh et al., 2006).

Recruitment and diagnosis

We recruited pAD and CT study participants prospectively from the North Los Angeles area for our IRB-approved research. We obtained informed consent from each study participant and all procedures complied with the Privacy of Personal Health Information Act and the Declaration of Helsinki principles for the use of human subjects in research. A neurologist obtained a complete medical history for each participant using a structured interview. A neuropsychologist administered a battery of cognitive assessments including the MMSE and ADAS-cog tests (Mendiondo et al., 2000; Weyer et al., 1997) to decide mental and cognitive deficits that fulfilled the criteria for pAD. We used guidelines approved by the American Academy of Neurology and the AMA for inclusion and exclusion criteria (Knopman et al., 2001) and the designation of probable AD is based on clinical criteria proposed by the Department of Health and Human Services Task Force on Alzheimer's disease (McKhann et al., 1984). In the pAD group, all participants had a history of progressive dementia of more than 12 months duration, had impairment of memory plus 2 or more areas of cognition, had no disturbance of consciousness and had disease onset between ages 40 and 90. We excluded pAD participants who had symptoms of clinical stroke, systemic disorders or other brain diseases that could account for the progressive deficits in memory and cognition. We also excluded participants if they had infection, fever, bleeding disorder, treatment, and other acute medical conditions that precluded lumbar puncture. Age- and gender-matched control subjects had no classifiable brain disorder after complete neurological and psychiatric evaluation. Comorbid conditions such as hypertension were recorded but were not a reason for exclusion.

Participants were not excluded from this study if they were taking prescription medications. However, a careful record of all prescription medications was kept. Of the 16 study participants included in this study, 7 pAD subjects were on a reversible acetylcholinesterase inhibitor, 2 CT and 4 pAD subjects were on selective serotonin reuptake inhibitors (SSRI), 3 CT and 3 pAD women were on estrogen replacement therapy, and 3 pAD subjects were on K^+/Na^+ blockers. Other prescription drugs included antihistamines (2 CT, 1pAD), Ca^{2+} channel blocker (1 CT, 1 pAD), 5HT1 receptor antagonist (2 pAD), alpha1 adrenergic blocker (2 pAD), cysteinyl leukotriene receptor inhibitor (1 CT), hydroxymethylglutaryl-coenzyme A reductase inhibitor (1 pAD) and a selective cyclooxygenase inhibitor (1 pAD).

Sample collection

We collected CSF by lumbar punctures, whole blood by venipuncture and midstream urine as described (Fonteh et al., 2007). We stored all samples in aliquots at $-80\,^{\circ}C$ until needed for analysis.

Determination of protein concentrations

A micro titer plate-based Coomasie protein assay using bovine serum albumin (0–100 µg/ml) as standard was used to measure protein concentrations (Zuo and Lundahl, 2000). Briefly, 20 µl of diluted CSF (20×), diluted plasma (500×) and urine (1×) were added to 96 well micro titer plate in triplicates. Coomassie dye (BioRad, Hercules, CA) was diluted (5×) and 200 µl added to each well. After 5 min, the OD at 595 nm was obtained using a microplate reader (Molecular Devices, Sunnyvale, CA) and protein concentrations in each sample determined using Softmax software (Molecular Devices).

Extraction, derivatization and $LCMS^2$ of FAA and DPs

Solid phase extraction followed by a derivatization (Husek, 1998) procedure using the EZ-Faast amino acid analyses kit from Phenomenex was performed on samples from CT and pAD subjects as described (Fonteh et al., 2006). Precursor ions of FAA and DPs and selected reaction monitoring (SRM) parameters (collision energy and tube lens voltage) were optimized for each FAA and DP (Fonteh et al., 2007). $LCMS^2$ was then performed and concentrations of FAA and DPs determined in CSF, plasma and urine from CT and pAD subjects.

Data analyses

Data are presented as the mean ± SEM and comparisons are made between CT and pAD. We used Prism (GraphPad Software, San Diego, CA) for graphical presentation and Statview (Abacus Concepts, Berkeley, CA) to compare the concentrations of FAAs and DPs using a *t*-test for unpaired data and the Mann–Whitney tests where appropriate. p values less than 0.05 were considered significant.

Results

Clinical classification and protein concentrations

Biochemical studies involving human subjects are often complex because of clinical heterogeneity. Our immediate goal was to identify changes in FAA and DP concentrations in a selected age- and gender-matched CT and pAD cohort. Our long-term goal is study these identified biochemical pathways in a larger sample size that would include pAD classification based on disease severity to better dissect disease-specific changes. In these first studies, 16 gender- and aged-matched study participants were recruited. As shown on Table 1, there was no significant difference in the mean age of the pAD and the CT group. Also, no age differences were found between gender groups (data not shown). A battery of neuropsychological tests that included MMSE and ADAS-Cog complemented

Table 1. Comparison of age, mental status, cognitive function and protein concentrations in study population

Parameters	CT ($n=8$)	pAD ($n=8$)	p value
Age (years)	$79.500 + 1.927$	$77.875 + 2.601$	0.6235
MMSE	$29.000 + 0.463$	$11.375 + 3.173$	$<0.0001^*$
ADAS-COG	$6.320 + 1.325$	$43.079 + 8.543$	0.0008^*
CSF protein (mg/ml)	$0.426 + 0.032$	$0.413 + 0.043$	0.8125
Plasma protein (mg/ml)	$120.125 + 2.386$	118.362 ± 6.198	0.7946
Urine protein (mg/ml)	$0.088 + 0.012$	$0.135 + 0.026$	0.1295

$^*p<0.05$; Age- and gender-matched (4 males and 4 females) study participants were selected as being CT or having pAD based on neurological examination and neuropsychological testing (MMSE and ADAS-cog). CSF, plasma and urine were collected and protein concentrations determined. The mean $\pm$ SEM and the p value obtained using a Student's t-test for unpaired data are shown for each variable.

neurological examination and diagnosis. Mean MMSE score of the pAD study participants was significantly lower than that of the CT group (Table 1). Mean ADAS-Cog score of the pAD group was significantly higher than that of the CT group (Table 1).

As part of the clinical evaluation, we recorded prescription drug use. pAD participants (3.5 ± 1.4 prescriptions) were more likely ($p=0.012$) to be taking prescription medication than CT subjects (1.1 ± 0.8 prescriptions). Acetylcholinesterase inhibitors, serotonin inhibitors and antihypertensives accounted for most prescription drugs taken by pAD subjects. These initial studies show that one can distinguish pAD from CT subjects based on neuropsychological scores and as expected, pAD subjects take more prescription medications than CT subjects.

We next determined the protein concentrations in CSF, plasma and urine. Although the mean protein levels in CSF and plasma were slightly lower in pAD than control subjects, these were not statistically different between the two groups (Table 1). The mean concentration of protein in urine was 53.4% higher in pAD subjects but this was not significantly different from that of CT subjects and was not associated with increased plasma urea. There were no gender differences in the mean protein concentrations for CT or pAD subjects (data not shown). These studies suggest the protein intake and excretion of our study groups are similar.

FAA and DP concentrations in CSF, plasma and urine

Using LCMS^2 with isotope dilution, we identified several important FAAs in human body fluids from subjects without any known neurological disease (CT). We quantitatively measured 21 FAAs and 2 DPs in CSF, 31 FAAs and 6 DPs in plasma and 23 FAAs and 5 DPs in urine (Fonteh et al., 2007). To determine changes in FAA and DP metabolism, we compared these metabolites between gender- and aged-matched subjects with pAD and CT. To aid the interpretation, these are divided into biochemical or functional families to include: 1) Histidine (imidazole)-containing FAAs or DPs that have antioxidant properties. 2) Aromatic-containing FAAs that are neurotransmitters. 3) FAAs and DPs associated with urea metabolism/detoxification and nitric oxide (NO) formation. 4) Glutamate-derived FAAs and DPs. 5) Aspartate and serine-derived FAAs. Results outlining differences in these FAA families in CT and pAD samples are described below. We have presented the concentrations of FAA and DP in pAD samples as supplementary data and will dwell on percentage changes when CT samples were compared to pAD (Fig. 1).

Concentration of histidine-containing FAAs and DPs

In CSF from pAD subjects, the concentration of histidine-containing FAAs were lower than that of CT subjects (Fig. 1). Although not significant, the mean concentration of histidine (Fig. 2A) and methyl-histidine (Fig. 2B) were lower in pAD plasma. The mean concentration of the DP, carnosine was significantly lower ($p=0.0289$) in pAD than in CT (Fig. 2C). The mean concentration of another DP, anserine, was 71% higher in pAD plasma than CT (Fig. 2D). However, the combined DP concentration was still significantly lower ($p=0.0246$) in pAD than CT plasma.

In urine, histidine, 3-methyl-histidine and carnosine concentrations were higher in pAD than CT (Fig. 1). In contrast, 1-methyl-histidine and anserine concentrations were lower in pAD urine. Total histidine and methyl-histidine concentration was higher in pAD urine while total carnosine and anserine concentrations were lower in pAD than CT (data not shown). Together, these data suggest that precursors of carnosine and anserine are lower in CSF and plasma, concomitant with a decrease in total DP concentration. Female study participants accounted for most of the decrease in plasma carnosine concentration (data not shown). There is increased urinary excretion of histidine-containing FAAs in pAD, perhaps a clearance from the degenerating brain. However, we need further research to validate and define the precise cause of these changes in CSF and plasma.

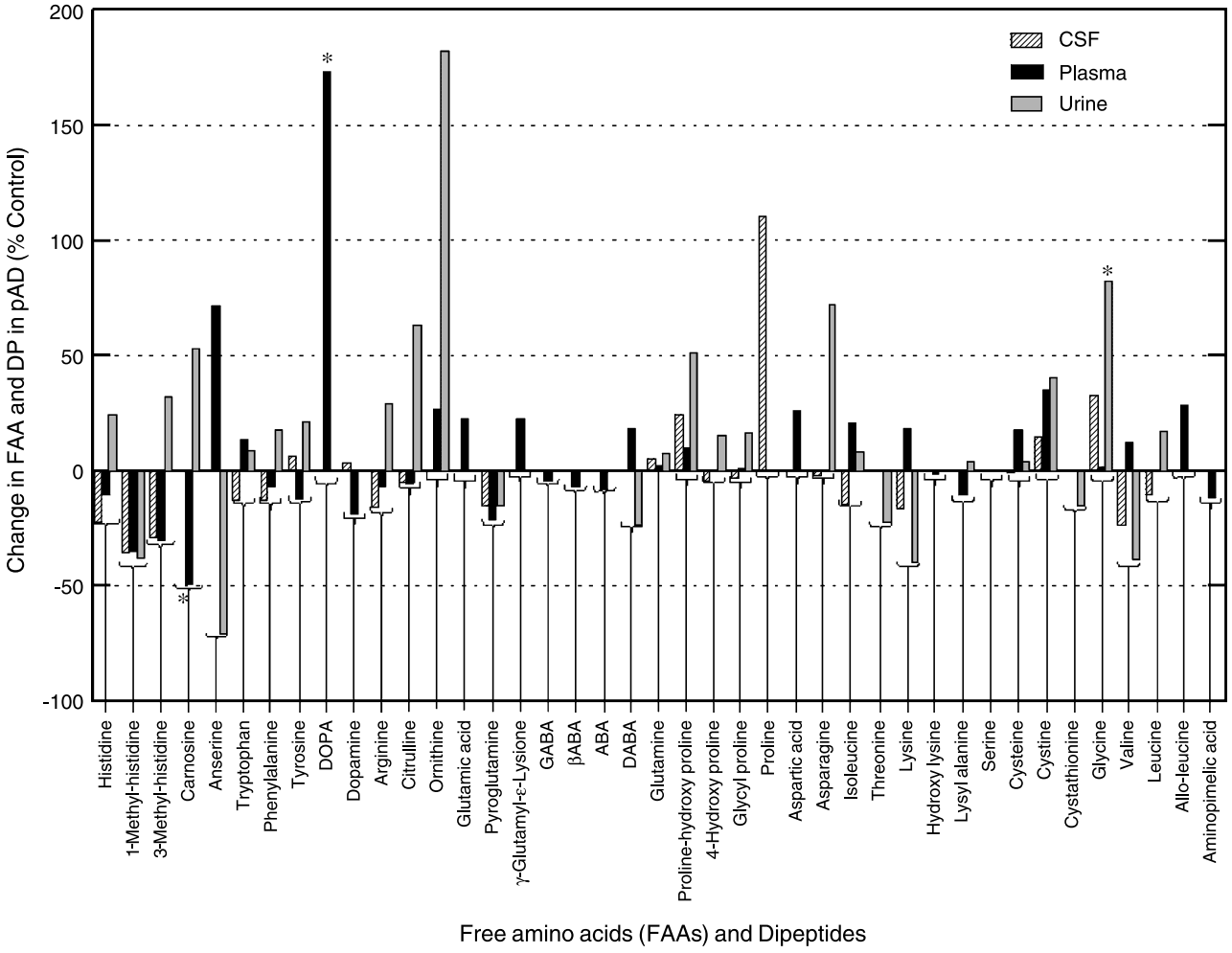

Fig. 1. Changes in FAA and DP concentrations in pAD samples. We extracted and derivatized FAAs and DPs from 200 µl CSF, 100 µl plasma and 200 µl urine. We performed LCMS^2 and calculated mole quantities (nmol/dl) of FAAs and DPs in samples from subjects without or with pAD. We then calculated the change in the mean concentration of FAA and DPs in pAD ($n = 8$) compared to CT ($n = 8$). These data are expressed as the % change in pAD compared to CT ($^*p < 0.05$)

Concentration of aromatic-containing FAAs

Tryptophan, phenylalanine, tyrosine, L-DOPA and dopamine were the major aromatic-containing FAA measured in CSF, plasma or urine (Fig. 1). In CSF, there was no significant difference between CT and pAD concentrations of aromatic FAAs (Fig. 1). While tryptophan, tyrosine and phenylalanine concentrations in plasma were not altered in pAD (Fig. 1), mean plasma L-DOPA concentration was significantly higher ($p = 0.003$) in pAD than CT (Fig. 3A). In contrast, the mean dopamine concentration was lower in pAD than in CT plasma (Fig. 3B). The ratio of L-DOPA to tyrosine was significantly higher in pAD than CT (Fig. 3C) while the mean dopamine to L-DOPA ratio was significantly lower in pAD (Fig. 3D). These differences in enzyme substrate and product ratios suggest there are changes in tyrosine hydrolase and L-DOPA decarboxylase activities in pAD. Only small changes in aromatic FAA concentrations were found in urine (Fig. 1), although we were not able to measure urinary L-DOPA and dopamine in these experiments. Together, these data show changes in peripheral dopamine biosynthesis in pAD subjects likely credited to enzymes in the dopamine biosynthetic pathway.

FAAs involved in urea metabolism and NO synthesis

CSF arginine and citrulline concentrations were slightly lower in CSF from pAD (Fig. 1). Total arginine and citrulline was highest in CSF from CT_M (6092.77 ± 472.47 nmol/dl) and this was significantly higher ($p = 0.0086$) than the concentration in CT_F ($4044.15 \pm$

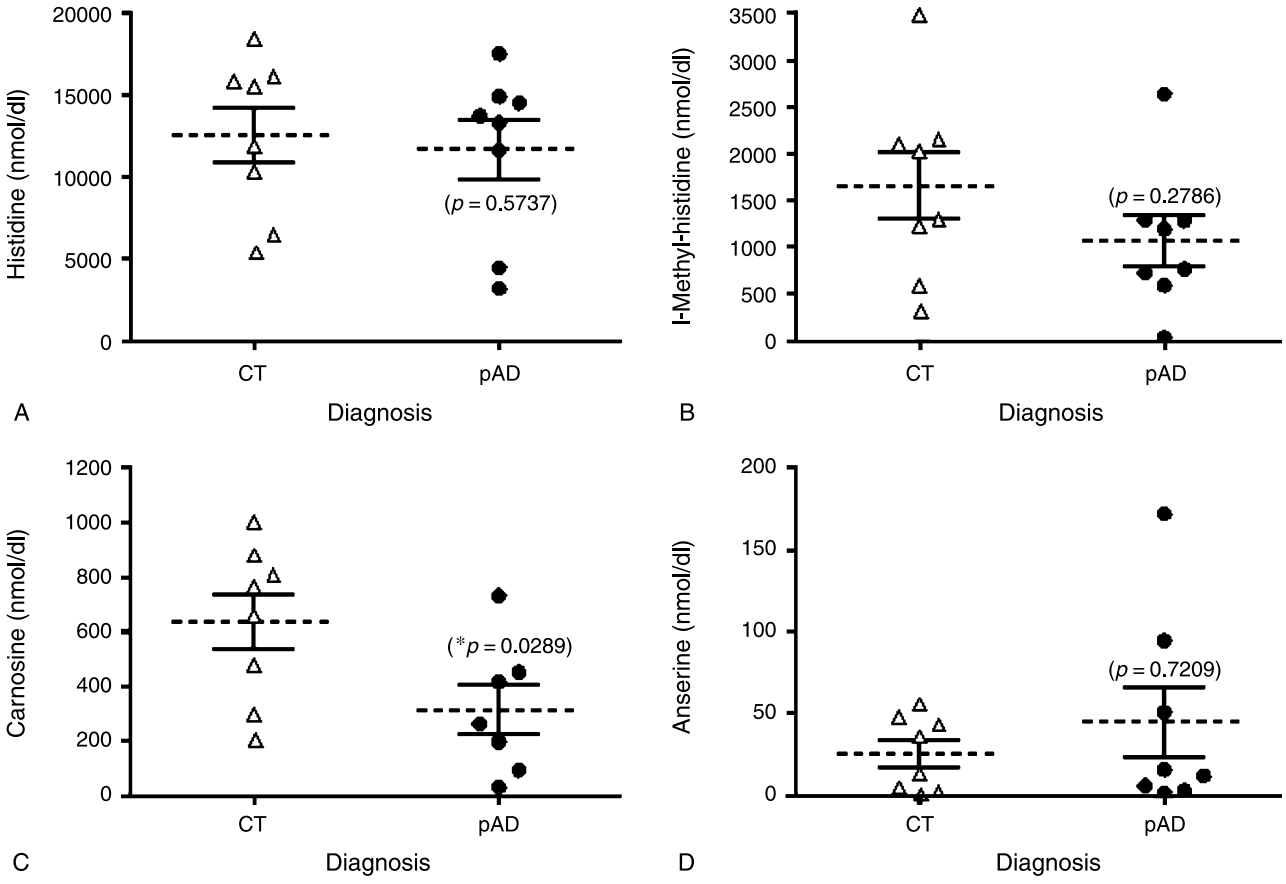

Fig. 2. Plasma concentration of histidine-containing FAAs and DPs. Concentrations of histidine (**A**), 1-methyl-histidine (**B**), carnosine (**C**) and anserine (**D**) in plasma from age- and gender-matched CT and pAD subjects were determined using LCMS^2. These data are individual plasma concentrations of CT ($n = 8$) and pAD ($n = 8$) subjects, with the mean concentrations (dotted line) $\pm$ SEM. p values comparing pAD with CT are shown for each plot ($^*p < 0.05$)

249.83 nmol/dl). CT and pAD difference for males (1972.99 nmol) was ~23 fold higher than the difference for females (83.16 nmol). In plasma, ornithine was 26.8% more abundant in pAD than in CT while the other FAAs are were not altered (Fig. 1). In contrast to the CSF and plasma, urine arginine, citrulline and ornithine concentrations were all higher in pAD than CT (Fig. 1). Total concentration of these FAAs was significantly ($p = 0.0109$) higher in pAD (54262.90 ± 5757.35 nmol/dl) than CT (28.94 ± 6.44 nmol/dl). These data suggest that male subjects account for the differences in urea metabolism or NO synthesis in CSF, while differences in urine are not gender-dependent.

Glutamate metabolism

Of the many glutamate-derived FAAs detected in CSF, plasma and urine, only slight changes were found in pAD (Fig. 1). Concentrations of several proline-containing amino acids were increased in pAD urine. These data show there may be differences in proline metabolism but not in other glutamate-derived metabolites in pAD.

Aspartate and serine family of FAA

In CSF, only modest differences between CT and pAD samples were noted in the concentrations of aspartate- and serine derived FAAs (Fig. 1). In plasma, isoleucine, lysyl alanine, lysine and cysteine were the highest concentration of FAAs or DP from the aspartate or serine families. Compared with CT plasma, few differences were observed in the concentrations of these compounds except for aspartic acid, cysteine and alloleucine, which were more than 25% higher in pAD (Fig. 1). In urine, there was an increase in asparagine, cysteine and glycine and a decrease in lysine and valine levels in pAD. Although

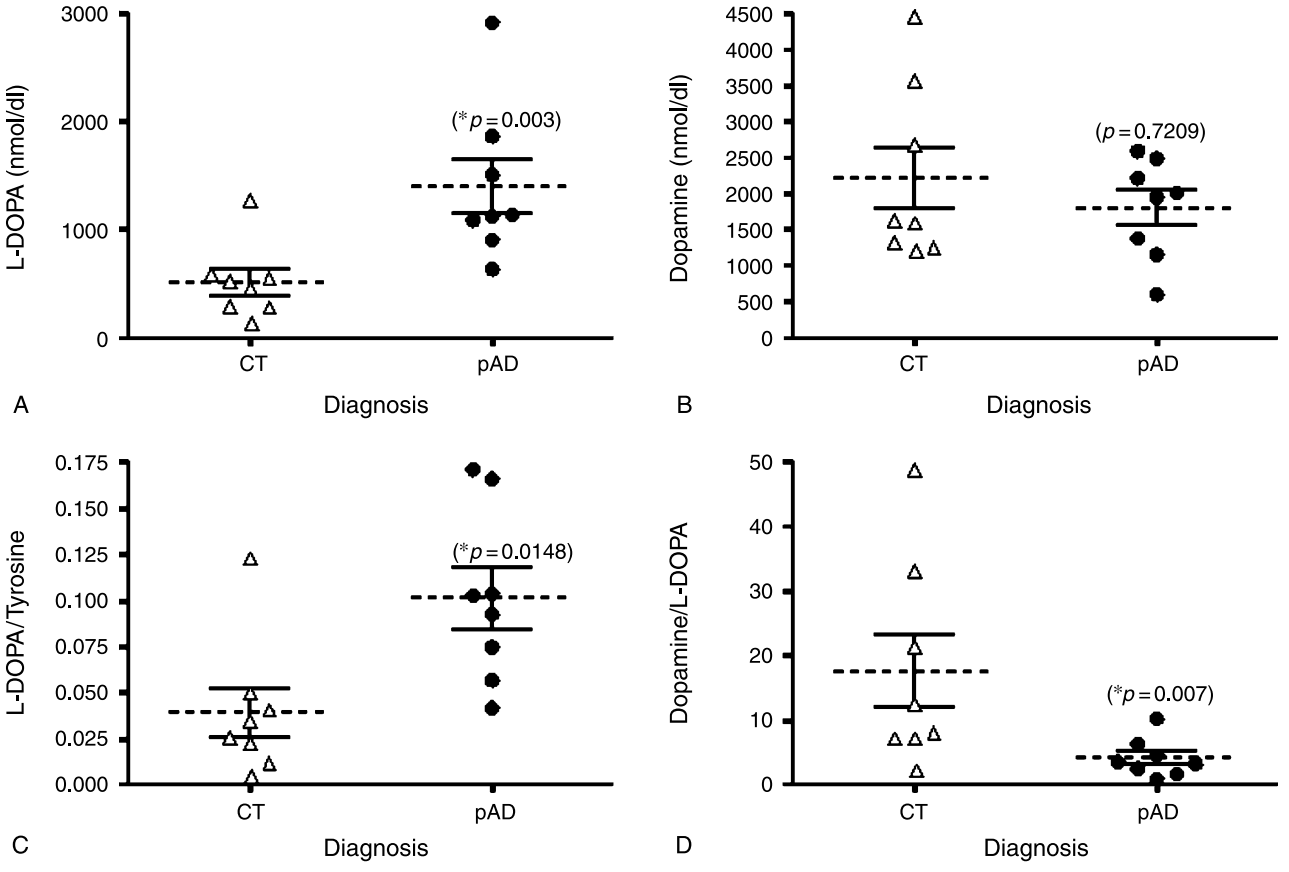

Fig. 3. Changes in plasma L-DOPA. Concentrations of L-DOPA (**A**), dopamine (**B**) and the ratios of L-DOPA/tyrosine (**C**) and dopamine/L-DOPA (**D**) in plasma from age- and gender-matched CT and pAD subjects were determined using LCMS^2. These data are concentrations of CT ($n = 8$) and pAD ($n = 8$), with the mean concentration (dotted line) $\pm$ SEM. p values comparing pAD with CT are shown for each plot ($^*p < 0.05$)

urine glycine concentrations were lower than those of other FAAs, it increased significantly ($p < 0.0054$) in pAD (15.6 ± 1.7 nmol/dl) compared with CT (8.5 ± 1.3 nmol/dl). Together, these data show the metabolism of aspartate or serine-derived FAAs is not altered in CSF or plasma while excretion of glycine in urine is higher in pAD than CT.

Discussion

AD is a complex disease that is influenced by genetics, the environment, age, head injury, gender or other demographic factors (Cummings et al., 1998). Several biochemical pathways involving neurotransmission and receptors may be influenced as AD progresses. Since amino acids play important roles in neurotransmission and receptor signaling pathways, biochemical pathways involving amino acids and dipeptides may change in AD (Csernansky et al., 1996; Greenamyre and Young, 1989; Perry et al., 1987; Pomara et al., 1992). Using LCMS^2 on age and gender-matched samples, we have revealed several new insights into FAA biochemical pathways in AD. The concentrations of FAAs or DPs involved in anti-oxidation (carnosine), neurotransmission (L-DOPA and dopamine), urea cycle/detoxification or NO formation (arginine, citrulline, ornithine) and inhibitory FAA (glycine) are significantly altered in samples from pAD compared with CT. Together, these data show the importance of FAAs and DPs in pAD pathogenesis and provide a scientific reason for designing novel strategies to control FAA metabolism in AD patients. The influence of prescription drugs on changes in FAA and DP concentrations cannot be excluded. In the present study, pAD participants took >2X more prescription drugs than CT. A careful review of the literature and known modes of drug action does not suggest that these classes of

prescription medications are likely to influence any of the biochemical pathways involving the FAAs and DPs described below.

The concentration of FAAs and DPs in CSF or plasma will be dictated in part by how much protein one consumes and by metabolic processes that control the absorption, transport, degradation and excretion of these molecules. Similarly, concentrations of FAAs and DPs in urine will be influenced by the rate of excretion of these molecules. These processes have significant ramifications on human health since FAAs and DPs control processes ranging from neurotransmission by receptor-mediated signaling, prevention of oxidation and detoxification by urea excretion. In this study, the protein concentrations of plasma, CSF or urine are only slightly different between CT and pAD study participants. If uptake does not contribute significantly to differences between CT and pAD, it is likely that proteolysis, deamination and other biochemical reactions that control FAA may be important. Given the increase in protein concentration in urine from pAD in this age- and gender-matched sample when none of the subjects had high urea or creatine on routine blood testing, it is likely there is increased degradation of proteins in AD. This would not be surprising because of the overall loss of brain tissue in AD (Wang et al., 2002).

An important difference between pAD and CT is the decrease in imidazole-containing FAA and DP in CSF, plasma and urine. Histidine is an important chelator of divalent ions and is normally found in the binding sites of heme proteins (Walker, 2004). Carnosine is a DP consisting of β-alanine and histidine obtained from the diet or synthesized in skeletal muscle or the brain by carnosine synthetases (Bonfanti et al., 1999). Carnosine prevents peroxidation of lipids, proteins and carbohydrates by reactive oxygen radicals, serves as an antioxidant, is a quencher of 4-hydroxy-2-nonenal and malonaldehyde, and prevents glycation of proteins (Hipkiss, 2002; Kohen

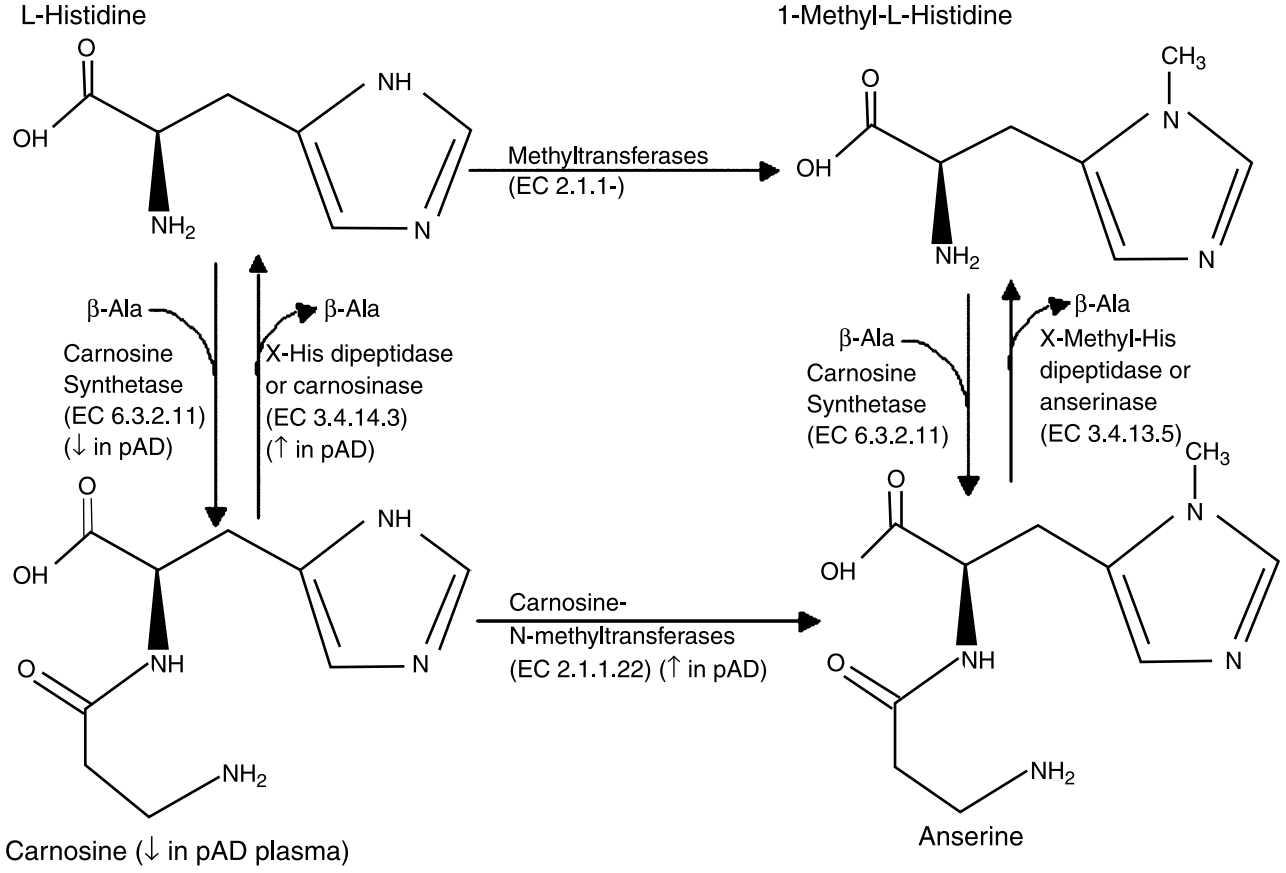

Fig. 4. Pathway for histidine metabolism. Arrows stand for the proposed changes [decrease (↓) or increase (↑)] in FAAs and DPs in body fluids and the proposed changes in enzyme activity and/or levels that likely account for these changes

et al., 1988; Preston et al., 1998; Stvolinskii et al., 2003). In whole cell studies using neurons, carnosine prevents cell death (Marchis et al., 2000). These important properties of carnosine have led to suggestions that it is an anti-aging agent and various nutritional supplements claim its use against AD, PD, autism and other neurodegenerative diseases (Boldyrev et al., 1999; Stuerenburg, 2000). Our study showing a decrease in histidine in CSF from pAD and in carnosine in plasma from pAD implies that pAD subjects cannot easily control peroxidation events. Such a decreased capacity to prevent oxidation will result in an increase in degenerating nerve cells, resulting in decline in cognitive and mental function.

Various mechanisms may account for the decrease in plasma carnosine in pAD. First, the uptake from dietary sources may be limited in pAD subjects. Second, carnosine may be excessively consumed in response to AD pathology. Third, the biosynthesis of carnosine may be different in pAD than in CT (Fig. 4). For example, carnosine synthetase activity may be lower in pAD than CT or the rate of breakdown of carnosine by carnosinase may be higher in pAD than in CT (Fig. 4). In pAD, there is an increase in plasma anserine concomitant with a decrease in urine while the decrease in carnosine accompanies an increase in urine. These changes may reflect a link between the biosynthesis and excretion of anserine and carnosine. Future validation studies are required to determine which of these mechanisms account for the decrease in carnosine, and whether these levels can be influenced or restored in pAD subjects.

Changes in catecholamine metabolism are also evident in pAD samples compared with CT. Dopamine may be derived from phenylalanine or tyrosine by enzymes depicted in Fig. 5. Once formed, dopamine acts on several receptor subclasses to influence CNS and peripheral events (Kohli, 1990; Mayerhofer et al., 1999; Memo et al., 1986). Most studies have linked dopamine

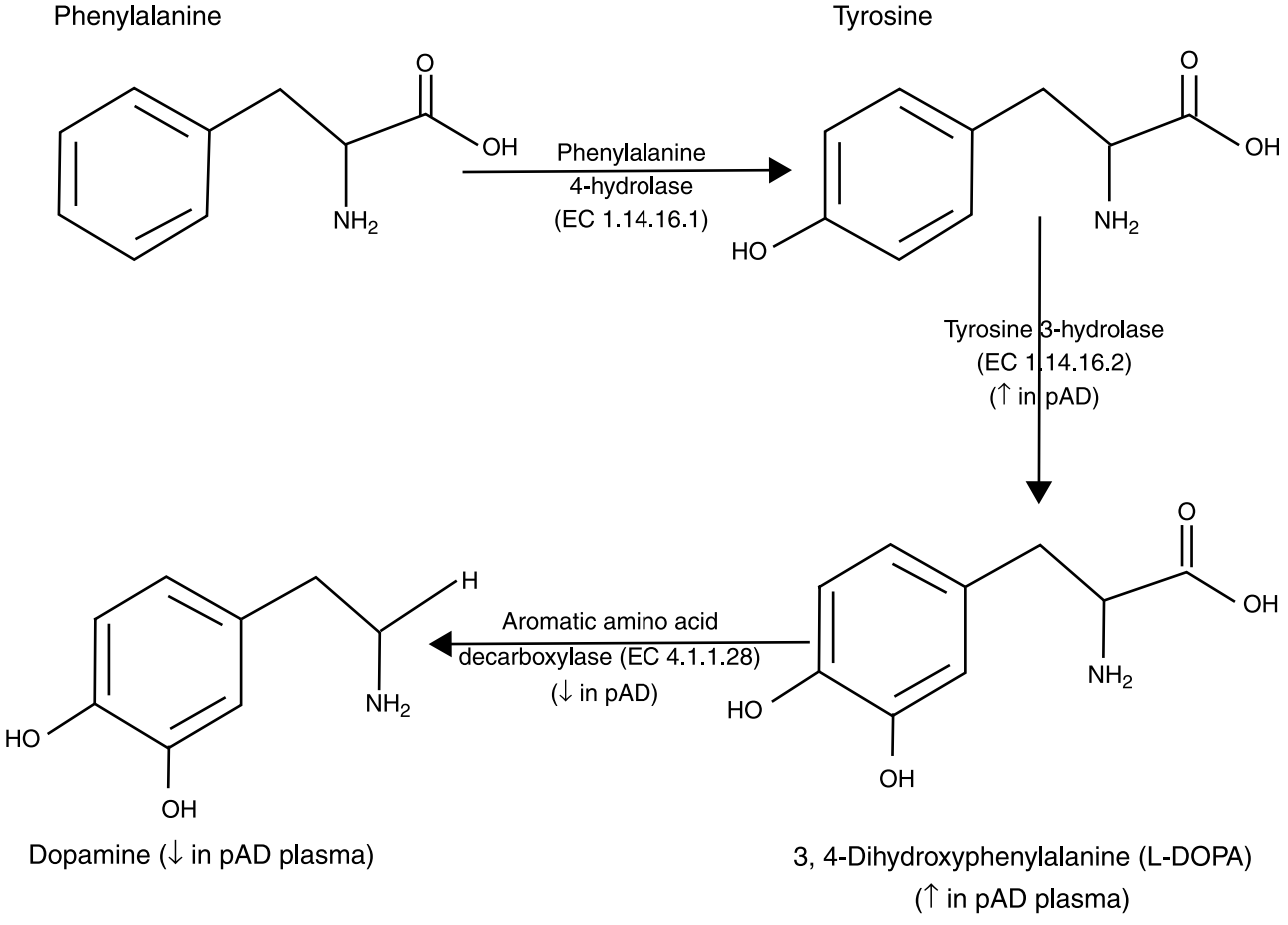

Fig. 5. Pathway for dopamine metabolism. Arrows stand for the proposed changes [decrease (↓) or increase (↑)] in FAAs in plasma and the proposed changes in enzyme activity and/or levels that likely account for these changes

to Parkinson's disease (Agid et al., 1979; Caronti et al., 1999; Corsini et al., 1980; Date and Ohmoto, 1996; Grunblatt et al., 2000; Zhang et al., 2000). Only a few studies have examined a possible link between dopamine biosynthesis and AD. Disturbances in the biosynthesis of monoaminergic neurotransmitters and their precursors have been reported in postmortem brain samples from AD subjects (Storga et al., 1996). A significant decrease in dopamine D_2 receptors in peripheral blood lymphocytes of AD patients was shown (Barbanti et al., 2000). The present studies show there are no significant difference in dopamine and its immediate precursors in the CSF. In contrast, L-DOPA concentration is significantly increased in plasma from pAD while dopamine is reduced. While most studies have examined a CNS role for dopamine, its effects on the peripheral nervous system are not as widely studied. Peripheral dopamine is mainly derived from peripheral sympathetic nerve terminals and studies suggest that dopamine is a third catecholaminergic neurotransmitter that complements the sympathetic neurotransmitter, norepinephrine, or the adrenomedullary hormone, epinephrine (Yoneda et al., 1985; Lackovic et al., 1982; Goldstein et al., 1995). Peripheral dopamine is associated with hypertension, memory improvement by autoreceptor activation (White et al., 1993), control of hemodynamics and inhibition of circulating beta-endophin levels (Barili et al., 1996; Bolme et al., 1977; Glavin and Hall, 1995; Kohli et al., 1991; Kuchel, 1999; Liu et al., 2001; Marazziti et al., 1992; Murphy et al., 2001; Singh et al., 2003; Snider, 1975). Because of these important functions of peripheral dopamine, the increase in L-DOPA and decrease in plasma dopamine shown in this study will likely have physiological and therapeutic implications in AD. Biochemical pathways that may account for the decrease in plasma dopamine concomitant with the increase in L-DOPA observed in this study include an increase in tyrosine hydrolase activity or a decrease in DOPA decarboxylase activity in pAD (Fig. 5). Since tyrosine hydroxylase is the rate-limiting enzyme in this pathway (Fitzpatrick, 1991), a likely explanation for these data would be that L-DOPA decarboxylase is inhibited in pAD. Increased conjugation of dopamine in plasma and its subsequent excretion may also account for the decrease in plasma concentration. Although it is important to establish the mechanism of the increase in L-DOPA and decrease in dopamine in plasma of pAD, there is the possibility that stimulation of decarboxylase or use of modulators of catecholamine biosynthesis may be a helpful AD treatment.

Glutamic acid is an important excitatory neurotransmitter (Fonnum, 1984; Nedergaard et al., 2002; Weinberg, 1999). In the brain, it is used for energy formation and for the biosynthesis of the inhibitory mediator, GABA (Berl and Waelsch, 1958; Vogel et al., 1975). Glutamic acid also forms glutamine in a process responsible for detoxifying the brain of ammonia. High concentrations of glutamic acid are associated with headaches and other neurological conditions (D'Andrea et al., 1991; Gallai et al., 2003). Glutamine is an inactive FAA in the CSF, which has been proposed to serve to buffer biological fluids and to serve as a nitrogen carrier (Lee et al., 1998). This process results in detoxifying the brain as nitrogen is funneled into the urea cycle in the liver for excretion. The increase in glutamine concentration in the CSF, plasma and urine of pAD subjects compared with CT may suggest increased amino acid metabolism needing more buffering capacity and more detoxification that would be expected with the loss of brain tissue. Further evidence of changes in detoxification of nitrogen is provided by a decrease in arginine and citrulline in CSF accompanied by a slight increase in plasma and a greater increase in urine. While the biochemical pathways leading to the increase in arginine, citrulline and ornithine in urine are not clear, measurement of these metabolites may serve as biomarkers of pAD.

Proline is another glutamic acid-derived amino acid used mainly for collagen formation and is thus critical in maintaining muscle, joints and tendons (Gould and Woessner, 1957). Proline hydroxyproline is a DP that is utilized for collagen formation. There is an increase in proline hydroxyproline in CSF, plasma and urine. Since proline levels are normally associated with degenerative diseases such as arthritis, the increased turnover in pAD subjects who do not have arthritis may reflect more brain degeneration than is occurring in CT subjects.

Glycine is an inhibitory neurotransmitter that is active in the spinal cord and the lower brain stem (Schofield, 1996). In this study, glycine concentrations consistently increase in pAD CSF and urine samples compared with CT. This increase may contribute to the symptoms of pAD. Given the effects of glycine and other FAAs mediated by various receptors, more studies will be needed to find out whether there are changes in the expression of these receptors in the brain of pAD subjects.

Overall, the strength of $LCMS^2$ in clarifying complex biochemical pathways is illustrated. FAAs or DPs that are altered in pAD may serve as metabolic markers or metabolic risk factors. Future investigations

looking at disease severity and on approaches to restore or control FAA and DP metabolism in AD are now warranted by these initial discovery studies. For example, studies based on dietary supplementation with carnosine or specific inhibitors targeting dopamine biosynthesis may restore concentrations of these molecules and may prevent disease progression and/or ameliorate AD symptoms.

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Appendix: Supplementary materials

Table I. FAA and DP concentrations in pAD samples

Amino acids	CSF (nmol/dl)	Plasma (nmol/dl)	Urine (nmol/dl)
Histidine	1440.62 ± 632.90	11668.00 ± 1815.72	46379.50 ± 6615.54
1-Methyl-histidine	259.86 ± 113.27	1066.92 ± 268.21	23879.40 ± 6141.94
3-Methyl-histidine	247.66 ± 157.80	516.08 ± 127.07	36759.65 ± 2580.93
Carnosine	ND	328.40 ± 91.30*	28603.29 ± 15669.25
Anserine	ND	45.70 ± 21.49	12116.43 ± 7884.92
Tryptophan	139.31 ± 32.35	3306.352 ± 899.580	1803.15 ± 355.75
Phenylalanine	591.10 ± 79.50	7177.74 ± 921.45	2076.10 ± 279.78
Tyrosine	2718.74 ± 483.68	14377.43 ± 1549.44	16025.73 ± 2308.09
DOPA	ND	1400.84 ± 253.68*	ND
Dopamine	34.823 ± 10.298	1802.84 ± 245.52	ND
Arginine	616.98 ± 145.91	9134.37 ± 1310.35	3161.76 ± 313.76
Citrulline	2737.28 ± 264.50	9497.67 ± 1118.66	32351.72 ± 4352.72
Ornithine	ND	6835.01 ± 2243.52 (+)	18749.52 ± 7706.66
Glutamic acid	NR	3318.85 ± 1126.55	NR
Pyroglutamine	8.85 ± 2.43	26.12 ± 2.37	253.55 ± 74.53
γ-Glutamyl-ε-lysine	ND	16.29 ± 3.92	ND
GABA	ND	184.11 ± 21.59	ND
βABA	ND	272.15 ± 50.28	ND
ABA	ND	1979.45 ± 217.41	ND
DABA	ND	11635.85 ± 999.12	55181.11 ± 7696.82
Glutamine	114661.24 ± 1066.76	22834.59 + 1197.97	1772.45 ± 738.34
Proline-hydroxyproline	8.63 ± 2.02	999.32 ± 139.45	1512.15 ± 296.02
4-Hydrox proline	28.51 ± 5.57	566.39 ± 67.36	329.69 ± 26.35
Glycyl proline	8.66 ± 1.57	23.18 ± 3.80	53.76 ± 8.89
Proline	68.60 ± 35.57	NR	ND
Aspartic acid	ND	1267.54 ± 286.01	ND
Asparagine	177.66 ± 16.02	NR	405.57 ± 70.32
Isoleucine	240.97 ± 65.57	149515.99 ± 37297.20	200.34 ± 65.51
Threonine	ND	NR	38.348 ± 4.939
Lysine	4464.69 ± 1084.58	21677.07 ± 5450.43	8381.49 ± 2649.70
Hydroxy lysine	ND	50.45 ± 2.88	ND
Lysyl alanine	ND	113839.27 ± 24960.53	532952.54 ± 63321.17
Serine	ND	21.18 ± 2.43	ND
Cysteine	130.14 ± 64.05	1281.97 ± 217.90	3043.92 ± 573.25
Cystine	784.14 ± 249.21	20920.33 ± 4593.49	109491.95 ± 18221.10
Cystathionine	ND	103.15 ± 22.15	2557.881 ± 752.357
Glycine	28.967 ± 12.291	15.25 ± 1.12	15.55 ± 1.66*
Valine	413.27 ± 122.09	5826.51 ± 1474.97	1119.59 ± 189.07
Leucine	1145.55 ± 151.30	NR	1379.89 ± 171.70
Allo-leucine	ND	4439.66 ± 962.29	ND
Aminopimelic acid	ND	216.45 ± 47.07	ND

ND Not detected; *NR* not resolved; * $p < 0.05$ compared to aged and gender-matched controls; We extracted and derivatized FAAs and DPs from 200 µl CSF, 100 µl plasma and 200 µl urine as described in Materials and methods. We performed $LCMS^2$ and calculated mole quantities (nmol/dl) of FAAs and DPs in samples from subjects with pAD. These data are mean ± SEM of 8 pAD subjects (4 males and 4 females) with an average age of 79.5 + 1.93 years

Table II. Changes in FAA and DP concentrations in pAD samples

Amino acids	CSF (Change, % CT)	Plasma (Change, % CT)	Urine (Change, % CT)
Histidine	−22.30	−10.59	24.46
1-Methyl-histidine	−35.42	−35.25	−38.20
3-Methyl-histidine	−28.72	−30.24	32.14
Carnosine	ND	−49.80*	53.00
Anserine	ND	71.64	−71.08
Tryptophan	−13.05	13.36	8.56
Phenylalanine	−12.93	−6.87	17.69
Tyrosine	6.46	−12.24	21.36
DOPA	ND	173.06*	ND
Dopamine	3.36	−18.77	ND
Arginine	−15.79	−7.10	29.19
Citrulline	−5.11	−5.43	63.0
Ornithine	ND	26.78	182.38
Glutamic acid	NR	22.19	NR
Pyroglutamine	−15.27	−21.44	−15.10
γ-Glutamyl-ε-lysine	ND	22.60	ND
GABA	ND	−4.62	ND
βABA	ND	−6.98	ND
ABA	ND	−8.26	ND
DABA	ND	18.15	−23.31
Glutamine	5.41	2.25	7.70
Proline-hydroxyproline	24.21	10.21	50.94
4-Hydrox proline	−4.35	−0.33	14.97
Glycyl proline	−2.93	0.86	16.62
Proline	110.03	NR	ND
Aspartic acid	ND	26.30	ND
Asparagine	−2.31	NR	71.91
Isoleucine	−14.38	20.39	8.30
Threonine	ND	NR	−22.55
Lysine	−16.57	18.32	−40.04
Hydroxy lysine	ND	−1.19	ND
Lysyl alanine	ND	−10.52	3.83
Serine	ND	0.15	ND
Cysteine	−1.12	17.93	4.16
Cystine	14.69	35.19	40.39
Cystathionine	ND	0.19	−15.54
Glycine	32.67	1.44	82.43*
Valine	−23.39	12.10	−38.76
Leucine	−10.58	NR	17.24
Allo-leucine	ND	28.64	ND
Aminopimelic acid	ND	−11.76	ND

ND Not detected; *NR* not resolved; * $p < 0.05$ compared with aged and gender-matched CT. We extracted and derivatized FAAs and DPs from 200 µl CSF, 100 µl plasma and 200 µl urine as described in Materials and methods. We performed LCMS[2] and calculated mole quantities (nmol/dl) of FAAs and DPs in samples from subjects without or with pAD. We then calculated the change in the mean concentration of FAA and DPs in pAD ($n = 8$) compared to CT ($n = 8$). These data are expressed as the % change

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