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Free D- and L-amino acids in ventricular cerebrospinal fluid from Alzheimer and normal subjects

G. Fisher[1], N. Lorenzo[1], H. Abe[2], E. Fujita[3], W. H. Frey[4], C. Emory[4], M. M. Di Fiore[5], and A. D'Aniello[6]

[1] Department of Chemistry, Barry University, Miami Shores, Florida, U.S.A.
[2] Laboratory of Marine Biochemistry, Graduate School of Agricultural Life Science, The University of Tokyo, Bunkyo-Ku, Tokyo, Japan
[3] Department of Food Science and Nutrition, Kyoritsu Women's University, Chiyoda-Ku, Tokyo, Japan
[4] Alzheimer's Treatment and Research Center, Health Partners Foundation, St. Paul, Minnesota, U.S.A.
[5] Department of Life Science, Second University of Naples, Caserta, Italy
[6] Department of Biochemistry and Molecular Biology, Stazione Zoologica "A Dohrn", Napoli, Italy

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Summary. Free D-Ser, D-Asp and total D-amino acids were significantly higher ($p < 0.05$) in Alzheimer (AD) ventricular CSF than in normal CSF. There was no significant difference in the total L-amino acids between AD and normal CSF, but L-Gln and L-His were significantly higher ($p < 0.05$) in AD-CSF. The higher concentrations of these D- and L-amino acids in AD ventricular CSF could reflect the degenerative process that occurs in Alzheimer's brain since ventricular CSF is the repository of amino acids from the brain.

Keywords: D- and L-Amino acids – Cerebrospinal fluid – Ventricular CSF – Alzheimer's disease

Introduction

The most prevalent optical isomers of amino acids are stereometrically the L-configuration. However, recently, D-Asp has been found in Alzheimer brain neurofibrillary tangles (Fisher et al., 1992a), and high levels of D-Asp and D-Ala have been found in proteins of white and gray matter of Alzheimer brain (Fisher et al., 1992b; D'Aniello et al., 1992). In both these cases, the level of these D-amino acids was higher in the Alzheimer brains than in normal brains. Similarly, D-Asp has also been found in the Alzheimer β-amyloid core proteins (Roher et al., 1993). In addition to the presence of these amino acids in proteins, D-Asp, D-Ala, and D-Ser have also been found in free forms in normal and Alzheimer brain tissue (Fisher et al., 1991; Nagata et al., 1995) and

also in normal and Alzheimer lumbar cerebrospinal fluid (CSF) (Fisher et al., 1994).

Much attention has been given to the determination of free amino acids of normal and Alzheimer patients in brain tissue (Tarbit et al., 1980) and in lumbar CSF by several researchers (Smith et al., 1985; Proctor et al., 1988; Degrell et al., 1989; Gelot et al., 1990; Kapaki et al., 1991; Pomara et al., 1992; Tohgi et al., 1992; Martinez et al., 1993). However, to our knowledge no studies have previously been reported on ventricular cerebrospinal fluid. For this reason we undertook a study to determine the concentration of free amino acids (D- and L- forms) in ventricular CSF of normal and Alzheimer persons.

Materials and methods

CSF samples

Normal and Alzheimer ventricular cerebrospinal fluids were obtained from the National Neurological Research Specimen Bank, Wadsworth VA Medical Center, Los Angeles, California. Ventricular CSF was obtained at autopsy following opening of the cranial cavity and withdrawal of the CSF by syringe from the third ventricle. Samples were stored frozen until analyzed. Twenty-one Alzheimer's samples were analyzed, ages 51–89 years old, mean 75.2 ± 10.4, and eleven normal subjects (deaths due to myocardial infarction or carcinoma) were analyzed, ages 58–86 years old, mean 70.1 ± 7.8. On chart review, the Alzheimer's disease cases met retrospective clinical criteria for primary degenerative dementia according to the *Diagnostic and Statistical Manual of Mental Disorders* – Third Version: Revised (American Psychiatric Association, 1987). The pathologic diagnosis of Alzheimer's disease was based on the presence of an age-adjusted moderate-to-severe number of neuritic plaques in the neocortex similar to published criteria without evidence of other neurologic disorders. As controls, CSF samples were taken at autopsy from individuals who were free of Alzheimer's disease or other known neurological illness.

Amino acid analysis

The ventricular CSF was deproteinized with 70% perchloric acid (PCA) in ratio of 8 μl PCA to 1 ml of CSF, and then centrifuged at 15,000 rpm for 5 min. 25–100 μl of the supernatant were neutralized with 1 M KOH to give pH 7–8, kept at 0°C for 30 min and centrifuged. The supernatants were utilized for amino acid analysis in two laboratories (Italy and Japan).

In the Italian laboratory amino acids were determined by the HPLC method used in our previous work (Fisher et al., 1994) with pre-column derivatization with OPA-Thiol (*o*-phthaldialdehyde/β-mercaptoethanol) and separation on a C-18 column using a citrate-acetonitrile gradient and fluorescence detection. A standard mixture of 19 amino acids (Sigma) plus γ-aminobutyric acid (GABA) and taurine, each at the concentration of 1 nmol/μl, was chromatographed under the same conditions, and the respective amino acid areas were used to quantify the amino acid concentrations of the samples. Under these conditions His + Gln as well as Tau + GABA eluted as single peaks, respectively. These amino acids were separated into individual peaks by the OPA-NAC (*o*-phthaldialdehyde/N-acetyl-L-cysteine) HPLC method, as described by Aswad (1984). Determinations of D-Asp, D-Ala and the total D-amino acids were carried out by an enzymatic method using beef kidney D-amino acid oxidase and octopus D-aspartate oxidase, as described by the method of D'Aniello et al. (1993a,b). D-Asp was also determined by the chiral HPLC method of Aswad (1994).

In the Japanese laboratories the amino acids were determined by two different HPLC methods. In the first method D- and L-amino acids were analyzed by derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) as described by the method of Okuma and Abe (1994). In the second method D- and L-Ser were analyzed by derivatization with N-tertbutyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde (Boc-L-cysteine/OPA), as described by Hashimoto et al. (1992).

Statistical analyses

The data were analyzed for statistical differences by two-sample T-tests using the statistical program MINI-TAB.

Results

D-Amino acids

The concentrations of D-Asp, D-Ala, and the total D-amino acid concentration were determined previously in the ventricular CSF of 8 Alzheimer's subjects (ages 74.5 ± 9.3 yr) and 8 aged normal persons (ages 60.1 ± 15.6 yr) (Table 1) (Fisher et al., 1994). Using samples from the present set, D-Ser and D-Arg were determined in the Japanese lab on 10 Alzheimer's (aged 78.6 ± 13.8 yr) and 5 normals (aged 66.0 ± 7.4 yr). Total D-amino acids were found to be significantly higher (1.5-fold, $p = 0.025$) in AD-CSF compared to normal CSF (26.4 nmol/ml vs 17.9 nmol/ml, respectively). D-Ser and D-Asp were also significantly higher in AD-CSF (D-Ser 5-fold increase, $p = <0.05$; D-Asp 2.7-fold increase, $p < 0.05$). D-Ala was slightly higher in AD-CSF than in normal

Table 1. D-Amino acids in Alzheimer and normal ventricular CSF

Amino acid	Alzheimer		Normal		Ratio Alz/Nrml
	nmol/ml	(%) D/(D + L)	nmol/ml	(%) D/(D + L)	
D-Ser[a]	9.0 ± 4.1	10.10	1.8 ± 1.2	4.13	5.0**
D-Asp[b]	3.3 ± 2.1	1.68	1.2 ± 0.8	0.61	2.72*
D-Ala[b]	0.8 ± 0.4	0.80	0.7 ± 0.3	0.70	1.14
D-Arg[a]	3.9 ± 6.8	11.04	4.4 ± 6.5	16.42	0.89
Total D-AAs[b]	26.4 ± 7.6	2.68	17.9 ± 5.4	2.07	1.47*

Figures represent the mean ± standard deviation of the mean. (%) = [D/(D + L)] × 100. The term total D-amino acids means the sum of D-Asp, D-Glu, D-Met, D-Pro, D-Phe, D-Tyr, D-Ala, D-Leu, D-Ile, D-Val, D-Ser, D-Arg, D-His, D-Thr, D-Lys, and D-Trp.
p values refer to two-sample t-test comparison of Alzheimers to normals **$p < 0.01$, *$p < 0.05$.
[a] From current analyses on 10 Alz (78.6 ± 13.8 yr) and 5 normals (66.0 ± 7.4 yr). [b] From previous analyses (Fisher et al., 1994) on 8 Alz (74.9 ± 9.3 yr) and 8 normals (60.1 ± 15.6 yr).

CSF (1.1-fold), but this increase was not statistically significant. Contrary to what was found for D-Ser and D-Asp, the D-Arg concentration was lower in the Alzheimer than normal ventricular CSF. However, this difference was not statistically significant.

L-Amino acids

The concentrations of 21 free amino acids including GABA and Tau are shown in Table 2. There was no significant difference in the concentrations of most individual L-amino acids as well as the sum total of the L-amino acids between normal and Alzheimer's subjects. The ratio of Alzheimer's to normal for individual amino acids was about 1.0, as determined in both the Italian and Japanese labs. However, by the OPA-Thiol method (Italian lab) two amino acids, His and Gln, were higher in the AD-CSF than normal CSF. Both His and Gln showed two-fold statistically significant ($p < 0.05$) elevation in the Alzheimer compared to the normal subjects. When expressed as percent of the total amino acids, Gln constitutes 18.8% of the AD-CSF amino acids compared to 8.5% of the normal CSF amino acids, and His constitutes 3.8% of the AD total versus 2.5% of the normal. His and Gln were also found to be elevated in the AD CSF by the Japanese lab, but the differences were not statistically significant. The Japanese analyses, however, did show a statistically significant ($p < 0.05$) increase for Phe in AD compared to normals. The differences between the Italian and Japanese labs may be due to the respective methods used and/or to the different numbers of samples.

Discussion

The finding that L-Gln is elevated in Alzheimer's ventricular CSF may be of particular interest since L-Gln has recently been found to be significantly elevated in lumbar CSF of Parkinson's patients (Mally et al., 1997), and Gln is metabolized within a neuron to Glu, which is a neurotransmitter. Therefore, the concentrations of Gln could be relevant to the changes taking place within a neuron in Alzheimer's disease (Proctor et al., 1988). Since ventricular CSF contains free amino acids and other compounds which come from various areas of the brain, the chemical composition of ventricular CSF closely represents the general chemical composition of the brain. Previously, we found that AD cerebral cortex proteins and AD neurofibrillary tangles contain significantly higher levels of D-amino acids (D-Asp and D-Ala) than in normal brains (Fisher et al., 1992a,b; D'Aniello et al., 1992). However, the levels of free D-Asp are significantly lower ($p < 0.01$) in AD hippocampus, frontal, temporal and parietal cortices (D'Aniello et al., 1998). Thus, the increased levels of free D-amino acids found in AD-CSF could be a reflection of the degenerative process occurring in the Alzheimer brain proteins.

Table 2. Total free amino acid concentrations of normal and Alzheimer ventricular CSF

	OPA-THIOL method		Ratio Alz/Nrml	FLEC method		Ratio Alz/Nrml
	Alzheimer n = 21 75.2 ± 10.4 yr 11.8 ± 6.1 hr PMI nmol/ml	Normal n = 11 70.1 ± 7.8 yr 15.2 ± 7.3 hr PMI nmol/ml		Alzheimer n = 10 78.6 ± 13.8 yr 13.2 ± 5.6 hr PMI nmol/ml	Normal n = 5 66.0 ± 7.4 yr 10.9 ± 4.6 hr PMI nmol/ml	
Glu	719 ± 538	544 ± 461	1.32	433 ± 282	268 ± 262	1.62
Ala	396 ± 271	537 ± 631	0.74	277 ± 153	152 ± 74	1.82
Gln	592 ± 535	244 ± 142	2.43*	39 ± 35	27 ± 10	1.44
His	120 ± 78	73 ± 44	1.64*	17 ± 7	12 ± 9	1.42
Gly	201 ± 132	185 ± 136	1.09	64 ± 45	53 ± 44	1.21
Ser	121 ± 81	109 ± 47	1.11	76 ± 43	44 ± 23	1.73
Lys	90 ± 126	62 ± 59	1.46	6.8 ± 5.5	8.1 ± 3.3	0.84
Leu	70 ± 48	70 ± 28	1.00	27 ± 18	28 ± 26	0.96
Phe	53 ± 40	68 ± 58	0.78	34 ± 28	11 ± 4	3.09*
Arg	88 ± 78	77 ± 109	1.13	33 ± 24	26 ± 14	1.27
Thr	71 ± 53	74 ± 44	0.97	36 ± 18	31 ± 17	1.1
Val	49 ± 29	56 ± 38	0.87	29 ± 15	22 ± 11	1.3
GABA	98 ± 102	178 ± 272	0.55	30 ± 36	22 ± 18	1.36
Asp	138 ± 244	144 ± 163	0.96	52 ± 41	86 ± 123	0.60
Tyr	76 ± 86	147 ± 213	0.52	ND	ND	
Asn	65 ± 53	35 ± 48	1.88	15 ± 12	8.9 ± 6.0	1.69
Tau	120 ± 158	129 ± 280	0.93	60 ± 49	35 ± 23	1.71
Ile	31 ± 18	83 ± 93	0.37	11 ± 7	13 ± 10	0.85
Met	58 ± 72	70 ± 99	0.84	3.7 ± 5.2	8.6 ± 6.6	0.43
Pro	ND	ND		28 ± 22	23 ± 17	1.22
Orn	ND	ND		1.0 ± 1.4	1.6 ± 0.6	0.62
Sum	3,158 ± 1,680	2,884 ± 2,258	1.09	1,274 ± 847	871 ± 690	1.46

Figures are means ± standard deviation; *ND* not determined; p values refer to two-sample t-test comparison of Alzheimers to normals; *$p < 0.05$.

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References

Aswad D (1984) Determination of D- and L-aspartate in amino acid mixtures by high performance liquid chromatography after derivatization with *o*-phthaldialdehyde. Anal Biochem 137: 405–407

D'Aniello A, Vetere A, Fisher GH, Cusano G, Chavez M, Petrucelli L (1992) Presence of D-alanine in proteins of normal and Alzheimer human brain. Brain Res 592: 44–48

D'Aniello A, Petrucelli L, Gardner C, Fisher G (1993a) Improved method for hydrolyzing proteins and peptides without inducing racemization and for determining their true D-amino acid content. Anal Biochem 213: 290–295

D'Aniello A, D'Onofrio G, Pischetola M, D'Aniello G, Vetere A, Petrucelli L, Fisher GH (1993b) Biological role of D-amino acid oxidase and D-aspartate oxidase: effects of D-amino acids. J Biol Chem 268: 26941–26949

D'Aniello A, Lee JM, Petrucelli L, Di Fiore M (1998) Regional decreases of free D-aspartate levels in Alzheimer's disease. Neurosci Lett (In press)

Degrell I, Hellsing K, Nagy E, Niklasson F (1989) Amino acid concentrations in cerebrospinal fluid in presenile and senile dementia of Alzheimer type and multi-infarct dementia. Arch Gerentol Geriatr 9: 123–135

Fisher GH, D'Aniello A, Vetere A, Padula L, Cusano G, Man EH (1991) Free D-aspartate and D-alanine in normal and Alzheimer brain. Brain Res Bull 26: 983–985

Fisher GH, Payan IL, Chou S-J, Man EH, Cerwinski S, Martin T, Emory C, Frey WH (1992a) Racemized D-aspartate in Alzheimer neurofibrillary tangles. Brain Res Bull 28: 12713–127131

Fisher GH, D'Aniello A, Vetere A, Cusano G, Chavez M, Petrucelli L (1992b) Quantification of D-aspartic acid in normal and Alzheimer brains. Neurosci Lett 143: 215–218

Fisher GH, Petrucelli L, Gardner C, Emory C, Frey WH, Amaducci L, Sorbi S, Sorrentino G, Borghi M, D'Aniello A (1994) Free D-amino acids in human cerebrospinal fluid of Alzheimer disease, multiple sclerosis, and healthy control subjects. Molec Chem Neuropathol 23: 115–124

Gelot MA, Jeandel C, Belleville F, Nabet P, Penin F, Cuny G (1990) Acides amines du liquid cephalo-rachidien dans la maladie d'Alzheimer. Ann Biol Clin 48: 182–184

Hashimoto A, Nishkawa T, Oka T, Takahashi K, Hayashi T (1992) Determination of free amino acids and enatiomers in rat brain and serum by high performance liquid chromatography after derivatization with N-tert-butyloxycarbonyl-L-cysteine and *o*-phthaldiadehyde. J Chromatog 582: 41–48

Kapaki E, Libidaki G, Segditsa J, Tegos S, Zournas CH, Papageorigou C (1991) Determination of cerebrospinal fluid amino acids in patients with Alzheimer's disease. Med Sci Res 19: 35–36

Mally J, Szalai Z, Stone TW (1997) Changes in the concentration of amino acids in serum and cerebrospinal fluid of patients with Parkinson's disease. J Neurol Sci 151: 159–162

Martinez M, Frank A, Diez-Tejedor E, Hernandez A (1993) Amino acid concentrations in cerebrospinal fluid and serum in Alzheimer's disease and vascular dementia. J Neural Transm 6: 1–9

Okuma E, Abe H (1994) Simultaneous determination of D- and L-amino acids in the nervous system of crustaceans using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase ion-pair high performance liquid chromatography. J Chromatogr 660: 243–250

Pomara N, Singh R, Deptula D, Chou J C-Y, Schwartz MB, LeWitt PA (1992) Glutamate and other CSF amino acids in Alzheimer's disease. Am J Psychiatry 149: 251–254

Procter AW, Palmer AM, Francis PT, Lowe SL, Neary D, Murphy E, Doshi R, Bowen DM (1988) Evidence of glutamatergic denervation and possible abnormal metabolism in Alzheimer's disease. J Neurochem 50: 790–801

Roher AE, Lowenson JD, Clarke S, Wokow C, Wang R, Cotter RJ, Reardon IM, Zurcher-Neely HA, Heinrikson RL, Ball MJ, Greenberg BD (1993) Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. J Biol Chem 268: 3072–2083

Smith CC, Bowen DM, Francis PT, Snowden JS, Neary D (1985) Putative amino acid transmitters in lumbar cerebrospinal fluid of patients with histologically verified Alzheimer's dementia. J Neurol Neurosurg Psychiatry 48: 469–471

Tarbit I, Perry EK, Perry RH, Blessed G, Tomlinson BE (1980) Hippocampal free amino acids in Alzheimer's disease. J Neurochem 35: 1246–1249

Tohgi H, Abe T, Takahashi S, Kimura M (1992) A selective reduction of excitatory amino acids in cerebrospinal fluid of patients with Alzheimer type dementia compared with vascular dementia of the Binswanger type. Neurosci Lett 141: 5–8

Authors' address: George H. Fisher, Ph.D., Professor of Chemistry, Barry University, Miami Shores, FL 33161, U.S.A., Internet: gfisher@diana.barry.edu; Fax (305) 899-3479

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