CSF excitatory amino acids and severity of illness in Alzheimer's disease

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Article abstract—Researchers have proposed that increased release of excitatory amino acids (EAAs) is involved in the pathogenesis of dementia of the Alzheimer type (DAT), and CSF EAA concentrations have been measured to obtain evidence in support of this hypothesis. However, previous comparisons of CSF EAA concentrations in patients with DAT and in controls have yielded inconsistent results, perhaps because patient samples have been heterogeneous as to dementia severity. To determine whether there are changes in CSF concentrations of EAAs related to severity of illness in patients with DAT, we measured CSF concentrations of glutamate, aspartate, and taurine in 32 subjects with DAT, in whom we also assessed the severity of illness using clinical and neuropsychological measures, and 11 age-matched controls. The results suggested that increased CSF aspartate and glutamate concentrations, as well as decreased taurine concentrations, may occur in some persons with more advanced symptoms of DAT.

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Dementia of the Alzheimer type (DAT) is the major cause of dementia in late life.[1] The neuropathology of DAT is characterized by the degeneration and loss of neurons from many brain regions, including the cerebral cortex, hippocampus, and basal forebrain.[2] Pyramidal neurons are the predominant cell type affected in the former two structures. The acidic amino acid, glutamate (GLU), is considered the major excitatory neurotransmitter for the pyramidal neurons of the cerebral cortex and hippocampus[3] and has both neuroexcitatory and neurotoxic properties.[4] Aspartate (ASP), another amino acid neurotransmitter abundantly present in the brain, demonstrates the same excitatory and excitotoxic properties as GLU. Excessive release of these excitatory amino acids (EAAs) may be a mechanism whereby neuronal loss may occur in DAT.[4]

Despite such hypotheses, there are few antemortem studies of EAAs in persons with DAT. The analysis of CSF EAA concentrations in individuals with DAT may offer an indirect assay of EAA levels in some brain areas, such as the neocortex and hippocampus, which are proximal to CSF spaces.[5] However, prior studies of CSF EAA concentrations in persons with DAT and controls have yielded inconsistent results, perhaps because patients with mixed severities of illness were combined, and stage-specific alterations were obscured. In addition, a variety of methods were used to obtain the CSF, deproteinate the CSF, and assay the CSF samples for EAAs. The purpose of this study was to quantify CSF EAA concentrations and severity of illness in DAT patients, and to use optimal methods for collection, storage and assaying of CSF samples. We also quantified CSF concentrations of taurine (TAR) to provide for a non-EAA comparison.

Methods. *Subject selection and assessment.* Twenty-nine subjects with DAT and eleven nondemented controls gave informed consent for the collection of CSF. All control and DAT subjects had previously been registered into longitudinal studies at the Memory and Aging Project of the Washington University Alzheimer's Disease Research Center and had been interviewed, along with their informants, to determine the presence or absence of dementia, and, when present, its stage using the standardized Washington University Clinical Dementia Rating (CDR).[6-8] At entry and annually thereafter, an expert clinician rated the subjects' cognitive abilities in each of six categories: memory, orientation, judgment and problem solving, function in community affairs, function in home and hobbies, and function in personal care. In each of these categories, impairment was rated on a five-point scale (none = 0, questionable = 0.5, mild = 1, moderate = 2, severe = 3). The scores from each of these categories (*Boxes*) were then summed to obtain a quantitative summary of severity of illness (*Sum of Boxes*). Previous studies have shown this measure to be highly sensitive in detecting progression of the illness over time.[9,10] From a further compilation of these six category ratings, a global CDR was also derived, i.e., a rating of no dementia (CDR 0), questionable dementia (CDR = 0.5), mild dementia (CDR 1), moderate dementia (CDR 2), or severe dementia (CDR 3).

Interrater reliability for the CDR is high, i.e., Kendall's tau B = 0.91; kappa = 0.74, weighted kappa = 0.87.[11] The diagnostic criteria for DAT[12] have been linked to the CDR

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and have been validated by autopsy with 96% accuracy for Alzheimer's disease.[13,14] Longitudinal data have been collected on subjects entered with questionable dementia (i.e., CDR 0.5), and the majority of them have been shown to progress to the more severe stages of DAT.[15] In addition, subjects designated as CDR 0.5, who have come to autopsy, have had Alzheimer's disease confirmed histologically.[16] The Sum of Boxes score and the CDR were derived without reference to the subject's performance on neuropsychological tests.

Independently of the clinical assessments, the subjects were also administered a battery of neuropsychological tests[17,18] as part of their annual assessment. For more severely demented subjects, testing was constrained by the inability to understand test instructions or perform the tasks (floor effects); therefore, sample sizes for the neuropsychological data reported here varied between 30 and 34 of the total sample of 40 subjects. Because of the multidisciplinary and longitudinal nature of the overall research effort of the Center, neuropsychological data from many of the participants of this study have been included in other reports.

Three tests from the neuropsychological battery were chosen to represent three types of cognitive abilities often found to be disturbed in patients with DAT.[18] Tests were administered according to their test manual instructions unless otherwise noted. The Associate Learning subtest of the Wechsler Memory Scale (WMS)[19] was used to measure episodic memory.[20] Scores on this test can range from 0 to 21; lower scores indicate greater impairment. The WMS Associate Learning subtest was chosen for this study rather than the WMS Logical Memory subtest because the latter is subject to extreme floor effects in moderate to severe dementia. The 60-item–version Boston Naming Test[21] was used to assess semantic memory. All items were administered, beginning with the first one; no phonemic cues were given. Scores can range from 0 to 60 items correctly named; lower scores indicate greater impairment. Trailmaking A[22] was used to assess visuomotor performance, sequenced cognition, and attention. A maximum of 180 seconds was allowed. Scores in this sample ranged from 25 to 180 seconds; higher scores indicate greater impairment.

Subjects rated as CDR 0, 0.5, 1, and 2 participated in this study within 4 months of their clinical assessment and staging. Since CDR 3 participants were expected to remain CDR 3 for the remainder of their illness, their participation was not limited by the above criterion. All subjects were at least aged 55 years and English-speaking. The exclusion criteria[12] were (1) comorbidity of dementia with any other psychiatric, neurologic, or relevant general medical illness, (2) diagnosis of primary depression, or concurrent mood disorder of any kind that fulfilled DSM-III–R[23] criteria, within the past 5 years, and (3) current treatment with drugs known to alter CSF EAA concentrations.[5,24]

Collection of CSF. CSF samples were obtained via lumbar puncture, performed generally in the lateral decubitus position, between 8:00 and 9:00 AM following overnight bed rest and fasting. When CSF could not be obtained in the lateral decubitus position, the subject was briefly raised to the sitting position. A total of 30 mL of CSF was collected, and a 2-mL aliquot comprising the third and fourth mL collected was selected for EAA analyses. This early aliquot was chosen since EAAs in CSF have not yet been shown to follow a cisternal-to-lumbar gradient.[5] Each CSF aliquot selected for the analysis of CSF EAA concentrations was passed through a 0.2-μm filter immediately following collection for deproteination. Deproteination by acidification was avoided since acidification without subsequent neutralization may elevate and confound CSF GLU concentrations by promoting the conversion of CSF glutamine to GLU.[25] Moreover, preliminary experiments were performed using CSF samples obtained other than those collected for this study to determine the effect of even brief (i.e., 10 minutes) acidification on CSF GLU concentrations, and demonstrated approximate 100% increases in CSF GLU concentrations and 10% decreases in CSF glutamine concentrations. Filtration, as described, was finally chosen for deproteination because it did not measurably alter CSF GLU or glutamine concentrations. After filtration, each aliquot was immediately frozen at −70° C for storage until the time of assay.

Quantification of CSF EAAs. GLU and ASP concentrations were quantified in duplicate from the designated 2-mL aliquot using a modification of an existing method.[26] L-dopa (Sigma, St. Louis, MO) was added to each sample prior to preparation for assay as an internal standard. Precolumn amino acid derivitization was achieved by mixing 20 μL of reconstituted sample with 40 μL of an opthalaldehyde (Eastman-Kodak, Rochester, NY)/B-mercaptoethanol (Sigma, St. Louis, MO) reagent. Two minutes later, a 20-μL sample of the supernatant was injected directly into a Beckmann integrated HPLC system, equipped with an ESA Coulochem 5100A detector, an ESA 5011 analytical cell, and an ESA HR-80 reverse phase column. The amino acids were eluted using a mobile phase consisting of 0.1 M Na_2HPO_4 and 25% methanol at 1.2 mL/min, and detected electrochemically at 0.60 V. The identities of GLU, ASP, and TAR in CSF were verified by adding authentic compounds to pilot CSF samples, not part of this dataset. Mean values from the duplicate assays were used in all data analyses.

Data analysis. Prior to a final analysis of the data, the clinical database of the Washington University Alzheimer's Disease Research Center was checked to determine whether any data collected subsequent to the subjects' lumbar puncture called the original diagnosis and eligibility for this study into question. The length of follow-up ranged from 19 to 35 months. This check revealed that one control subject was diagnosed as developing questionable DAT (i.e., CDR 0.5) 11 months after the lumbar puncture. One subject with questionable DAT was diagnosed with dementia associated with depression 15½ months after the lumbar puncture. The results are therefore presented in the text and in the figures and table with and without inclusion of these two cases.

Preliminary examination of the data revealed that the distribution of ASP and GLU values did not satisfy the assumption of normality. Also, both the Sum of Boxes and CDR scores are not continuous measures with regular intervals between score values. Therefore, nonparametric rank-order correlations (Kendall's tau, corrected for ties) were calculated to test for significant relationships between the variables. The p values (two-tailed) less than 0.05 were considered to be significant.

Results. The mean age of the subjects was 73 years (range, 53 to 90 years). Grouped by CDR scores, the subjects did not differ with respect to mean age (ANOVA with age as dependent variable, F = 0.630, p = NS). Furthermore, these five groups did not differ significantly with respect to gender (i.e., CDR 0 = 6 M/5 F, CDR 0.5 = 4 M/3 F, CDR 1 = 7 M/3 F, CDR 2 = 3 M/3 F, CDR 3 = 2 M/4 F). CSF ASP and GLU concentrations were not significantly correlated with age (CSF ASP: tau = −0.090, p = NS; CSF GLU: tau = 0.034; p = NS). However, CSF TAR concentrations were significantly correlated with age (tau = −0.234; p = 0.034).

Scattergrams of CSF ASP, GLU, and TAR concentrations versus the Sum of Boxes scores are shown in figure 1. In general, CSF GLU concentrations were lower than CSF ASP concentrations, and lower than CSF GLU concentrations reported by some other investigators.[27] Presumably, the lower GLU concentrations observed in this study were attributable to our choice of filtration as a method for sample deproteination (see Discussion).

Correlations between increasing Sum of Boxes scores and higher CSF ASP concentrations (tau = 0.218, p = 0.048) and between lower CSF TAR concentrations (tau adjusted for ties = −0.231, p = 0.036) were statistically significant. A trend was also found between increasing Sum of Boxes scores and higher CSF GLU concentrations (tau = 0.202, p = 0.067). When the two questionable cases were excluded from the analysis the relationships between the Sum of Boxes scores and CSF amino acid concentrations were strengthened (i.e., for CSF ASP: tau = 0.259, p = 0.022; for CSF GLU: tau = 0.247, p = 0.029; for CSF TAR: tau = −0.247, p = 0.029). However, the scattergrams in figure 1 reveal substantial variance in CSF concentrations in patients with higher Sum of Boxes scores. In particular, there appear to be outlying subjects with several-fold elevations in both CSF ASP and GLU concentrations. The correlations between CSF amino acid concentrations and CDR scores (CSF ASP and CDR: tau = 0.184, p = 0.094; CSF GLU and CDR: tau = 0.158, p = 0.150; CSF TAR and CDR: tau = −0.264, p = 0.016) were generally weaker than those observed using the Sum of Boxes scores. Again, when the two questionable cases were excluded, these correlations were strengthened (i.e., for CSF ASP: tau = 0.215, p = 0.057; for CSF GLU: tau = 0.196, p = 0.083; for CSF TAR: tau = −0.279, p = 0.014). However, with or without the questionable cases, only the correlation between CSF TAR and CDR scores achieved statistical significance.

Correlations between CSF amino acid concentrations and performance on the selected neuropsychological tests were either absent or weak (table). After exclusion of the questionable subjects, a significant correlation was found between higher CSF GLU concentrations and poorer performance on the Trailmaking A test, and between lower CSF TAR concentrations and poorer performance on the Associate Learning test. However, the scattergrams shown in figure 2 again show that a few subjects with outlying CSF EAA values account for the significance of these correlations, particularly in the case of the correlation between CSF GLU concentrations and Trailmaking A test performance.

CSF GLU and ASP concentrations were strongly intercorrelated (tau = 0.631, $p < 0.0001$) (figure 3). In contrast,

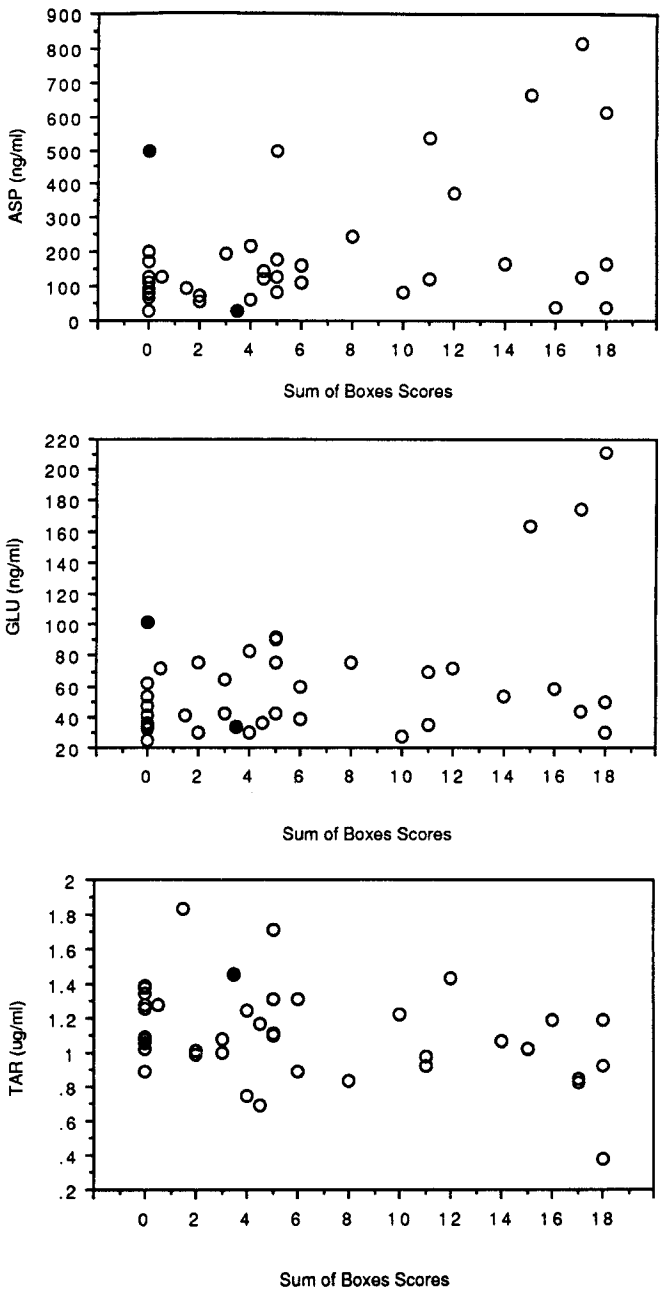

Figure 1. Scattergrams illustrating CSF amino acid concentrations and clinical severity of illness scores in subjects with DAT and controls. Sum of Boxes scores represent a clinical index of severity of illness. The two patients with questionable diagnoses are indicated as filled circles (see Methods).

CSF TAR concentrations were not significantly correlated with either CSF ASP (tau = −0.154, p = 0.162) or CSF GLU concentrations (tau = −0.082, p = 0.456).

Discussion. These results do not support the conclusion that there are progressive elevations of CSF EAA concentrations with increasing (or decreasing) severity of illness in subjects with DAT. Rather, the data suggest that there is considerable variability of CSF EAA concentrations in DAT patients, and that in a few subjects with more severe illness, CSF EAA

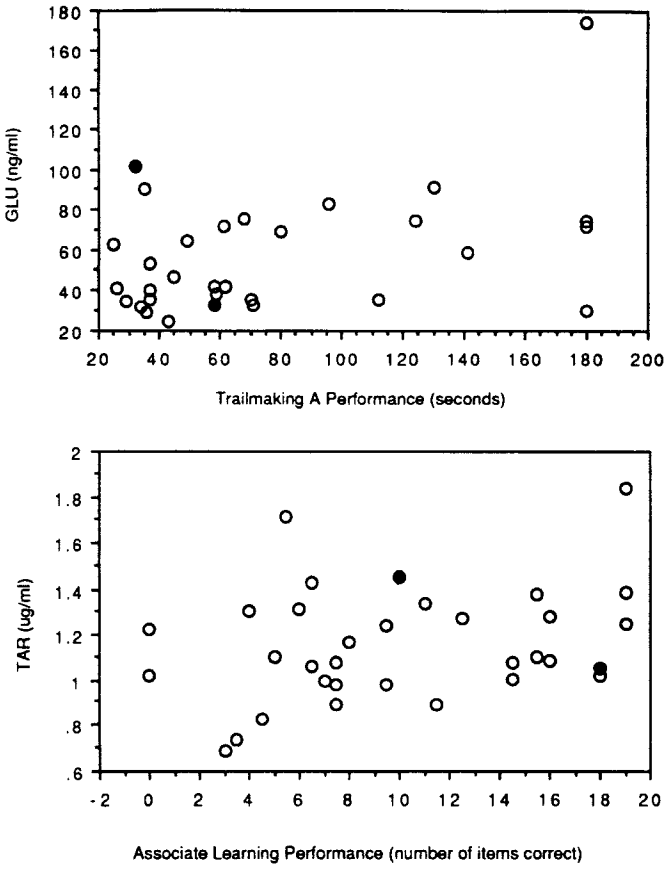

Figure 2. Scattergrams illustrating statistically significant CSF amino acid values and neuropsychological test performance scores (see the table) in subjects with DAT and controls. The two cases with questionable diagnoses are indicated as filled circles (see Methods).

concentrations may be abnormally elevated compared with patients with less severe illness and controls. This pattern of results was present most clearly using the clinical Sum of Boxes scores. The analysis of relationships between the selected neuropsychological measures and CSF EAA concentrations was largely negative, probably because some of the more severely ill subjects could not be tested. The findings were similar for both CSF ASP and GLU. However, this point should not be surprising, given that the CSF concentrations of these two EAAs were highly intercorrelated.

In contrast to CSF ASP and GLU, CSF TAR concentrations appeared to be decreased in some DAT subjects with more severe illness. Again, variability among CSF TAR concentrations in subjects with more severe illness largely accounted for these findings. Also, CSF TAR alone was correlated (inversely) with age, despite the absence of a relationship between clinical severity, as assessed by Sum of Boxes or CDR scores, and age in this sample of subjects. Contrasting findings with regard to CSF ASP, GLU, and TAR concentrations suggest that the observed differences in CSF EAA concentrations in DAT subjects were probably not related to general phenomena that might have altered the concentration of all

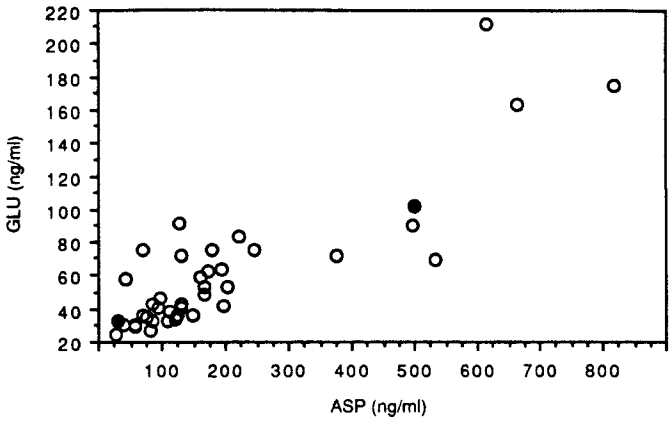

Figure 3. Scattergram illustrating the CSF ASP and GLU concentrations in subjects with DAT and controls (see text). The two patients with questionable diagnoses are indicated as filled circles (see Methods).

amino acids or substances in CSF. For example, dilutional changes in the concentrations of CSF substances caused by enlargement of the CSF spaces would likely have been uniform reductions, as was observed for CSF TAR.

These results may help to resolve discrepancies in previous observations.[27] Prior CSF GLU concentrations in DAT subjects have yielded mixed results, perhaps because of small sample sizes, varying methods for CSF collection, storage and assay, and the practice of combining DAT subjects with varying severity of illness into a single cohort. Two studies found no significant alterations in lumbar CSF GLU concentrations in mixed-stage groups of subjects (N = 11 and 16) with DAT compared with controls.[28,29] However, another report, in which all subjects (N = 15) had exhibited the disease for at least 3 years (but were not systematically staged), found significant reductions in CSF GLU.[30] Finally, Martinez et al.[31] found increases in CSF ASP but decreases in CSF GLU in subjects with DAT (N = 13) compared with controls.

Pomara et al.[32] examined CSF EAA concentrations in DAT subjects specifically staged as having mild and moderate dementia (N = 10), and found significant elevations in CSF GLU concentrations com-

Table *Correlations between degree of cognitive impairment and CSF amino acid concentrations*

Cognitive test	CSF ASP	CSF GLU	CSF TAR
Associate Learning Test	−0.140(−0.190)	−0.090(−0.137)	0.221(0.251*)
Boston Naming Test	−0.065(−0.108)	−0.115(−0.169)	0.018(0.039)
Trailmaking A†	0.133(0.187)	0.207(0.271*)	−0.224(−0.257)

Numbers in parentheses are correlation coefficients (Kendall's tau) calculated after excluding the two questionable cases (see Methods for details).

* $p < 0.05$.

† For this test, higher scores indicate more impairment.

pared with age-matched controls. Furthermore, these investigators found a similar but statistically nonsignificant trend ($p = 0.09$) towards increased CSF ASP concentrations. However, Toghi et al.[33] (N = 13), although they also excluded severely ill subjects, found increases in CSF ASP but decreases in CSF GLU compared with controls. In only one prior study, Smith et al.[28] addressed the association between CSF EAA concentrations and cognitive impairment in patients with DAT (N = 11). They reported a correlation between increasing CSF GLU concentrations and more severe cognitive impairment in subjects with DAT, although they did not find significant group differences in CSF GLU concentrations between biopsy-verified DAT patients and controls.

Taken together, these studies suggest that increases in CSF EAA concentrations may occur at least in some subjects with DAT and that the degree of elevation may vary with the severity of the disease. Our data are not inconsistent with this conclusion. However, the variability of our values in more severely ill patients suggests that there is not a smoothly progressive relationship between CSF EAA concentrations and severity of illness in DAT. Our findings, obtained in the largest sample to date, suggest that such relationships may be due to a small number of abnormal values.

Only Pomara et al.[32] have reported decreases in CSF TAR concentrations in DAT subjects. Martinez et al.[31] who also quantified this amino acid, found no difference in CSF TAR concentrations between a small (N = 13), mixed group of DAT subjects and controls. Further comparisons of a variety of CSF amino acid concentrations will be necessary to determine whether this additional finding is due to nonspecific factors, such as dilution, or specific pathophysiologic mechanisms. In either case, our data suggest that other CSF amino acids may be altered in DAT.

CSF concentrations of neuroactive substances are presumed to reflect their concentrations in the brain. This claim is plausible for the cortex given its size and proximity to CSF spaces. McGale et al.[5] reported that there were no differences between the ventricular and lumbar CSF concentrations for most amino acids, including ASP and GLU. This evidence suggests that the measurement of ASP, GLU, and TAR concentrations in lumbar CSF can provide a valid measure of their concentrations near the brain. However, no studies in humans or animals have as yet examined the correlations between CSF ASP, GLU, and TAR concentrations in lumbar or ventricular CSF and specific brain areas. The results of such studies would be critical in interpreting the meaning of CSF EAA changes in disease states.

Among methodologic problems encountered in assaying CSF concentrations of amino acids is the potential for chemical conversion during processing or storage of the CSF sample. For CSF GLU, this is a potentially serious problem, since glutamine can be converted into GLU by addition of perchloric and other acids, as has been done to deproteinate CSF samples.[25] Furthermore, concentrations of CSF glutamine exceed CSF GLU concentrations by 100-fold.[5] In this study, CSF samples were deproteinated using filtration rather than by acidification to avoid this problem (see Methods). Perhaps as the result of our choice of this method, our CSF GLU concentrations were considerably lower than those reported in some previous studies.[28,32,33] Moreover, we found CSF GLU concentrations to be lower than CSF ASP concentrations, whereas the reverse is generally true in samples taken directly from brain.[5] In addition to the consideration of methods for deproteination, the relative concentrations of CSF GLU and ASP might also be affected by methods for the collection of CSF, in particular procedures for aliquoting.

CSF EAA concentrations might become elevated because of increases in synthesis and release of EAAs, decreased reuptake of CSF EAAs after release, or changes in the movement of EAAs across the brain/CSF and CSF/blood barriers. Procter et al.[34] have previously reported abnormalities of CSF EAA metabolism in postmortem brain samples from individuals with DAT. If such processes, particularly changes in release or reuptake, result in a group of DAT subjects with elevated CSF EAA concentrations, there are also at least two possible explanations for this finding. First, increased accumulation of CSF EAA may signal relatively late events in some patients with DAT that also contribute to end-stage neuronal damage and death. Similar to the way in which excitotoxic neuronal injury has been proposed as a subsequent complication of ischemic injury to the brain,[35] excitotoxicity in DAT may be a final pathway to the end stage of neural deterioration and triggered by other earlier neurotoxic processes. Second, the processes responsible for increased accumulation of CSF EAA may occur throughout the course of DAT in some subjects, but only become observable late in the illness when the disease is widespread and the amount of involved brain tissue in proximity to CSF spaces is large. With regard to the consideration of these two possibilities, the normal control who developed questionable DAT after undergoing a lumbar puncture had CSF ASP and GLU concentrations nearly as great as the most severely demented subjects and twice greater than any other control subject. This case, if not simply spurious, would seem to support the early emergence of increases in CSF EAA rather than either of these two possibilities.

These findings need to be replicated in an independent and larger sample where one can determine whether DAT patients with elevated CSF EAA concentrations are a genuine subgroup and not simply explained by a few spurious values. Methodologic factors that may affect CSF EAA concentrations should be studied further. If there is a subgroup of DAT subjects with elevated CSF EAA concentrations, the clinical, cognitive, and other neurobiologi-

cal characteristics of such patients should be carefully defined. Furthermore, correlations between increased concentrations of EAAs in CSF and neuropathology in specific brain regions should be studied. If increases in the presence of EAAs in brain tissue do play a specific pathogenetic role in the course of DAT in even a few patients, pharmacologic strategies to mitigate EAA release or EAA effects on postsynaptic sites of action might be useful to treat their illness.

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