INTRODUCTION

Aberrant glutamate stimulation has been proposed as a mechanism by which synapses and neurons are injured in Alzheimer's disease (AD) (**1**). Excitatory amino acid transporter 2 (EAAT2; also called GLT-1) is the major glutamate transporter in forebrain that is responsible for a number of essential neuroprotective and regulatory functions that include preventing glutamate-mediated injury to neurons and synapses, as well as regulating normal synaptic input specificity (2-5). A number of reports indicate that EAAT2 levels are significantly reduced in AD (6-8), thus raising the possibility that glutamate dyshomeostasis may play a significant role in AD pathogenesis. In addition, EAAT2 is oxidatively damaged by exposure to amyloid-beta (Aβ) (9-13). EAAT2 oxidation has been shown to impair glutamate uptake and also promote formation of high molecular weight EAAT2 oligomers that are insoluble in detergents such as Triton X-100 (14-16).

These findings suggest the hypothesis that AD pathogenesis may disrupt EAAT2 via mechanisms that recapitulate those of other key AD-related molecules, most notably Aβ that undergoes oxidation, misfolding, and aggregation. Although the studies cited above establish that EAAT2 is biochemically and functionally damaged by Aβ-related processes, the potential disease relevance of such findings have not been examined in AD patients and there is currently little evidence at the protein level addressing whether aberrant EAAT2 expression is related to the degree of cognitive loss and associated pathology. To address this important issue we measured Triton X-100-insoluble and Tritron X-100-soluble EAAT2 in the hippocampus and frontal cortex of more than 100 clinically and pathologically well-characterized normal controls, patients with Clinical Dementia Rating (CDR)=0.5 (17), and later-stage AD patients.

MATERIALS AND METHODS

Patients: Subjects came from the Alzheimer's Disease Center (ADC), Oregon Health and Sciences University (OHSU), and Alzheimer's Disease Research Center (ADRC) at the University of Washington (Table 1). Control subjects and subjects with clinical dementia rating CDR=0.5 (intended to approximate mild cognitive impairment) were participants in brain aging studies at the Oregon Aging/ADC. Subjects received annual neurologic and neuropsychologic evaluation, with CDR assigned by an experienced clinician. Controls had normal cognitive and functional examinations. CDR=0.5 subjects were functionally intact on enrollment and progressed to a global CDR=0.5 (no sub-scores greater than 0.5) at their last evaluation, within a year of autopsy. AD subjects were diagnosed by clinical team consensus conference, met NINDS-ADRDA diagnostic criteria for clinical AD, had CDR=>1.0, and neuropathologic confirmation at autopsy (after informed consent). Tissue use conformed to IRB-approved protocols. Neuropathologic assessment conformed to NIA-Reagan consensus criteria (18). The AD group included subjects with probable AD, moderate-to-frequent neuritic plaques, and Braak stage V-VI neurofibrillary tangles. Controls were clinically non-demented subjects with sparse or no neuritic plaques and neurofibrillary tangles ≤ Braak stage II. We excluded AD patients and controls with Lewy body disease involving the brainstem (including substantia nigra), amygdala, middle frontal gyrus, and patients with vascular brain disease manifested by grossly observed arterial territorial infarcts, grossly observed lacunar infarcts, or microvascular infarcts. PD patients had expected clinical signs, symptoms, and midbrain, but not cerebral cortex Lewy body pathology.

ELISAs: Brain samples were homogenized, sequentially extracted in 10 mM Tris, 1 mM EGTA, 1 mM DTT, 10% sucrose, and then extracted three times with 1% Triton X-100 as previously described (19). Remaining detergent-insoluble material was extracted with 70%

formic acid. Formic acid extracts of detergent-insoluble proteins were resolubilized and adsorbed onto 96-well plates as described elsewhere (19). EAAT2/GLT-1 was detected with antibodies AB12 and GLT-1A (20). PS1 was detected with PS1 N-terminal fragment antisera (21). Aβ was detected with 4G8 (Covance/Signet Laboratories, Deadham, MA). Total tau was detected using anti-tau antibody (Dako, Carpinteria, CA). ELISA plates were developed using standard methods with horseradish peroxidase (HRP)-conjugated antibodies and tetramethylbenzidine substrate.

Immunohistochemistry and Biochemistry: Standard immunohistochemical methods were used to evaluate EAAT2 staining in paraffin-embedded postmortem brain sections. Slides were incubated with AB12 and developed using 3,3'-Diaminobenzidine (Vector Laboratories, Burligame, CA). Double immunostaining labeled EAAT2 (DAB brown chromogen) and either total tau (Tau-2; Sigma, St. Louis, MO) or Aβ (4G8) labeld with Vector Red (Vector Laboratories, Burligame, CA). Counter-stains were omitted in double-label experiments. A Nikon Optiphot-2 microscope/Insight QE digital camera was used. Image acquisition was performed using Spot imaging software (Diagnostics Instruments, Sterling Heights, MI) and formatted with Photoshop. For each experiment, images were acquired and digitally processed under identical conditions. Digital image processing was limited to linear brightness and contrast adjustments performed identically on experimental and control images.

Mouse brain extracts were solublized in Laemmli sample buffer. Total Triton X-100 soluble protein concentrations were determined by the BCA method (Pierce, Rockford, IL) and western blotted with AB12 or GLT-1A, and detected using HRP-conjugated antibodies and chemiluminescence.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS): Equal amounts of formic acid-extracted protein were prepared from 5 AD subjects (mean age: 80 years; gender:

3 female and 2 male; CERAD NP score: Moderate or Frequent; Braak Stage: VI), pooled, dried, dissolved in bicarbonate buffer, reduced, and treated with iodoacetamide before being subjected to trypsin digestion as described previously (22). Eluted peptides were resuspended in 0.1% formic acid, separated by two-dimensional microcapillary high-performance LC, and amino acid sequences of separated peptides determined by tandem MS (ThermoFinnigan, San Jose, CA). Proteins from the mixture were identified automatically using the SEQUEST program, which searched spectral data against the International Protein Database. Sensitivity and specificity of protein identifications were determined by PeptideProphet and ProteinProphet as described previously (22).

Statistics: Data were analyzed with Analysis of Variance (ANOVA), Pearson correlation tests, and Fisher exact tests using SPSS 15.0 (SPSS, Chicago, IL). We predicted that detergent-insoluble EAAT2 would be highest in later stage AD cases, lower in CDR=0.5 cases, and lowest in normal controls that were predicted to be comparable to PD patients lacking concurrent AD pathology. To specifically test this *a priori* prediction well-established ANOVA trend analyses methods (23, 24) were used with the trend weights -2, -2, 1, and 3 corresponding to the PD, Normal controls, CDR=0.5, and AD, groups, respectively. In keeping with accepted methods these trend weights were selected because they are orthogonal integers (sum to zero) and represent the originally predicted relationships among the study groups (23, 24). Specifically, we predicted that the levels of insoluble EAAT2 in the PD and normal control groups would be both, comparable to each other, and lower than the other two groups (–2, -2; PD and Normal controls). The insoluble EAAT2 levels of the CDR=0.5 group were predicted to be higher than PD and normal controls but lower than the AD group, while the AD group was expected to have the highest insoluble EAAT2 levels (+1, +3 for the CDR=0.5 and AD groups respectively). These trend analyses followed only statistically significant omnibus ANOVA results.

RESULTS

Detection of detergent-insoluble EAAT2 in AD by mass spectrometry

To examine whether detergent-insoluble EAAT2 accumulates in AD we performed mass spectrometry (MS) on tryptic digests of total detergent-insoluble proteins pooled from five autopsy-confirmed AD patients. A total of 348 statistically significant ($p<0.05$) sequence-to-spectra matches corresponding to EAAT2 tryptic peptide fragments were identified (Figure 1A). These fragments clustered in six primary domains located throughout EAAT2 (Figure 1B), suggesting the entire molecule was represented in the detergent-insoluble protein fraction from AD brains.

These data confirm that Triton X-100-insoluble EAAT2 is present in the brains of AD patients. Despite the structural accuracy of LC-MS-MS, this approach is not well suited for quantitative comparisons between affected and control subjects. Thus, in order to examine whether EAAT2 detergent-insolubility is elevated in AD we developed two independent EAAT2 enzyme-linked immunosorbent assays (ELISAs). This approach was adopted to facilitate quantification of the relatively large number of samples to be tested (102 cortex and 53 hippocampus samples measured in triplicate).

Specificity of EAAT2 detection

AB12 is a polyclonal antibody that recognizes an amino-terminus epitope common to all EAAT2/GLT-1 isoforms, while GLT-1A is a polyclonal antibody that recognizes the C-terminal domain of the dominant EAAT2 isoform expressed in brain (20). These antibodies were tested under a variety of conditions using brain tissue from GLT-1 wild-type and GLT-1 knockout (KO) mice. Figure 2 shows that AB12 and GLT-1A selectively recognized EAAT2/GLT-1 by ELISA (Figure 2A). In addition, we verified that EAAT2/GLT-1 levels

measured by these ELISAs increased as a linear function of protein assayed (linear regression for AB12 ELISA: $r^2 = 0.8799$, $F[1,23]=168.6$, $p<0.0001$; and GLT-1A ELISA: $r^2 = 0.9567$, $F[1,23] = 507.8$, $p<0.00001$). AB12 and GLT-1A also detected GLT-1 by immunohistochemistry (Figure 2B), and recognized Triton X-100-soluble GLT-1 via western blots (Figure 2C) from GLT-1 wild-type, but not GLT-1 knock-out mice. These data prove that AB12 and GLT-1A specifically recognize EAAT2.

Detergent-insoluble EAAT2 aberrantly accumulates in AD:

Frontal cortex and hippocampus tissue samples were obtained from a well-characterized cohort of AD, CDR=0.5 (intended to approximate mild cognitive impairment), and normal control patients. Parkinson's disease (PD) subjects served as non-AD neurodegenerative disease controls (Table 1). Tissue samples were serially extracted three times with Triton X-100 to remove detergent-soluble proteins. Residual detergent-insoluble proteins were then solublized with formic acid. Neutralized formic acid-extracted proteins and the first Triton X-100-soluble fraction were analyzed by ELISAs to determine the relative levels of detergent-insoluble and detergent-soluble EAAT2.

In hippocampus, detergent-insoluble EAAT2 levels were elevated in AD patients compared to normal controls and PD patients, while detergent-insoluble EAAT2 levels in CDR=0.5 patients were intermediately elevated between the controls and later-stage AD patients (Figures 3 A, B). These differences in detergent-insoluble EAAT2 levels among groups detected either with AB12 or GLT-1A ELISAs were statistically significant ($F[3,49] = 7.107$, $p<0.0005$ and $F[3,49] = 6.733$, $p<0.0007$, respectively). These data suggest that EAAT2 becomes increasingly detergent-insoluble as AD-related pathology progresses from a less demented state represented by PD patients and normal controls, to CDR=0.5, and then becomes most pronounced by later stage AD. This conclusion was supported by the outcome

of a contrast analysis (See Materials and Methods) testing the prediction that detergent-insoluble EAAT2 levels followed a statistically significant trend where: Normals = PD < CDR=0.5 < AD (AB12: t[49] = 3.685, p<0.001; and GLT-1A: t[49] = 3.353, *p*<0.002). The scatter plot in figure 3C shows that detergent-insoluble EAAT2 levels in hippocampus measured by AB12 and GLT-1A ELISAs were significantly positively correlated (r = 0.820, N=53, *p*<0.01), thereby confirming the close correspondence between two independent assays that show EAAT2 detergent insolubility increases as AD pathology increases.

In marked contrast to these findings, Triton X-100-insoluble PS1 levels (Figure 3D) in the same samples did not differ among groups (F[3,49] = 2.141, n.s.). PS1 was chosen for comparison because it is localized prominently in astrocytes (25), which express approximately 80% of the EAAT2 in hippocampus and because overall PS1 expression levels are not affected by AD (26). These data argue that the detergent insolubility displayed by EAAT2 was not the result of generalized, non-specific cellular injury. Moreover, PS1 is structurally similar to EAAT2. Each molecule has eight hydrophobic membrane-spanning domains with the N- and C- terminal domains localized intracellularly (27). Thus, the detergent insolubility profiles of EAAT2 obtained from the identical samples are unlikely to have arisen by non-specific protein-protein interactions that might have occurred during the extraction process.

We also examined detergent-soluble EAAT2 levels in the same hippocampal samples (Figures 3 E, F). In contrast to the significant trend of increasing EAAT2 detergent insolubility that attended increasing AD pathology, there was a statistically significant difference among groups in detergent-soluble EAAT2 levels detected by AB12 ELISAs (F[3,49] = 3.237, *p*<0.030) where detergent-soluble EAAT2 levels appeared to decrease with increasing AD pathology. Differences in detergent-soluble EAAT2 levels detected by GLT-1A ELISAs were not statistically significant (F[3,49] = 2.468, n.s.).

Because detergent-soluble EAAT2 levels were comparatively low in AD patients with respect to normal controls while the detergent-insoluble EAAT2 levels were markedly elevated in the same samples, it is highly unlikely that the increased detergent-insoluble EAAT2 measured in AD patients could have been due to incomplete extraction of Triton X-100-soluble EAAT2 or that it reflects non-specific EAAT2 associations with detergent-insoluble amyloid plaques or neurofibrillary tangles (NFTs). This conclusion was further supported by the findings that detergent-soluble EAAT2 levels were not significantly correlated with detergent-insoluble Aβ levels measured by 4G8 ELISAs (AB12 vs. Aβ: r = -0.167, N=53, n.s. and GLT-1A vs. Aβ: r = -0.162, N=53, n.s.). Similarly, detergent-soluble EAAT2 levels were not correlated with insoluble tau levels measured by tau-2 ELISAs (AB12 vs. tau: r = -0.141, N=53, n.s. and GLT-1A vs. tau: r = -0.131, N=53, n.s.), findings that argue against the possibility non-aggregating EAAT2 in the lysates associated non-specifically with the insoluble Aβ or insoluble tau.

As observed in hippocampus, Triton X-100-insoluble EAAT2 levels were markedly elevated in AD frontal cortex compared to PD and normal control subjects (Figure 4A,B). Detergent-insoluble EAAT2 levels measured by GLT-1A ELISAs in CDR=0.5 subjects again fell between controls and AD levels (Figures 4B), while the increased detergent-insoluble EAAT2 levels measured by AB12 ELISAs in CDR=0.5 cortex were less pronounced (Figure 4A). These differences in detergent-insoluble EAAT2 levels among groups were statistically significant (AB12 ELISA: $F[3,98] = 8.743$, $p<0.00003$; GLT-1A ELISA: $F[3,98] = 13.375$, $p<0.00001$). Detergent-insoluble EAAT2 levels in frontal cortex (Figure 4 A, B) followed the predicted trend: controls=PD < CDR=0.5 <AD confirmed by statistically significant contrast test outcomes for both AB12 and GLT-1A ELISAs ($t[98] = 3.100$, $p<0.002$ and $t[98] = 4.087$, $p<0.0001$, respectively). Figure 4C shows a significant positive correlation between detergent-

insoluble EAAT2 levels measured using AB12 vs. GLT-1A ($r = 0.801$, $N=102$, $p<0.01$). Triton X-100-insoluble PS1 levels in frontal cortex (Figure 4D) did not differ significantly among groups ($F[3,98]=1.651$, n.s.). In contrast to hippocampus (Figure 3 E,F), detergent-soluble EAAT2 expression levels in frontal cortex of normal controls, CDR=0.5, and AD patients (Figure 4 E, F) were similar (AB12 ELISA: $F[3,98] = 3.669$, $p<0.015$; GLT-1A ELISA $F[3,98] = 1.330$, n.s). Again, detergent-soluble EAAT2 levels in frontal cortex did not correlate with the levels of detergent-insoluble Aβ or tau measured in the same samples (AB12 vs. Aβ: $r = -0.160$, $N=102$, n.s.; GLT-1A vs. Aβ: $r = -0.008$, $N=102$, n.s.; AB12 vs. tau: $r = -0.105$, $N=102$, n.s.; and GLT-1A vs. tau: $r = -0.049$, $N=102$, n.s.), thus arguing against the possibility that incomplete extraction or non-specific EAAT2 protein-protein interactions occurred during tissue processing.

EAAT2 localization appears comparatively normal in AD

In normal brain tissue EAAT2 is localized primarily, but not exclusively (see Discussion), in fine astrocytic processes that densely ramify throughout peri- and extra-synaptic domains (28-29). Having shown that Triton X-100-insoluble EAAT2 complexes accumulate in AD hippocampus and frontal cortex as determined by our biochemical assays, we examined whether aberrant EAAT2 expression could be localized near amyloid plaques, in association with NFTs, or aberrantly accumulate in neuronal or astrocytic cell bodies.

Double-label immuno-staining failed to reveal a consistent morphological association between EAAT2 expression and Aβ deposits in AD, which argues against the idea that EAAT2 accumulated in association with senile plaques *in vivo* (Figure 5A,B). In keeping with the predominantly astrocytic expression pattern characteristic of EAAT2 (28-29), we found a paucity of EAAT2 immunoreactivity in neuronal cell bodies and dendritic processes in both AD and normal control brains (Figure 5C, D). The primary morphologically neuron-like EAAT2

expression pattern observed was that associated with apparent neurofibrillary ghost tangles in AD subjects that immunostained weakly for EAAT2 (not shown). Thus, we found limited evidence that EAAT2 was prominently associated with NFTs, a finding consistent with the fact that NFTs are localized primarily in neurons, but not in astrocytes of AD patients (30).

Close inspection of EAAT2 immunostaining attributable to morphologically distinct astrocyte-like cells revealed that EAAT2 was expressed in distal processes and to a lesser extent in cell bodies, both in AD and control brains. Nonetheless, astrocytes with prominent cell body EAAT2 immunostaining were more readily identified in AD frontal cortex than in normal control cortex (Figure 5E, F). Despite this potentially interesting non-quantitative distinction between AD and control subjects, the overall immunohistochemical findings suggested that EAAT2 localization was not dramatically altered in AD patients compared to controls.

DISCUSSION

Aberrant EAAT2 detergent-insolubility is a novel biochemical lesion in AD

Using two independent ELISA systems to measure detergent-insoluble EAAT2, along with corroborating mass spectrometry, we found that detergent-insoluble EAAT2 is aberrantly elevated in AD patients compared to controls and intermediately elevated in mildly impaired CDR=0.5 patients with autopsy-confirmed prodromal AD neuropathology. These data argue that EAAT2 detergent insolubility represents a progressive biochemical lesion of AD. These findings further suggest that EAAT2 belongs to class of specific proteins (Aβ and tau being the best-characterized examples) that display altered detergent solubility in AD, while other proteins (31), including PS1 do not. The findings in this report are in keeping with previous work showing that glial fibrillary acidic protein (31) becomes increasingly detergent-insoluble in AD. Taken together these findings support the idea that detergent insolubility is an aspect of the disease process not restricted to some neuronal molecules, but also includes specific astrocytic proteins as well.

In contrast to detergent-insoluble EAAT2, detergent-soluble EAAT2 levels were reduced in AD patients compared to normal controls. These data are in keeping with previous human studies (6-8) and in findings from two AD mouse models (32, 33). Protein detergent insolubility does not appear to be recapitulated in AD mice models (data not shown). Thus, while it is currently not possible to examine detergent-insolubility in mice, the effects of AD-related pathology on soluble EAAT2/GLT-1 expression in AD patients and transgenic mice are nonetheless mutually supportive.

Our findings cannot address the mechanisms by which detergent-insoluble EAAT2 accumulates in AD. However, potentially important insights come from data showing that glutamate transporters are sensitive to biological conditions that promote reactive oxygen

species (16, 34). Significantly, oxidative stress is an early and persistent feature of AD (35). Post-translational oxidative EAAT2 modifications both inhibit glutamate uptake (16), and promote formation of detergent-insoluble high molecular weight multimers (34). This correspondence between detergent insolubility and reduced uptake suggests the possibility that increased levels of detergent-insoluble EAAT2 observed in AD brain tissue may reflect increasing EAAT2 dysfunction. This idea is consistent with data showing that Aβ generates oxidative radicals that impair glutamate uptake (36-37). In addition, Aβ impairs glutamate uptake in synaptosomes (9-13).

Until recently, EAAT2 had been widely accepted as an astrocyte-specific glutamate transporter. It is now clear that EAAT2 is also expressed by neurons (38). Such findings, in conjunction with a report that EAAT2 is sporadically expressed in tau-positive cortical and hippocampal neurons of some AD patients (39), raised the question of whether increased AD-related detergent-insoluble EAAT2 reflects aberrant EAAT2 expression in neurons. We did observe EAAT2 immunoreactivity associated with apparent ghost tangles (neurofibrillary remnants of dead neurons). However, much more striking was the lack of EAAT2 perikaryon immunoreactivity in either AD or normal control patients. These findings suggest that EAAT2 does not markedly accumulate in somal or proximal dendritic regions of neurons in AD. It seems more likely that detergent-insoluble EAAT2 complexes accumulate in fine peri-synaptic distal astrocytic processes where the majority of EAAT2 is normally localized (28), but which are difficult to investigate using light microscopic methods. Our human postmortem specimens are not suitable for the electron microscopic approaches required to quantitatively address this issue.

EAATs regulates multiple critical neuroprotective functions in the brain and are disturbed in AD

A family of five Na^+-dependent high affinity glutamate transporters referred to as EAAT1-5 (also known as GLAST, GLT-1, EAAC1, EAAT4, and EAAT5, respectively) carry out the critical task of clearing glutamate, primarily into astrocytes, thereby maintaining glutamate at basal extracellular concentrations recently estimated to be in the low nanomolar range (40). The importance of rapidly clearing extracellular glutamate is illustrated by the consequences of injecting potent glutamate transport blockers in mice, which die quickly of apparent acute glutamate toxicity (41). EAAT1, EAAT2, and neuron-specific EAAT3 are the primary glutamate transporters in the hippocampus and cortex. EAAT4 is expressed primarily in the cerebellum, while EAAT5 is expressed mostly in the retina (42). Of these transporters, EAAT2 is responsible for the majority of glutamate clearance in forebrain and accounts for approximately 80% of the glutamate transporters in hippocampus (28, 29). The disproportionate expression of EAAT2 compared to other glutamate transporter subtypes is reflected by the dramatic phenotype of GLT-1 knockout (KO) mice, which die shortly after birth due to seizures (2). The phenotypes of EAAT1/GLAST KO and EAAT3/EAAC1 KO mice are subtler. EAAT1/GLAST KO mice develop normally, but display defects in motor coordination related to cerebellar function (43) and EAAT3/EAAC1 knock out mice also breed normally but develop age-related neuronal loss (44). Nonetheless, both EAAT1 and EAAT3 expression are disturbed in AD (6, 44-46). Thus, there is evidence that multiple members of the Na^+-dependent glutamate transporter family, in addition to EAAT2, may contribute to AD-related pathogenic processes.

In addition to the critical role EAAT2 plays in preventing excitotoxicity (2), EAAT2 regulates stimulus-specific synaptic plasticity (5). EAAT2 loss has also been shown to impair activity-dependent glucose utilization (47). Over time it is plausible that impaired EAAT2 functions, even if initially latent or mild, may synergistically combine to promote increasingly pathogenic cycles of CNS dysfunction. Interestingly, memantine, a drug hypothesized to temper excessive NMDA receptor activation has efficacy in treating AD (48). Such findings

lend additional credence to the notion that disturbed glutamatergic signaling may play a significant role in AD pathogenesis. The findings in this report offer further evidence that glutamate-related dysfunction may be an important feature of AD pathology and suggest the possibility that strategies aimed at enhancing the natural neuroprotective properties of astrocytes may open new therapeutic opportunities to treat AD.

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REFERENCES

1. Lipton SA. Pathologically activated therapeutics for neuroprotection. Nat Rev Neurosci 2007; 8:803-808
2. Tanaka K, Watase K, Manabe T, et al. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 1997; 276:1699-1702
3. Namura S, Maeno H, Takami S, et al. Inhibition of glial glutamate transporter GLT-1 augments brain edema after transient focal cerebral ischemia in mice. Neurosci Lett 2002; 324:117-120
4. Pardo AC, Wong V, Benson LM, et al. Loss of the astrocyte glutamate transporter GLT1 modifies disease in SOD1(G93A) mice. Exp Neurol 2006; 201:120-130
5. Tsvetkov E, Shin RM, Bolshakov VY. Glutamate uptake determines pathway specificity of long-term potentiation in the neural circuitry of fear conditioning. Neuron 2004; 41:139-151
6. Jacob CP, Koutsilieri E, Bartl J, et al. Alterations in expression of glutamatergic transporters and receptors in sporadic Alzheimer's disease. J Alzheimer's Dis 2007; 11:97-116
7. Masliah E, Alford M, DeTeresa R, et al. Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. Ann Neurol 1996; 40:759-766
8. Abdul, HM, Sama, MA, Furman, JL et al. Cognitive decline in Alzheimer's disease is associated with selective changes in calcineurin/NFAT signaling. J Neurosci 2009; 29: 12957-12969
9. Guo ZH, Mattson MP. Neurotrophic factors protect cortical synaptic terminals against amyloid and oxidative stress-induced impairment of glucose transport, glutamate transport and mitochondrial function. Cereb. Cortex. 2000; 10:50-57

10. Guo Z, Ersoz A, Butterfield DA, et al. Beneficial effects of dietary restriction on cerebral cortical synaptic terminals: preservation of glucose and glutamate transport and mitochondrial function after exposure to amyloid beta-peptide, iron, and 3-nitropropionic acid. J Neurochem 2000; 75:314-320

11. Keller JN, Pang Z, Geddes JW, et al. Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid beta-peptide: role of the lipid peroxidation product 4-hydroxynonenal. J Neurochem 1997; 69:273-284

12. Keller JN, Germeyer A, Begley JG, et al. 17Beta-estradiol attenuates oxidative impairment of synaptic Na+/K+-ATPase activity, glucose transport, and glutamate transport induced by amyloid beta-peptide and iron. J Neurosci Res 1997; 50:522-530

13. Lauderback CM, Hackett JM, Huang FF, et al. The glial glutamate transporter, GLT-1, is oxidatively modified by 4- hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1- 42. J Neurochem 2001; 78:413-416

14. Haugeto O, Ullensvang K, Levy LM, et al. Brain glutamate transporter proteins form homomultimers. J. Biol. Chem. 1996; 271:27715-27722

15. Trotti D, Rizzini BL, Rossi D, et al. Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. Eur J Neurosci 1997; 9:1236-1243

16. Trotti D, Rossi D, Gjesdal O, et al. Peroxynitrite inhibits glutamate transporter subtypes. J Biol Chem 1996; 271:5976-5979

17. Hughes CP, Berg L, Danziger WL, et al. A new clinical scale for the staging of dementia. Br J Psychiatry 1982; 140:566-572

18. Ball M, Braak H, Goethe J, et al. Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute

Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. Neurobiol Aging 1997; 18:S1-2

19. Yang W, Woltjer RL, Sokal I, et al. Quantitative Proteomics Identifies Surfactant-Resistant {alpha}-Synuclein in Cerebral Cortex of Parkinsonism-Dementia Complex of Guam but Not Alzheimer's Disease or Progressive Supranuclear Palsy. Am J Pathol 2007; 171:993-1002
20. Williams SM, Sullivan RK, Scott HL, et al. Glial glutamate transporter expression patterns in brains from multiple mammalian species. Glia 2005; 49:520-541
21. Yang Y, Kinney GA, Spain WJ, et al. Presenilin-1 and intracellular calcium stores regulate neuronal glutamate uptake. J Neurochem 2004; 88:1361-1372
22. Woltjer RL, Cimino PJ, Boutte AM, et al. Proteomic determination of widespread detergent-insolubility including A-beta but not tau early in the pathogenesis of Alzheimer's disease. FASEB J 2005; 19:1923-1925
23. Field A. Discovering Statistics Using SPSS. 2nd ed. London: Sage Publications, 2005: 777
24. Winer BJ, Brown DR, Michels KM. Statistical Principles in Experimental Design. 3rd ed: McGraw-Hill Inc, 1991: 1057
25. Cribbs DH, Chen LS, Bende SM, et al. Widespread neuronal expression of the presenilin-1 early-onset Alzheimer's disease gene in the murine brain. Am J Pathol 1996; 148:1797-1806
26. Johnston JA, Froelich S, Lannfelt L, Cowburn RF, et al. Quantification of presenilin-1 mRNA in Alzheimer's disease brains. FEBS Lett 1996; 394:279-284
27. Yernool D, Boudker O, Jin Y, et al. Structure of a glutamate transporter homologue from Pyrococcus horikoshii. Nature 2004; 431:811-818

28. Furness DN, Dehnes Y, Akhtar AQ, et al. A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: New insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). Neuroscience 2008; 157:80-94

29. Lehre KP, Danbolt NC. The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J Neurosci 1998; 18:8751-8757

30. Feany MB, Dickson DW. Neurodegenerative disorders with extensive tau pathology: a comparative study and review. Ann Neurol 1996; 40:139-148

31. Woltjer RL, Sonnen JA, Sokal I, et al. Quantitation and mapping of cerebral detergent-insoluble proteins in the elderly. Brain Pathol 2009; 19:365-374.

32. Malm TM, Iivonen H, Goldsteins, G et al. Pyrrolidine dithiocarbamate activates Akt and improves spatial learning in APP/PS1 mice without affecting beta-amyloid burden. J Neurosci 2007;27: 3712-3721

33. Masliah E, Alford M, Mallory M, et al. Abnormal glutamate transport function in mutant amyloid precursor protein transgenic mice. Exp Neurol 2000; 163:381-387

34. Trotti D, Danbolt NC, Volterra A. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol Sci 1998; 19:328-334

35. Moreira PI, Santos MS, Oliveira CR, et al. Alzheimer disease and the role of free radicals in the pathogenesis of the disease. CNS Neurol Disord Drug Targets 2008; 7:3-10

36. Harris ME, Carney JM, Cole PS, et al. beta-Amyloid peptide-derived, oxygen-dependent free radicals inhibit glutamate uptake in cultured astrocytes: implications for Alzheimer's disease. Neuroreport 1995; 6:1875-1879

37. Harris ME, Wang Y, Pedigo NW Jr, et al. Amyloid beta peptide (25-35) inhibits Na+-dependent glutamate uptake in rat hippocampal astrocyte cultures. J Neurochem 1996; 67:277-286

38. Chen W, Mahadomrongkul V, Berger UV, et al. The glutamate transporter GLT1a is expressed in excitatory axon terminals of mature hippocampal neurons. J Neurosci 2004; 24:1136-1148

39. Thal DR. Excitatory amino acid transporter EAAT-2 in tangle-bearing neurons in Alzheimer's disease. Brain Pathol 2002; 12:405-411

40. Herman MA, Jahr CE. Extracellular glutamate concentration in hippocampal slice. J Neurosci; 2007; 27:9736-9741

41. Shimamoto K, Sakai R, Takaoka K, et al. Characterization of novel L-threo-beta-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. Mol Pharmacol 2004; 65:1008-1015

42. Danbolt NC. Glutamate uptake. Prog Neurobiol 2001; 65:1-105

43. Watase K, Hashimoto K, Kano M, et al. Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. Eur J Neurosci 1998; 10:976-988

44. Aoyama K, Suh SW, Hamby AM, et al. Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nat Neurosci 2006; 9:119-126

45. Duerson K, Woltjer RL, Mookherjee P, et al. Detergent-Insoluble EAAC1/EAAT3 Aberrantly Accumulates in Hippocampal Neurons of Alzheimer's Disease Patients. Brain Pathol 2009; 19:267-278

46. Scott HL, Pow DV, Tannenberg AE, et al. Aberrant expression of the glutamate transporter excitatory amino acid transporter 1 (EAAT1) in Alzheimer's disease. J Neurosci 2002; 22:RC206

47. Voutsinos-Porche B, Bonvento G, Tanaka K, et al. Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. Neuron 2003; 37:275-286

48. Lipton SA. Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. Nat Rev Drug Discov 2006; 5:160-170