Published in final edited form as:

Neurobiol Dis. 2012 January ; 45(1): 381–387. doi:10.1016/j.nbd.2011.08.027.

Preferential accumulation of amyloid-beta in presynaptic glutamatergic terminals (VGluT1 and VGluT2) in Alzheimer's disease cortex

Sophie Sokolow^{1,2,3}, Sanh H. Luu¹, Karabi Nandy^{1,4}, Carol A. Miller⁵, Harry V. Vinters⁶, Wayne W. Poon⁷, and Karen H. Gyls^{1,2,3}

¹UCLA School of Nursing, Los Angeles, CA 90095, USA

²UCLA Center for the Advancement of Gerontological Nursing Sciences, Los Angeles, CA 90095, USA

³UCLA Brain Research Institute, Los Angeles, CA 90095, USA

⁴UCLA Department of Biostatistics, School of Public Health, Los Angeles, CA 90095, USA

⁵Departments of Pathology, Neurology and Program in Neuroscience, the Keck USC School of Medicine, Los Angeles, CA 90033, USA

⁶Dept. of Pathology and Laboratory Medicine and Neurology, UCLA School of Medicine, Los Angeles, CA 90095, USA

⁷Institute for Memory Impairments and Neurological Disorders, UC Irvine, Irvine 92697 CA

Abstract

Amyloid-beta (A β) is thought to play a central role in synaptic dysfunction (e.g. neurotransmitter release) and synapse loss. Glutamatergic dysfunction is involved in the pathology of Alzheimer's disease (AD) and perhaps plays a central role in age-related cognitive impairment. Yet, it is largely unknown whether A β accumulates in excitatory boutons. To assess the possibility that glutamatergic terminals are lost in AD patients, control and AD synaptosomes were immunolabeled for the most abundant vesicular glutamate transporters (VGluT1 and VGluT2) and quantified by flow cytometry and immunoblot methods. In post-mortem parietal cortex from aged control subjects, glutamatergic boutons are fairly abundant as approximately 40% were immunoreactive for VGluT1 (37%) and VGluT2 (39%). However, the levels of these specific markers of glutamatergic synapses were not significantly different among control and AD cases. To test the hypothesis that A β is associated with excitatory terminals, AD synaptosomes were double-labeled for A β and for VGluT1 and VGluT2, and analyzed by flow cytometry and confocal microscopy. Our study demonstrated that A β immunoreactivity (IR) was present in glutamatergic terminals of AD patients. Quantification of A β and VGluT1 in a large population of glutamatergic nerve terminals was performed by flow cytometry, showing that 42% of VGluT1 synaptosomes were immunoreactive for A β compared to 9% of VGluT1 synaptosomes lacking A β -IR. Percentage of VGluT2 synaptosomes immunoreactive for A β (21%) was significantly higher than VGluT2 synaptosomes lacking A β -IR (9%). Moreover, A β preferentially affects

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Corresponding author: Sophie Sokolow, PhD, MPharm UCLA School of Nursing, 700 Tiverton Avenue, Factor Bldg #5-946 Los Angeles, CA, 90095 USA Phone: +1-310-206-3390 Fax: +1-310-206-7703 ssokolow@sonnet.ucla.edu.

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VGluT1 (42% positive) compared to VGluT2 terminals (21%). These data represent the first evidence of high levels of A β in excitatory boutons in AD cortex and support the hypothesis that A β may play a role in modulating glutamate transmission in AD terminals.

Introduction

Alzheimer's disease (AD) is the most common form of dementia, affecting > 5 million Americans (Hebert et al., 2003). Abnormal amyloid-beta (A β) metabolism, tau hyperphosphorylation and synapse loss are characteristic features of AD. Although the molecular triggers of AD are unknown, extensive studies indicate that A β plays a principal role in AD pathogenesis (Kuo et al., 1996; McLean et al., 1999; Naslund et al., 2000).

Presynaptic buildup of soluble A β is suggested as a proximal cause of synapse dysfunction in AD (Fein et al., 2008; Shankar et al., 2007; Shankar et al., 2008). Natural human A β dimers are able to (i) mediate loss of dendritic spines and excitatory synapses; (ii) inhibit long-term potentiation (LTP) and facilitate long-term depression (LTD) in normal rodent hippocampus, and (iii) impede learned behavior in normal rats (Shankar et al., 2007; Shankar et al., 2008). The degree of clinical impairment strongly correlates to the decrease of electrophysiologically active synapses especially glutamatergic pyramidal synapses (Shankar et al., 2007; Shankar et al., 2008; Terry et al., 1991). Nonetheless, a direct link between presynaptic A β and the loss of excitatory function has yet to be established.

Glutamate is a key neurotransmitter in primary perception and cognition. It is the principal excitatory neurotransmitter in the neocortex and hippocampus, major brain areas affected in AD. Furthermore, glutamate is associated with excitotoxicity and LTP. Despite the central role of glutamatergic synapses in learning and memory, their involvement in AD pathology remains unclear. Many neurochemical studies have associated a dysfunction of the glutamatergic system with AD pathology, including reduction of glutamic acid content in AD brain (Procter et al., 1988), reduced receptor binding (Cross et al., 1987) and decreased cortical and hippocampal glutamate uptake which was interpreted as a result of glutamatergic synapse loss (Cowburn et al., 1988; Hardy et al., 1987). Recently, synthetic A β peptide species were shown to potentiate K⁺-induced glutamate release from normal rodent hippocampus (Kabogo et al., 2010).

With the availability of VGluT specific antibodies, immunohistochemical investigation of excitatory boutons has become possible. VGluT1 and VGluT2 are presynaptic proteins responsible for glutamate transport into synaptic vesicles and are found in functionally different subtypes of neurons (Freneau et al., 2004; Herzog et al., 2001; Takamori et al., 2000; Varoqui et al., 2002). Several immunohistochemical studies yielded conflicting results in VGluT1 and VGluT2 expression in AD post-mortem brain tissue. Glutamatergic presynaptic bouton density has been reported as elevated in midfrontal gyrus of patients with mild cognitive impairment (MCI) and depleted in mild- and severe stage of AD (Bell et al., 2007). Another group observed an important reduction in VGluT1 and VGluT2 expression in AD prefrontal cortex; VGluT1 reduction was correlated to the clinical dementia rating score (Kashani et al., 2008). Others found reduced VGluT1 levels in parietal and occipital cortex but not in frontal cortex of AD patients; VGluT2 expression was not modified in these brain areas (Kirvell et al., 2006). In a more recent investigation, VGluT1 expression and cognitive scores were correlated but no difference was observed in VGluT1 expression in prefrontal and temporal cortex of control and AD patients (Kirvell et al., 2010). Because of the discrepancy between studies, the involvement of excitatory synapses in AD remains unclear, despite their central role in clinical symptoms that characterize the disease.

In this study, we aimed to (i) detect alterations in glutamate transporter levels by examining large population of AD and control synaptosomes using flow cytometry analysis, and (ii) determine the degree to which A β is present in excitatory terminals. In the present experiments, A β , VGluT1 and VGluT2 were quantified by flow cytometry and confocal microscopy in surviving terminals of AD parietal cortex (Brodmann area A7). This area of the CNS was previously reported to be severely affected in AD and abundantly expressing A β (Fein et al., 2008; Kirvell et al., 2006).

Methods

Human brain specimens

A total of 15 individuals were studied including 8 with AD (7 females, 1 males, age 86.0 ± 3.3 yr; mean postmortem delay: 6.9 ± 0.8 h) and 7 aged cognitively normal controls (4 females, 3 males, age 89.4 ± 3.3 yr, mean postmortem delay: 7.4 ± 1.4 h). Brain specimens from parietal cortex (Brodmann area A7) were obtained at autopsy from the Alzheimer's disease Research Centers at the University of Southern California, the University of California at Los Angeles and the University of California at Irvine. Table 1 shows the demographic characteristics of the participants, postmortem interval (PMI), Braak stage and frequency of plaques, neutrophil threads, gliosis and neuron loss.

Synaptosome preparation

Synaptosomes were prepared from cryopreserved human brain tissue. Samples (1 to 3 g) were minced and slowly frozen on the day of autopsy in 10% dimethyl sulfoxide and 0.32 M sucrose and stored at -80°C until homogenization. The crude synaptosome fraction was prepared as previously described (Fein et al., 2008). Briefly, the minced tissue was homogenized in 10 volume of 0.32 M sucrose containing protease and phosphatase inhibitors. The homogenate was first centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 10,000 g for 20 min to obtain the crude synaptosomal pellet (P-2) which contains synaptic terminals that have resealed into functional spheres during homogenization in sucrose. Aliquots of P-2 are routinely cryopreserved in 0.32 M sucrose and stored at -80°C , according to protocols previously described for optimal preservation (Dodd et al., 1986).

Western blotting analysis

Protein concentrations in synaptosome-enriched fractions (SEF) were determined using BCA assays (Thermo Scientific, Waltham, MA). Samples were not boiled, as VGluT proteins aggregate after boiling. Non-boiled samples were electrophoresed on 8% SDS-page gels (Expedeon, San Diego, CA). Membranes were blocked for 1 h at room temperature in 5% BSA, followed by incubation overnight at 4°C with the primary antibodies in PBS containing 0.01% Tween 20 (PBS-T) and 1.5% (W/V) BSA and 0.05% sodium azide: VGluT1 1:1000 (Abcam, Cambridge, MA) and VGluT2 1:1000 (Synaptic Systems, Germany). Membranes were then incubated for 1 h with an anti-rabbit IgG horseradish peroxidase (HRP)-conjugated (1:30,000; Jackson ImmunoResearch, West Grove, PA). Membranes were incubated with SuperSignal West Dura substrate (Thermo Scientific, Waltham, MA) and exposed to an OptiChemi HR Camera 600 (UVP Imaging, Upland, CA). Quantification of proteins was performed following optimal exposure time using the VisionWorksLS Image Acquisition and Analysis software (UVP Imaging, Upland, CA). To strip the immunoblots, membranes were incubated at room temperature in 0.1 M glycine at pH 2.5. At the end of each experiment, membranes were rinsed in large volumes of PBS-T and equal loading of protein was verified by staining PVDF membranes with Coomassie Blue. Control and AD samples were analyzed simultaneously within the same blot. All experiments were performed in duplicate.

Immunolabeling of synaptosomal fraction

P-2 aliquots were immunolabeled for flow cytometry analysis according to an intracellular antigen staining method (Gyls et al., 2000). Pellets were fixed in 0.25% buffered paraformaldehyde (PAF) and permeabilized in 0.2% Tween20/PBS (15 min., 37°C). VGluT1-2 antibodies were labeled directly with Zenon kit Alexa Fluor 488 or 647 reagents according to manufacturer (Invitrogen, Carlsbad, CA). This mixture was added to P-2 aliquots and incubated at RT for 30 min. Pellets were washed 2 times with 1 ml 0.2% Tween20/PBS, then resuspended in PBS buffer for flow cytometry analysis. The synaptosomal pellet was dispersed for all washes and for incubations with fixative, detergent, antibodies and then collected by centrifugation ($1310 \times g$ at 4°C). Control and AD synaptosomes were immunolabeled in the same experiment.

Fluo-4NW

Synaptosomal preparation was resuspended in a physiologic salt solution (PSS) consisting of 145 mM NaCl, 10 mM HEPES, 5 mM KCl, 1.2 mM MgCl₂, 1.2 mM CaCl₂ and 10 mM glucose. Synaptosomes were incubated for 30 min at 37°C, with the loading solution containing the calcium indicator dye fluo-4NW (Molecular Probes, Eugene, OR). Synaptosomes were then washed for 5 min and the synaptosomal pellet was incubated for an additional 15 min with the PSS solution in order to allow complete esterase cleavage of the fluo-4 dye. After a final wash, the synaptosomal pellet was resuspended in PSS and analyzed by flow cytometry analysis.

Flow cytometry

For flow cytometry analysis, immunolabeled synaptosomes were transferred to a flow tube and analyzed as previously described (Gyls et al., 2000). Data was acquired using a BD-Calibur I analytic flow cytometer (Becton-Dickinson, San Jose, CA) equipped with argon 488 nm and 635 nm diode lasers. Ten thousand particles were collected and analyzed for each sample. Debris was excluded by establishing a size threshold set on forward light scatter. Alexa-488 and fluo-4NW were detected by the FL1 photomultiplier tube detector, whereas Alexa 647 was assessed by the FL4 photomultiplier tube detector. Analysis was performed using FCS Express software (DeNovo Software, Los Angeles, CA).

Confocal microscopy

Synaptosome-enriched fractions were immunolabeled as described above and washed, then dispersed with a pipette and spread on slides. Slides were dried, coverslipped with Prolong Antifade (Molecular Probes, Eugene, OR), and stored at 4°C. VGluT1-2 antibodies were coupled with Alexa 488 and 10G4, were labeled with Alexa 568. Confocal fluorescence and differential interference contrast images of synaptosomes were taken using a 100X 1.4 Planapo objective lens on a Leica SP2 Confocal Inverted Microscope (Heidelberg, Germany) equipped with argon laser (488 nm excitation) and diode pump (561 nm excitation).

Statistical data analysis

For simple statistical analysis, means $\pm$ SEM were calculated using SigmaPlot (Systat Software, San Jose, CA) and One-way ANOVA was used for multiple comparisons of means. For comparisons of VGluT1 and VGluT2 between normal and AD cases, a mixed model ANOVA was fit to the data, treating VGluT as a within-subject factor and the two groups as a between-subject factor. Groups and VGluT were considered as fixed effects while subjects were considered as random effects. Treating subjects as random allowed for the covariances in the model to vary due to differences among subjects, although subjects were still assumed to be independent. Interaction between factors was also included in the

model. In the event where there were significant interaction between group and VGluT, we investigated further into their simple effects and differences in the least square means. A similar mixed model ANOVA was fit to compare A β in VGluT1 and VGluT2 among AD cases. VGluT and A β were both considered to be within-subject fixed effects while subjects were treated as random. Descriptive statistics were obtained and mixed models were built using SAS 9.2. (SAS Institute Inc., Cary, NC).

Results

Quantification of VGluT1 and VGluT2 terminals in human parietal cortices

As synaptic terminals are not well resolved by light microscopy, even at the highest magnification, we analyzed immunolabeled synaptosomes by flow cytometry (FACS). This method quantifies multiple parameters on each nerve terminal in a sample (Fein et al., 2008), including fluorescence and forward scatter (FSC), which is proportional to the size of a particle. We have previously reported that non-synaptosomal elements are excluded by drawing a size-based gate that includes only particles that are ~ 0.75 to 1.5 microns (Gyls et al., 2004a; Gyls et al., 2007). In the present study, ten thousand size-gated particles were acquired using an acquisition gate based on size standard beads (Gyls et al., 2004a; Gyls et al., 2007). Only specific immunolabeled particles were included in a gate drawn on background labeling in the presence of an isotype-specific control antibody (Fig. 1A). The difference in protein level was expressed as a change in the number of positive particles (Gyls et al., 2004a). Background labeling is illustrated for a representative sample in Fig. 1A. The degree of synaptosomal purity is illustrated in Fig. 1B: synaptosomes were acquired based on size and analyzed as percentage of immunopositive particles for the synaptosomal-associated protein SNAP-25 (Fein et al., 2008). Fig. 1C shows a representative dot plot of a total synaptosome preparation and size standards used to determine the size exclusion. Synaptosome integrity was evaluated using fluo-4NW, a Ca²⁺ sensitive fluorescent dye. Fluo-4NW is an acetoxymethyl (AM) ester derivative capable of permeating cell as an uncharged molecule. Once inside the cell, cytoplasmic esterases cleave the ester group, resulting in a charged form of the dye that cannot leak out of intact cells. Among 10,000 synaptosomes labeled with the fluorescent dye, 99% displayed an intense fluorescent signal (Fig. 1D). This indicates that synaptosomal membranes were intact in our preparation.

Synaptosomes from aged control and AD were immunolabeled with VGluT isotype-specific antibodies and analyzed by flow cytometry. Representative density plots of VGluT1 and VGluT2 immunofluorescence in one normal and one AD patient are shown in Fig. 1E-H. The number of immunofluorescent particles included in the rectangular gate represents the synaptosomes specifically immunolabeled with isotype-specific antibodies for VGluT1 (Fig. 1E and G) and VGluT2 (Fig. F and H). Glutamatergic terminals were fairly abundant in human parietal cortex of aged control and AD cases and there was a significant VGluT by group interaction (Fig. 1I). The mean number of VGluT1 synaptosomes was significantly higher in AD (49%, N = 6) as compared to normals (37%, N = 4, $p < 0.05$). A reduced number of VGluT2 terminals was noted for the AD cases but the difference was not significant. Samples were also dual labeled for VGluT1 and VGluT2 to determine the degree of VGluT1 and VGluT2 co-localization, which revealed that ~18% of glutamatergic terminals contained both VGluT1 and VGluT2 (data not shown). Taken together with the data in Fig. 1 I, these results indicate that approximately 60-70% of synaptic terminals in parietal cortex are glutamatergic.

Our Western blot analysis showed that the concentration of the VGluT1 and VGluT2 in SEF were subject to intergroup variation with a trend for reduced VGluT1 and VGluT2 levels. However these differences did not reach significance for the region studied with this method (Fig. 2). In contrast to flow data, Western blot analysis showed an apparent decrease in

VGluT1 immunoreactivity in AD versus controls. This difference was not significant and can be justified by the difference in methods: indeed Western blot is a semi-quantitative technique and less accurate than flow cytometry. Cases used for flow cytometry and Western Blot analyses are shown in Table 1.

Co-localization of A β in glutamatergic terminals

Confocal analysis reveals that A β is co-localized in glutamatergic boutons immunoreactive for VGluT1 (Fig. 3A-B) and VGluT2 (Fig. 3E-F). Arrowheads showed co-localization of A β and VGluT1-2 in a subset of synaptosomes (Fig. 3C and 3G); differential contrast images (Fig. 3D and 3H) revealed the spherical structure of the synaptosome particles.

The degree of synaptic co-localization was then quantified by flow cytometry. Samples were double labeled for A β and VGluT1-2 specific antibodies and only size gated particles were examined in order to ensure ~ 90% synaptosomal purity (Gyls et al., 2004a). Ten thousand synaptosomes were collected. The number of single-positive (A β ⁻/VGluT1-2⁺) and double-positive (A β ⁺/VGluT1-2⁺) synaptosomes was analyzed using quadrant analysis (Fein et al., 2008). Positive immunofluorescent synaptosomes were identified by drawing a quadrant that excludes background labeling in the lower right, upper left and upper right quadrants (Fig. 4A). This method allowed us to quantify (i) glutamatergic synaptosomes which are immunoreactive only for VGluTs (A β ⁻/VGluT1-2⁺); (ii) non glutamatergic synaptosomes positive for A β only (A β ⁺/VGluT1-2⁻) and (iii) glutamatergic synaptosomes immunoreactive for A β (A β ⁺/VGluT1-2⁺) (Fig. 4B-E). The degree of A β co-localization in glutamatergic terminals of a representative AD case is illustrated in Fig. 4B (A β /VGluT1) and Fig. 4C (A β /VGluT2), and for a typical normal control sample in Fig. 4D and 4E.

In AD parietal cortex, A β preferentially accumulates in VGluT1 synaptosomes (42% of A β ⁺/VGluT1⁺ N = 6) as compared to non-VGluT1 synaptosomes (27% of A β ⁺/VGluT1⁻, N = 6). For both transporters, the number of glutamatergic terminals accumulating A β (42% of A β ⁺/VGluT1⁺ and 21% A β ⁺/VGluT2⁺) was significantly higher than the number of glutamatergic terminals unaffected by A β 9% of A β ⁻/VGluT1⁺ and 9% of A β ⁻/VGluT2⁺. Lastly, using a mixed model ANOVA, we found a significant interaction between VGluT and A β signifying that A β s accumulates preferentially in VGluT1 surviving terminals (42% of A β ⁺/VGluT1⁺) compared to VGluT2 (21% of A β ⁺/VGluT2⁺) (Fig. 4F).

Discussion

Glutamate dysfunction mediated by A β oligomeric species is thought to be an important aspect of AD pathogenesis but the underlying mechanisms remain intangible. Abnormal synaptic plasticity induced by A β is also attributed to early memory loss that precedes neuronal degeneration (Selkoe, 2002). Several studies reported the co-localization of A β with postsynaptic NMDA-receptors (Dewachter et al., 2009; Lacor et al., 2007) but it remains unclear whether A β is present within presynaptic glutamatergic terminals. The present results highlight colocalization of A β and vesicular glutamate transporters in the synaptic compartment of human diseased brain.

The major finding of our study is that the percentage of VGluT1 synaptosomes containing A β (42% of A β ⁺/VGluT1⁺) was almost twice higher than the percentage of non-glutamatergic synaptosomes affected by A β (27% of A β ⁺/VGluT1⁻); i.e., indicating that a significant pool of surviving glutamatergic terminals have accumulated A β . Moreover, for both transporters, the number of glutamatergic terminals accumulating A β was two to four times higher than the number of glutamatergic terminals devoid of A β (A β ⁻/VGluT1-2⁺); i.e., most surviving excitatory terminals in AD neocortex are likely to be affected by A β . We also showed up regulation of VGluT1 but not VGluT2 in AD cortical synapses and a

preferential A β buildup in VGluT1 surviving terminals. The elevation in VGluT1 positive terminals in AD may be a feedback response to the higher A β levels in this population, which would be expected to differentially affect glutamatergic function. VGluT1 and VGluT2 have been considered as separate neuronal populations but our data suggest that there may be expression of both transporters and some overlap of function in a minor subpopulation of terminals. Consistent with this observation, VGluT1 mRNA is expressed in pyramidal neurons in all layers of the human cortex whereas VGluT2 mRNA was only found in the superficial layers (II and III). This expression pattern suggests that VGluT1 and VGluT2 are associated with specific efferent circuits (i.e. cognition, emotion, sensation) (McCullumsmith and Meador-Woodruff, 2003).

Increased interstitial A β has been associated with synaptic activity *in vivo* in mouse models (Cirrito et al., 2008; Cirrito et al., 2005) and human patients (Brody et al., 2008). Therefore the presynaptic accumulations observed in the present experiments may contribute to production and synaptic release of A β by glutamatergic neurons. Release of A β by glutamatergic synapses is also indicated by the finding that activation of group II metabotropic glutamate receptors (mGluRs) triggered secretase activity and synaptic A β release in TgCRND8 mice (Kim et al., 2010). Glutamate as well as A β release may be affected; for example, age-related reduction in stimulated glutamate release has been observed in APP/PS1 transgenic mice (Minkeviciene et al., 2008); moreover, spontaneous excitatory release may also be affected in AD (Pratt et al., 2011). Glutamatergic signaling and function is also regulated by interactions of receptors like NR2B with post-synaptic scaffolding proteins, which may ultimately enhance presynaptic glutamate release (Proctor et al., 2011). Another mechanism by which the presence of A β in glutamatergic terminals may induce synaptotoxicity is through the inhibition of glutamate reuptake. The resulting elevation of extracellular glutamate followed by desensitization would be expected to ultimately activate perisynaptic NMDA-receptors and mGluRs (Li et al., 2009).

In this report, the A β assembly states associated with VGluT1-2 are more likely to be a 56 kD oligomeric species and small oligomers < 20 kD, which are prominent in synaptic terminals (Fein et al., 2008; Lesné et al., 2006; Sokolow et al., 2011). Indeed, our group previously reported a strong correlation between presynaptic A β and several oligomeric species of A β peptide in AD synaptosomes (Fein et al., 2008; Sokolow et al., 2011). Selkoe and his collaborators have suggested that functional synaptic changes in AD lead to synaptic failure and are responsible for the cognitive deficits prior to neurodegeneration (Selkoe, 2002). In rat hippocampus, secreted A β dimers were capable of selectively blocked LTP of excitatory synaptic transmission, an electrophysiological correlate of learning and memory (Shankar et al., 2008; Walsh et al., 2002). Inhibition of LTP by A β oligomers has also been attributed to NMDA-receptor-dependent pathways (Shankar et al., 2007). Hence, analogous effects caused by oligomeric A β buildup in glutamatergic boutons might contribute to AD synaptotoxicity and subsequently cognitive impairment in AD patients. More recently, Puzzo et al. have suggested that at picomolar concentrations A β can actually play a positive role on neurotransmission and synaptic plasticity (Puzzo et al., 2008). Since the pathological concentration of A β oligomers is unknown, A β peptides may trigger synaptic potentiation in AD glutamatergic synapses, perhaps at the early stages of the disease. This is consistent with the work of Abramov *et al.* who demonstrated that release of endogenous A β peptides play a critical role in regulating synaptic function and mediate presynaptic potentiation (Abramov et al., 2009). In their report, excitatory synapses were shown to be highly sensitive to changes in A β levels (Abramov et al., 2009). Moreover, GluR2 subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) increased as MMSE declined in incipient AD synaptoneurosomes (Williams et al., 2009). Within this context, it will be important to examine glutamatergic synaptic A β levels in a larger set of early AD cases (Braak and Braak I-III) as well as comparing glutamatergic synaptic A β levels in brain

regions differently affected by the disease progression (e.g. entorhinal cortex vs. motor cortex).

Analyzing intact nerve terminals, we did not observe loss of glutamatergic endings in AD compared to aged controls. Although other investigators have observed reduced levels of VGluT1 in AD parietal cortex (Kirvell et al., 2006), the discrepancy could be explained by the difference in methods. Integrity of AD synaptosomes was demonstrated by the size of the acquired particles and the conservation of presynaptic markers in synaptosomes affected by A β (Gylys et al., 2004a; Gylys et al., 2004b) as well as by the retention of the viability dye, fluo-4AM, in this report. The fact that in AD cortex we did not observe any loss of VGluT1-2 boutons does not rule out the possibility that A β -bearing terminals are abnormally active compared to A β -free synapses. Indeed, unusual patterns of neuronal activity, such as reduced glutamatergic excitatory currents and increased proportion of hyperactive and hypoactive neurons were reported in mouse models of AD (Busche et al., 2008; Palop et al., 2007; Palop and Mucke, 2010). In AD patients, aberrant neural network activity was also documented (Buckner et al., 2005).

Taken together, the present results support the hypothesis that accumulation of A β in glutamatergic terminals may have a direct effect on surviving glutamatergic terminals and glutamate function in AD cortex. Complimentary studies using animal models of AD are needed to determine the functional effects of A β accumulation in glutamatergic boutons over the course of AD pathology.

Conclusion

Our report demonstrates the presence of A β in cortical glutamatergic terminals and underlies their important contribution in AD pathology. These findings emphasize the importance of elucidating the synaptic cascade triggered by A β in order to prevent glutamate dysfunction and cognitive loss. This study supports the valuable therapeutic benefits of targeting glutamatergic neurons to treat Alzheimer's disease.

Acknowledgments

We are thankful Mr. David Moom for technical assistance. This work was supported by NIH AG27465 to KHG, by NIA AG18879 to CAM. HVV is supported by the Daljit S. and Elaine Sarkaria Chair in Diagnostic Medicine. Tissue was obtained from the Alzheimer's Disease Research Center Neuropathology Cores of USC (NIA 050 AG05142), UCLA (NIA P50 AG 16970), and UC Irvine (NIA P50 AG016573). Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility supported by NIH CA16042 and AI 28697, and by the JCCC, the UCLA AIDS Institute, the David Geffen School of Medicine and the Chancellor's Office at UCLA. The authors wish to thank the families who generously donated the brain samples for the present research program.

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Highlights

>We quantify glutamatergic terminals in Alzheimer's disease and aged control brain.>Glutamatergic boutons are fairly abundant in pathologic and aged control cortex.
>We examine levels of Abeta in glutamatergic boutons in Alzheimer's disease cortex. >
High levels of Abeta are found in excitatory boutons in Alzheimer's disease cortex.

Fig. 1.

Flow cytometry analysis of VGLUT1 and VGLUT2 in control and AD synaptosomes. Human cortical synaptosomal preparation immunolabeled with (A) non-immune IgG and (B) SNAP-25. Positive control sample labeled for SNAP-25 with side scatter dot plot showing polystyrene size standards (C). Synaptosome integrity assessed with the fluorescent dye, Fluo-4NW (D). Representative density plots of normal (E and F) and AD (G and H) synaptosomes labeled for VGLUT1 (E-G) and VGLUT2 (F-H). The forward scatter parameter on the X-axis is proportional to size. Synaptic distribution of VGLUT1 and VGLUT2 in control and AD parietal cortex are shown (I). Mixed model ANOVA, VGLUT * group interaction [$F = 66.90$; $P < 0.001$], values represent means $\pm$ SEM. *, $P < 0.05$ between VGLUT1-IR in AD compared to controls.

Fig. 2.

Representative immunoblots of VGLUT 1 (A) and VGLUT2 (B) expression in SEF isolated from Brodmann area A7 in 3 aged normal controls and 3 Alzheimer's disease (AD) patients. (C) Densitometric analyses of VGLUT1 and VGLUT2 proteins. Data were expressed as percentage of the control group, taken as 100%. Values are means ± SEM. N = 6 normal and 8 AD cases for VGLUT1 (100.0 ± 6.9 vs. 83.6 ± 6.5 , $P > 0.05$). N = 5 normal and 5 AD cases for VGLUT2 (100.0 ± 15.4 vs. 67.7 ± 19.0 , $P > 0.05$).

Fig. 3.

Confocal analysis of VGLUT1 and A β co-localization (A-C). Washed synaptosomes isolated from a 95 y/o AD case (parietal cortex) were dual labeled for VGLUT1 (A) and A β (B). Merge image, arrowheads showing the co-localization of VGLUT1 and A β in a subset of synaptosomes (C). VGLUT2 and A β co-localization (E-G). Synaptosomal preparation dual labeled for VGLUT2 (E) and A β (F). Merge image, arrowheads showing the co-localization of VGLUT2 and A β in a subset of synaptosomes (G). Differential interference contrast images (D and H).

Fig. 4.

Co-localization of VGLUT1-2 and Aβ. (A) Quadrant analysis of human cortical synaptosomal preparation immunolabeled with non-immune IgG. A representative sample of AD parietal cortex double labeled for VGLUT1 and Aβ (B) and VGLUT2 and Aβ (C). Quadrant analysis for one representative aged normal control sample double labeled for VGLUT1 and Aβ (D) and VGLUT2 and Aβ (E). Glutamatergic synaptosomes containing Aβ (Abeta⁺/VGLUT⁺) are in the upper right quadrant, non-glutamatergic synaptosomes positive for Aβ (Abeta⁺/VGLUT⁻) are in the lower right quadrant and glutamatergic synaptosomes lacking Aβ (Abeta⁻/VGLUT⁺) are in the upper left quadrant. Numbers represent the percentage of synaptosomes in each quadrant. (F) Aβ variation in single-labeled (Abeta⁺/VGLUT⁻ only, silver bars; Abeta⁻/VGLUT⁺ only, grey bars) and dual-labeled (Abeta⁺/VGLUT⁺, black bars) fractions of six AD parietal cortices. Due to the low Aβ levels in aged normal controls, this analysis was only performed in AD cases. Mixed model ANOVA, VGLUT * Aβ interaction [$F = 7.27$; $P < 0.01$]. Values represent means $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$, dual-labeled compared to single-labeled fractions; ### $P < 0.0001$ Abeta⁺/VGLUT1⁺ dual-labeled fractions compared to Abeta⁺/VGLUT2⁺ dual-labeled fractions.

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Table 1

Clinical and neuropathological characteristics of patients and controls Neuropil threads and plaques are reported for parietal cortex and refer to silver (Gallyas) stain. The plaque number includes plaques with and without cores; sparse (< 5/field), mod (6 to 20/field), freq (21 to 30/field).

Cases	Sex	Age (yr)	Ethnicity	PMI (h)	MMSE (1-30)	CDR (1-3)	Parietal cortex atrophy	Neuritic plaques	Neurofib. changes (neuropil threads)	Neuronal loss	Gliosis
Normal											
726[#]	F	97	Caucasian	5.5	ND	ND	Mild	None	None	None	None
779^{*,#}	F	89	Caucasian	8.4	29	ND	Moderate	None	None	None	None
789^{*,#}	F	105	Caucasian	6.0	30	0.5	Mild	None	None	None	None
810[*]	M	81	Hispanic	3.0	27	ND	Mild	None	None	None	None
824^{*,#}	F	87	Caucasian	7.0	29	0	None	None	None	None	None
1509[#]	M	82	ND	14.9	ND	ND	None	None	None	None	None
1209[#]	M	85	ND	6.8	ND	ND	None	None	None	None	None
AD											
825[#]	III	68	Caucasian	9.75	ND	ND	None	Moderate	Moderate	None	None
782^{*,#}	IV-V	89	Caucasian	9.0	ND	ND	Mild	Moderate	Sparse	None	None
787^{*,#}	IV	87	Hispanic	6.0	ND	ND	Mild	Moderate	Sparse	None	Sparse
745^{*,#}	V	92	Caucasian	4.8	ND	ND	Mild	Moderate	Sparse	None	None
776^{*,#}	V	99	Caucasian	7.5	ND	ND	Mild	Sparse	None	None	None
1009[#]	VI	82	ND	4.5	ND	ND	Frequent	Frequent	Frequent	Sparse	Sparse
102^{*,#}	VI	80	ND	ND	ND	ND	Moderate	Moderate	Frequent	None	None
028^{*,#}	VI	91	ND	ND	ND	ND	Moderate	Frequent	Frequent	None	None

Abbreviations: CDR, cognitive dementia rating; F, female; M, male; ND, not documented; PMI, post-mortem interval.

* Samples used for flow cytometry analysis.

Samples used for Western Blot analysis.