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Oxidative stress and hippocampal synaptic protein levels in elderly cognitively intact individuals with Alzheimer's disease pathology

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Abstract

Neuritic amyloid plaques and neurofibrillary tangles are hallmarks of Alzheimer's disease (AD) and are major components used for the clinical diagnosis of this disorder. However, many individuals with no cognitive impairment (NCI) also present at autopsy with high levels of these AD pathologic hallmarks. In this study, we evaluated 15 autopsy cases from NCI individuals with high levels of AD-like pathology (HPNCI) and compared them to age- and postmortem-matched cohorts of individuals with amnesic mild cognitive impairment (aMCI) and NCI cases with low AD-like pathology (LPNCI). Individuals classified as HPNCI or aMCI had a significant loss of both presynaptic and postsynaptic proteins in the hippocampus compared to the LPNCI cohort. In addition, these two groups had a significant increase in three different markers of oxidative stress compared to the LPNCI group. The changes in levels of synaptic proteins strongly associated with levels of oxidative stress. These data suggest that cognitively older subjects without dementia but with increased levels of AD-like pathology may represent a very early preclinical stage of AD.

Keywords

Synapses; neurodegeneration; dementia; aging; amyloid; hippocampus; temporal lobe

1. Introduction

Alzheimer's disease (AD) is a progressive dementing disorder that is most often characterized in its early clinical stages by subtle changes in memory and verbal fluency. Two primary pathological hallmarks, neurofibrillary tangles (NFT) and neuritic plaques (NP), define this neurodegenerative disorder. NFTs, composed of hyperphosphorylated tau, are present early in the mesial temporal cortex, hippocampus, and amygdala. In the early stages of the disease, NPs, composed primarily of beta amyloid, are observed in neocortical areas such as the frontal, parietal, and lateral temporal regions. As the disease progresses,

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Disclosure statement

None of the authors have any disclosures.

NFTs are observed in neocortical structures and the mesial temporal lobe manifests high densities of NPs. One of the great conundrums in AD research is that numerous studies report various degrees of AD-like pathology in older individuals without significant cognitive change (Arriagada, et al., 1992, Bennett, et al., 2006, Crystal, et al., 1988, Davis, et al., 1999a, Dickson, et al., 1992, Galvin, et al., 2005, Haroutunian, et al., 1998, Hulette, et al., 1998, Jellinger and Attems, 2012, Katzman, et al., 1988, Knopman, et al., 2003, Mitchell, et al., 2002, Morris and Price, 2001, Mufson, et al., 1999, Mufson, et al., 2016, Price, et al., 1991, Price, et al., 2009, SantaCruz, et al., 2011, Schmitt, et al., 2000, Tomlinson, et al., 1968, Troncoso, et al., 1998, Troncoso, et al., 1996). In most of these studies there is considerable overlap between the degree of pathology observed in individuals with no cognitive impairment and that found in individuals with amnesic mild cognitive impairment (aMCI), which is believed to be a transition between normal cognition and early AD (Mufson, et al., 2012). Such findings suggest that these lesions are most likely accumulating for a long time before onset of any dementia. The question is whether or not this increased accumulation of neuropathologic lesions is truly a forerunner of AD, and thus these individuals are preclinical AD (PCAD), or are these neuropathological lesions “relatively” harmless accumulations that may in fact be simply age-related (Mufson, et al., 2016).

In addition to these hallmarks, there is considerable brain atrophy, neuronal loss and appreciable amounts of synapse loss as the disease progresses. We and others have shown that synapse loss occurs in both the neocortex and hippocampal formation and closely associates with cognitive decline (DeKosky and Scheff, 1990, Scheff and Price, 2006, Scheff, et al., 2015, Scheff, et al., 2007, Scheff, et al., 2011, Sze, et al., 1997, Terry, et al., 1991). Synaptic dysfunction appears to be one of the seminal events leading to aMCI (Mufson, et al., 2012, Scheff, et al., 2012). The precise etiology of synaptic failure remains to be identified. There is strong evidence that oxidative damage and microtubule/actin changes are important early events in the progression of the disease leading to synaptic dysfunction. Some of the most progressive ideas suggest that AD begins early in adulthood, likely within the mesial temporal lobe, and when a critical level of specific cellular/molecular crisis has been attained, the individual begins to manifest AD symptoms.

What role oxidative damage plays in the onset and early progression of AD is unclear. It appears that oxidative stress, defined as an imbalance between reactive oxygen species and antioxidant systems is an early event in the disease process (Ansari and Scheff, 2010, Keller, et al., 2005, Nunomura, et al., 2001, Pratico and Sung, 2004). Although it is still to be determined where oxidative stress originates, the interplay with hyperphosphorylated tau, β -amyloid, and mitochondrial dysfunction appear to be important (Fariss, et al., 2005, Luque-Contreras, et al., 2014, Stamer, et al., 2002, Yan, et al., 2013, Zhao and Zhao, 2013). Recent evidence clearly demonstrates that A β and tau pathologies co-occur at synapses and may be responsible for a loss of synaptic integrity (Takahashi, et al., 2010). There is also strong evidence that oxidative stress as a result of A β and tau may cause synaptic dysfunction in the early stages of AD (Kamat, et al., 2014).

The severity of AD is defined by the distribution of NFTs according to Braak staging (Braak and Braak, 1991, Braak and Braak, 1995) and the severity of NP pathology as defined by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra, 1997, Mirra,

et al., 1991). While the combination of NFTs and NPs defines AD, synaptic loss does not (Scheff, et al., 2014). NFTs alone are not a certain indicator of AD and are found in other disease states (Arai, et al., 2001, Brat, et al., 2001, Buee, et al., 2000, Cairns, et al., 2007, Dickson, et al., 2011, Goedert, 2004, Nelson, et al., 2009, Noda, et al., 2006, Williams, 2006). This AD hallmark is routinely found in the mesial temporal lobe but rarely in the neocortex of cognitively intact elderly individuals (Bouras, et al., 1994). The present set of studies was undertaken to evaluate possible synaptic change in the hippocampus, a component of the medial temporal cortical memory circuit, in cognitively normal individuals with high levels of AD-like pathology. In addition, this region of the brain was assessed for possible changes in hippocampal oxidative stress that might be linked with any change in synaptic integrity.

2. Methods

Postmortem human brains

Tissue was examined from 52 individuals (mean age 86.2 ± 7.0 years; range 69 to 98) who were participants in either the University of Kentucky Alzheimer's Disease Center (UKADC) (Davis, et al., 1999b), or the Rush Religious Orders Study (RROS), a longitudinal clinical-pathologic study of aging and AD composed of older Catholic nuns, priests, and brothers (Bennett, et al., 2002, DeKosky, et al., 2002, Mufson, et al., 2000). The Human Investigations Committee of the University of Kentucky College of Medicine and the Rush University Medical Center approved the studies. Individuals included in these studies agreed to annual clinical evaluation and brain donation at the time of death. For all subjects, cognitive test scores were available within the last year of life. Subjects were categorized as no cognitive impairment (NCI; $n = 48$) or aMCI ($n = 15$), based on cognitive testing prior to death using previously reported criteria for aMCI (Bennett, et al., 2002, Mufson, et al., 1999). The neuropathological assessment did not contribute to the diagnosis of aMCI with the exceptions of the standard exclusion criterion noted below. Eight of the aMCI subjects had bilateral hippocampal sclerosis (Ighodaro, et al., 2015) while none of the NCI subjects did. The NCI subjects were without a history of dementia or other neurological disorders and were further subdivided based on neuropathologic evaluation at autopsy. The NCI cohort was divided into a low AD-type pathology (LP) and a high AD-type pathology (HP). Individuals in the LP-NCI group had Braak scores 0-II and a CERAD score (Mirra, et al., 1991) of negative to possible. Individuals in the HP-NCI group had a Braak score of III-V and a CERAD score of probable to definite. None of the individuals in the HPNCI cohort had a Braak score of VI. None of the subjects in the current study had primary age-related tauopathy (PART) (Jellinger, et al., 2015).

Standard criteria for exclusion included the presence of 1) significant cerebral stroke regardless of antemortem date, 2) large cortical infarcts identified in the postmortem neuropathologic evaluation, 3) significant trauma within 12 months before autopsy, 4) seizure within the last 6 months before autopsy, 5) on a respirator longer than 12 hours before death, 6) in coma longer than 12 hours immediately before death, 7) currently undergoing radiation therapy for CNS tumor, and 8) Lewy bodies in the area of interest. None of the subjects used in the present set of investigations were known to have any

additional neurologic or psychiatric conditions. None of the NCI subjects used in this study were TDP-43 positive (Geser, et al., 2010).

Clinical Evaluations

Details of the RROS and UKADC have been published elsewhere (Bennett, et al., 2002, Schmitt, et al., 2000, Schmitt, et al., 2012). All subjects have detailed mental status testing and neurologic and physical examinations annually. Subjects were followed for 2 to 21 years (mean 9 ± 5 years). For the MCI cohort, all 15 subjects were amnesic without multi domain involvement. Tissue from questionable cases was not included in the study.

Neurochemical assessment

At autopsy, brain tissue was processed as previously described (Markesbery, et al., 2006, Mufson, et al., 2000). Tissue from the hippocampal formation was evaluated for changes in both presynaptic and postsynaptic proteins as well as soluble A β_{1-42} concentration (Murphy, et al., 2007). Hippocampal tissue samples included the CA1-CA3 region, the hilar region, dentate gyrus, and a portion of the subiculum.

Biochemical analysis

All tissue samples were frozen on dry ice or in liquid nitrogen at time of autopsy and stored at -80°C until used for analysis. Tissues were homogenized using a Bio-Gen RO-200 homogenizer (Pro Scientific, Oxford, CT) in a lysis buffer containing 10mM HEPES, 137mM NaCl, 0.6 mM MgSO₄, 4.6mM KCL, 1.1 mM KH₂PO₄, pepstatin A, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride. Samples were centrifuged at 1000g for 10 min/ 4°C to remove cell debris, and the collected supernatant was centrifuged at 15,000g for 10 min/ 4°C . Supernatants were used for the analyses. Total protein concentration was determined by the BCA method (Sigma, St. Louis, MO).

Assessment of synaptic proteins

Synaptic proteins were evaluated by Western blot as previously described (Ansari, et al., 2008a, Ansari, et al., 2008b). Supernatants were probed for possible changes in synapsin I (Millipore, Temecula, CA AB 1543), synaptophysin (Millipore, Temecula, CA AB 9272), postsynaptic density-95 (PSD-95) (Santa Cruz Biotech, CA sc-28941), synapse associated protein 97 (SAP-97) (Santa Cruz Biotech sc-25661), and drebrin (Sigma, St. Louis, MO D3816). For the analysis, 25 μg of protein was loaded with the appropriate marker (β -actin) (Santa Cruz Biotech, Santa Cruz, CA sc-47778) on a gradient gel (4-20% Tris-HC), followed by transfer to polyvinylidene fluoride membrane using a semidry transfer system (Bio-Rad, Hercules, CA) in transfer buffer (25mM Tris, 150 mM glycine) at 15V for 2h. The membrane was blocked with 5% milk or BSA in Tris/saline buffer + Tween 20 (TBST). Primary antibodies were added and incubated overnight at 4°C . Blots were washed three times in TBST and incubated for 1h with alkaline phosphatase conjugated secondary antibodies. The membrane was washed three times in TBST for 5 min and developed in Sigma Fast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (Informer Technologies, Walnut, CA). Membranes were also incubated with an antibody for beta-actin as a loading control and synaptic proteins

levels were normalized to beta-actin levels prior to statistical analysis. All assays were run in triplicate and median value was used for analysis.

Assessment of oxidative stress

Protein oxidation (protein carbonyls; PC), lipid peroxidation (4-hydroxynonenal; 4-HNE) and protein nitration (3-nitrotyrosine; 3-NT) were assessed following a standard previously described protocol (Ansari, et al., 2008a). Detection of PC used the OxyBlot Protein Oxidation detection kit (Millipore, Billerica, MA, S7150) with modification. Briefly, normalized samples (4mg/ml) were combined with 12% sodium dodecyl sulfate diluted 2,4-dinitrophenyl hydrazine, and incubated for 20 min at room temperature. Samples were neutralized with NaOH and loaded on a nitrocellulose membrane under vacuum using a slot-blot apparatus. The membrane was blocked with 3% BSA in PBS/Tween and incubated with 1:100 dilution of anti-DNP polyclonal (Millipore, Billerica, MA). The membrane was subsequently incubated with an anti-rabbit IgG alkaline phosphatase secondary antibody (Sigma, St. Louis, MO, A 3687). Following three washes in PBS/Tween, the membranes were developed with Sigma Fast tablets (BCIP/NBT substrate, B 5655). Blots were scanned with Adobe PhotoShop and quantified with Sion image (PC version of Macintosh-compatible NIH image). No non-specific binding of antibody to the membrane was observed.

Estimation of 4-HNE was similar to that used for PC with normalized samples mixed with a modified Laemmli buffer containing Tris base and glycerol. Samples (250 ng) were loaded onto the nitrocellulose membrane in the slot-plot apparatus. The membranes were incubated with a 1:5000 dilution of anti-HNE polyclonal antibody (Millipore, Billerica, MA, AB 5605) for 1h 30min. The membranes were then washed and treated with the anti-rabbit IgG alkaline phosphatase secondary antibody at a dilution of 1:8000. The membranes were developed with Sigma Fast tablets and quantified identical to PC. Faint background staining due to antibody alone was sometimes observed; however, each sample had a control and this effect was well controlled.

For 3-NT, the samples were treated identical to 4-HNE with the exception that a 1:2000 dilution of anti-3-NT polyclonal antibody (Sigma, St. Louis, MO, N 0409) was used. The same anti-rabbit IgG secondary antibody was used in a 1:8000 ratio. No non-specific binding of antibody to the membrane was observed.

Measurement of soluble A β 42

Measurements for soluble A β were identical to that used previously (Murphy, et al., 2007). Briefly, soluble amyloid- β_{1-42} (A β 42) peptide concentration was quantified in sodium dodecyl sulfate (SDS)-soluble A β fractions, assayed using a fluorescent-based ELISA (Biosource, Camarillo, CA) with a capture antibody specific for the NH₂ terminus of human A β (amino acids 1-16). Values for detection antibodies specific for the neopeptide at the 42-amino acid end of A β were determined from standard curves using synthetic A β 42 peptide (Biosource)) and expressed as picomoles per gram wet weight. This ELISA assay detects both A β monomers and oligomers.

Statistical Analysis

The relationship between dependent variables and diagnostic group was examined with an analysis of variance (ANOVA) using Statview 5.0 (SAS Institute, Cary, NC). Prior to using the ANOVA, data were subjected to Levene's test for homogeneity of variance. In all cases, the null hypothesis was supported by the Levene's test. If a significant ANOVA was found, the Fischer-Hayter test (Hayter, 1986) was used to identify pairs of diagnostic groups that differed significantly. Because the diagnostic groups were heterogeneous in their clinical features, the relationship between dependent variables (synaptic proteins and oxidative stress) evaluation was examined using Spearman correlation. Level of significance was set at $p < 0.05$.

3. Results

Demographics

Subject characteristics grouped by consensus diagnosis are presented in Table 1. The three different groups did not differ significantly ($p > 0.05$) in gender, age, postmortem interval (PMI), or education, eliminating these variables as possible major contributors for observed differences. As expected there was a significant difference in the Mini-Mental State Examination [$F(2,49) = 9.689$, $p < 0.001$]. Post hoc analysis revealed that the aMCI group differed significantly ($p < 0.001$) from the other two cohorts, which were not different from each other. Because a criterion for placement into the HPNCI group was a Braak score of III or greater, it was inappropriate to test if a group difference existed between the LPNCI and HPNCI cohorts for this variable. A Chi-square statistic was used to determine if the HPNCI and aMCI groups differed in their Braak distribution, Braak scores were partitioned into three different ranges (0-II; III-IV; V-VI) and evaluated with a Chi-square statistic. The HPNCI and aMCI groups ($\chi^2_{(1)} = 0.186$; $p > 0.5$) were not significantly different.

Synaptic proteins

Five different synaptic proteins were evaluated for each of the subjects representing three different possible stages of the disease progression. As can be seen in Figure 1A, the disease progression affected all five proteins examined.

Presynaptic

Synapsin-1 is a major protein in the presynaptic portion of the synapse. Analysis showed a significant difference between clinical groups [$F(2,49) = 9.804$, $p < 0.0005$]. Levels were significantly lower in both the HPNCI ($p < 0.01$) and aMCI ($p < 0.001$) compared to the LPNCI cases (Figure 1B). The aMCI cohort did not differ from the HPNCI group ($p > 0.1$). Similar results were found when assessing levels of the synaptic vesicular protein, synaptophysin, [$F(2,49) = 4.683$, $p < 0.05$]. Compared to the LPNCI cohort, both the aMCI ($p < 0.05$) and HPNCI ($p < 0.05$) groups displayed significantly lower levels of this synaptic protein (Figure 1B). These two groups were not significantly different from each other ($p > 0.1$).

A subsequent analysis was carried out to determine if there were any age-related changes, regardless of group designation, in the quantitative analysis of the presynaptic proteins. These analysis failed to show any significant association ($p > 0.05$).

Postsynaptic

Quantitative analysis of drebrin, an actin binding synaptic scaffolding protein found in dendritic spines, revealed a significant change [$F(2,49) = 14.826$, $p < 0.0001$] in the hippocampus with both the HPNCI and aMCI cohorts significantly lower ($p < 0.001$) compared to LPNCI. The aMCI and HPNCI groups did not differ statistically ($p > 0.1$) (Figure 1C). SAP-97, a membrane-associated phosphoprotein that participates in AMPA-type glutamate receptors, displayed a significant change [$F(2,49) = 12.582$, $p < 0.0001$] with both the HPNCI and aMCI cohorts demonstrating a significant decline ($p < 0.005$) compared to LPNCI (Figure 1C). There was no significant difference between the HPNCI and aMCI groups ($p > 0.1$). Similar to drebrin, the core scaffolding component of the postsynaptic element, PSD-95, showed a significant change depending on group designation [$F(2,49) = 8.956$, $p < 0.005$]. Post hoc testing revealed that only the aMCI cohort was significantly different from LPNCI ($p < 0.005$) while the HPNCI group ($p > 0.05$) was not significantly lower. The HPNCI and aMCI groups did not differ statistically ($p > 0.1$).

A subsequent analysis was carried out to determine if there were any age-related changes, regardless of group designation, in the quantitative analysis of the postsynaptic proteins. The postsynaptic protein, PSD-95 showed a significant association with the subject's age ($r = .338$; $p < 0.05$). A possible age-related association with the other two proteins (drebrin, SAP-97) were not found to be significant ($p > 0.05$).

ApoE genotype and synaptic changes

Apolipoprotein E (*APOE*) genotype ($\epsilon 2/2$, $\epsilon 2/3$, $\epsilon 3/3$, $\epsilon 3/4$, $\epsilon 4/4$) was determined for each of the cohorts (Table 2). The aMCI group had significantly more individuals with at least one $\epsilon 4$ allele compared to the NCI groups combined ($p < 0.01$). Changes in synaptic proteins were further grouped by whether or not the individual had any *APOE* $\epsilon 4$ allele. Unpaired t-tests failed to identify a significant difference between these two groups for any of the synaptic proteins analyzed. These results indicate that *APOE* $\epsilon 4$ genotype did not influence changes in synaptic proteins in the hippocampus.

Oxidative stress

Levels of protein oxidation, lipid peroxidation, and protein nitration were evaluated as markers of oxidative stress using slot blots (Figure 2). Analysis of PC demonstrated a significant difference between groups [$F(2,49) = 65.119$, $p < 0.0001$]. Group comparison showed that both the HPNCI ($p < 0.001$) and aMCI ($p < 0.001$) had higher levels compared to the LPNCI cohort. In addition, the aMCI group had significantly greater level ($p < 0.001$) than the HPNCI group (Figure 3). Similar to PC, the levels of 4HNE were also significantly different among the three groups [$F(2,49) = 43.621$, $p < 0.0001$]. The LPNCI group had significantly lower levels compared to both the HPNCI ($p < 0.0001$) and the aMCI ($p < 0.001$) cohorts. Groups HPNCI and aMCI were not significantly different from each other ($p > 0.05$) (Figure 3). Levels of 3-NT were significantly elevated [$F(2,49) = 60.702$, $p < 0.001$]

with the LPNCI group significantly lower than HPNCI ($p < 0.001$) and aMCI ($p < 0.001$). These two groups were not significantly different from each other ($p > 0.1$).

Association between synaptic proteins and oxidative stress

We evaluated the possible relationship between changes in synaptic proteins and levels of different markers of oxidative stress (Figure 4). There was a significant association for all the synaptic proteins and each of the three different markers of oxidative stress. As levels of oxidative stress increased, the levels of synaptic proteins decreased. This relationship held true for both the presynaptic and postsynaptic proteins. The only nonsignificant correlation was between the PC and synaptophysin (Table 3).

Soluble A β_{1-42}

Analysis of the soluble A β_{1-42} demonstrated a significant group effect [$F(2,49) = 3.985$, $p < 0.03$]. Subsequent analysis revealed that the levels in the aMCI cohort were significantly higher ($p < 0.05$) than those observed in the two NCI groups, which were not significantly different from each other ($p > 0.1$) (Figure 5). Analysis failed to reveal a significant association between soluble A β_{1-42} and MMSE ($p > 0.1$). To determine whether or not the levels of A β_{1-42} were associated with changes in CERAD, we arbitrarily assigned a numerical value to the different CERAD stages and used a Spearman rank correlation. The analysis revealed a significant association ($r = .336$, $p < 0.05$) such that as the levels of A β_{1-42} increased so did the CERAD levels indicating definite AD.

4. Discussion

The present findings support and extend previous results concerning synaptic change in the human hippocampus during the progression of AD. Consistent with previous studies, we observed a decline in synaptic integrity in a cohort of individuals classified as aMCI. Similar to our ultrastructural study of stratum radiatum in region superior (Scheff, et al., 2007), we found a significant loss of synaptic proteins in the aMCI group. One major difference between these two studies is that the lysate in the present investigation included the hippocampus proper, the dentate gyrus, and portions of the subiculum. We also monitored five different synaptic proteins – 2 presynaptic (synapsin-1, synaptophysin) and 3 postsynaptic (drebrin, PSD-95, SAP-97). An earlier study (Counts, et al., 2012) evaluating the hippocampus in MCI found a significant loss of drebrin but no change in synaptophysin. Another early evaluation assessing possible hippocampal change in a small cohort of MCI subjects reported a significant loss of PSD-95 (Sultana, et al., 2010). The present study also differed from previous reports of possible synaptic changes by evaluating a relatively large cohort of individuals with no cognitive impairment but with postmortem findings of relatively high levels of AD-like pathology. These subjects had a Braak score of III-V and a CERAD composite of probable or definite AD. This HPNCI subjects revealed significantly lower levels of all synaptic proteins compared to an age- and postmortem-matched cohort of individuals with no or low AD-like pathology (Figure 1). The levels in the HPNCI cohort were equivalent to those found in the aMCI group with the exception of PSD-95. The different cohorts of subjects used in the present set of studies were well characterized and most had been followed and annually tested for more than 8 years. The individuals in the

aMCI group were originally recruited as cognitively normal and subsequently followed longitudinally (Schmitt, et al., 2012).

Three different markers of oxidative stress were evaluated in the same lysate used to determine the levels of the different synaptic proteins. Protein carbonyls and 3-NT are well respected measures of oxidative stress (Dalle-Donne, et al., 2003, Darwish, et al., 2007) and have been demonstrated in the hippocampus in numerous neurodegenerative diseases including AD (Aksenov, et al., 2001, Hensley, et al., 1995). In addition a large number of studies have used 4-HNE as a measure of lipid peroxidation and showed AD-related elevations in several mesial temporal lobe structures (Lovell, et al., 1995, Markesbery and Lovell, 1998, Montine, et al., 1998). Our analysis also revealed a significant change in the levels of oxidative stress. All three markers revealed a significant increase in both the HPNCI and aMCI cohorts compared to the LPNCI group. For the PC and 3-NT measurements there was extremely limited overlap with the LPNCI cohort (Figure 3). The analysis revealed that while the HPNCI group differed from aMCI for PC levels, these two groups were not statistically different when comparing 4-HNE and 3-NT. It has long been thought that oxidative stress is one of earliest events in the pathogenesis of AD (Nunomura, et al., 2001) and multiple studies have reported oxidative stress is significantly elevated in several brain regions of aMCI individuals (Ansari and Scheff, 2010, Ansari and Scheff, 2011, Butterfield, et al., 2006, Keller, et al., 2005, Markesbery, et al., 2005, Pratico and Sung, 2004, Williams, et al., 2006). The levels of oxidative stress closely associate with changes in cognition (Ansari and Scheff, 2010, Ansari and Scheff, 2011). Our observation that elevated oxidative stress is present in the HPNCI cohort supports the previously proposed idea that oxidative stress is an early component of AD progression. While it is unclear to what extent this oxidative stress might exacerbate changes in synaptic function, as shown in Figure 4, there is an extremely strong association between the oxidative stress markers and synaptic proteins. We previously showed that levels of oxidative stress closely associate with changes in cognition (Ansari and Scheff, 2010, Ansari and Scheff, 2011).

There are multiple potential sources for oxidative stress including mitochondrial dysfunction (Fariss, et al., 2005). The mitochondrial cascade hypothesis was proposed as a possible early contributor to the onset of the pathological alternations contributing to AD (Swerdlow and Khan, 2004), and, although it has undergone some revision, it continues to receive substantial support especially regarding the relationship to beta amyloid and tau (Casley, et al., 2002, Gibson, et al., 2010, Hroudova, et al., 2014, Simoncini, et al., 2015, Swerdlow, et al., 2014, Wang, et al., 2008, Wang, et al., 2014). Mitochondria play an important role in cellular homeostasis and are responsible for most of the energy supplies required by cells including synaptic function by neurons (Bereiter-Hahn, 2014). Mitochondria are also a major contributor of cellular reactive oxygen species as a result of electron transport in the oxidative phosphorylation chain and as such can alter cellular gene expression leading to pathogenic mutations that could alter total cellular function (Dhillon and Fenech, 2014, Picard, 2015). Such mitochondrial function could not only lead to a disruption of cellular energy but also a transformation of synaptic proteins leading to an alteration in synaptic morphology and number (Roberts, et al., 2015). Recent mitochondrial proteomics research suggests that synapses may have a high sensitivity to oxidative stress as a result of decreased mitochondrial superoxide dismutase (Volgyi, et al., 2015). An important

subcomponent of the mitochondrial permeability transition pore, 1Q binding protein, was elevated in synaptic as compared to nonsynaptic mitochondria (Volgyi, et al., 2015). Opening of this pore leads to a decrease in calcium homeostasis, a loss of membrane potential, and increased oxidative stress. Mitochondria are also affected early in transgenic AD mouse models (Calkins, et al., 2011, Du, et al., 2010, Gillardon, et al., 2007). Other sources of oxidative stress may be changes in the transmembrane NADPH-oxidase complex and/or alterations in the endoplasmic reticulum (Ansari and Scheff, 2011, Cahill-Smith and Li, 2014, Gao, et al., 2012, Li, et al., 2015, Mota, et al., 2015, Zhang, et al., 2015).

An important variable to consider in synaptic protein loss is the epigenomics, which includes factors such as changes in brain DNA methylation that is associated with not only AD but a variety of different diseases. (Bennett, et al., 2015, Lardenoi, et al., 2015, Mastroeni, et al., 2010, Mastroeni, et al., 2011, Yu, et al., 2015). Such changes have been linked to changes in AD-related mitochondrial energy metabolism (Salminen, et al., 2015) and also synaptic function (Berchtold, et al., 2013, Berchtold, et al., 2014, Mastroeni, et al., 2015). Downregulation of important synaptic genes could significantly alter synaptic homeostasis and plasticity in both normal aging and AD leading to subtle changes in cognition that might be difficult to detect early in the disease progression.

Previous studies have shown an association between the pathologic hallmarks of AD (NFT and NP) and cognitive change (Nelson, et al., 2012). Our work (DeKosky and Scheff, 1990, Scheff, et al., 2015, Scheff, et al., 2007, Scheff, et al., 2006, Scheff, et al., 2011) and others (Coleman and Yao, 2003, Terry, et al., 1991) have reported a strong association between cognitive function and synaptic homeostasis supporting the idea of a cause-effect relationship. As the number of synapses or synaptic proteins decline, there is a change in cognition. Although synaptic loss is not a unique hallmark of AD (Scheff, et al., 2014), it does associate stronger with cognitive change compared to the presence of NFT and NP accumulation. At present it is unclear why some subjects with high levels of AD-like pathology have cognitive impairment (aMCI) and others, with very similar levels of neuropathology do not. Recent cellular and molecular studies have demonstrated a remarkable amount of structural remodeling in the hippocampus during the progression of the disease (Mufson, et al., 2015). The concept of cognitive or brain reserve is often also suggested to account for the differences. Brain reserve (Katzman, et al., 1988, Stern, 2009) postulates that these individuals have a greater quantity of either neurons or synapses at the onset of the disease process and thus can perform cognitive tasks because the total loss has not reached a specific threshold. On the alternative side, cognitive reserve takes a more active approach by recruiting other regions of the brain not as severely affected to help perform the task and perhaps making new connections (Stern, 2009). There is considerable evidence for neuropil plasticity in the hippocampus during the course of AD progression (Mufson, et al., 2015). The occurrence of high pathology and low synaptic proteins in the HPNCI group does not appear to be related to the presence in APOE ϵ 4 (see Table 2). Both NCI cohorts had significantly less individuals with the ϵ 4 allele compared to the aMCI group in which 60% were ϵ 4 positive.

Hippocampal sclerosis (HS) refers to atrophy of the hippocampal formation resulting from CA1/subiculum neuronal loss and increased gliosis (Nag, et al., 2015). It has recently been

postulated as a possible preclinical stage of AD (Sperling, et al., 2011) although it is also a consequence of normal aging (Nelson, et al., 2011, Nelson, et al., 2013, Neltner, et al., 2014) and may not be associated with any type of dementia. Early studies have linked HS to cognitive decline in the elderly (Beach, et al., 2003, Corey-Bloom, et al., 1997, Dickson, et al., 1994). In the present investigation, each subject was probed for TDP-43 pathology as a marker of HS (Ighodaro, et al., 2015, Nelson, et al., 2011). None of the NCI subjects were positive to TDP-43 while over 60% of the aMCI group tested positive and all showed signs of cognitive impairment. Since there is a considerable amount of AD-like pathology in the HPNCI cohort without detectable cognitive impairment, can some of this be expected as part of the “normal” aging process or is it “abnormal” aging and on the cusp of the transition to mild cognitive impairment?

One surprising observation in the present study is the relatively low levels of soluble $A\beta_{1-42}$ in the HPNCI cohort compared to the aMCI group that also showed a significant loss of the different synaptic proteins compared to the LPNCI group. This would support findings that this form of soluble $A\beta$ does not have a generalized effect on all parts of the synaptic connections. There is a substantial amount of research documenting the relationship between small soluble $A\beta_{1-42}$ and changes in cognition (Walsh and Selkoe, 2007). It is thought that soluble oligomers disrupt hippocampal synaptic plasticity by altering the induction of long term potentiation through effects on the postsynaptic receptors (Li, et al., 2010). Prior studies have demonstrated that the presynaptic element is most likely unaffected by the oligomers (Cheng, et al., 2009, Shankar, et al., 2008, Townsend, et al., 2006). The present loss of synaptic proteins and increases in oxidative stress in the hippocampus might possibly be upstream events from the increase in s $A\beta_{1-42}$. As part of our analysis we found a significant association between soluble $A\beta_{1-42}$ and the individuals CERAD score in the aMCI cohort. This might possibly indicate the onset of an additional factor at the time of transition to this cognitive state. In the presnet study, individuals with high AD-like pathology very similar to that observed in the aMCI cohort had very low levels of soluble $A\beta_{1-42}$ and no cognitive impairment. It may be that the current cognitive tests are not sensitive enough to detect impairment or the fact that when soluble $A\beta_{1-42}$ is detectable it triggers changes in brain structures responsible for the cognitive change. This paradox clearly awaits further study.

Study limitations

The present study has limitations that should be considered when generalizing the results. The sample size for each cohort, while larger than in many investigations of this type, is still small and subject to possible unintended sampling bias. It is not a sample that is population based but rather one of convenience. Although the groups were matched based on age, postmortem interval, and education, the participants are volunteers who are well educated. The results represent a single cross section of any possible progression despite that the participants were clinically and cognitively evaluated longitudinally. The results have greater utility since the participants were volunteers and not recruited from a memory-disorders clinic. All subjects were originally enrolled as cognitive unimpaired and over the age of 70. However, it is unclear to what extent different pharmaceuticals may have had an effect on the results since this factor was not considered in the analysis.

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Figure 1.

Changes in synaptic protein levels in the hippocampus. Five different synaptic proteins (2 presynaptic and 3 postsynaptic) were analyzed by Western blot with beta actin used as a loading control on the gels (A). Scatter plots showing changes in different synaptic proteins within the hippocampus for each subject from the different cohorts representing different possible stages in the progression of the disease: Low pathology no cognitive impairment (LPNCI), high pathology no cognitive impairment (HPNCI), amnesic mild cognitive impairment (aMCI). (B) Antibodies directed against presynaptic proteins synapsin-1 and synaptophysin are shown. (C) Antibodies directed against postsynaptic proteins drebrin, SAP-97, and PSD-95. Horizontal lines indicate group medians. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, # $p < 0.001$ compared to LPNCI.

Oxidative Stress Markers

Figure 2.

Representative slot-blots for (A) protein carbonyl, (B) 4-hydroxynonenal, and (C) 3-nitrotyrosine fractions from the hippocampus from individuals classified as no cognitive impairment and low AD-like pathology (LP-NCI), no cognitive impairment and high AD-like pathology (HP-NCI), and amnesic mild cognitive impairment (aMCI). The slot-blot shows alterations in staining for the different cohorts for each of the different markers of oxidative stress.

Figure 3.

Scatterplots showing changes in the markers of hippocampal oxidative stress among the different cohorts in the present study. The low pathology no cognitive impairment (LPNCI) cohort consistently demonstrated significantly lower levels of oxidative stress compared to the high pathology no cognitive impairment (HPNCI) and amnesic mild cognitive impairment (aMCI) groups for all three of the different markers of oxidative stress. Horizontal lines indicate group medians. *p<0.01, **p<0.001 compared to LPNCI, #p<0.001 compared to HPNCI.

Figure 4.

Correlations between different synaptic proteins and markers of oxidative stress in the hippocampus. Almost every comparison showed a negative association between levels of oxidative stress and the levels of various synaptic proteins. One exception was the lack of any observed association between levels of protein carbonyls and levels of the presynaptic marker synaptophysin. Abbreviations: AD, arbitrary units; PSD-95, postsynaptic density-95; SAP-97, synapse associated proteins 97.

Figure 5.

Scatterplots showing the levels of soluble A β ₁₋₄₂ in the hippocampus as a function of the different cohorts evaluated. There was no significant difference observed between the two groups with individuals labeled as no cognitive impairment (NCI) ($p > 0.1$). However, both the low pathology and high pathology NCI groups were significantly lower than the amnesic mild cognitive impairment (aMCI) cohort. Horizontal lines indicate group medians. * $p < 0.05$

Table 1

Demographics and clinical characteristics

Group	n	Age (yrs) at death	PMI (h)	Gender M/F	MMSE	Braak (range)	CERAD plaques	Education (yrs)	APOE $\epsilon 4$
LPNCI	22	84.5 $\pm$ 8.9	2.6 $\pm$.8	8/14	29.0 $\pm$.9	0-II	0-A	16.1 $\pm$ 2.4	2/22
HPNCI	15	85.7 $\pm$ 5.2	3.7 $\pm$ 1.6	5/10	28.9 $\pm$ 1.4	III-V	B-C	16.1 $\pm$ 2.7	2/15
aMCI	15	85.7 $\pm$ 5.7	3.1 $\pm$.8	6/9	26.4 $\pm$ 3.0	II-VI	A-C	15.6 $\pm$ 1.9	9/15

Mean $\pm$ standard deviation

CERAD, Consortium to Establish a Registry in AD : 0 not AD; A low likelihood; B Intermediate likelihood; C high likelihood

PMI, post mortem interval; MMSE, Mini-Mental State Examination; APOE, apolipoprotein E

Table 2

Distribution of ApoE categories by group

ApoE	LPNCI	HPNCI	aMCI
E2/2	0	0	1 (7%)
E2/3	1 (5%)	2 (13%)	0
E2/4	0	0	0
E3/3	12 (86%)	11 (73%)	5 (33%)
E3/4	2 (9%)	2 (13%)	9 (60%)
E4/4	0	0	0

Table 3Correlations^{*} between synaptic proteins and oxidative stress

	Protein Carbonyls	4-Hydroxynonenal	3-Nitrotyrosine
Drebrin	r =.516, p < 0.0001	r =.559, p<0.0001	r =.544, p< 0.0001
SAP 97	r =.439, p<0.005	r =.485, p <0.0005	r =.494, p<0.0005
PSD 95	r =.357, p<0.01	r =.440, p<0.005	r =.337, p<0.05
Synapsin	r =.554, p<0.0001	r =.555, p< 0.0001	r =.686, p<0.0001
Synaptophysin	r =.206, p>0.1	r =.301, p<0.05	r =.362, p<0.01

* All correlations were run using Spearman's rho statistic