Published in final edited form as:

J Alzheimers Dis. 2010 ; 19(1): 97–109. doi:10.3233/JAD-2010-1216.

Changes in the Expression of Genes Associated with Intraneuronal Amyloid Beta and Tau in Alzheimer's Disease

Robert K. Fujimura^{*,a}, Teresita Reiner^a, Fangchao Ma^{a,g}, Virginia Phillips^f, Alicia de las Pozas^a, Dennis W. Dickson^f, Bernard A. Roos^{a,b,c,d}, Guy A. Howard^{a,b,e}, and Carlos Perez-Stable^{*,a,b}

^aGeriatric Research, Education, and Clinical Center and Research Service, Bruce W. Carter Veterans Affairs Medical Center, Miami, FL, USA 33125

^bDivision of Gerontology & Geriatric Medicine, Department of Medicine, University of Miami Miller School of Medicine, Miami, FL, USA 33136

^cDepartment of Neurology, University of Miami Miller School of Medicine, Miami, FL, USA 33136

^dStein Gerontological Institute, Miami Jewish Home and Hospital, Miami, FL, USA 33137

^eDepartment of Biochemistry & Molecular Biology, University of Miami Miller School of Medicine, Miami, FL, USA 33136

^fDepartment of Pathology, Mayo Clinic, Jacksonville, FL, USA 32224

Abstract

The clinical hallmark of Alzheimer's disease (AD) is impairment of cognition associated with loss of synapses, accumulation of amyloid β (A β) both within neurons and as extracellular deposits, and neurofibrillary degeneration composed of phospho-tau. Neurons in the hippocampus are among those that are most vulnerable. The purpose of this study was to investigate the expression of genes associated with cognition, synapse, and mitochondrial function in hippocampal neurons of AD compared to normal individuals. Neurons from the hippocampus with intraneuronal A β immunoreactivity were captured with laser microdissection; RNA was extracted; and levels of brain-derived neurotrophic factor (BDNF), TrkB (BDNF receptor), dynamin-1 (DYN), and cytochrome C oxidase subunit II (COX2) were assessed with quantitative real time-polymerase chain reaction. We found no significant differences in the expression of these genes in AD between neurons associated with A β compared to those lacking A β immunoreactivity. Double immunofluorescence microscopy showed the number of hippocampal neurons coexpressing A β or phospho-tau and either BDNF, TrkB, or DYN was similar in AD and controls. Our results suggest that intraneuronal A β or phospho-tau do not have obligatory effects on reducing the expression of genes important for memory and cognition in hippocampus of AD.

Keywords

A β ; BDNF; COX2; DYN; double immunofluorescence; qRT-PCR; phospho-tau

*Corresponding authors: Robert K. Fujimura, Ph.D. and Carlos Perez-Stable, Ph.D. Bruce W. Carter VA Medical Center, GRECC (11-GRC), 1201 NW 16 Street, Miami, FL 33125 USA. Tel.: +1 305 575-3388; Fax: +1 305 575 3365; fujimurr@bellsouth.net or cperez@med.miami.edu..

^gCurrent Address: Department of Public Health, Division of Epidemiologic Studies, Illinois Department of Public Health, Springfield, IL, USA 62761

INTRODUCTION

The clinical hallmark of Alzheimer's disease (AD) is impairment of memory and cognition that correlates with accumulation of A β within neurons as well as in extracellular deposits referred to as senile plaques [1–3]. A β is derived from amyloid precursor protein (APP) by regulated intramembranous proteolysis and eventually is broken down or eliminated [4]. In AD, these processes are disrupted. Accumulation of soluble forms of A β in the hippocampus and amygdala appears to be one of the earliest events that lead to cognitive impairment [5,6]. More recently, injection of a natural soluble form of A β into rat brain rapidly and potentially impaired cognitive function without inducing permanent neurological deficits [7]. These results led to the hypothesis that low amounts of diffusible forms of A β can directly reduce expression of neuronal genes involved in memory and cognition.

Neuronal synaptic dysfunction is a significant factor contributing to memory loss in AD [8–12]. Evidence suggests that A β may affect hippocampal synaptic efficacy before pathological structural degeneration [13,14]. For example, treatment of hippocampal neurons *in vitro* with A β results in decreases in expression of dynamin-1 (DYN), a protein important in synaptic function [15]. Similarly, mitochondrial genes are up-regulated in APP transgenic mice because of oxidative damage at the earliest stage, indicating impairment of mitochondrial energy metabolism by A β [16]. A β peptides also cause leaks in the mitochondrial membrane, resulting in the release of cytochrome c, activation of caspase-3, and apoptotic death of neurons [17,18]. It is possible that intraneuronal A β has direct effects on expression of synaptic or mitochondrial genes as an early event in the molecular pathogenesis of AD [3,13,16,19,20].

We hypothesize that if intraneuronal A β directly reduces the expression of neuronal genes associated with neuronal growth and survival, as well as synaptic and mitochondrial functions, hippocampal neurons containing A β immunoreactivity will have lower expression of these genes compared to neurons not associated with intraneuronal A β . Brain-derived neurotrophic factor (BDNF) and its receptor TrkB are representative genes important for memory and cognition that were analyzed in this study [21–23]. We also analyzed DYN, important for synaptic vesicle recycling and acquisition of memory, and cytochrome C oxidase subunit II (COX2), important for mitochondria function [24,25]. It is clear from previous studies that the expression of BDNF, TrkB, and DYN is lower in brains of AD compared to controls [26–31]. Cytochrome oxidase activity is decreased, but the expression of mitochondrial gene, such as COX2, is increased, possibly due to compensatory effects on surviving neurons [19,25,32,33]. At present, it is not clear to what extent the changes in the expression of these genes are due to direct effects of intraneuronal A β .

In the present study, we used laser capture microdissection to isolate hippocampal neurons associated with intraneuronal A β immunoreactivity, as well as neurons without A β , in brains of AD and normal controls and determined the levels of BDNF, DYN, and COX2 by qRT-PCR. We found no significant differences in the expression of these genes in neurons with or without A β immunoreactivity in AD. Using double immunofluorescence microscopy (DIF), we confirmed there were hippocampal neurons with A β immunoreactivity that express BDNF, TrkB, or DYN in both AD and controls. Similar to previous reports, we found lower levels of BDNF, TrkB, and DYN in AD that were correlated with an overall increase in intraneuronal A β .

We then investigated whether hippocampal neurons with phosphorylated tau (P-tau) immunoreactivity express lower levels of BDNF, TrkB, and DYN than neurons without P-tau immunoreactivity. Tau is a microtubule-binding protein that becomes abnormally phosphorylated in AD and accumulates to form neurofibrillary tangles [34,35]. Our results

using DIF were similar to A β , with no significant difference in gene expression for BDNF or DYN in neurons with and without P-tau immunoreactivity in AD and controls. We conclude that neither A β nor P-tau has obligatory intracellular effects on reducing the expression of genes important for memory and cognition in hippocampus of AD.

MATERIALS AND METHODS

Human hippocampal tissue

Both cryosections and paraffin-embedded hippocampal tissue sections (10 μ m thick) were obtained from AD and age-matched controls. At the time of autopsy 0.5-cm thick pieces of hippocampus were placed in OCT medium and flash frozen in isopentane chilled with liquid nitrogen. Sections were cut at 10 μ m thickness and stored at -80°C before analysis. Paraffin sections were obtained from additional AD cases and controls that had been fixed for 10–14 days in neutral buffered formaldehyde before embedding. All cases were confirmed by standardized clinical and neuropathologic methods that included quantitative assessment of senile plaques and neurofibrillary tangles with thioflavin-S fluorescent microscopy in multiple cortical sections and the hippocampus. Tissue was obtained from Mayo Clinic, Jacksonville, FL. Pathological information on the cases is summarized in Table 1. The time interval from death to freezing or fixation of tissue was between 2 and 11 hours.

RNA from laser capture microdissection of neurons with and without A β immunoreactivity

All procedures were carried out in an RNase-free environment, and all solutions contained placental RNase inhibitor (40 U/100 μ l) from Invitrogen (Carlsbad, CA). Slides were processed singly with rapid staining and washed briefly with phosphate-buffered saline (PBS) after each step. For immunostaining prior to laser capture, we followed the procedures of Fend F, et al. and D'Andrea, et al [36,37]. The procedure is as follows: cryosections of AD or controls were thawed, fixed with 75% ethanol for 30 sec, and then immunostained for 5 min at room temperature for A β using a rabbit polyclonal antibody (0.83 μ g/ μ l) from Invitrogen diluted to 1:625 with 1.5% goat serum in PBS. Labeled cells were identified by incubating for 2 min at 37°C with fluorescein isothiocyanate (FITC)-labeled swine anti-rabbit secondary antibody (DAKO, Carpinteria, CA) diluted to 1:10 with 1.5% goat serum in PBS. Slides were pretreated with 0.05% pontamine and 0.1% Triton X-100 to minimize background. Subsequently, neurons were morphologically identified by staining with 1% Nissl (Sigma, St. Louis, MO; ref. 38) for 30 sec, dehydrated with xylene, and stored in a vacuum desiccator until microdissection. Sections were microdissected using a Leica laser capture microdissection (LCM) unit at the Imaging Laboratory, University of Miami Miller School of Medicine (Miami, FL). From an AD case, A β positive and negative Nissl stained neurons were collected into separate tubes (Fig. 1). Neurons from normal controls often displayed only Nissl staining. On average, ~500 neurons of each category were collected per slide for AD and ~1000 neurons per slide for the controls. Neurons from six slides for each case were pooled. Three AD cases (AD1, AD2, AD3) and four controls (C1, C2, C3, C4) were processed (Table 1).

RNA from pooled neurons for each case was extracted using an Absolutely RNA Nano kit from Stratagene (La Jolla, CA) and treated with RNase-free DNase I (Invitrogen) to remove DNA. The concentration for each RNA preparation was determined using the Ribogreen kit (Invitrogen). RNA was asymmetrically amplified through two cycles using a Message Amp aRNA kit from Ambion (Austin, TX). In addition, total RNA from an age-matched normal case from the hippocampus tissue was obtained from Ambion.

Reverse transcription and quantitative real time -polymerase chain reaction (qRT-PCR)

Four µg or less of RNA isolated from LCM neurons or hippocampus tissue was reverse transcribed to cDNA with random primers using the First Strand cDNA Synthesis kit for RT-PCR from Roche Applied Sciences (Indianapolis, IN) in a thermo-cycler GeneAmp 9600 PCR from Perkin-Elmer (Waltham, MA). Primer-pair sequences specific for BDNF (Qiagen, Chatsworth, CA), DYN (Operon Huntsville, AL), and COX2 (TIB Mol Biol, Adelphia, NJ) were designed using published literature or the Primer 3 program (<http://frodo.wi.mit.edu>) (Table 2). Ribosome elongation factor 1 α (EF1 α) was used as the reference gene for the calculation of Ro [39] (Table 2). Quantitative RT-PCR was performed using the Light Cycler DNA Master SYBR Green I kit with varying amounts of cDNA (1 to 200 ng) and a Light Cycler system from Roche Applied Sciences (Indianapolis, IN). The qRT-PCR and melting point (Mp) profiles were obtained. Each product was confirmed for the expected size by agarose gel electrophoresis. For EF1 α and COX2, PCR was started with denaturation at 95°C, 30 sec, followed by amplification for 45 cycles at 95°C (0 sec, 20°C/sec), annealing at 50°C (10 sec, 5°C/sec), and elongation at 72°C (10 sec, 2°C/sec), and linked to detection at 80°C (0 sec, 20°C/sec). For BDNF and DYN, target genes with small copy number, PCR was started with a two-step denaturation at 50°C for 2 min, 95°C for 2 min, followed by amplification for 45 cycles at 95°C 15 sec, 58°C 30 sec, 72°C 45sec, and detection at 80°C [40]. The qRT-PCR profile was obtained by the instrument software using the fluorescent intensity at the detection temperature for each cycle. Negative controls were without reverse transcriptase (RT), resulting in no PCR product. The EF1 α reference gene was always analyzed using the same conditions as the target gene for calculation of Ro. Age-matched hippocampus RNA was also included in each run and used as a reference (Ro=1). Each sample was analyzed at least three times from at least three independent experiments.

Calculation of Ro and statistical methods

Ro, the initial ratio of target RNA of a specimen to EF1 α RNA, was obtained by the equation derived by Pfaffl [41]. Ro for each specimen was averaged from at least three independent experiments. The amplification efficiency (E), whose log is the slope for the logarithmic phase of each amplification curve, was obtained by the PSI-plot software from Poly Software International (Salt Lake City, UT) or by the Microsoft Excel program [42]. Crossing point (Cp) or the threshold of logarithmic amplification was obtained as the second derivative maximum using the instrument software. Statistical analyses as described by Fujimura, et al. [43] showed that the ratios of target genes to the reference gene EF1 α (Ro) for each set of specimens were independent of cDNA concentrations. Therefore, the significant differences observed in Ro between specimens were due to differences in amounts of target gene RNA. For each target gene, the analysis of variance was performed to compare Ro values between AD (n=3) and controls (n=4). Significance level was set at 0.05 for all tests. A post-hoc power analysis showed that with a two-sided 0.05 significance level and assuming no interclass correlation, there is an 80 percent and 88 percent power to detect an effect size of 1.75 and 2.00, respectively.

Double immunofluorescence (DIF)

For DIF, additional paraffin-embedded hippocampal sections from AD and controls were used (Table 1). Endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol for 5 min. For antigen retrieval, sections were incubated in hot 10 mM citrate buffer (pH 6.0) for 20 min. Immunofluorescence for A β (Abcam, Cambridge, MA; ab10148), P-tau (clone AT8; Pierce Biotechnology, Rockford, IL), BDNF (Santa Cruz Biotechnology, Santa Cruz, CA; H-117), TrkB (Cell Signaling, Beverly, MA; clone 80G2), and DYN (BD Biosciences, San Jose, CA; clone 41) was done using a 1:50 dilution of rabbit polyclonal or mouse monoclonal antibodies for 30 min at room temperature. After incubation with antibodies to

either A β or P-tau, antibody binding was detected with biotinylated anti-rabbit or mouse IgG (1/200 dilution; 20 min at room temperature; Vector Laboratories, Burlingame, CA), a 1:300 dilution of FITC-avidin DCS (Vector Laboratories) for 5 min, followed by avidin/biotin blocking for 15 min, and then immunostaining with BDNF, TrkB, or DYN and detection with biotinylated anti-rabbit or mouse IgG and Texas Red DCS for 5 min. Pseudocolor images were captured using a Nikon fluorescence microscope with FITC and Texas Red filters using IP Lab software (Fairfax, VA) and merged using Adobe Photoshop (San Jose, CA). The number of A β , P-tau, BDNF, TrkB, and DYN positive cells from 3–16 high-power fields (x200) were chosen from the pyramidal layer in areas with the highest density of immunostaining for three AD (AD5, AD6, AD7) and three controls (C5, C6, C7). The number of cells coexpressing A β /TrkB, A β /DYN, P-tau/BDNF, and P-tau/DYN was determined by counting yellow cells from the merged images. For negative controls, we used the same concentration of mouse or rabbit IgG (Santa Cruz Biotechnology) instead of specific primary antibodies, resulting in lack of immunostaining.

Western blot analysis

Total human hippocampus tissue lysates from normal and AD and from normal prostate was purchased from ProSci, Inc. (Poway, CA). After separation of 5!10 μ -g protein by SDS-PAGE, proteins were transferred by electrophoresis to Immobilon-P membrane and incubated in 5% nonfat dry milk, TBS, and 0.1% Tween-20 for 1 hour. The same antibodies used for DIF were diluted 1/1,000 in 5% nonfat dry milk, TBS, and 0.1% Tween-20 and incubated overnight at 4EC. Membranes were washed in TBS and 0.1% Tween-20 and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1/2,000 dilution; Santa Cruz) for 1 hour, washed in TBS and 0.1% Tween-20, and analyzed by exposure to X-ray film using enhanced chemiluminescence plus (ECL plus, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Staining of total protein with Coomassie blue was used as protein loading controls. X-ray films were scanned using an Epson Perfection 2450 Photo scanner.

RESULTS

The expression levels of BDNF, DYN, and COX2 in A β -positive and A β -negative neurons

We hypothesized that A β -associated neurons would have lower expression levels of genes essential for memory, as well as synaptic and mitochondrial functions, compared to A β -free neurons. To test this hypothesis, we obtained frozen sections of hippocampal tissue from AD and controls (Table 1). A β -positive and A β -negative Nissl-positive neurons were obtained by LCM from three AD and four control cases and analyzed by qRT-PCR for the expression of BDNF, DYN, and COX2 (Figs. 1, 2). The results showed that for each AD case, there were no significant differences in the levels of BDNF, DYN, or COX2 between A β -positive and A β -negative neurons (Fig. 2). In addition, the changes in expression of COX2 and DYN were not necessarily linked to changes in expression of BDNF. For BDNF, a gene essential for memory and cognition, the expression in Nissl-positive neurons of the controls was much higher ($R_o = 10$) compared to total hippocampal RNA, while in AD the expression in Nissl-positive neurons was much lower ($R_o = 0.5$) (Fig. 2A). For TrkB, a receptor for BDNF, the expression in AD cases was also very low ($R_o \leq 0.01$) (data not shown). For DYN, a gene essential for synapses, the expression was similar between AD and controls, but much lower than in total hippocampal RNA ($R_o \leq 0.1$) (Fig. 2B). For mitochondrial COX2, the expression was similar in controls compared to total hippocampal RNA ($R_o = 1$), with substantial variation in AD (Fig. 2C). Thus the changes in the expression of these three target genes did not appear to be linked. We conclude that there may not always be a direct relationship between memory and cognitive impairment and mitochondrial or synaptic dysfunction in AD, contrary to the expectation from the literature [3,6,8–13,18–20].

Hippocampal cells coexpressing A β and BDNF, TrkB, or DYN are present in AD and control cases

We used DIF to determine if hippocampal neurons with A β immunoreactivity that expressed BDNF, TrkB, or DYN were present in both AD and controls. As expected, we found more neurons with intracellular A β immunoreactivity in AD compared to controls and more neurons immunoreactive for BDNF, TrkB, and DYN in controls compared to AD (Figs. 3, 4, 7). The number of hippocampal neurons double immunostained for A β and TrkB or A β and DYN (yellow cells in the merged images) was similar in AD and controls (Fig. 7B). These results support the qRT-PCR analysis, in that expression of BDNF and DYN is similarly detected in hippocampal neurons of AD cases that are both A β -positive and A β -negative.

Hippocampal cells coexpressing P-tau and BDNF or DYN in AD and controls

In addition to A β , P-tau, the major structural component of neurofibrillary tangles, has been hypothesized to have an important role in neuronal dysfunction in AD [31,34,35]. We used DIF to determine if hippocampal neurons that were immunoreactive for P-tau also expressed BDNF or DYN. Our results showed, as predicted, more neurons with P-tau immunoreactivity in AD compared to normal controls (Figs. 5, 7A). Similar to the results with A β immunoreactivity, the number of hippocampal neurons double-immunolabeled for P-tau and BDNF or for P-tau and DYN (yellow cells in the merged images) was similar in AD and controls (Figs. 5, 7B). There were fewer double-labeled neurons for P-tau and DYN (0.9–1.3 cells/hpf) compared to A β and DYN (2.3–2.5 cells/hpf) in both AD and controls (Fig. 7B); however, the differences were not statistically significant ($P = 0.1$). The specificity of the antibodies used for DIF were confirmed by Western blot analysis of human hippocampus protein lysates from normal and AD compared to proteins from human prostate (Fig. 6). Overall, these results suggest that intraneuronal accumulation of A β and P-tau protein in hippocampal neurons does not have a strong negative effect on expression (mRNA and protein) levels of BDNF, TrkB, or DYN in either AD or controls.

DISCUSSION

The underlying molecular mechanisms of AD remain to be elucidated. Two prevailing hypotheses for the progressive neurologic deterioration in AD are increases in A β in senile plaques and P-tau in neurofibrillary tangles [2,3,35,44]. More recent evidence in transgenic mouse and rat models suggest that the soluble oligomers of A β are the toxic species in AD [5–7]. It is not clear in human cases of AD if the increases in A β or P-tau in hippocampal neurons directly decreases the expression of genes important for memory and cognition, such as BDNF and TrkB, or genes important in synaptic and mitochondrial function, such as DYN and COX2, respectively. In the present study, our results using LCM and qRT-PCR analysis of RNA showed no differences in the expression of these genes (i.e., BDNF, DYN, and COX2) in neurons of the hippocampus of AD and controls that displayed intraneuronal A β immunoreactivity compared to neurons without A β immunoreactivity (Fig. 2). Utilizing DIF in AD and controls, we also found a similar number of hippocampal neurons coexpressing A β /TrkB, A β /DYN, P-tau/BDNF, and P-tau/DYN (Figs. 3–5, 7B). Therefore, our data suggest that the accumulation of intracellular A β and P-tau in hippocampal neurons does not always play a major role in directly reducing expression of BDNF, TrkB, DYN, or COX2.

Data suggesting that A β can directly reduce the expression of DYN come from experimental treatment of fetal rat hippocampal neurons grown in culture [15]. A small but significant decrease in DYN protein in hippocampal extracts isolated from the Tg2576 transgenic mouse model of AD has also been demonstrated [15]. These mice contain a double mutation in APP that increases production of A β and produces some of the characteristics of AD

pathology, in particular, senile plaque formation [45]. The reduction of DYN protein in cultured rat hippocampal neurons and possibly in the Tg2576 transgenic mice may be due to A β -mediated increases in calpain degradation of DYN [15]. This potential mechanism of A β -mediated increase of calpain degradation of DYN protein may explain why our data in human hippocampal tissue sections from AD show no difference in DYN mRNA levels compared to control cases, whereas our results utilizing DIF show reduced levels of DYN protein in AD compared to controls (Figs. 2, 3B, 7A). However, if the A β -mediated increase in calpain degradation of DYN protein is a major mechanism, it is difficult to explain why our results show similar numbers of dual-labeled A β /DYN hippocampal neurons in AD and controls (Figs. 3B, 7B).

Additional evidence in fetal rat cultured cortical neurons suggests that A β can interfere with the ERK and AKT signaling pathways that activate BDNF transcription factors [46]. Another study found that treatment of a human neuroblastoma cell line with soluble A β peptides decreases phosphorylated cAMP response element-binding protein (CREB), a regulator of BDNF transcription, and BDNF total mRNA [40]. In the present report, our data are similar to those of other previously published reports showing that BDNF mRNA and protein levels are reduced in AD compared to controls [21–23]. Because our data showed that the expression level of BDNF mRNA was similar in hippocampal neurons with intraneuronal A β compared to neurons free of A β immunoreactivity (Fig. 2A), it is likely that intraneuronal A β -mediated reduction of transcription factors regulating BDNF is not a major feature of AD.

Our results in hippocampal tissue sections are contrary to the hypothesis that intraneuronal A β directly reduces the expression of genes important in memory and cognition. However, it is possible that the hippocampus in AD is permeated with soluble oligomeric A β peptides that are not visible with immunostaining. We also investigated by DIF whether P-tau was associated with reduced levels of BDNF, TrkB, or DYN in hippocampal neurons of AD and controls. The abnormal accumulation of P-tau is hypothesized to disrupt transport of proteins and growth factors by interfering with microtubule-dependent axoplasmic transport [35,44]. The results with DIF, using the AT8 monoclonal antibody that specifically recognizes P-tau but not nonphosphorylated tau protein, were similar to the results with DIF for A β (Figs. 5, 7). However, in view of our negative findings, future studies with greater sample sizes are warranted.

There are no reports that the overexpression of P-tau has a direct effect in reducing the levels of DYN or BDNF, as has been reported for A β in cultured neuronal cells [15,40,46]. In contrast, one report shows that BDNF stimulation of differentiated P19 mouse embryonic carcinoma cells results in a rapid decrease in tau phosphorylation, suggesting that BDNF activation of its receptor, TrkB, has a role in lowering P-tau protein [47]. Therefore, one possibility is that reduction of BDNF protein increases P-tau, while P-tau does not directly reduce BDNF. However, if the reduction of BDNF is correlated with increased P-tau, it is difficult to explain why our results show similar numbers of hippocampal neurons coexpressing P-tau and BDNF in both AD and controls (Figs. 5A, 7B). There were fewer P-tau labeled neurons coexpressing BDNF and these cells were present in the same proportion in the AD and control cases. It would be of interest to determine if these cells are the same cells that coexpress A β and BDNF.

Perhaps a better way to determine whether A β and P-tau protein are maintained at harmless levels in healthy controls is to study animal models. Injection of a natural soluble form of A β peptide into rat brain rapidly and potently impairs cognitive function without inducing permanent neurological deficits, although the effect on genes important in cognitive function was not reported [7]. Numerous transgenic mouse models are available that overexpress

mutated APP or mutated tau that develops amyloid plaques and neurofibrillary tangles, respectively [48,49]. The advent of inducible transgenic mouse models that target neuronal cells with APP or tau in a controlled and timed manner may be required to determine whether genes important for memory and cognition, like BDNF, TrkB, and DYN, are reduced [48–51]. Although the neuropathology of transgenic mouse models appears similar to that in AD, synaptic changes at the molecular level may not completely recapitulate the pathology in humans [48].

To analyze the expression levels of genes associated with memory and cognition, we obtained hippocampal tissue from AD and controls 2 to 11 hours after death. This analysis reduces variables associated with cultured neuronal cells and the differences observed in animal models compared to human AD. Our results show that in AD and controls, the reduction in intracellular levels of BDNF, TrkB, DYN, and COX2 does not correlate with intraneuronal A β and P-tau. There may be an important indirect effect of A β and P-tau that reduces the levels of the expression of these genes. An alternative proposal is that A β and P-tau are not the primary cause of AD but that increased oxidative stress caused by other pathogenic factors may lead to the increases in A β and P-tau, and possibly decrease the expression of neural genes [16,52].

Acknowledgments

This project was initiated while R.K.F. was in Dr. Paul Shapshak's laboratory (Department of Psychiatry, University of Miami Miller School of Medicine). We thank Dr. Carol Petito (Department of Pathology, University of Miami Miller School of Medicine) and Brigitte Shaw (Imaging Laboratory, Diabetes Research Institute, University of Miami Miller School of Medicine) for advice and guidance with LCM. We thank David Vasquez and Blanca Rodriguez for excellent technical assistance and Virginia Roos for editorial assistance. This work was supported in part by the South Florida Veterans Affairs Foundation for Research and Education and by the Department of Veterans Affairs. G.A.H. is the recipient of a VA Senior Research Career Scientist award.

REFERENCES

- [1]. Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, Buxbaum JD. Correlation between elevated levels of amyloid β -peptide in the brain and cognitive decline. *JAMA*. 2000; 283:1571–1577. [PubMed: 10735393]
- [2]. Selkoe DJ, Schenk D. Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. *Ann Rev Pharmacol Toxicol*. 2003; 43:545–584. [PubMed: 12415125]
- [3]. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297:353–356. [PubMed: 12130773]
- [4]. Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E4: a causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc Natl Acad Sci USA*. 2006; 103:5644–5651. [PubMed: 16567625]
- [5]. Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular A β and synaptic dysfunction. *Neuron*. 2003; 39:409–421. [PubMed: 12895417]
- [6]. Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM. Intraneuronal A β causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron*. 2005; 45:675–688. [PubMed: 15748844]
- [7]. Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, Ashe KH. Natural oligomers of the amyloid- β protein specifically disrupt cognitive function. *Nature Neurosci*. 2005; 8:79–84. [PubMed: 15608634]
- [8]. Flood DG, Coleman PD. Hippocampal plasticity in normal aging and decreased plasticity in Alzheimer's disease. *Prog Brain Res*. 1990; 83:435–443. [PubMed: 2203107]
- [9]. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol*. 1991; 30:573–580.

- [10]. Dickson DW, Crystal HA, Bevona C, Honer W, Vincent I, Davies P. Correlations of synaptic and pathological markers with cognition of the elderly. *Neurobiol Aging*. 1995; 16:285–298. [PubMed: 7566338]
- [11]. Sze CI, Troncoso JC, Kawas C, Mouton P, Price DL, Martin LJ. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. *J Neuropathol Exp Neurol*. 1997; 56:933–944. [PubMed: 9258263]
- [12]. Masliah E, Mallory M, Alford M, DeTeresa R, Hansen LA, McKeel DW Jr, Morris JC. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology*. 2001; 56:127–129. [PubMed: 11148253]
- [13]. Selkoe DJ. Alzheimer's disease is a synaptic failure. *Science*. 2002; 298:789–791. [PubMed: 12399581]
- [14]. Yao PJ. Synaptic frailty and clathrin-mediated synaptic vesicle trafficking in Alzheimer's disease. *Trends Neurosci*. 2004; 27:24–29. [PubMed: 14698607]
- [15]. Kelly BL, Vassar R, Ferreira A. Beta-amyloid-induced dynamin 1 depletion in hippocampal neurons: a potential mechanism for early cognitive decline in Alzheimer's disease. *J Biol Chem*. 2005; 280:31746–31753. [PubMed: 16002400]
- [16]. Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA. Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol*. 2001; 60:759–767. [PubMed: 11487050]
- [17]. Reddy PH, McWeeney S, Park BS, Manczak M, Gutala RV, Partovi D, Jung Y, Yau V, Searles R, Mori M, Quinn J. Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and apoptotic genes is an early cellular change in Alzheimer's disease. *Hum Mol Genet*. 2004; 13:1225–1240. [PubMed: 15115763]
- [18]. Takuma K, Yan SS, Stern DM, Yamada K. Mitochondrial dysfunction, endoplasmic reticulum stress, and apoptosis in Alzheimer's disease. *J Pharmacol Sci*. 2005; 97:312–316. [PubMed: 15750290]
- [19]. Reddy PH. Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. *J Neurochem*. 2006; 96:1–13. [PubMed: 16305625]
- [20]. Keating DJ. Mitochondrial dysfunction, oxidative stress, regulation of exocytosis and their relevance to neurodegenerative diseases. *J Neurochem*. 2008; 104:298–305. [PubMed: 17961149]
- [21]. Yamada K, Nabeshima T. Brain-derived neurotrophic factor/TrkB signaling in memory processes. *J Pharmacol Sci*. 2003; 91:267–270. [PubMed: 12719654]
- [22]. Lee JL, Everitt BJ, Thomas KL. Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science*. 2004; 304:839–843. [PubMed: 15073322]
- [23]. Reichardt LF. Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci*. 2006; 361:1545–1564. [PubMed: 16939974]
- [24]. Yao PJ, Zhu M, Pyun EI, Brooks AI, Therianos S, Meyers VE, Coleman PD. Defects in expression of genes related to synaptic vesicle trafficking in frontal cortex of Alzheimer's disease. *Neurobiol Dis*. 2003; 12:97–109. [PubMed: 12667465]
- [25]. Onyango I, Khan S, Miller B, Swerdlow R, Trimmer P, Bennett P Jr. Mitochondrial genomic contribution to mitochondrial dysfunction in Alzheimer's disease. *J Alzheimers Dis*. 2006; 9:183–193. [PubMed: 16873965]
- [26]. Phillips HS, Hains JM, Armanini M, Laramie GR, Johnson SA, Winslow JW. BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron*. 1991; 7:695–702. [PubMed: 1742020]
- [27]. Connor B, Young D, Yan Q, Faull RLM, Synek B, Dragunow M. Brain-derived neurotrophic factor is reduced in Alzheimer's disease. *Mol Brain Res*. 1997; 49:71–81. [PubMed: 9387865]
- [28]. Salehi A, Verhaagen J, Dijkhuizen PA, Swaab DF. Co-localization of high-affinity neurotrophin receptors in nucleus basalis of Meynert neurons and their differential reduction in Alzheimer's disease. *Neuroscience*. 1996; 75:373–387. [PubMed: 8931004]

- [29]. Allen SJ, Wilcock GK, Dawbarn D. Profound and selective loss of catalytic TrkB immunoreactivity in Alzheimer's disease. *Biochem Biophys Res Commun*. 1999; 264:648–651. [PubMed: 10543986]
- [30]. Kish SJ, Bergeron C, Rajput A, Dozic S, Mastrogiacono F, Chang LJ, Wilson JM, DiStefano LM, Nobrega JN. Brain cytochrome oxidase in Alzheimer's disease. *J Neurochem*. 1992; 59:776–779. [PubMed: 1321237]
- [31]. Wolozin BL, Pruchnicki A, Dickson DW, Davies P. A neuronal antigen in the brains of Alzheimer patients. *Science*. 1986; 232:648–650. [PubMed: 3083509]
- [32]. Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, Johnson AB, Kress Y, Vinters HV, Tabaton M, Shimohama S, Cash AD, Siedlak SL, Harris PL, Jones PK, Petersen RB, Perry G, Smith MA. Mitochondrial abnormalities in Alzheimer's disease. *J Neurosci*. 2001; 21:3017–3023. [PubMed: 11312286]
- [33]. Manczak M, Park BS, Jung Y, Reddy PH. Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease: implications for early mitochondrial dysfunction and oxidative damage. *Neuromolecular Med*. 2004; 5:147–162. [PubMed: 15075441]
- [34]. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci USA*. 1986; 83:4913–4917. [PubMed: 3088567]
- [35]. Chun W, Johnson GV. The role of tau phosphorylation and cleavage in neuronal cell death. *Front Biosci*. 2007; 12:733–756. [PubMed: 17127334]
- [36]. Fend F, Emmert-Buck MR, Chaqui R, Cole K, Lee J, Liotta LA, Ratfeld M. Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am J Pathol*. 1999; 154:61–66. [PubMed: 9916919]
- [37]. D'Andrea MR, Nagele RG, Wang H-Y, Peterson PA, Lee DH. Evidence that neurons accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. *Histopathology*. 2001; 38:120–134. [PubMed: 11207825]
- [38]. Luo L, Salunga RC, Guo H, Bittner A, Joy KC, Galindo JE, Xiao H, Rogers KE, Wan JS, Jackson MR, Erlander MG. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat Med*. 1999; 5:117–122. [PubMed: 9883850]
- [39]. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci*. 2004; 117:2971–2981. [PubMed: 15173316]
- [40]. Garzon DJ, Fahnestock M. Oligomeric amyloid decreases basal levels of brain-derived neurotrophic factor (BDNF) mRNA via specific downregulation of BDNF transcripts IV and V in differentiated human neuroblastoma cells. *J Neurosci*. 2007; 27:2628–2635. [PubMed: 17344400]
- [41]. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29:e45. [PubMed: 11328886]
- [42]. Ramakers C, Ruijter JM, Deprez RM, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett*. 2003; 339:62–66. [PubMed: 12618301]
- [43]. Fujimura RK, Khamis I, Shapshak P, Goodkin K. Regional quantitative comparison of multispliced to unspliced ratios of HIV-1 RNA copy number in infected human brain. *J NeuroAIDS*. 2004; 2:45–60. [PubMed: 16873205]
- [44]. Schindowski K, Belarbi K, Buée L. Neurotrophic factors in Alzheimer's disease: role of axonal transport. *Genes Brain Behav*. 2008; 7:43–56. [PubMed: 18184369]
- [45]. Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. *Science*. 1996; 274:99–102. [PubMed: 8810256]
- [46]. Tong L, Balazs R, Thornton PL, Cotman CW. Beta-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons. *J Neurosci*. 2004; 24:6799–6809. [PubMed: 15282285]

- [47]. Elliott E, Atlas R, Lange A, Ginzburg I. Brain-derived neurotrophic factor induces a rapid dephosphorylation of tau protein through a PI-3 Kinase signaling mechanism. *Eur J Neurosci.* 2005; 22:1081–1089. [PubMed: 16176349]
- [48]. Duyckaerts C, Potier M-C, Delatour B. Alzheimer disease models and human neuropathology: similarities and differences. *Acta Neuropathol.* 2008; 115:5–38. [PubMed: 18038275]
- [49]. Beglopoulos V, Shen J. Gene-targeting technologies for the study of neurological disorders. *Neuromolecular Med.* 2004; 6:13–30. [PubMed: 15781974]
- [50]. Janus C. Conditionally inducible tau mice – designing a better mouse model of neurodegenerative diseases. *Genes Brain Behav.* 2008; 7(Suppl 1):12–27. [PubMed: 18184367]
- [51]. Nissen A, Mocanu M, Eckermann K, Khlistunova I, Biernat J, Drexler D, Petrova O, Zhou L, Rune G, Mandelkow E-M. O4-01-02: Neurofibrillary tangles and neuronal loss in an inducible transgenic mouse model expressing a mutant repeat domain of tau. *Alzheimer's and Dementia.* 2008; 4(Suppl):T183–T184.
- [52]. Castellani RJ, Zhu X, Lee HG, Smith MA, Perry G. Molecular pathogenesis of Alzheimer's disease: reductionist versus expansionist approaches. *Int J Mol Sci.* 2009; 10:1386–1406. [PubMed: 19399255]

Fig. 1.

LCM of A β -associated and non-associated neurons from AD hippocampal tissue section. Left panel, immunostaining using FITC-conjugated A β antibody. Middle panel, Nissl stain of neurons (dark areas). Right panel, laser capture of Nissl-stained neurons that overlap with A β immunostaining were microdissected and collected as A β -associated neurons. Nissl-stained neurons without A β immunostaining were collected as A β -free neurons.

Fig. 2.

Quantitative RT-PCR analysis of hippocampal A β -associated and A β -free neurons for BDNF, DYN, and COX2. (A) Expression of BDNF showing higher levels (Ro) in three control cases compared to three AD cases. There were no significant differences in BDNF mRNA between A β -associated (A β) compared to non-associated (N) neurons. (B) Expression of DYN showing similar levels (Ro) in controls (average of three cases) compared to two AD cases and similar levels between A β compared to N neurons in AD cases. (C) Expression of COX2 showing variable differences between controls and AD cases and similar levels between A β compared to N neurons in AD cases. Data were expressed relative to reference total hippocampal (HC) RNA (Ro = 1.0) for BDNF, DYN, and COX2 from at least three independent experiments done in triplicate (*columns*, mean; *bars*, SD). Numbers below the bar refer to the individual C and AD cases (see Table 1).

Fig. 3.

Hippocampal cells coexpressing A β and BDNF (A) or DYN (B) are present in AD and control cases. Representative DIF of A β /BDNF and A β /DYN showing higher levels of A β (green) in AD (AD6, AD7) compared to controls (C7, C6); higher levels of BDNF and DYN (red) in controls compared to AD; and similar numbers of A β /BDNF and A β /DYN (yellow cells in merge) in controls compared to AD ($\times 200$). Arrows point to examples of coexpressing cells.

Fig. 4.

Hippocampal cells coexpressing A β and TrkB are present in AD and control cases. Representative DIF of A β /TrkB showing high levels of A β (green) in AD (AD5) compared to control (C5); higher levels of TrkB (red) in control compared to AD; and similar numbers of A β /TrkB (yellow cells in merge) in controls compared to AD ($\times 200$). Arrows point to examples of coexpressing cells. The bottom two panels are the negative controls using nonspecific mouse or rabbit IgG as primary antibodies and the appropriate FITC-avidin or Texas Red DCS. No immunostaining was detected.

Fig. 5.

Hippocampal cells coexpressing P-tau and BDNF (A) or DYN (B) are present in AD and control cases. Representative DIF of P-tau/BDNF and P-tau/DYN showing higher levels of P-tau (green) in AD (AD5, AD6) compared to controls (C7, C5); higher levels of BDNF and DYN (red) in control compared to AD; and similar numbers of P-tau/BDNF and P-tau/DYN (yellow cells in merge) in controls compared to AD ($\times 200$). Arrows point to examples of coexpressing cells.

Fig. 6.

Western blot analysis showing specificity of antibodies used in DIF. Aβ (~4kd) peptide and P-tau (55–74 kd) proteins were more highly expressed in human hippocampal AD compared to normal (N) and prostate (Pr) protein lysates. In contrast, BDNF (14 kd) and DYN (100 kd) were more highly expressed in normal compared to AD hippocampus and prostate. Truncated noncatalytic TrkB (TrkB-t; 95kd) was higher in AD compared to control hippocampus but not detected in prostate. Coomassie blue stain of total protein was the loading control.

Fig. 7.

Quantitative analysis of DIF. (A) Number of cells immunostaining for A β and P-tau (columns) per 3–16 random areas with the highest density of immunostaining per high-powered fields (hpf, $\times 200$) was determined in AD ($n = 3$) and compared to control ($n = 3$) cases. Results showed significant increases in cells immunostaining for A β and P-tau in AD compared to control cases (*, $P < 0.009$; Student's t -test). (B) Number of cells coexpressing A β /TrkB, A β /DYN, P-tau/BDNF, and P-tau/DYN (columns) per 3–16 random highest density of immunostaining high-powered fields (hpf, $\times 200$) was determined in AD and compared to control cases. Results showed no significant differences. There were fewer cells coexpressing P-tau/DYN (0.9–1.3 cells/hpf) compared to cells coexpressing A β /DYN (2.3–2.5 cells/hpf) in AD and control cases but the results were not significant ($P = 0.1$). Bars, SD.

Table 1

Pathological data of AD and control cases

Case	Analysis	Age	SP ^a	NFT ^b	Braak stage ^c
Controls					
C1	LCM/qRT-PCR	92	0	1	III
C2	LCM/qRT-PCR	84	0	1	I
C3	LCM/qRT-PCR	91	0	0	I
C4	LCM/qRT-PCR	90	0	0	II
C5	DIF ^d	86	0	2	II–III
C6	DIF	82	0	2	II–III
C7 ^e	DIF	87	14	15	III
AD					
AD1	LCM/qRT-PCR	81	16	29	VI
AD2	LCM/qRT-PCR	89	24	41	VI
AD3	LCM/qRT-PCR	77	22	38	V
AD4	LCM/qRT-PCR	86	10	27	VI
AD5	DIF	93	18	20	V
AD6	DIF	84	14	30	VI
AD7	DIF	87	28	35	V

^aSP, density of senile plaques in hippocampus (average number per 1.25 mm²)

^bNFT, density of neurofibrillary tangles in the hippocampus (average number per 0.25 mm²)

^cBraak neurofibrillary tangles stage: 0 – VI

^dDIF, Double immunofluorescence microscopy

^eC7, pathological aging; neurologically normal individual with SP and NFT, but low Braak stage

Table 2Oligonucleotide primers used with RNA from hippocampal neurons with or without A β immunoreactivity

Gene and Sequence	Reference	Product, bp	Mp, °C
BDNF	Primer 3	158	85
Forward:	5'-GAGCTGAGCGTGTGTGACAG-3'		
Reverse:	5'-ATGGGATTGCACTTGGTCTC-3'		
Dynamin 1	Primer 3	126	84
Forward:	5'-AGACCATCCGGAATCTTGTG-3'		
Reverse:	5'-GCAGCTCCGAGAAGATGAAC-3'		
COX2	Primer 3	129	84
Forward:	5'-ACAGACGAGGTCAACGATCC-3'		
Reverse:	5'-AGATTAGTCCGCCGTAGTCG-3'		
EF	[39]	236	86
Forward:	5'-AGGTGATTATCCTGAACCATCC-3'		
Reverse:	5'-AAAGGTGGATAGTCTGAGAAGC-3'		