Published in final edited form as:

Eur J Neurosci. 2007 October ; 26(8): 2131–2141. doi:10.1111/j.1460-9568.2007.05797.x.

Involvement of astroglial ceramide in palmitic acid-induced Alzheimer-like changes in primary neurons

Sachin Patil¹, Joseph Melrose¹, and Christina Chan^{1,2}

¹Department of Chemical Engineering and Material Science, Michigan State University, East Lansing, MI 48824, USA

²Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA

Abstract

A high-fat diet has been shown to significantly increase the risk of the development of Alzheimer's disease (AD), a neurodegenerative disease histochemically characterized by the accumulation of amyloid beta (A β) protein in senile plaques and hyperphosphorylated tau in neurofibrillary tangles. Previously, we have shown that saturated free fatty acids (FFAs), palmitic and stearic acids, caused increased amyloidogenesis and tau hyperphosphorylation in primary rat cortical neurons. These FFA-induced effects observed in neurons were found to be mediated by astroglial FFA metabolism. Therefore, in the present study we investigated the basic mechanism relating astroglial FFA metabolism and AD-like changes observed in neurons. We found that palmitic acid significantly increased de-novo synthesis of ceramide in astroglia, which in turn was involved in inducing both increased production of the A β protein and hyperphosphorylation of the tau protein. Increased amyloidogenesis and hyperphosphorylation of tau lead to formation of the two most important pathophysiological characteristics associated with AD, A β or senile plaques and neurofibrillary tangles, respectively. In addition to these pathophysiological changes, AD is also characterized by certain metabolic changes; abnormal cerebral glucose metabolism is one of the distinct characteristics of AD. In this context, we found that palmitic acid significantly decreased the levels of astroglial glucose transporter (GLUT1) and down-regulated glucose uptake and lactate release by astroglia. Our present data establish an underlying mechanism by which saturated fatty acids induce AD-associated pathophysiological as well as metabolic changes, placing 'astroglial fatty acid metabolism' at the center of the pathogenic cascade in AD.

Keywords

Alzheimer's disease; astroglia; ceramide; fat; rat

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease mostly affecting basal forebrain, cortex and hippocampus, whereas cerebellum is relatively spared (Smith, 1998; Giasson *et al.*, 2002). Pathologically, AD is characterized by extracellular deposits of amyloid beta (A β) protein and intracellular accumulation of neurofibrillary tangles (Mattson, 2004). A β possesses aggregating properties leading to the formation of senile plaques, a characteristic feature of AD (Glennner, 1988). A β aggregates exert toxicity to nerve cells and thus have been considered to play a central role in AD-associated neurodegeneration (Yankner *et al.*, 1990; Mattson *et al.*, 1992; Chang & Suh, 2005). Neurofibrillary tangles, the other major characteristic of AD pathology, consist of paired helical filaments of microtubule-associated tau protein, which is hyperphosphorylated in AD (Lee *et al.*, 2000). The hyperphosphorylation of tau disrupts the cytoskeleton of neurons, which leads to their degeneration thus playing an important role in AD pathology (Mattson *et al.*, 1992). In addition to these pathophysiological changes, AD is also characterized by abnormal metabolic changes; glucose metabolism in AD brain is decreased by 46% compared with healthy individuals and is considered to precede the neuropathological changes associated with the disease (Hoyer, 1991).

Despite these data the causal mechanisms leading to these pathophysiological and metabolic abnormalities associated with AD are not well understood. Various studies have implicated the possible involvement of saturated fatty acids in the pathogenesis of AD. Epidemiological studies suggest that a high-fat diet is a major risk factor for the development of AD and the degree of saturation of fatty acids is critical in determining the risk for AD (Grant, 1999; Solfrizzi *et al.*, 2005; Scarmeas *et al.*, 2006). This notion is further supported by various *in-vivo* studies where mice fed a western, high-fat (21–40%)/high-cholesterol (0.15–1%) diet developed AD-like pathophysiological changes in their brain (Levin-Allerhand *et al.*, 2002; Shie *et al.*, 2003; Oksman *et al.*, 2006). In addition, various vascular diseases, such as diabetes mellitus, hypertension and obesity, pose a significant risk for developing AD and are characterized by increased levels of saturated free fatty acids (FFAs) in the plasma (Skoog *et al.*, 1996; Arvanitakis *et al.*, 2004; Monti *et al.*, 2004; Whitmer Rachel *et al.*, 2005). Similarly, traumatic brain injury has been established as an independent risk factor for AD (Guo *et al.*, 2000) and is characterized by elevated levels of palmitic and stearic acids in the brain (Homayoun *et al.*, 1997), with palmitic acid (PA) increasing from ~ 60 to 180 μ M and stearic acid from ~ 50 to 350 μ M (Lipton, 1999). Finally, apolipoprotein E4 is an important genetic risk factor for AD and its risk may be further increased by a hyperlipidemic lifestyle (Petot *et al.*, 2003).

Previously, we have shown a direct causal role of saturated fatty acids, palmitic and stearic fatty acids, in the amyloidogenic processing of amyloid precursor protein and the hyperphosphorylation of tau in neurons mediated by astroglia (Patil & Chan, 2005; Patil *et al.*, 2006). The aim of the present study was to further our mechanistic understanding of the role of astroglial FFA metabolism in causing the aforementioned pathophysiological changes as well as the abnormal glucose metabolism associated with AD.

Materials and methods

Isolation and culture of primary rat cortical neurons and astroglia

Primary cortical neurons were isolated from 1-day-old Sprague-Dawley rat pups and cultured as described by Chandler *et al.* (1993). All procedures were performed according to guidelines approved by the Institutional Animal Care and Use Committee at Michigan State University. In brief, the animal heads were submerged in ice-cold 70% ethanol briefly and decapitated. The brains were removed and subjected to the procedure for isolating the neuronal cells. The cells were plated on poly-L-lysine-coated six-well plates at the concentration of 2×10^6 cells/well in fresh cortical medium [Dulbecco's modified Eagle's medium (this and all other media are from Invitrogen, CA, USA) supplemented with 10% horse serum (Sigma, St Louis, MO, USA), 25 mM glucose, 10 mM HEPES (Sigma), 2 mM glutamine (BioSource International, CA, USA), 100 IU/mL penicillin and 0.1 mg/mL streptomycin]. After incubating for 3 days (37 °C, 5% CO₂), the medium was completely replaced with 3 mL of cortical medium supplemented with 5 µM cytosine-β-arabinofuranoside (Cal-biochem, CA, USA). Subsequently, after 2 days, the neuronal culture was switched back to cortical medium without cytosine-β-arabinofuranoside. The experiments were performed on 6–7-day-old neuronal cultures. To obtain primary cultures of astroglial cells, the cortical cells from 1-day-old Sprague-Dawley rat pups were cultured in astroglial medium Dulbecco's modified Eagle's medium/Ham's F12 medium (1 : 1), 10% fetal bovine serum (Biomed, CA, USA), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (Pshenichkin & Wise, 1995). The cells were plated on poly-L-lysine-coated, six-well plates at the concentration of 2×10^6 cells/well. The culture medium was changed every 2 days. After 8–10 days of culture (37 °C, 5% CO₂) in excess of 95% pure astroglial culture was obtained, which was used for the experiments. At 24 h prior to the treatment of astroglia with fatty acids, the medium was changed from astroglial medium to neuronal cell culture medium.

Ceramide measurement

Astroglia were washed with ice-cold phosphate-buffered saline and lipids were then extracted by using the chloroform/methanol method as described by Bligh & Dyer (1959). The organic phase was dried under N₂ and the ceramide was measured after its deacylation to sphingolipid base and derivatization with *o*-phthaldehyde as described earlier (Merrill *et al.*, 1988). Briefly, the dried lipids from the organic phase were resuspended in 500 µL of 1 N KOH in methanol and incubated at 100 °C for 1 h to deacylate ceramide to free sphingolipid bases. The lipids were then dissolved in 50 µL of methanol and 50 µL of *o*-phthaldehyde reagent was added. *o*-Phthaldehyde reagent was prepared by mixing 99 mL of boric acid (3% w/v in water, pH 10.5), 1 mL of ethanol containing 50 mg *o*-phthaldehyde (Sigma) and 50 µL of β-mercaptoethanol (Sigma). The derivatized sample aliquots (20 µL) were quantified by high-performance liquid chromatography using a Nova Pak C18 column (60 Å, 4 µm, 3.9 × 150 mm; Waters, MA, USA). Fluorescent-labeled lipids were eluted isocratically by using methanol/5 mM potassium phosphate (pH 7.0) (90 : 10, v/v) at a flow rate of 0.6 mL/min and detected with fluorescence detector (excitation wavelength 340 nm, emission wavelength 455 nm). A standard curve obtained by running known

amounts of ceramide (type III, from bovine brain sphingomyelin; Sigma) was used as a comparison to determine ceramide levels in the experimental samples.

Lactate dehydrogenase assay

The secreted and intracellular levels of lactate dehydrogenase (LDH) were measured to determine the cell toxicity in astroglial cells by using a cytotoxicity detection kit (Roche, IN, USA). The cytotoxicity was determined as a percentage, i.e. the fraction of LDH released into the medium ($\text{LDH}_{\text{medium}}$), relative to the total LDH ($\text{LDH}_{\text{medium}}$ and intracellular LDH):

$$\text{LDH}_{\text{release}}(\%) = 100 \times \text{LDH}_{\text{medium}} / (\text{LDH}_{\text{medium}} + \text{LDH}_{\text{intracellular}})$$

Immunostaining of reactive oxygen species

Intracellular reactive oxygen species (ROS) were detected by staining with the oxidant-sensitive dye 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein (DCF) diacetate (DA) (Molecular Probes, OR, USA). H_2DCFDA is cleaved of the ester groups by intracellular esterases and converted into the membrane-impermeable, nonfluorescent derivative H_2DCF . Oxidation of H_2DCF by ROS results in highly fluorescent 2,7-dichlorofluorescein (Sauer *et al.*, 2001). The cells were incubated for 30 min at 37 °C with 2 μM 5-(6)-chloromethyl-2',7'-dichlorodi-hydrofluorescein diacetate in Hanks' balanced salt solution without phenol red (Invitrogen). The cells were then washed three times with phosphate-buffered saline and analysed with confocal microscopy (Zeiss LSM, 5 Pa).

Western blot analyses

For western blotting, the following antibodies were used: BACE1 (Chemicon, CA, USA), AT8 (Pierce Biotechnology, Rockford, IL, USA), PHF-1 (from Dr P. Davies, Albert Einstein College of Medicine, New York, NY, USA), Tau-1 (Chemicon), glycogen synthase kinase 3 (GSK-3) α/β (Chemicon), phospho-GSK-3 α/β (Sigma), MAP Erk1/2 (Cell Signaling Technology, MA, USA), phospho-MAP Erk1/2 (Cell Signaling), cyclin-dependent kinase 5 (cdk5) (Santa Cruz Biotechnology, CA, USA), p35/p25 (Santa Cruz Biotechnology), GLUT1 (Novus Biologicals, CO, USA) and actin (Sigma). To extract membrane proteins (BACE1 and GLUT1), cells were washed three times with ice-cold Tris-buffered saline (25 mM Tris, pH 8.0, 140 mM NaCl and 5 mM KCl) and lysed for 20 min by scraping into ice-cold radioimmunoprecipitation assay buffer [1% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) deoxycholate, 50 mM Tris, pH 7.2, 150 mM NaCl, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride, all chemicals from Sigma] (Wen *et al.*, 2004). To extract all other proteins, radioimmunoprecipitation assay buffer containing 1% (v/v) Triton, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) deoxycholate, 20 mM Tris, pH 7.4, 150 mM NaCl, 100 mM NaF, 1 mM Na_3VO_4 , 1 mM EDTA, 1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (all chemicals from Sigma) was used (Williamson *et al.*, 2002). The total cell lysate was obtained by centrifugation at 13 000 g for 15 min at 4 °C. The total protein concentration was measured by a BCA protein assay kit (Pierce Biotechnology). Equal amounts of total protein from each condition were run at 200 V on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Bio-Rad, CA, USA).

The separated proteins were transferred to nitrocellulose membranes for 1 h at 100 V and incubated at 4 °C overnight with the appropriate primary antibodies (1 : 1000 BACE1, 1 : 200 AT8, 1 : 200 PHF-1, 1 : 2000 Tau-1, 1 : 1000 GSK-3 α / β , 1 : 1000 phospho-GSK-3 α / β , 1 : 1000 MAP Erk1/2, 1 : 1000 phospho-MAP Erk1/2, 1 : 1000 cdk5, 1 : 1000 p35/p25, 1 : 500 GLUT1 and 1 : 2000 actin). Blots were washed three times in phosphate-buffered saline-Tween and incubated with appropriate horseradish peroxidase-linked secondary antibodies (Pierce Biotechnology) diluted in phosphate-buffered saline-Tween for 1 h at room temperature (25°C). After washing three times in phosphate-buffered saline-Tween, blots were developed with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and imaged with ChemiDoc (Bio-Rad). QUANTITY ONE software (Bio-Rad) was used to quantify the signal intensity of the protein bands.

ELISA measurements of amyloid beta 40 and amyloid beta 42

For A β measurements, the media and neuronal cells were collected after 24 h of treatment. The media were treated with protease inhibitor cocktail (Sigma) and cleared by brief centrifugation (1500 g, 5 min, 4 °C). A β 40 and A β 42 in the media were measured by using colorimetric sandwich ELISA according to the manufacturer's instructions (Wako Chemicals, VA, USA). The cells were lysed and the intracellular protein was measured by using the BCA protein assay kit (Pierce Biotechnology), which was used to normalize the A β 40 and A β 42 values.

Measurement of glucose uptake and lactate production

For measurement of glucose uptake and lactate production, the conditioned media were collected immediately after experimental treatment and centrifuged for 10 min at 1500 g to remove any cell debris. The glucose and lactate concentrations in the media were measured by using enzymatic glucose (Stanbio Laboratories, TX, USA) and lactate (Trinity Biotech, MO, USA) assays. The glucose uptake and lactate production were calculated by using the differences between the metabolite concentrations in the media before and after the treatment. The data were normalized by using total intracellular protein levels.

Measurement of intracellular ATP in astroglia

To measure the intracellular ATP levels, astroglia were washed with ice-cold phosphate-buffered saline and then lysed with 0.7% perchloric acid. The perchloric acid was neutralized by using 0.7 N NaOH. The neutralized samples were used to measure intracellular ATP levels with a luciferase-based chemiluminescent assay (Molecular Probes).

Data analyses

Data are shown as means $\pm$ SD for the indicated number of experiments. Student's *t*-test and one-way ANOVA with Tukey's post-hoc method were used to evaluate statistical significances between different treatment groups. Statistical significance was set at $P < 0.05$.

Results

Palmitic acid metabolism by astroglia and increased de-novo synthesis of ceramide

Previously, we showed that saturated FFAs, PA and stearic acid, had no direct deleterious effects on primary rat cortical neurons; however, these FFAs, through their metabolism by primary rat cortical astroglia, exerted AD-like pathological effects (increased amyloidogenesis and tau hyperphosphorylation) in cortical neurons (Patil & Chan, 2005; Patil *et al.*, 2006). This led us to further investigate the FFA metabolism by cortical astroglia. In the present study, we treated cortical astroglia with 0.2 mM PA for 24 h and measured the cellular levels of ceramide with high-performance liquid chromatography. We found that the cellular level of ceramide was significantly increased in PA-treated astroglia as compared with untreated astroglia ($74 \pm 10\%$, $P < 0.05$, $n = 3$, Fig. 1). The PA treatment of astroglia and consequent increase in ceramide levels did not cause cellular stress in astroglia (Fig. 2) or affect the astroglial cell viability compared with controls as measured by percentage LDH release (Fig. 3). Treatment with 300 μM H_2O_2 was used as a positive control and significantly increased LDH release from astroglia ($45 \pm 1\%$, $P < 0.05$, $n = 3$, Fig. 3). This is supported by a previous study (Gorina *et al.*, 2005). The PA-induced increase in the astroglial ceramide synthesis was completely blocked by incubating the astroglia with 2 mM L-cycloserine (L-CS) (Fig. 1). L-CS inhibits serine palmitoyltransferase, an enzyme that catalyses the first committed step of *de-novo* synthesis of ceramide.

Involvement of astroglial ceramide in palmitic acid-induced amyloidogenesis and tau hyperphosphorylation in neurons

Here, we treated cortical astroglia with 0.2 mM PA for 24 h and then used the astroglia-conditioned media to treat cortical neurons for 24 h. After 24 h of treatment, the media were collected for measurement of secreted $\text{A}\beta$ levels and the neurons were washed, lysed and the total cellular protein was used for western blot analysis of the different proteins. As shown in Fig. 4, the treatment of neurons with the conditioned media from PA-treated astroglia significantly increased BACE1 levels ($62 \pm 7\%$, $P < 0.05$, $n = 3$). BACE1 is a rate-limiting enzyme in the amyloidogenic processing of amyloid precursor protein leading to the formation of C-terminal fragments of amyloid precursor protein (C99), which are further processed by γ -secretase to form $\text{A}\beta$; a slight increase in BACE1 levels leads to a dramatic increase in the production of $\text{A}\beta_{40/42}$ (Li *et al.*, 2006). In this context, we found that the PA-astroglia-treated neurons significantly increased secretion of $\text{A}\beta_{40}$ ($178 \pm 13\%$, $P < 0.05$, $n = 3$) and $\text{A}\beta_{42}$ ($208 \pm 25\%$, $P < 0.05$, $n = 3$) as compared with controls (Fig. 4). In addition, the phosphorylation of tau was significantly increased in neurons treated with the conditioned media from PA-treated astroglia as observed by immunoblotting with two different antibodies, PHF-1 ($28 \pm 4\%$, $P < 0.05$, $n = 3$, Fig. 5) and AT8 ($47 \pm 2\%$, $P < 0.05$, $n = 3$, Fig. 5). The PHF-1 and AT8 antibodies recognize tau protein hyperphosphorylated at AD-specific phosphoepitopes; PHF-1 recognizes tau phosphorylated at Ser396 and Ser404 (Otvos *et al.*, 1994), whereas AT8 is specific for tau phosphorylated at Ser202 and Thr205 (Goedert *et al.*, 1995). Ser202, Ser396 and Ser404 are three of the nine abnormal tau phosphorylation sites associated with neurofibrillary tangles in AD (Gong *et al.*, 1994). In addition to PHF-1 and AT8 antibodies, we also carried out immunoblotting with Tau-1 antibody, which detects all of the isoforms of dephosphorylated tau, thus acting as a

negative control. As expected, Tau-1 was significantly decreased in PA-astroglia-treated neurons as compared with control-astroglia-treated neurons (Fig. 5). As PA treatment was found to elevate *de-novo* synthesis of ceramide in astroglia, we wanted to investigate a possible involvement of astroglial ceramide in the observed PA-astroglia-induced amyloidogenesis and tau hyperphosphorylation in neurons. For this, we treated astroglia with 2 mM L-CS together with 0.2 mM PA for 24 h and then transferred the astroglia-conditioned medium to the neurons (24 h treatment). The inhibition of astroglial ceramide synthesis by L-CS treatment blocked the PA-astroglia-induced BACE1 up-regulation, A β production and hyperphosphorylation of tau (Figs 4 and 5). To eliminate the possibility that the observed inhibitory effects of L-CS may be a direct effect of L-CS in the astroglia-conditioned media on neurons, in a separate experiment we added 2 mM L-CS to the conditioned media from astroglia just before transferring to neurons. We found that this treatment of neurons (but not the astroglia) with L-CS did not inhibit the PA-astroglia-induced pathophysiological changes observed in neurons (data not shown).

We further studied the possible activation of various AD-related kinases (GSK-3 α/β , cdk5 and MAPK Erk1/2) (Gomez-Ramos *et al.*, 2003), one or more of which may be responsible for the observed PA-astroglia-induced tau hyperphosphorylation, and potentially activated by ceramide. An increase in the phosphorylation of these enzymes was used as an indicator of their activation. As shown in Fig. 6A, levels of phosphorylated GSK-3 α/β (Tyr279/Tyr216) were significantly increased in PA-astroglia-treated neurons as compared with controls, without any significant change in the levels of total GSK-3 α/β . GSK-3 α phosphorylated at Tyr279 and GSK3- β phosphorylated at Tyr216 suggest increased activity (Emamian *et al.*, 2004). In addition to activating GSK-3 α/β , PA treatment also increased the cleavage of cdk5 activator p35 to p25 (Fig. 6B). p25 accumulates in the brains of AD patients and the conversion of p35 to p25 suggests augmented activity of cdk5 (de la Monte *et al.*, 2000; Lee *et al.*, 2000). Finally, there was no change in the levels of total and phosphorylated MAPK Erk1/2 in the PA-astroglia-treated neurons as compared with control-astroglia-treated neurons (Fig. 6C), suggesting no change in its activity. The treatment of astroglia with 2 mM L-CS inhibited both the PA-astroglia-induced increase in the level of phosphorylated GSK-3 α/β and the cleavage of p35 to p25 (Fig. 6). To further evaluate the possible involvement of these enzymes in the PA-astroglia-induced tau hyperphosphorylation in neurons, we treated neurons with the pharmacological inhibitors of these enzymes, 10 mM LiCl₂ (for GSK-3 α/β), 10 μ M roscovitine (for cdk5) and 30 μ M PD98059 (for MAPK Erk1/2). The treatment with LiCl₂, but not roscovitine and PD98059, inhibited the PA-astroglia-induced hyperphosphorylation of tau in neurons (Fig. 7).

Palmitic acid-induced abnormal glucose metabolism in astroglia

In addition to the observed involvement of PA in causing AD-like pathophysiological changes described above, we wanted to study its potential effect on glucose metabolism, which is severely compromised in AD. To study the effects of PA on cellular glucose metabolism, neurons and astroglia were treated with 0.2 mM of PA for 24 h. As shown in Fig. 8A, there was no change in the glucose uptake and lactate production in neurons treated with PA as compared with the untreated neurons. In the astroglia, however, PA treatment significantly decreased basal glucose uptake and lactate release (Fig. 8B). Interestingly, the

level of astroglial glucose transporter (GLUT1) was found to be significantly down-regulated in PA-treated astroglia as compared with the untreated astroglia ($51 \pm 2\%$, $P < 0.05$, $n = 3$, Fig. 9). To further investigate the perturbed astroglial metabolism due to PA treatment, we measured cellular ATP production in astroglia. We expected PA treatment to decrease astroglial ATP production in the light of our observation that PA decreases glucose uptake. However, we found that there was an increase in ATP production in the PA-treated astroglia as compared with the untreated astroglia (Fig. 10). The increased ATP production in PA-treated astroglia may be due to the uptake and increased oxidation of PA by astroglia. In support of this, it has previously been shown that, although PA inhibits glucose oxidation, PA oxidation itself makes up for this and thereby prevents ATP loss, which otherwise would be expected due to the reduced glucose oxidation (Smith *et al.*, 1999; King *et al.*, 2001). Furthermore, it has been shown that, although glucose utilization is reduced in AD brain, oxygen consumption and CO₂ production are unchanged or even increased as compared with healthy controls (Fukuyama *et al.*, 1994; Hoyer, 1998). Thus, it has been hypothesized that substrates other than glucose (e.g. FFAs and endogenous amino acids) may be oxidized in AD brain (Hoyer, 1991), which may partially compensate for the energy deficit observed in AD brain due to decreased glucose metabolism (Pettegrew *et al.*, 1995).

Discussion

In the present study, we investigated the underlying mechanism by which saturated fatty acids, through their uptake and metabolism by astroglia, lead to AD-like pathophysiological and metabolic changes. We found that saturated fatty acid (PA) induced *de-novo* synthesis of ceramide in primary astroglia. These data are in agreement with a previous report that also demonstrated significant uptake of PA by astroglia followed by transient increase in *de-novo* ceramide synthesis (Blazquez *et al.*, 2000).

Increased ceramide levels have been suggested to be central to AD pathology; ceramide levels were found to be elevated in AD brain as compared with brains of healthy controls and the levels were higher in the affected regions (cortex and hippocampus) as compared with the unaffected regions (cerebellum) of the AD brain (Han *et al.*, 2002; Cutler *et al.*, 2004). At the subcellular level, immunohistochemical analysis of AD brain showed abnormal expression of ceramides in the astroglia (Satoi *et al.*, 2005). Furthermore, the exogenous addition of ceramide to neurons has been shown to induce A β production (Puglielli *et al.*, 2003). Our findings reported here, however, are the first to demonstrate a direct causal role of astroglial PA metabolism and endogenously synthesized ceramide in astroglia, in causing A β production and tau hyperphosphorylation in neurons, two characteristic signatures of AD pathology. The PA-induced, *de-novo*-synthesized ceramide was also found to be involved in activating two of the oxidative stress-dependent kinases (cdk5 and GSK-3) as shown by increased cleavage of p35 to p25 and increased levels of phosphorylated GSK-3, respectively. Previously, we showed that conditioned media from FFA-treated astroglia caused oxidative stress in neurons (Patil & Chan, 2005). The increased oxidative stress may increase intracellular Ca²⁺ accumulation in neurons (Numakawa *et al.*, 2007), thus activating Ca²⁺-dependent cysteine protease (calpain), which induces cleavage of p35 to p25 (Lee *et al.*, 2000). p25 has been shown to be accumulated in the brains of patients with AD and its increased level has been associated with augmented activity of

cdk5, which in turn may lead to the hyperphosphorylation of tau. However, in the present study, cotreatment of neurons with a potent cdk5 inhibitor, roscovitine, did not inhibit the observed PA-astroglia-induced hyperphosphorylation of tau, thus suggesting that PA-astroglia-induced hyperphosphorylation of tau in primary rat cortical neurons is independent of the observed activation of the cdk5 pathway. Our results agree with a previous report that showed that, in primary rat hippocampal neurons, cleavage of p35 to p25 and subsequent activation of cdk5 were not involved in the hyperphosphorylation of tau (Kerokoski *et al.*, 2002). However, through the use of the pharmacological GSK-3 inhibitor LiCl₂, we found that GSK-3 played a role in the PA-astroglia-induced tau hyperphosphorylation observed in neurons. GSK-3 was previously shown to be involved in causing both tau hyperphosphorylation and A β production, and is thus central and essential in the development of AD; GSK-3 β is involved in hyperphosphorylation of tau and its other isoform GSK-3 α is involved in A β production by regulating the activity of γ -secretase (Phiel *et al.*, 2003; Takashima, 2006). These studies, together with our present data, further emphasize the central role of PA-induced abnormal astroglial ceramide metabolism in causing AD-like pathophysiological characteristics.

In addition to the aforementioned pathophysiological changes, AD is also characterized by abnormal metabolic changes. In the patients who are genetically pre-disposed to AD, cerebral metabolic changes occur well before any pathophysiological manifestation of the disease (Gibson, 2002). Among them, decreased cerebral glucose metabolism is a distinct characteristic of AD brain (Mosconi, 2005; Ogawa *et al.*, 2005). In AD brain, glucose utilization and ATP formation are decreased significantly (approximately 46 and 19%, respectively) as compared with healthy controls (Hoyer, 1991). The decreased glucose metabolism and energy production may play a central role in causing A β production and tau hyperphosphorylation (Liu *et al.*, 2004; Planel *et al.*, 2004; Hoyer *et al.*, 2005; Velliquette *et al.*, 2005; Gong *et al.*, 2006). At the subcellular level, although neurons are traditionally considered to be primary users of glucose in the brain (Chih *et al.*, 2001), more recent studies suggest a central role of astroglia in the cerebral glucose utilization, in which case astroglia take up glucose and produce lactate, the latter supplied to neurons as a fuel for their metabolic activities and energy production (Pellerin & Magistretti, 1994; Chih *et al.*, 2001; Kasischke *et al.*, 2004). Thus, the decreased glucose metabolism observed in AD brain may suggest decreased glucose uptake and metabolism of glucose by the astroglia. In this regard, we found that PA treatment significantly decreased glucose uptake and lactate release by astroglia. The observed down-regulation in glucose uptake by astroglia in the presence of PA may be attributed to the PA-induced down-regulation of astroglial glucose transporter (GLUT1) levels. Interestingly, AD brain is characterized by significant reductions in GLUT1 levels (Simpson *et al.*, 1994) and the disease severity is associated with a progressive decline in GLUT1 gene expression (Mandelin, 2000). Thus, together these data may suggest a central role of saturated FFAs in causing abnormal glucose metabolism in addition to the AD-like pathophysiological changes discussed earlier.

Alzheimer's disease is a peculiar neurodegenerative disease in that the observed pathophysiological and metabolic damage is found to be region-specific and cell type-specific; basal forebrain, cortex and hippocampus are affected, whereas cerebellum is

relatively spared and the cholinergic neurons are the type of neurons most affected (Beffert *et al.*, 1999; Szutowicz *et al.*, 2006). Here, it is interesting to note that different brain regions show differential FFA metabolic activities; the activity of fatty acyl-CoA synthetase is 10× lower in cerebellum as compared with the other affected brain regions (Szutowicz & Lysiak, 1980). Fatty acyl-CoA synthetase is the first enzyme involved in cellular FFA metabolism, which converts fatty acid to fatty acyl-CoA, which is then utilized by the cells in catabolic (e.g. β -oxidation) and anabolic (e.g. ceramide synthesis) pathways (Pei *et al.*, 2003). These studies, together with our present data, may suggest that, under pathologically elevated levels of saturated FFAs, cerebellum may be less likely to be affected by abnormal FFA metabolism due to its lower activity level of fatty acyl-CoA synthetase as compared with the other (affected) brain regions and thus may explain, in part, the region-specific damage observed in AD brain. Furthermore, we previously showed that FFA metabolism by astroglia results in increased ROS production in neurons (Patil & Chan, 2005; Patil *et al.*, 2006). The elevated ROS, in turn, were found to be involved in causing BACE1 up-regulation and tau hyperphosphorylation in neurons (Patil & Chan, 2005; Patil *et al.*, 2006), suggesting a central role of oxidative stress in FFA-induced damage. Thus, elevated FFA metabolism associated with higher fatty acyl-CoA synthetase activity in the affected regions (basal forebrain, cortex and hippocampus) may lead to increased oxidative stress in these regions compared with unaffected regions (cerebellum). In this context, it is interesting to note that, in AD brains, oxidative stress markers are higher in the affected regions than in the unaffected regions (Sultana *et al.*, 2006a,b). The increased oxidative stress may be damaging, particularly to the basal forebrain cholinergic neurons as these neurons have been shown to lack an important antioxidative enzyme, seleno-glutathione peroxidase (Trepanier *et al.*, 1996). This may account, in part, for the cell type-specific damage observed in AD pathology; a substantial loss of basal forebrain cholinergic neurons is a distinct feature of AD (Wu *et al.*, 2005). Furthermore, these basal forebrain cholinergic neurons, through their long ascending projections, innervate cortical and hippocampal regions (McKinney *et al.*, 1983) and the increased oxidative stress in these regions of AD brain may lead to the degeneration of these projections from the cholinergic neurons. In this context, it is also interesting to note that A β plaques, hallmarks of AD, have been shown to be specifically located where the projection of the basal forebrain cholinergic neurons degenerates (Arendt *et al.*, 1985; Ransmayr *et al.*, 1989).

Based on our findings, we propose the following sequence of events by which saturated FFAs may play a central role in the pathogenesis of AD (Fig. 11). Various risk factors associated with AD, e.g. high-fat diet, diabetes, brain trauma, etc., may account for the elevated levels of saturated FFAs in AD brain. The saturated FFAs are taken up and metabolized by astroglia, down-regulating astroglial GLUT1 levels and glucose metabolism. Furthermore, astroglial FFA metabolism results in the increased production of ROS in neurons. This may be due to the increased levels of ceramides in FFA-treated astroglia; ceramide may induce the secretion of cytokines (Lieb *et al.*, 1997) or other signaling molecules, e.g. nitric oxide (Pahan *et al.*, 1998), by astroglia, which may induce ROS production in neurons (Floyd *et al.*, 1999; Wei *et al.*, 2000). The increased oxidative stress in neurons causes BACE1 up-regulation and GSK-3 activation, which in turn results in the increased A β production and the hyperphosphorylation of tau, respectively.

In summary, we found that, under pathological levels of PA, its metabolism by astroglia is involved in causing the AD-like pathophysiological as well as metabolic changes. In particular, PA-induced *de-novo* synthesis of ceramide in astroglia was found to play a causal role in increasing A β production and hyperphosphorylating tau in neurons, two main pathophysiological changes associated with AD. It would be a significant step forward in AD research if saturated FFAs were shown to exert their risk for the development of AD through a similar mechanism *in vivo*. Thus, we plan to further investigate astroglial FFA metabolism *in vivo*, which may help in uncovering more clues that may be translated into potential targets for therapeutic intervention in AD.

Acknowledgments

We thank Dr P. Davies (Albert Einstein College of Medicine, New York, NY, USA) for kindly providing PHF-1 antibody. We also thank Deebika Balu for her help with the isolation of primary rat neuronal cells. This work was funded in part by the Michigan State University (MSU) Foundation, National Science Foundation (BES nos 0331297 and 0425821), National Institutes of Health (1R01GM079688-01), Environmental Protection Agency (RD83184701), Whitaker Foundation and Quantitative Biological Modelling Initiative (QBMI) at MSU.

Abbreviations

Aβ	amyloid beta
AD	Alzheimer's disease
cdk5	cyclin-dependent kinase 5
DA	diacetate
DCF	dichlorodihydrofluorescein
FFA	free fatty acid
GSK-3	glycogen synthase kinase 3
L-CS	L-cycloserine
LDH	lactate dehydrogenase
PA	palmitic acid
ROS	reactive oxygen species

References

- Arendt T, Bigl V, Tennstedt A, Arendt A. Neuronal loss in different parts of the nucleus basalis is related to neuritic plaque formation in cortical target areas in Alzheimer's disease. *Neuroscience*. 1985; 14:1–14. [PubMed: 3974875]
- Arvanitakis Z, Wilson RS, Bienias JL, Evans DA, Bennett DA. Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. *Arch Neurol*. 2004; 61:661–666. [PubMed: 15148141]
- Beffert U, Cohn JS, Petit-Turcotte C, Tremblay M, Aumont N, Ramassamy C, Davignon J, Poirier J. Apolipoprotein E and β -amyloid levels in the hippocampus and frontal cortex of Alzheimer's disease subjects are disease-related and apolipoprotein E genotype dependent. *Brain Res*. 1999; 843:87–94. [PubMed: 10528114]

- Blazquez C, Galve-Roperh I, Guzman M. De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. *FASEB J*. 2000; 14:2315–2322. [PubMed: 11053253]
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37:911–917. [PubMed: 13671378]
- Chandler LJ, Newsom H, Sumners C, Crews F. Chronic ethanol exposure potentiates NMDA excitotoxicity in cerebral cortical neurons. *J Neurochem*. 1993; 60:1578–1581. [PubMed: 8455043]
- Chang KA, Suh YH. Pathophysiological roles of amyloidogenic carboxy-terminal fragments of the β -amyloid precursor protein in Alzheimer's disease. *J Pharmacol Sci*. 2005; 97:461–471. [PubMed: 15821343]
- Chih CP, Lipton P, Roberts EL. Do active cerebral neurons really use lactate rather than glucose? *Trends Neurosci*. 2001; 24:573–578. [PubMed: 11576670]
- Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, Troncoso JC, Mattson MP. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci USA*. 2004; 101:2070–2075. [PubMed: 14970312]
- Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA. Convergent evidence for impaired AKT1-GSK3 β signaling in schizophrenia. *Nat Genet*. 2004; 36:131–137. [PubMed: 14745448]
- Floyd RA, Hensley K, Jaffery F, Maidt L, Robinson K, Pye Q, Stewart C. Increased oxidative stress brought on by pro-inflammatory cytokines in neurodegenerative processes and the protective role of nitron-based free radical traps. *Life Sci*. 1999; 65:1893–1899. [PubMed: 10576433]
- Fukuyama H, Ogawa M, Yamauchi H, Yamaguchi S, Kimura J, Yonekura Y, Konishi J. Altered cerebral energy metabolism in Alzheimer's disease: a PET study. *J Nucl Med*. 1994; 35:1–6. [PubMed: 8271029]
- Giasson BI, Ischiropoulos H, Lee VMY, Trojanowski JQ. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Rad Biol Med*. 2002; 32:1264–1275. [PubMed: 12057764]
- Gibson GE. Interactions of oxidative stress with cellular calcium dynamics and glucose metabolism in Alzheimer's disease. *Free Rad Biol Med*. 2002; 32:1061–1070. [PubMed: 12031890]
- Glenner GG. Alzheimer's disease: its proteins and genes. *Cell (Camb, MA, USA)*. 1988; 52:307–308.
- Goedert M, Jakes R, Vanmechelen E. Monoclonal antibody AT8 recognizes tau protein phosphorylated at both serine 202 and threonine 205. *Neurosci Lett*. 1995; 189:167–170. [PubMed: 7624036]
- Gomez-Ramos A, Diaz-Nido J, Smith MA, Perry G, Avila J. Effect of the lipid peroxidation product acrolein on tau phosphorylation in neural cells. *J Neurosci Res*. 2003; 71:863–870. [PubMed: 12605413]
- Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K. Alzheimer's disease abnormally phosphorylated τ is dephosphorylated by protein phosphatase-2B (calcineurin). *J Neurochem*. 1994; 62:803–806. [PubMed: 8294942]
- Gong CX, Liu F, Grundke-Iqbal I, Iqbal K. Impaired brain glucose metabolism leads to Alzheimer neurofibrillary degeneration through a decrease in tau O-GlcNAcylation. *J Alz Dis*. 2006; 9:1–12.
- Gorina R, Petegnief V, Chamorro A, Planas AM. AG490 prevents cell death after exposure of rat astrocytes to hydrogen peroxide or proinflammatory cytokines: involvement of the Jak2/STAT pathway. *J Neurochem*. 2005; 92:505–518. [PubMed: 15659221]
- Grant WB. Dietary links to Alzheimer's disease: 1999 update. *J Alz Dis*. 1999; 1:197–201.
- Guo Z, Cupples LA, Kurz A, Auerbach SH, Volicer L, Chui H, Green RC, Sadovnick AD, Duara R, DeCarli C, Johnson K, Go RC, Growdon JH, Haines JL, Kukull WA, Farrer LA. Head injury and the risk of AD in the MIRAGE study. *Neurology*. 2000; 54:1316–1323. [PubMed: 10746604]
- Han X, Holtzman DM, McKeel DW Jr, Kelley J, Morris JC. Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. *J Neurochem*. 2002; 82:809–818. [PubMed: 12358786]
- Homayoun P, De Turco EBR, Parkins NE, Lane DC, Soblosky J, Carey ME, Bazan NG. Delayed phospholipid degradation in rat brain after traumatic brain injury. *J Neurochem*. 1997; 69:199–205. [PubMed: 9202311]

- Hoyer S. Abnormalities of glucose metabolism in Alzheimer's disease. *Ann NY Acad Sci.* 1991; 640:53–58. [PubMed: 1776759]
- Hoyer S. Risk factors for Alzheimer's disease during aging. Impacts of glucose/energy metabolism. *J Neur Transm Suppl.* 1998; 54:187–194.
- Hoyer A, Bardenheuer HJ, Martin E, Plaschke K. Amyloid precursor protein (APP) and its derivatives change after cellular energy depletion. An in vitro-study. *J Neur Transm.* 2005; 112:239–253.
- Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science (Wash, DC, USA).* 2004; 305:99–103.
- Kerokoski P, Suuronen T, Salminen A, Soininen H, Pirttilä T. Cleavage of the cyclin-dependent kinase 5 activator p35 to p25 does not induce tau hyperphosphorylation. *Biochem Biophys Res Comm.* 2002; 298:693–698. [PubMed: 12419309]
- King LM, Sidell RJ, Wilding JR, Radda GK, Clarke K. Free fatty acids, but not ketone bodies, protect diabetic rat hearts during low-flow ischemia. *Am J Physiol Heart Circ Physiol.* 2001; 280:H1173–H1181. [PubMed: 11179061]
- Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature.* 2000; 405:360–364. [PubMed: 10830966]
- Levin-Allerhand JA, Lominska CE, Smith JD. Increased amyloid- β levels in APPSWE transgenic mice treated chronically with a physiological high-fat high-cholesterol diet. *The journal of nutrition Health Aging.* 2002; 6:315–319.
- Li Y, Zhou W, Tong Y, He G, Song W. Control of APP processing and A β generation level by BACE1 enzymatic activity and transcription. *FASEB J.* 2006; 20:285–292. [PubMed: 16449801]
- Lieb K, Fiebich BL, Hull M, Berger M, Bauer J. Potent inhibition of interleukin-6 expression in a human astrocytoma cell line by tenidap. *Cell Tissue Res.* 1997; 288:251–257. [PubMed: 9082960]
- Lipton P. Ischemic cell death in brain neurons. *Physiol Rev.* 1999; 79:1431–1568. [PubMed: 10508238]
- Liu F, Iqbal K, Grundke-Iqbal I, Hart GW, Gong CX. O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proc Natl Acad Sci USA.* 2004; 101:10 804–10 809.
- Mandelin AM. Alzheimer's disease severity is associated with a progressive decline in glut1 gene expression: role of posttranscriptional regulation. *Diss Abstr Int B.* 2000; 2002:62, 4951.
- Mattson MP. Pathways towards and away from Alzheimer's disease. *Nature.* 2004; 431:107.
- Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. β -Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci.* 1992; 12:376–389. [PubMed: 1346802]
- McKinney M, Coyle JT, Hedreen JC. Topographic analysis of the innervation of the rat neocortex and hippocampus by the basal forebrain cholinergic system. *J Comp Neurol.* 1983; 217:103–121. [PubMed: 6875049]
- Merrill AH Jr, Wang E, Mullins RE, Jamison WCL, Nimkar S, Liotta DC. Quantitation of free sphingosine in liver by high-performance liquid chromatography. *Anal Biochem.* 1988; 171:373–381. [PubMed: 3407935]
- de la Monte SM, Ganju N, Feroz N, Luong T, Banerjee K, Cannon J, Wands JR. Oxygen free radical injury is sufficient to cause some Alzheimer-type molecular abnormalities in human CNS neuronal cells. *J Alz Dis.* 2000; 2:261–281.
- Monti LD, Landoni C, Setola E, Galluccio E, Lucotti P, Sandoli EP, Origgi A, Lucignani G, Piatti P, Fazio F. Myocardial insulin resistance associated with chronic hypertriglyceridemia and increased FFA levels in Type 2 diabetic patients. *Am J Physiol.* 2004; 287:H1225–H1231.
- Mosconi L. Brain glucose metabolism in the early and specific diagnosis of Alzheimer's disease. *Eur J Nucl Med Mol Imag.* 2005; 32:486–510.
- Numakawa Y, Matsumoto T, Yokomaku D, Taguchi T, Niki E, Hatanaka H, Kunugi H, Numakawa T. 17 β -Estradiol protects cortical neurons against oxidative stress-induced cell death through reduction in the activity of mitogen-activated protein kinase and in the accumulation of intracellular calcium. *Endocrinology.* 2007; 148:627–637. [PubMed: 17082253]

- Ogawa M, Watabe H, Teramoto N, Miyake Y, Hayashi T, Iida H, Murata T, Magata Y. Understanding of cerebral energy metabolism by dynamic living brain slice imaging system with [18F]FDG. *Neurosci Res* (Amst, Netherlands). 2005; 52:357–361.
- Oksman M, Iivonen H, Högges E, Amtul Z, Penke B, Leenders I, Broersen L, Luetjohann D, Hartmann T, Tanila H. Impact of different saturated fatty acid, polyunsaturated fatty acid and cholesterol containing diets on beta-amyloid accumulation in APP/PS1 transgenic mice. *Neurobiol Dis*. 2006; 23:563–572. [PubMed: 16765602]
- Otvos L Jr, Feiner L, Lang E, Szendrei GI, Goedert M, Lee VMY. Monoclonal antibody PHF-1 recognizes Tau protein phosphorylated at serine residues 396 and 404. *J Neurosci Res*. 1994; 39:669–673. [PubMed: 7534834]
- Pahan K, Sheikh FG, Khan M, Namboodiri AMS, Singh I. Sphingomyelinase and ceramide stimulate the expression of inducible nitric-oxide synthase in rat primary astrocytes. *J Biol Chem*. 1998; 273:2591–2600. [PubMed: 9446561]
- Patil S, Chan C. Palmitic and stearic fatty acids induce Alzheimer-like hyperphosphorylation of tau in primary rat cortical neurons. *Neurosci Lett*. 2005; 384:288–293. [PubMed: 15939536]
- Patil S, Sheng L, Masserang A, Chan C. Palmitic acid-treated astrocytes induce BACE1 upregulation and accumulation of C-terminal fragment of APP in primary cortical neurons. *Neurosci Lett*. 2006; 406:55–59. [PubMed: 16904262]
- Pei Z, Oey NA, Zuidervart MM, Jia Z, Li Y, Steinberg SJ, Smith KD, Watkins PA. The acyl-CoA synthetase ‘bubblegum’ (lipidosin): further characterization and role in neuronal fatty acid β -oxidation. *J Biol Chem*. 2003; 278:47 070–47 078.
- Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci USA*. 1994; 91:10 625–10 629.
- Petot GJ, Traore F, Debanne SM, Lerner AJ, Smyth KA, Friedland RP. Interactions of apolipoprotein E genotype and dietary fat intake of healthy older persons during mid-adult life. *Metab Clin Exp*. 2003; 52:279–281. [PubMed: 12647263]
- Pettegrew JW, Klunk WE, Kanal E, Panchalingam K, McClure RJ. Changes in brain membrane phospholipid and high-energy phosphate metabolism precede dementia. *Neurobiol Aging*. 1995; 16:973–975. [PubMed: 8622789]
- Phiel CJ, Wilson CA, Lee VMY, Klein PS. GSK-3 α regulates production of Alzheimer’s disease amyloid- β peptides. *Nature* (Lond, UK). 2003; 423:435–439. [PubMed: 12761548]
- Planel E, Miyasaka T, Launey T, Chui DH, Tanemura K, Sato S, Murayama O, Ishiguro K, Tatebayashi Y, Takashima A. Alterations in glucose metabolism induce hypothermia leading to tau hyperphosphorylation through differential inhibition of kinase and phosphatase activities: implications for Alzheimer’s disease. *J Neurosci*. 2004; 24:2401–2411. [PubMed: 15014115]
- Pshenichkin SP, Wise BC. Okadaic acid increases nerve growth factor secretion, mRNA stability, and gene transcription in primary cultures of cortical astrocytes. *J Biol Chem*. 1995; 270:5994–5999. [PubMed: 7890729]
- Puglielli L, Ellis BC, Saunders AJ, Kovacs DM. Ceramide stabilizes β -site amyloid precursor protein-cleaving enzyme 1 and promotes amyloid β -peptide biogenesis. *J Biol Chem*. 2003; 278:19 777–19 783.
- Ransmayr G, Cervera P, Hirsch E, Ruberg M, Hersch LB, Duyckaerts C, Hauw JJ, Delumeau C, Agid Y. Choline acetyltransferase-like immunoreactivity in the hippocampal formation of control subjects and patients with Alzheimer’s disease. *Neuroscience*. 1989; 32:701–714. [PubMed: 2601840]
- Satoi H, Tomimoto H, Ohtani R, Kitano T, Kondo T, Watanabe M, Oka N, Akiguchi I, Furuya S, Hirabayashi Y, Okazaki T. Astroglial expression of ceramide in Alzheimer’s disease brains: a role during neuronal apoptosis. *Neuroscience* (Oxf, UK). 2005; 130:657–666.
- Sauer H, Klimm B, Hescheler J, Wartenberg M. Activation of p90RSK and growth stimulation of multicellular tumor spheroids are dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *FASEB J*. 2001; 15:2539–2541. [PubMed: 11641267]
- Scarmeas N, Stern Y, Tang MX, Mayeux R, Luchsinger JA. Mediterranean diet and risk for Alzheimer’s disease. *Ann Neurol*. 2006; 59:912–921. [PubMed: 16622828]

- Shie FS, LeBouef RC, Jin LW. Early intraneuronal A β deposition in the hippocampus of APP transgenic mice. *Neuroreport*. 2003; 14:123–129. [PubMed: 12544843]
- Simpson IA, Chundu KR, Davies-Hill T, Honer WG, Davies P. Decreased concentrations of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. *Ann Neurol*. 1994; 35:546–551. [PubMed: 8179300]
- Skoog I, Lernfelt B, Landahl S, Palmertz B, Andreasson LA, Nilsson L, Persson G, Oden A, Svanborg A. 15-year longitudinal study of blood pressure and dementia. *Lancet*. 1996; 347:1141–1145. [PubMed: 8609748]
- Smith MA. Alzheimer disease. *Int Rev Neurobiol*. 1998; 42:1–54. [PubMed: 9476170]
- Smith JM, Solar SM, Paulson DJ, Hill NM, Broderick TL. Effect of palmitate on carbohydrate utilization and Na/K-ATPase activity in aortic vascular smooth muscle from diabetic rats. *Mol Cell Biochem*. 1999; 194:125–132. [PubMed: 10391132]
- Solfrizzi V, D'Introno A, Colacicco AM, Capurso C, Del Parigi A, Capurso S, Gadaleta A, Capurso A, Panza F. Dietary fatty acids intake: possible role in cognitive decline and dementia. *Exp Gerontol*. 2005; 40:257–270. [PubMed: 15820606]
- Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, Merchant M, Markesbery WR, Butterfield DA. Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: an approach to understand pathological and biochemical alterations in AD. *Neurobiol Aging*. 2006a; 27:1564–1576. [PubMed: 16271804]
- Sultana R, Poon HF, Cai J, Pierce WM, Merchant M, Klein JB, Markesbery WR, Butterfield DA. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. *Neurobiol Dis*. 2006b; 22:76–87. [PubMed: 16378731]
- Szutowicz A, Lysiak W. Regional and subcellular distribution of ATP-citrate lyase and other enzymes of acetyl-CoA metabolism in rat brain. *J Neurochem*. 1980; 35:775–785. [PubMed: 6109001]
- Szutowicz A, Bielarczyk H, Gul S, Ronowska A, Pawelczyk T, Jankowska-Kulawy A. Phenotype-dependent susceptibility of cho-linergic neuroblastoma cells to neurotoxic inputs. *Metab Brain Dis*. 2006; 21:149–161. [PubMed: 16724269]
- Takashima A. GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J Alz Dis*. 2006; 9:309–317.
- Trepanier G, Furling D, Puymirat J, Mirault ME. Immunocytochemical localization of seleno-glutathione peroxidase in the adult mouse brain. *Neuroscience (Oxf)*. 1996; 75:231–243.
- Velliquette RA, O'Connor T, Vassar R. Energy inhibition elevates β -secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: Possible early events in Alzheimer's disease pathogenesis. *J Neurosci*. 2005; 25:10 874–10 883. [PubMed: 15634762]
- Wei T, Chen C, Hou J, Xin W, Mori A. Nitric oxide induces oxidative stress and apoptosis in neuronal cells. *Biochim Biophys Acta Mol Cell Res*. 2000; 1498:72–79.
- Wen Y, Onyewuchi O, Yang S, Liu R, Simpkins JW. Increased beta-secretase activity and expression in rats following transient cerebral ischemia. *Brain Res*. 2004; 1009:1–8. [PubMed: 15120577]
- Whitmer RA, Gunderson EP, Barrett-Connor E, Quesenberry CP Jr, Yaffe K. Obesity in middle age and future risk of dementia: a 27 year longitudinal population based study. *BMJ (Clin Res Ed)*. 2005; 330:1360.
- Williamson R, Scales T, Clark BR, Gibb G, Reynolds CH, Kellie S, Bird LN, Varndell LM, Sheppard PW, Everall I, Anderton BH. Rapid tyrosine phosphorylation of neuronal proteins including tau and focal adhesion kinase in response to amyloid- β peptide exposure: involvement of src family protein kinases. *J Neurosci*. 2002; 22:10–20. [PubMed: 11756483]
- Wu CK, Thal L, Pizzo D, Hansen L, Masliah E, Geula C. Apoptotic signals within the basal forebrain cholinergic neurons in Alzheimer's disease. *Exp Neurol*. 2005; 195:484–496. [PubMed: 16085017]
- Yankner BA, Duffy LK, Kirschner DA. Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science*. 1990; 250:279–282. [PubMed: 2218531]

Fig. 1.

Palmitic acid (PA)-induced, *de-novo* synthesis of ceramide in astroglia. Astroglia were treated for 24 h with 0.2 mM PA or 4% bovine serum albumin [control (C)], after which cellular lipids were extracted for ceramide determination by high-performance liquid chromatography. PA significantly increased ceramide synthesis in astroglia, which was completely inhibited by treatment of astroglia with 2 mM L-cycloserine (L-CS), an inhibitor of *de-novo* synthesis of ceramide. Data are taken from three different experiments and are expressed as mean $\pm$ SD. One-way ANOVA with Tukey's post-hoc method was used for analysing the differences between treatment groups. * $P < 0.05$ compared with control; # $P < 0.05$ compared with PA treatment.

Fig. 2.

Immunostaining of intracellular reactive oxygen species (ROS) in astroglia. Astroglia were treated for 24 h with 0.2 mM of palmitic acid (PA) or 4% bovine serum albumin (control) and then stained with 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate for intracellular ROS detection and examined with confocal fluorescence microscopy (Zeiss LSM, 5 Pa). Objective lens magnification, 40 $\times$.

Fig. 3.

Measurement of lactate dehydrogenase (LDH) release from astroglia treated with palmitic acid (PA). Treatment for 24 h with 0.2 mM PA failed to liberate LDH from astroglia as compared with controls. In contrast, 1 h treatment of astroglia with 300 mM H₂O₂ (positive control) induced significant LDH liberation after 24 h as compared with both control and PA-treated cells. Data are taken from three different experiments and are expressed as mean $\pm$ SD. One-way ANOVA with Tukey's post-hoc method was used for analysing the differences between treatment groups. * $P < 0.05$ compared with control.

Fig. 4.

Involvement of astroglial ceramide in palmitic acid (PA)-astroglia-induced BACE1 up-regulation and amyloid beta ($A\beta$) protein production in neurons. Astroglia were treated with 0.2 mM of PA or 4% bovine serum albumin [control (C)] for 24 h, followed by transfer of the astroglia-conditioned media to neurons (24 h treatment). (A) Immunoblot shows increased level of BACE1 in neurons treated with PA-astroglia-conditioned media compared with controls and the abnormal protein elevation was inhibited by treatment of astroglia with 2 mM L-cycloserine (L-CS). Histogram corresponding to BACE1 blot represents quantitative determinations of intensities of the relevant bands normalized to actin. (B) PA-astroglia-treated neurons show increased production of $A\beta$ 40 and $A\beta$ 42 as compared with controls, which was inhibited by treatment of astroglia with 2 mM L-CS. Data represent mean $\pm$ SD of three independent experiments. One-way ANOVA with Tukey's post-hoc method was used for analysing the differences between treatment groups. * $P < 0.05$ compared with control; # $P < 0.05$ compared with PA treatment.

Fig. 5.

Involvement of astroglial ceramide in palmitic acid (PA)-astroglia-induced tau hyperphosphorylation in neurons. In neurons treated with conditioned media from PA-treated astroglia, tau was found pathologically hyperphosphorylated as shown by immunoblotting with PHF-1 and AT8 antibodies. Tau-1 detects dephosphorylated tau, thus showing decreased levels in PA-astroglia-treated neurons. These PA-astroglia-induced tau abnormalities were blocked by inhibiting astroglial ceramide synthesis with 2 mM L-cycloserine (L-CS). Histograms corresponding to PHF-1 and AT8 blots represent quantitative determinations of intensities of the relevant bands normalized with actin. Data represent mean $\pm$ SD of three independent experiments. One-way ANOVA with Tukey's post-hoc method was used for analysing the differences between treatment groups. * $P < 0.05$ compared with control (C); # $P < 0.05$ compared with PA treatment.

Fig. 6.

Palmitic acid (PA)-astroglia-induced activation of Alzheimer's disease-specific kinases in neurons is mediated by astroglial ceramide. Conditioned media from PA-treated astroglia activated (A) glycogen synthase kinase 3 (GSK-3) [increased levels of phosphorylated GSK-3 (P-GSK-3)] and (B) cyclin-dependent kinase 5 (cdk5) (increased cleavage of p35 to p25) but not (C) MAP Erk1/2 [no change in the levels of phosphorylated MAP Erk1/2 (P-MAP Erk1/2)]. The treatment of astroglia with 2 mM L-cycloserine (L-CS) inhibited PA-astroglia-induced activation of both GSK-3 and cdk5. Data are representative of three different experiments. C, control.

Fig. 7.

Glycogen synthase kinase 3 (GSK-3) is involved in palmitic acid (PA)-astroglia-induced tau hyperphosphorylation in neurons. Astroglia-conditioned media were transferred to neurons, with or without various kinase inhibitors, i.e. 10 mM LiCl₂ (GSK-3 inhibitor), 10 mM roscovitine (cyclin-dependent kinase 5 inhibitor) and 30 mM PD98059 (mitogen-activated protein kinase inhibitor). Immunoblot analysis with PHF-1 and AT8 antibodies shows that only LiCl₂ inhibited the observed PA-induced tau hyperphosphorylation in neurons. Histogram data represent mean $\pm$ SD of three independent experiments. One-way ANOVA and Tukey's post-hoc method was used for analysing the differences between treatment groups. * P < 0.05 compared with control; # P < 0.05 compared with PA treatment. C, control.

Fig. 8.

Palmitic acid (PA) down-regulates glucose uptake and lactate release by astroglia. The cortical neurons and astroglia were treated for 24 h with 0.2 mM of PA or 4% bovine serum albumin. (A) In neurons, PA treatment did not change glucose uptake and lactate production. (B) PA treatment significantly decreased glucose uptake and lactate production by astroglia. Data represent mean $\pm$ SD of six experiments. Student's *t*-test was used for analysing the differences between the two treatment groups. * $P < 0.05$ compared with respective control. C, control.

Fig. 9.

Palmitic acid (PA) down-regulates GLUT1 level in astroglia. Astroglia were treated for 24 h with 0.2 mM of PA or 4% bovine serum albumin. The immunoblot analysis shows that PA treatment significantly decreased the levels of GLUT1 as compared with the untreated astroglia. β -actin is shown as a marker for protein loading. The histogram represents quantitative determinations of intensities of the relative bands normalized with actin. Data represent mean $\pm$ SD of three independent experiments. Student's *t*-test was used for analysing the differences between the two treatment groups. * $P < 0.05$ compared with respective control (C).

Fig. 10.

Measurement of intracellular ATP in astroglia. The cortical astroglia were treated for 24 h with 0.2 mM of palmitic acid (PA) or 4% bovine serum albumin. PA treatment increased cellular ATP production in astroglia. Data represent mean $\pm$ SD of three experiments. Student's *t*-test was used for analysing the differences between the two treatment groups. **P* < 0.05 compared with respective control (C).

Fig. 11.

Proposed cellular mechanism by which astroglial free fatty acid (FFA) metabolism leads to Alzheimer's disease (AD)-like physiological and metabolic changes. The elevated levels of saturated FFAs associated with various risk factors for AD down-regulate glucose metabolism in astroglia (decrease in GLUT1 levels, glucose uptake and lactate production in astroglia). Furthermore, FFA-treated astroglia secrete factor(s) that induce reactive oxygen species (ROS) production in neurons. The increased ROS production leads to up-regulation of BACE1 levels and glycogen synthase kinase 3 (GSK-3) activity, which in turn cause amyloidogenic processing of amyloid precursor protein (APP) and hyperphosphorylation of tau, respectively. The FFA-induced ceramide synthesis in astroglia plays a central role in causing the above-mentioned AD-like pathophysiological changes in neurons.