This is to certify that the
thesis entitled

REGIONAL DISPARITIES IN EXPRESSING ALZHEIMER'S DISEASE NEUROPATHOLOGY – AN INFERENTIAL STUDY

presented by

DEEBIKA BALU

has been accepted towards fulfillment
of the requirements for the

MASTER OF SCIENCE degree in CHEMICAL ENGINEERING AND MATERIAL SCIENCE

Christine Chan
Major Professor's Signature

11/26/07
Date

REGIONAL DISPARITIES IN EXPRESSING ALZHEIMER'S DISEASE NEUROPATHOLOGY – AN INFERENTIAL STUDY

By

DEEBIKA BALU

A THESIS

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in partial fulfillment of the requirements
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ABSTRACT

REGIONAL DISPARITIES IN EXPRESSING ALZHEIMER'S DISEASE NEUROPATHOLOGY – AN INFERENTIAL STUDY

By

DEEBIKA BALU

Alzheimer's disease (AD) is a progressive, neuro-degenerative disease triggered by multiple factors. Modification of two major neuronal proteins, Amyloid Beta and Tau, contributes to formation of senile plaques and neuro-fibrillary tangles in the brain. Such pathological changes lead to neuronal dysfunction. It is interesting to note that the part of the brain involved in cognition and memory i.e. the cortex and the hippocampus are the ones that are severely affected compared to the cerebellum. The cerebellum (another part of the brain) is mostly involved in motor neuronal activities such as muscle movements, posture and equilibrium.

Saturated free fatty acids such as palmitic acid and stearic acid have been shown to induce the pathological changes observed in AD through astroglia mediated oxidative stress in cortical neurons. It is known that the de novo synthesized ceramides signals that cascade of events that eventually lead to oxidative stress in cortical astrocytes. Post-mortem brain analysis studies in AD patients suggest higher levels of ceramides in cortex and hippocampus compared to cerebellum. The results of the current study suggest that the cerebellar astrocytes proliferate in the presence of free fatty acids by production of lower levels of ceramides. The study was undertaken to study the variation in the behavior of the neurons and astrocytes from two different brain regions and there is indeed a marked difference in the astrocytic cell fate (cell death or proliferation) in the presence of free fatty acids.

ACKNOWLEDGEMENTS

Mom and Dad, I could not have accomplished this without you; thank you for the support and encouragement throughout the years. I thank my family and my friends for supporting me through tough times.

I sincerely thank my advisor, Dr. Christina Chan for her help and guidance. I cannot convey how much I appreciate your dedication, constructive criticism and motivation for my scientific training over the past 2 years. I would also like to thank the members of my guidance committee, Drs. Patrick Walton and Mark Worden for their thoughtful advice and critique in the preparation of this work.

I wish to thank the faculty and students of the Department of Chemical Engineering for contributing to my scientific development by providing an excellent training atmosphere here at MSU. Last, but certainly not the least, to the members of the lab, whose friendship and scientific feedback has been very supporting over the past few years. All of you have made this an amazing graduate school experience.

This research was made possible by the help and support I received from many people. It might be possible that I may not have explicitly named all the people without whose help I would not have been able to present my research as a thesis, yet I do remember their contributions to my research and my life.

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LIST OF ABBREVIATIONS

ACh : Acetyl Choline

ACS : Acetyl Co-enzymeA Synthetase

AD : Alzheimer's disease

APP : Amyloid Precursor Protein

ATP : Adenosine Triphosphate

BACE : Beta Amyloid Cleaving Enzyme

Co-A : Co-enzyme A

FACL : Fatty Acyl CoA Ligase

FFA : Free fatty acid

NFT : Neuro-fibrillary Tangles

PA : Palmitic Acid

PET : Positron Emission Tomography

ROS : reactive oxygen species

PBS : phosphate buffered saline

CHAPTER 1

1. INTRODUCTION

1. 1 Alzheimer's disease and its impact

In the recent report released by the Alzheimer's disease Association, it has been estimated that there are about 5.1 million Americans suffering from Alzheimer's disease and the number is expected to increase to 11 million by 2050 if no cure would be found by then (Alzheimer's Association Report 2007). The report reveals the financial, social and emotional impact that the disease has on the patients, their families, the society and the nation. The United States Federal Government spends about $148 million annually on the direct and indirect costs associated with Alzheimer's disease (AD) and other related dementia. There is no cure or vaccine therapy available for the disease that devastates the life not only of the patient but also changes the life of their immediate family. Hence, it is necessary to find a cure in the near future or at least outline the high risk factors for this disease.

1.2 Pathophysiological and metabolic changes in Alzheimer's disease

Alzheimer's disease is a pathological condition marked by two hallmark protein abnormalities the Amyloid Plaques commonly known as the Senile Plaques and Neuro Fibrillary Tangles (Mattson M.P., 2004). The amyloid plaques are deposits of the Amyloid Beta (Aβ) Protein formed outside the neurons in the brain. This protein is derived from a long, transmembrane protein, amyloid precursor protein (APP). APP is first cleaved by β secretase (BACE1) forming the smaller, membrane bound C-terminal fragments of APP (CTFs), which are further cleaved by γ-secretase leading to the formation of Aβ protein (Vassar et al 1999).

The neurofibrillary tangles (NFT) are paired helical filaments that are composed of the tau protein. The Tau proteins are the microtubule-associated proteins expressed in neuronal cells. They play an essential role in the assembling the microtubules and in regulating axonal transport. Aggregation of specific sets of these Tau proteins is the pathological symptom of many neuro-degenerative diseases such as Alzheimer's disease. When hyperphosphorylated, the Tau proteins form the tangles that disrupt basic cell functions and ultimately cell death. Furthermore, the density of the NFT's has been shown to correlate well with the severity of dementia in AD (Bierer et al 1995).

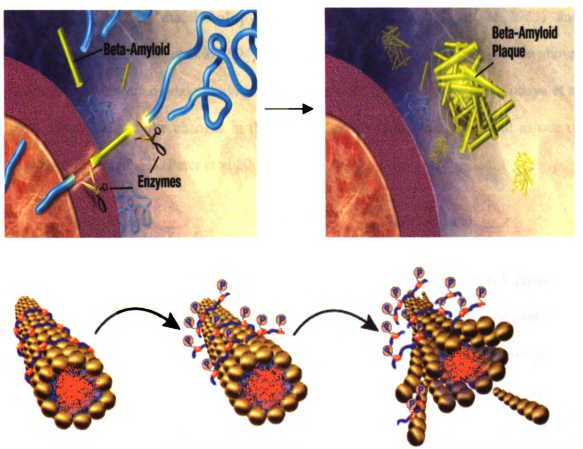


Fig1.1 Protein abnormalities in AD (Beta Amyloid formation image Courtesy of Alzheimer's Disease Education and Referral center). *Images in thesis are presented in color.*

In addition to the above mentioned pathological changes, AD is also characterized by certain metabolic changes such as decreased cerebral glucose metabolism (46% decrease) and reduced Adenosine Triphosphate (ATP) formation (about 19% decrease) compared to the age matched healthy controls (Hoyer et al 1991). Positron Emission Tomography (PET) scans that allow in vivo imaging of the brain metabolism show decreased cerebral glucose metabolism in patients suffering from AD. Further, this decrease correlates with the regions that are severely affected in AD (Munch et al 1998). Few studies have suggested that glucose hypometabolism could be an important marker for early diagnosis of AD (Mosconi 2005). The activities of various metabolic enzymes have been shown to be decreased in AD, mainly of pyruvate dehydrogenase (Sorbi et al 1983) and cytochrome oxidase (Parker et al 1994). Furthermore, autopsy studies in AD brain shows decreased cytochrome oxidase activity specifically in NFT-bearing neuron (Mutisya et al 1994). Finally, metabolic changes in the cerebral cortex have been suggested as one of the early markers of AD (Peter et al 2004).

1.3 AD and free fatty acids

Most of the research that has been conducted so far has determined certain risk factors for AD. The prime risk factors that have been identified for AD include high fat diet, brain trauma, Type 2 Diabetes, and ageing. High fat diet is a significant risk factor for the development of AD and the degree of saturation of fatty acids is an important criterion in determining the risk (Grant 1999). Further, few in vivo studies where the rats fed with high fat diet developed AD like changes in their brain (Levin et al 2002). Also, diabetes mellitus is a significant risk factor for AD (Arvanitakis et al 2002), and is characterized

by elevated plasma levels of saturated free fatty acids (FFAs). Similarly, brain trauma has been established as an independent risk factor for AD (Guo et al 2000).

All of these risk factors except ageing (which is a natural process accompanied by increased oxidative stress levels) is well marked by the elevated levels of free fatty acids in the plasma. Hence free fatty acids have a direct or indirect effect in causing AD. It is interesting to note that in AD brain, the NFTs are enriched in fatty acids such as Palmitic and Stearic acids. Recent finding suggests that saturated free fatty acids such as Palmitic Acid and Stearic Acid cause pathological changes such as tau hyperphosphorylation and BACE-1 upregulation in cortical neurons (Patil et al 2005, 2006).

1.4 AD and its effect on the different brain regions

The pathophysiological changes such as presence of Amyloid plaques and neurofibrillary tangles are mainly confined to those regions of the brain involved in cognition, thinking and memory i.e. the cortical and the hippocampal regions. The cerebellum is the part of the brain involved in involuntary muscle co-ordination, equilibrium, posture and motor learning. It is relatively spared from the changes that occur during the pathogenesis of AD. Although there are few reports that suggest that cerebellum does get affected in severe form of AD and there are Amyloid plaques scattered in the cerebellum in the late stages of the disease (Yamaguchi et al 1989).

In addition, glucose metabolism in the cerebellar region also has been shown to be reduced depending on severity of the disease (Ishii et al 1998). Free fatty acids, before

they enter the cellular membranes are activated to their respective Co-Enzyme A (Co-A) derivative by a group of membrane associated enzymes called acyl CoA synthetases (Lehninger Principles of Biochemistry, Fourth Edition). The activity of the acyl CoA synthetases has been shown to ten times higher in the cortex compared to the cerebellum (Szutowicz et al 1983). Interestingly, this might explain the relatively lesser effect of free fatty acids on cerebellar neurons in causing AD-like changes.

1.5 Aims of the study

The present study is an effort to understand the effect of saturated free fatty acids specifically free fatty acids in causing pathological and metabolic changes occurring AD (if any) in the cerebellum. It is a comprehensive study explaining the regional disparities in expressing AD-like symptoms in the brain. It is shown in this study that the cerebellar astrocytes are metabolically less active than the cortical astrocytes and the cerebellar neurons also show changes similar to cortical neurons on exposure to Palmitic acid at a higher concentration. We have investigated the mechanism through which the cerebellar astrocytes remain less susceptible to the effect of free fatty acids. This has led us to determine the differences in free fatty acid metabolism in the cortex and the cerebellum. This could be used to determine the exact point at which the free fatty acid metabolism differs and hence could be used to determine right therapeutic drug targets for AD.

1.6 Organization of the thesis

In the current study, we were interested in focusing on two different aspects of cerebellar metabolism. In chapter 2, the results of treating the neurons and astrocytes (both from the cortex and from the cerebellum) are presented. Additionally, a hypothetical experiment to demonstrate the higher activity of cortical astrocytes compared to the cerebellar astrocytes has been described. The dose response of the cerebellar neurons to palmitic acid conditioned astrocytes media was also studied. There exists a difference in free fatty acid metabolism between the astrocytes and the neurons from the cortical and cerebellar regions. It was identified the difference arises due to differential expression of Acyl CoA Synthetase 3 (ACS3) – an isomer group enzymes called Acyl CoA Synthetases that activate the free fatty acids to enter the metabolic cycle inside the neurons and astrocytes.

Chapter 3 described the experiments that were performed to elucidate the metabolic differences between the cortical and cerebellar astrocytes. Interestingly, since glucose metabolism is being suggested as an early marker for AD, we studied the glucose metabolism in cortical and cerebellar astrocytes treated with varying concentration of Palmitic acid. It was also identified that the difference in the glucose metabolism is due to the differential expression of GLUT 1 receptor protein – a set of glucose transporter proteins that regulate the entry of glucose molecules through the cell walls of the astrocytes.

CHAPTER 2

2. EFFECT OF SATURATED FREE FATTY ACIDS ON CORTICAL AND CEREBELLAR NEURONS AND ASTROCYTES

2.1 Introduction

Elevated levels of free fatty acids is a common feature in most of the prime risk factors of AD that include Type 2 Diabetes, hyperlipidemia and brain trauma. Few studies have suggested a role for astroglia in mediating the oxidative stress pathway in causing AD like pathophysiological changes upon exposure to palmitic and stearic acids (Patil et al 2005, 2006). These studies mostly focus on the cortex or the hippocampus : the regions of the brain that are severely affected in AD. The cerebellum : the part of the brain involved in motor learning, equilibrium and body posture is the least affected, and this might explain as to why the AD patients can maintain their posture and equilibrium although the have severe problems in cognition and memory.

Hence we decided to elucidate the reason behind this regional disparity and the mechanism by which the cerebellar neurons do not undergo such changes upon exposure to saturated free fatty acids. It was determined that this is due to the difference in expression of Acyl CoA Synthetase 3 (ACS3): an isomer of acyl CoA synthetase (an enzyme that activates free fatty acids before its catabolism / anabolism inside a cellular membrane).

2.1.1 Free fatty acid metabolism: role of acyl CoA synthetases

Cells cannot metabolize free fatty acids directly. Free fatty acids are first activated to their respective CoAcyl derivatives by acyl CoA synthetase. This step is usually a branch point after which the FFAs could be metabolized ether anabolically or catabolically. The anabolic pathway leads to production of Triglycerides, ceramides, phospholipids and cholesterol synthesis, and the catabolically pathway leads to beta-oxidation in mitochondria or the peroxisomes. Formation of a thiol ester bond between the Fatty acid and the (thiol group of) Coenzyme A forms acyl CoA, and this reaction usually takes place in the outer membrane of the mitochondria. Various isoforms of this enzyme has been shown to be expressed in various levels in different parts of the body e.g liver, kidney, brain, and muscle cells. The regulation of free fatty acid uptake by the Fatty Acid Transporter protein (FATP) and the long chain acyl CoA Synthetase ligase (ACSL) has been well studied in adipocytes, and liver cells.

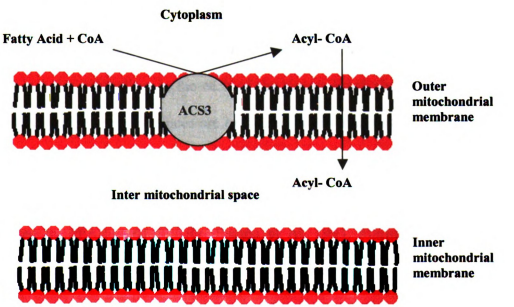


Fig 2.1 Free fatty acid metabolism. *Images in this thesis are presented in color.*

2.1.2 Fatty Acyl CoA Isomers:

Long-chain acyl-coenzyme A 1 synthetases (ACSs) catalyze the formation of acyl-CoA thioesters from fatty acids, ATP and CoA (Tanaka et al., 1979). Various groups have cloned and characterized the different isoforms in different species including humans (Ghosh et al., 1995; Minekura et al., 1997). Interestingly, five different enzymes with a common structure have been characterized in the rat, and each ACS appears to have a marked tissue distribution and a completely different regulation from those of the others (Suzuki et al., 1995).

For example, the rat ACS2 and ACS3 are predominantly expressed in brain (Fujino et al., 1996) whereas the Rat ACS1 is commonly found in liver, heart, and adipose tissue (Suzuki et al., 1990). Rat ACS4 and ACS5 are highly expressed in steroidogenic tissues and in small intestine, respectively (Kang et al., 1997). Irrespective of the organs or tissues in which they are highly expressed, the biological significance of all the five isoforms cannot be overlooked; their importance lies in the fact that all of them are essential in lipid metabolism in the each of the tissues listed above. The isoforms that have different substrate preferences are expressed in different tissues and are present in different subcellular locations. The expression of each of the rat isoforms is likely to be regulated independently. For example, ACS1 mRNA in different tissues is variously upregulated by PPARα and PPARγ (Suzuki et Al. 1995, Martin et al 1997) and by insulin, ACS2 in brain is upregulated by PPARβ (Kansara, et al 1996). These findings suggest that each of these isomers have distinct functions, possibly directing fatty acids to different metabolic pathways.

In human skin fibroblasts, triacsin C, which inhibits ACS1 and 4 but not ACS5, blocks *de novo* glycerolipid synthesis but does not affect lysophospholipid reacylation (Igal et al, 1997), suggesting that different ACS isoforms generate independent acyl-CoA pools which have specific fates. In humans, mutations in the FACL4 gene inactivate the enzyme are linked to mental retardation (Meloni et al, 2002), reasserting the unique function of this isoform in brain.

In our study we first determined if there is indeed any variation in the expression of tau and BACE-1 between the two regions of the brain namely the cortex and the cerebellum. If there does exist a difference in the expression, we could conclude that this could be only due to the differential regulation of free fatty acid metabolism (specifically saturated free fatty acids) in the two brain regions. Since, it has been shown that astrocytes and not neurons regulate the free fatty acid metabolism in the brain we determined the effect of saturated free fatty acids (Palmitic Acid) on the neurons and the astrocytes of both the brain regions.

2.2 Materials and Methods

2.2.1 Cell Culture

2.2.1.1 Cerebellar Neurons and Astrocytes Culture

Cultures of dissociated cerebellar granule cells were isolated from seven-day-old Sprague Dawley rat pups and cultured according to the methods established by Trenkner with some modifications (Trekner 1991). Cerebella were dissected out, freed of meninges, and incubated 15 minutes at room temperature in Ca-and Mg-free Hanks' balanced salt solution (CMF-HBSS) containing 1% trypsin. The cell solution was washed with 0.016% Deoxyribose Nuclease (Dnase) solution. The tissues were centrifuged for at 3000 rpm and the supernatant was discarded. The cerebella pieces were resuspended in the DNase solution and triturated using a glass Pasteur pipette. The cell solution was centrifuged through a gradient buffer containing 4% bovine serum albumin (BSA) in CMF-HBSS for 5 min at 3000 rpm. The supernatant was discarded and the cells were resuspended in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum, 25 mM KCl and 0.001mM Insulin from Bovine Pancreas (Sigma, MO). The cells were plated at a density of 1 x 10^6 cells per well in poly –l-Lysine (Trevigen Inc, MD) coated 6-well plates. Cultures were kept in a humidified atmosphere of 5% CO_2, 95% air at 37°C in a granule cell medium. Three days after incubation (37°C, 5% CO_2), the medium was subsequently replaced with 2ml of granule cell medium supplemented with 5 μM cytosine-β-arabinofuranoside (Arac from Calbiochem, CA, USA). After 2 days, the neuronal culture was switched back to granule cell medium without Arac. The experiments were performed on 6- to 7-day-old culture. One-half of the culture medium was exchanged with fresh medium twice a week.

To obtain astroglial cultures the cells were replaced with the medium without addition of Arac on the second day from the day of isolation.

2.2.1.2 Cortical Neurons and Astrocytes Culture

Cultures of primary cortical neurons and astrocytes were obtained from one-day-old Sprague–Dawley rat pups according to the published methods as described by Chandler et al with some modifications (Chandler et al 1993). The cells were plated on poly-l-lysine-coated, six-well plates at the concentration of 2×10^6 cells per well in fresh cortical medium DMEM (Invitrogen, CA) supplemented with 10% horse serum (Sigma, MO), 25 mM glucose, 10 mM HEPES (Sigma), 2 mM glutamine (Sigma), 100 IU/ml penicillin, and 0.1 mg/ml streptomycine. For neuronal cultures two days after incubation (37 °C, 5% CO_2), the medium was replaced with 2 ml of cortical medium supplemented with 5 μM cytosine-β-arabinofuranoside (Arac from Calbiochem, CA, USA) to prevent the proliferation of astrocytes and glial cells. After 2 days, the neuronal culture was switched back to cortical medium without Arac.

The experiments were performed on 6- to 7-day-old culture. To obtain primary cultures of astroglial cells, the cortical cells from one-day-old Sprague–Dawley rat pups were cultured in DMEM/Ham's F12 medium (1:1), 10% fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 0.1 mg/ml streptomycine. The cells were plated on poly-l-lysine coated, 6-well plates at the concentration of 2×10^6 cells per well. Cells were grown for 8–10 days (37 °C, 5% CO_2) and culture medium was changed every 2 days.

2.2.2 Free fatty acid treatment

Palmitic acid was used for the experiments as the representative fatty acids for the saturated fatty acids since it is the most abundant fatty acids of their class in the plasma. In case of severe brain injury, the levels of palmitic acid and Stearic acid in the brain increase to approximately 180 μM and 350 μM respectively (Lipton 1999). Traumatic brain injury has been established as a risk factor for AD and hence a concentration of 0.2-0.25 mM has been used in the present study and a control of 4% BSA in the cortical neuronal medium was used. The media in the seven to ten day old cultures

2.2.3. Western Blotting

To examine the effects of free fatty acids induced changes in BACE1 expression on the cerebellar neurons, the cells were treated either with 0.2mM and 0.5mM palmitic acid, or 4% BSA (control) for 24 hours. For western blotting, the cells were washed three times with ice-cold TBS (25mM Tris, pH 8.0, 140mM NaCl, and 5mM KCl) and lysed for 20 min by scraping into ice-cold Radioimmunoprecipitation assay (RIPA) buffer [1% (v/v) Nonidet P40, 0.1% (w/v) SDS, 0.5% (w/v) Sodium deoxycholate, 20mM Tris, pH 7.4, 150mM NaCl, 100mM NaF, 1mM Na_3VO_4, 1mM EDTA, 1mM EGTA, and 1mM PMSF. The cell lysate was obtained by centrifugation at 13,000 rpm for 20 min at 4°C. The total protein concentration was measured by bichinconic acid assay (BCA). Equal amounts of total protein from each condition were run at 200V on 10% Tris-HCl gels for phosphorylated tau and actin. The separated proteins were transferred to nitrocellulose membranes for 1 h at 100V and incubated at 4°C overnight with the appropriate primary antibodies [1:1000 BACE 1 (Chemicon, USA) 1:200 Actin]. Blots were washed three

times in PBS–Tween (PBS-T) and incubated with appropriate HRP-linked secondary antibodies diluted in PBS-T for 1 h. After three washes in PBS-T, blots were developed with the Pierce SuperSignal West Femto Maximum Sensitivity Substrate and imaged with the Bio- Rad ChemiDoc.

To examine the FFA-induced hyperphosphorylation of tau, primary rat cerebellar neurons were left untreated or treated with 0.25 mM of either palmitic or 4% BSA (control) for 24 h. After 24 h, the cells were lysed and Western blot analysis was performed to determine the cellular levels of hyperphosphorylated tau using a specific primary antibody AT8 (Pierce Biotechnology, IL). To determine the relative expression levels of the ACS3, cerebellar astrocytes and neurons; cortical astrocytes were cultured, lysed and Western blot analysis was done using primary polyclonal antibody for ACS3 (1:200 ACS3, Affinity Bioreagents Inc).

2.2.4 Measurement of Reactive Oxygen Species

Reactive oxygen species (ROS) were detected by staining with the dye 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFA, Molecular Probes, OR). Confluent cerebellar and cortical astrocytes were initially treated with 0.25μM palmitic acid or 4% BSA and the conditioned media from the respective astrocytes were transferred to the neurons. In other words, the conditioned media from the cortical astrocytes were transferred to the cortical astrocytes and the media from the cerebellar astrocytes were transferred to the cerebellar neurons. The treated neurons were incubated for 30 min at 37 °C with 2 μM H_2DCFA in DMEM (Catalog number 10313, Invitrogen)

without serum. The cells were then washed three times with PBS and the fluorescence was read from a fluorescence microscopy (Emission: 530 nm Excitation: 485 nm). To minimize the background due to cell clustering, before the incubation with the dye, the cells were kept in PBS and the fluorescence was noted (blank values). The absolute values for the fluorescence due to ROS formation was obtained after subtracting the blank values from the ones obtained after dye incubation.

2.2.5 Cell Proliferation Assay

The cerebellar astrocytes cultured in a 6-well plate were trypsinized and the cells were counted using a hemacytometer. The final density of the cell solution was made to be equal to approximately 10^5 cells per ml by diluting the cell solution with the astrocytes media. 100 □l of this cell solution was plated on the 96 well plate and incubated for 6 hours so that the cells are completely attached to the surface. The media was carefully removed and the cerebellar astrocytes were treated with 0.25 mM and 0.5 mM palmitic acid (PA) or with 4% BSA (control). The cell numbers were quantified with a Proliferation Assay (CyQuant NF Proliferation Assay, Invitrogen) using a fluorescence microplate reader. The CyQUANT NF assay is based on measurement of cellular DNA content via fluorescent dye binding. Because cellular DNA content is highly regulated, it is closely proportional to cell number. The extent of proliferation is determined by comparing the Absorbance values of the samples treated with Palmitic acid with untreated controls using a fluorescence spectrophotometer.

2.3 RESULTS AND DISCUSSION

2.3.1 Effect of palmitic acid on cerebellar neurons

There was no change in the levels of phosphorylated tau in rat cerebellar neurons treated directly with palmitic acid, as compared to controls (Fig 2.2). This could be because the neurons do not actively take up and metabolize fatty acids compared to the astrocytes (Blazquez et al 2000). This shows that the physiological concentration of palmitic acid during the progression of the disease i.e. at 0.25 mM do not have any effect on the cerebellar neurons.

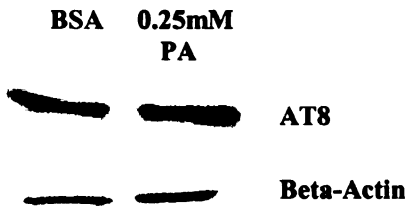


Fig 2.2 Direct treatment of cerebellar neurons with increasing concentration of palmitic acid. Primary rat cerebellar neurons were treated for 24 h with 0.25 mM of either palmitic acid (PA) or with 4% bovine serum albumin (BSA), carrier for FFAs (control). Cell lysates from fatty acid-treated and control cells were immunoblotted with AT8 antibody, which recognize phosphorylated tau. β-actin is shown as a marker for equal protein loading.

A number of studies suggests a central for astroglia mediated oxidative stress in causing pathological changes observed in AD (Patil et al 2005). Therefore, in the present study, we treated the rat cerebellar astrocytes with 0.25 mM palmitic or 0.5 mM palmitic acid for 24 hours and transferred the conditioned media to treat the cerebellar neurons for 24 hours. The conditioned media from FFA-treated astrocytes did not cause hyperphosphorylation of tau in the cortical neurons (Fig 2.3), as observed by Western blotting with AT8 antibody.

AT8 recognizes tau phosphorylated at Ser202 and Thr205 (Goedert et al 1995). Ser202 is one of the abnormal phosphorylation sites of the hyperphosphorylated tau associated with NFTs in AD (Gong et al 1994).

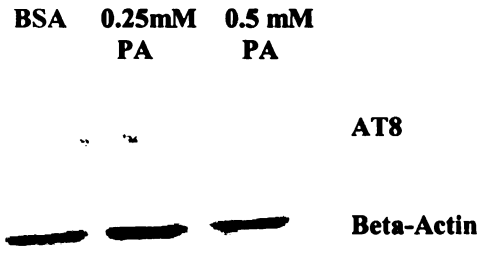
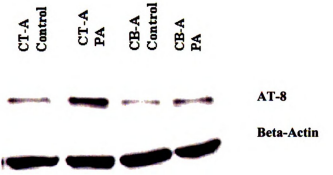


Fig 2.3 Treatment of cerebellar neurons with conditioned media from cerebellar astrocytes. Primary rat cerebellar astrocytes were treated for 24 h with 0.25 mM , 0.5 mM palmitic acid (PA) or with 4% bovine serum albumin (BSA), carrier for FFAs (control). The conditioned media from the three conditions were transferred to the neurons. Cell lysates from fatty acid-treated and control cells were immunoblotted with AT8 antibody, which recognize phosphorylated tau. β-actin is shown as a marker for equal protein loading.

The conditioned media from the cerebellar astrocytes did not cause any change in the tau phosphorylation levels in cerebellar neurons. Previous studies show that conditioned media from palmitic acid treated cortical astrocytes cause tau hyperphosphorylation and BACE1 upregulation in cortical neurons (Patil et al 2005, 2006). Hence, we studied the effect of conditioned media from palmitic acid treated cortical astrocytes on cerebellar neurons (Fig 2.4).

To further establish that the cortical astrocytes and not cerebellar astrocytes are involved in causing AD-related pathological changes, we treated the cerebellar astrocytes with 0.25mM palmitic acid or with 4% BSA, and transferred the media to cortical neurons. Tau phosphorylation levels (Fig 2.5) and BACE1 levels (Fig 2.6) were determined by Western blotting methods using specific primary antibodies.

A.



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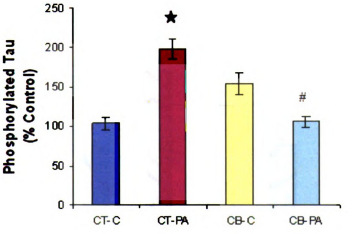
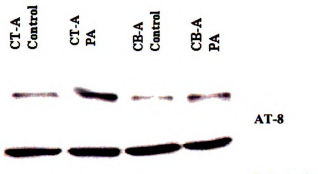


Fig 2.4 FFA induced tau hyperphosphorylation in cerebellar neurons. Astrocytes from the cortical and cerebellum were treated with 0.2 uM Palmitic Acid (PA) or 4% BSA (Control). The conditioned media from the astrocytes were transferred to the cerebellar neurons (24 hours treatment). A. Western Blot Analysis of hyper-phosphorylated tau using AT-8 antibody in cerebellar neurons B. Quantification of Western Blot Analysis of hyper-phosphorylated tau using phospho-specific AT-8 antibody in cerebellar neurons. Histograms corresponding to AT8 blots represent quantification of the relevant bands' intensities. All the histograms were normalized based on the control data of each individual set of experiments. Data represent mean±S.D. of three independent experiments. Student's t test was used to determine the differences between treatment groups (* $p < 0.01$ compared to the control, # $p < 0.05$ compared within treatment groups). *Images in this thesis are presented in color.*

A.



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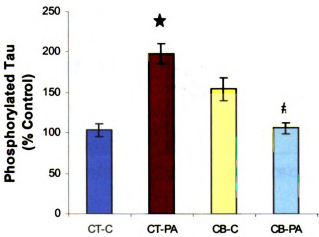
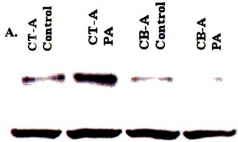


Fig 2.5 FFA induced tau hyperphosphorylation in cortical neurons. Astrocytes from the cortical and cerebellum were treated with 0.2 uM Palmitic Acid (PA) or 4% BSA (Control). The conditioned media from the astrocytes were transferred to the cortical neurons (24 hours treatment). A. Western Blot Analysis of hyper-phosphorylated tau using AT-8 antibody in cortical neurons B. Quantification of Western Blot Analysis of hyper-phosphorylated tau using phospho-specific AT-8 antibody in cerebellar neurons. Histograms corresponding to AT8 blots represent quantification of the relevant bands' intensities. All the histograms were normalized based on the control data of each individual set of experiments. Data represent mean±S.D. of three independent experiments. Student's t test was used to determine the differences between treatment groups (* $p < 0.01$ compared to the control, # $p < 0.05$ compared within treatment groups). *Images in this thesis are presented in color.*



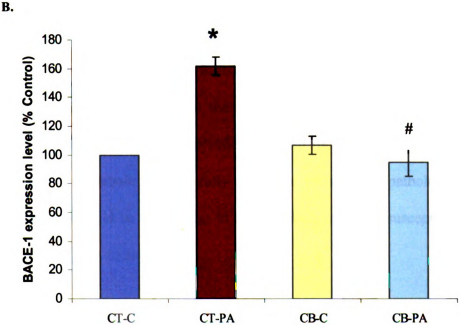
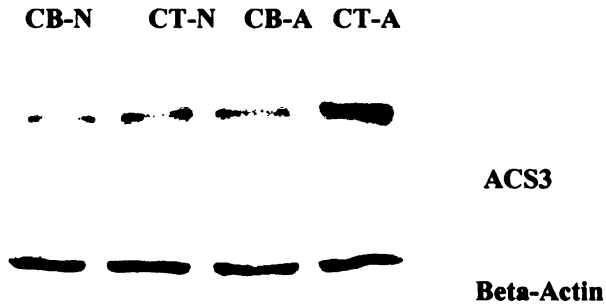


Fig 2.6 FFA induced BACE-1 upregulation in cortical neurons. Astrocytes from the cortical and cerebellum were treated with 0.2 uM Palmitic Acid (PA) or 4% BSA (Control). The conditioned media from the astrocytes were transferred to the cortical neurons (24 hours treatment). A. Western Blot Analysis of BACE-1 using specific antibody in cortical neurons B. Quantification of Western Blot Analysis of BACE-1 in cerebellar neurons. Histograms corresponding to BACE-1 blots represent quantification of the relevant bands' intensities. All the histograms were normalized based on the control data of each individual set of experiments. Data represent mean±S.D. of three independent experiments. Student's t test was used to determine the differences between treatment groups (* $p < 0.05$ compared to the control, # $p < 0.05$ compared within treatment groups). *Images in this thesis are presented in color.*

2.3.2 Regional disparities in expressing AD-pathological changes:

Few studies report the activity of the fatty acyl CoA Synthetase enzyme to be 10 times higher in the cortex compared to that of the cerebellum (Szutowicz et al, 1980). This suggests that the cerebellum is distinct from the cortex and the hippocampus in expressing the neuropathological changes because of its distinct activity in metabolizing Free Fatty Acids (FFA). Activity and expression levels are two different aspects in governing the reaction catalyzed by an enzyme. Therefore, we investigated the relative expression levels of the acyl CoA synthetase in the neurons and the astrocytes of both the cortex and cerebellum. It was found that the astrocytes generally have higher expression levels of ACS3 compared to the neurons. It is interesting to note that the cortical astrocytes have the highest expression levels of ACS3 (Fig 2.7), which explains its higher activity in metabolizing free fatty acids and causing the pathological changes in AD. Hence, this could be a reason as to why cerebellum is less susceptible to oxidative stress because of FFA metabolism.

A.



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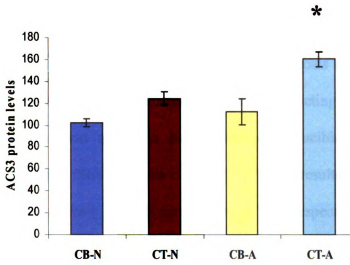


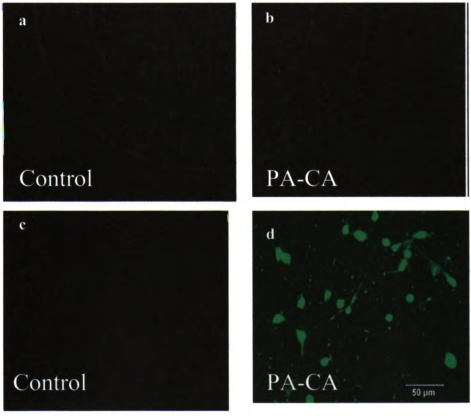
Fig 2.7 Relative expression levels of ACS3 in cortical, cerebellar astrocytes and neurons. A. Western Blot Analysis of ACS3 using ACS3 specific antibody B. Quantification of Western Blot Analysis of ACS3 protein levels in cerebellar and cortical neurons, cerebellar and cortical astrocytes. Histograms corresponding to ACS3 blots represent quantification of the relevant bands' intensities. Beta-Actin was used as a marker for equal protein loading. *Images in this thesis are presented in color.*

2.3.3 Involvement of Reactive Oxygen Species (ROS)

Fatty Acids induces the gene for fatty CoA synthetase in adipocytes (Gargiulo et al, 1999). This would lead to activation of Palmitate to Palmitoyl-CoA. If this occurs in neurons then it would increase the utilization of free fatty acids. In pancreatic beta cell line INS-1, Palmitate induces activation of Carnitine Palmitoyltransferase gene thereby increasing beta-oxidation and reducing the glucose oxidation. We know that Protein Kinase-C activation may lead to activation of NADPH oxidase and increase ROS production in vascular cells. Fatty acids are shown to be involved in PKC activation, leading to ROS production (Duval et al, 2003). If this enzyme is not activated in cerebellum, this might spare the cerebellum from the effects of oxidative stress.

There are few cell systems in which Ceramide synthesis has been shown to induce apoptotic pathways independent of ROS generation. In human granulose cells, palmitic and stearic acid has been shown to cause apoptosis independent of ROS production or Nitric Oxide (NO) production. In insulin secreting cells, palmitic acid causes ROS generation and apoptosis independent of inducible Nitric Oxide Synthase (iNOS) upregulation (Shimabukuro et al, 1998). These results indicate that saturated fatty acids induce apoptosis due to metabolism of the respective acyl CoA derivative. This is important in our study, as the acyl CoA synthetase is 10 times more active in cortex compared to the cerebellum. We have confirmed that the ROS species produced in the cortical neurons treated with conditioned media from palmitic acid exposed astrocytes to be higher than the cerebellar neurons treated in the similar manner with conditioned media from cerebellar astrocytes (Fig 2.8).

A.



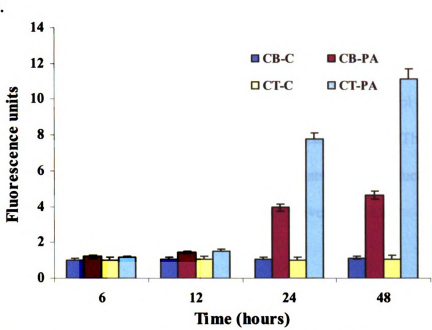


Fig 2.8 Intracellular ROS accumulation in cerebellar and cortical neurons. A. Fluorescence Microscopy images of intracellular ROS accumulation in neurons. a. Cerebellar neurons treated with media from control cerebellar astrocytes b. Cerebellar neurons treated with the media from PA conditioned cerebellar astrocytes c. Cortical neurons treated with media from control cerebellar astrocytes d. Cortical neurons treated with the media from PA conditioned cortical astrocytes B. Quantification of ROS produced in cortical and cerebellar neurons. Cortical and cerebellar astrocytes were treated with 0.25mM palmitic acid or 4% BSA (control). The conditioned media from cortical astrocytes were transferred to the cortical neurons and the ROS produced was measured in fluorescence units by using an oxidant-sensitive dye (H2=DCFA, Molecular Probes) for 48 hours in 6 hours intervals. Similarly the ROS levels were quantified using intensity of fluorescence produced on incubation with the dye. Thus a time dependent ROS production was investigated in the cortical and cerebellar astrocytes. Results presented are expressed as mean +/- SD of two independent time series experiments. *Images in this thesis are presented in color.*

We have shown that cortical astrocytes and not cerebellar astrocytes cause tau hyperphosphorylation and BACE1 upregulation in cortical and cerebellar neurons. This is due to the difference in relative expression of the enzyme ACS3 a free fatty acid activator. We have shown that it is highly expressed in cortical astrocytes compared to the cerebellar neurons. It is interesting to note that most of the isomers of this enzyme are highly expressed in proliferating cells such as astrocytes, leydig cells and in lymphocytes.

So what causes this marked difference in astrocytes metabolism upon treatment with free fatty acid. It has been shown that de novo synthesized ceramides causes apoptosis in astrocytes in a time dependent manner (Blazquez et al 2000). Further there a number of studies that suggests that ceramide plays an important role in the control of cell fate in the central nervous system under different pathophysiological situations. Thus, elevations of intracellular Ceramide levels, which may in turn be related to the induction of apoptotic cell death, have been shown to occur in neurodegenerative disorders.

The cerebellar astrocytes must possess a different mechanism to control and regulate the levels of intra-cellular ceramide. On the other hand, cortical astrocytes have been suggested to produce ceramides on treatment with palmitic acid and this causes oxidative stress in the neighboring neurons eventually leading to AD-like pathology (Patil et al 2006). The major metabolic routes that contribute to the maintenance and variation of the cellular ceramide include ceramide biosynthesis, by de novo pathway or sphingosine recycling, ceramide formation from complex sphingolipids degradation and ceramide catabolism. It is worthwhile to know that any mitogenic stimulus is followed by changes in ceramide levels. This is usually one of the key step in a series of reactions that are to follow. Furthermore, proliferation of astrocytes requires low cellular levels of ceramides (Riboni et al 2002). In particular, mitogenic stimuli, such as basic fibroblast growth factor (bFGF), rapidly down regulate the cellular levels of ceramide by stimulating sphingomyelin synthase. Moreover, although ceramide may significantly contribute to cell death in neurological disorders, the possible involvement of ceramide synthesis de novo in neural cell death / differentiation is yet unknown.

Hence, this suggests that the ceramides secreted by the cerebellar astrocytes could be at very low levels and hence this might cause the astrocytes to proliferate instead of inducing oxidative stress. This could be the reason as to why the cerebellum do not show pronounced neuro pathological changes as that of the cortex and hippocampus in progression of Alzheimer's disease. Interestingly, The central role of ceramides in AD is supported by the elevated levels of ceramides found in AD brain as compared to healthy controls; ceramide levels are higher in the affected regions i.e. cortex and hippocampus as compared to the unaffected areas i.e.cerebellum (Cutler et al 2004).

To determine if the treatment of the cerebellar astrocytes do indeed cause proliferation as opposed to cell death we treated the cerebellar astrocytes with 0.25mM and 0.5 mM palmitic acid (PA) or with 4% BSA(control) and quantified the number of cells with a Proliferation Assay (CyQuant NF Proliferation Assay, Invitrogen) using a fluorescence microplate reader.

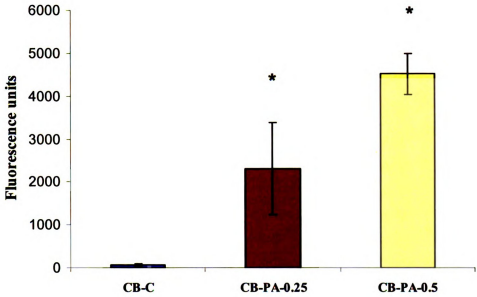


Fig 2.9 Proliferation of cerebellar astrocytes in the presence of palmitic acid. Astrocytes from the cerebellum were treated with 0.2 μM Palmitic Acid (PA) or 4% BSA and the proliferation was measured in fluorescence intensity units using the CyQuant NF Proliferation Assay (*$p < 0.05$ compared to the control). *Images in this thesis are presented in color.*

The cerebellar astrocytes indeed proliferate on treatment with palmitic acid for 24 hours (Fig 2.9). This explains the unique machinery of the cerebellum in preventing the neuronal and astrocytic cell death in AD or rather when the brain is under oxidative stress.

CHAPTER 3

3. EFFECT OF PALMITIC ACID IN GLUCOSE METABOLISM IN CEREBELLAR ASTROCYTES

3.1 INTRODUCTION

3.1.1 The concept of energy metabolism in brain

The brain consumes about 20% of the total energy produced in the body, which is relatively a large compared to its size. Most of the available energy is spent on pumping ions such as Sodium and Potassium across cellular membranes. This process is critical for the proper functioning of the central nervous system (CNS) because any abnormalities in energy metabolism in the brain will potentially have a harmful effect on the proper functioning of the tissues and the other physiological systems.

Most of the energy supplied to the brain comes through metabolism of glucose. Therefore, metabolic regulation and control of glucose levels is of central significance. Oxidation of the glycolytic product pyruvate yields the energy rich compound acetyl-CoA, which is used for production of Adenosine Tri-Phosphate (ATP), Acetyl Choline (ACh) and cholesterol. ATP is produced when glucose is oxidized in the Tri-Carboxylic Acid (TCA) cycle, which is used to drive the active ion transport across the membranes. ATP guarantees most cellular and molecular processes such as protein synthesis, maintenance of ion homeostasis, folding sorting and degradation of proteins and even maintenance of synaptic transmission. ATP is known to inhibit Insulin-degrading enzyme (IDE) activity, which is shown to be over-expressed in AD brain in accordance with decreased ATP levels (Camberos et al 2001).

Glucose is critical for the production of acetyl-CoA, a precursor of acetylcholine (Ragozzino et al, 1996), which is further confirmed by the fact that a decrease in glucose concentrations result in a decrease in brain acetylcholine (Gibson et al 1976). Hence, it is interesting to study the effect of glucose in affecting the proper functioning of the CNS.

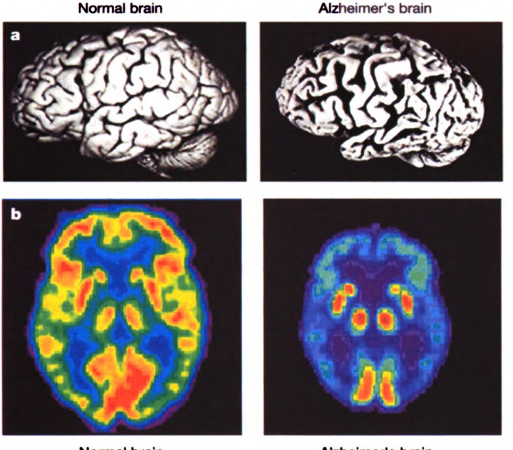


Fig. 3.1 Positron emission Tomography images of brain glucose utilization in a normal and an AD patient (Mattson et al, 2004). These images are currently used to distinguish AD from other types of dementias. The metabolic degeneration occurring in the brain of an AD patient could be accurately determined by PET images. Higher metabolic rate is indicated by the red color and the violet indicates lower metabolism. As shown above, in AD the cortex shows relatively lower metabolism compared to the age matched control as indicated by the blue spread over the entire brain section. In AD patient, the lower area of the brain (cerebellum) is shown in red indicating that the cerebellum remains relatively unaffected compared to the normal brain. *Images in this thesis are presented in color.*

3.1.2 Alzheimer's Disease and Type-2 Diabetes

Epidemiological evidence has shown that type 2 diabetes is a risk factor for AD (Ott et al., 1999). Since diabetes is a disease of glucose metabolism, what is the functional relationship between the two and how does type 2 diabetes increase the risk of AD?

Until recently, it was thought that insulin was not relevant to brain glucose metabolism. However, it is now agreed that insulin can cross the blood brain barrier (BBB) and that a small subset of CNS neurons in areas involved in cognitive function express insulin sensitive GLUT 4 and 8 receptors. The brain can also synthesize a small amount of insulin in situ (Watson and Craft, 2003). In addition, there is a physiological coupling between glucose and steroid levels via the hypothalamic–pituitary–adrenal axis, and abnormalities in peripheral glucose metabolism can indirectly elevate glucocorticoid levels. Glucocorticoids do enter the brain where they can alter both glucose metabolism and nerve cell viability (Sapolsky, 2000).

Although the molecular mechanisms are not yet understood, a number of studies suggest that elevated peripheral glucose levels and insulin resistance have a negative effect on memory and nerve cell viability. (1) Insulin resistance leads to a higher mortality rate from ischemia (LeMay et al., 1988). (2) AD patients with lower insulin levels have lower cognitive skills than those with normal levels (Craft et al., 1996). (3) The administration of insulin and glucose together enhances the memory of AD patients to a greater extent than glucose alone (Manning et al., 1993; Craft et al., 1996). (4) People with type 2 diabetes have impaired learning and more rapid memory decline with age than controls

(Ryan and Geckle, 2000). If one makes the assumption that there is a causal link between insulin and glucose utilization in the brain, then it follows that glucose metabolism is associated with cognitive skills.

3.1.3 Abnormalities in brain glucose metabolism and its impact on cellular and molecular mechanisms in sporadic dementia of Alzheimer type

We know that the onset of AD is usually associated with Amyloid plaques and neurofibrillary tangles but recently its has been shown that reduced cerebral glucose utilization is the most consistent feature of an AD brain (Schubert, 2005). Type 2 Diabetes which is a risk factor for AD is also characterized by reduced glucose uptake and hence increased blood glucose concentration (Bergman, 2000). Type 2 Diabetes is well studied compared to AD and it has been shown that there is a direct effect of Free Fatty Acids on muscle metabolism. For example, elevated FFAs are shown to suppress glucose uptake (Bergman, 2000), and the fact that Blood Brain Barrier (BBB) is not a barrier for FFAs (Abel, Handbook of Neurochemistry,1982) suggests a direct / indirect link between elevated levels of plasma FFAs and decreased cerebral glucose metabolism. Also, free fatty acids stored in the from of triacylglycerols in adipocytes, can also serve as energy sources (Lehninger Principles of Biochemistry, Edition 2). Thus, this form of free fatty acid can be utilized for supplementing the energy demand from peripheral tissues. Taking all these ideas together it is worthwhile to study effect of elevated levels of FFAs on the neuronal/ astroglial glucose uptake.

3.2 Materials and Methods :

3.2.1 Cell Culture and free fatty acid treatment

The astrocytes from two different brain regions were cultured as described in the previous chapter (Chapter 2). For the experiments, seven to ten day old cultures were used. The cortical and cerebellar astrocytes were treated with 0.2 mM, 0.5 mM palmitic acid or with 4% BSA (control) for 24 hours.

3.2.2 Measurement of glucose uptake by the astrocytes

To calculate the glucose uptake in particular, their concentrations in the media were measured by using enzymatic glucose assay (Stanbio Laboratories, TX). The glucose uptake was calculated by using the differences between the glucose concentrations in the media before and after the treatment. The media was collected and centrifuged for 5 minutes at 1730 rpm to eliminate the presence of any cell debris which might interfere with the assay.

3.2.3 Western Blotting

For Western blot analysis, cell lysates were obtained as described in previous chapter (Chapter 2). The total protein concentration was measured by BCA protein assay. Equal amounts of total protein from each condition were run at 200V on 10% Tris-HCl gels for 1 hour. The separated proteins were transferred to nitrocellulose membranes for 1 h at 100V and incubated at 4°C overnight with the appropriate primary antibodies [1:1000 GLUT1, 1:200 Actin]. Blots were washed three times in PBS–Tween (PBS-T) and incubated with appropriate HRP-linked secondary antibodies diluted in PBS-T for 1 h.

After three washes in PBS-T, blots were developed with the Pierce SuperSignal West Femto Maximum Sensitivity Substrate and imaged with the Bio- Rad ChemiDoc

3.3 Results and discussion

The complexity of nutrient utilization in the Central Nervous System has been a major field of research since brain utilizes about 20% of the glucose as a energy source. It was believed that glucose was the neuronal fuel under normal conditions, whereas ketones could substitute for glucose under hypoglycemic conditions (Cryer, 1981). This view has been challenged recently when it was shown that lactate could substitute as a fuel, during energy times of energy deficit. It has been shown that lactate is an energy substrate for neuronal functional recovery during hypoxia-ischemia (Schurr et al 1997). Insulin-induced hypoglycemia increases lactate utilization in the brain (Hellman et al 1992). In fact, the astrocyte-neuron lactate shuttle hypothesis suggests that neurons may actually prefer glial-derived lactate to glucose as a fuel for neuronal activity (Magistretti et al 1999). According to the hypothesis, neuronal activity increases extracellular glutamate, which stimulates glial anaerobic glycolysis and converts glucose to lactate. Lactate is transported out of glia by monocarboxylate transporter 1 and into neurons by monocarboxylate transporter 2, where it is metabolized to pyruvate via lactate dehydrogenase-1. This pyruvate then enters the neuronal tricarboxylic acid cycle to generate ATP (Chih et al 1991). In support of this hypothesis, a recent study showed that the human brain preferentially uses circulating lactate to sustain metabolism even at a normal glucose concentration (Smith et al, 2003). Fatty Acids can act as neuronal fuels (Kim et al, 2003).

We have hypothesized a correlation between the glucose metabolism and the AD related pathological changes. To test the hypothesis we treated the astrocytes cultured from the cortex and the cerebellum with palmitic acid and determined their glucose uptake. The experiment results suggest a significant decrease in glucose uptake upon Palmitic Acid treatment in cortical and not cerebellar astrocytes compared to control. We treated the primary cortical and cerebellar astrocytes with increasing concentrations of palmitic acid: 0.25 mM and 0.5 mM.

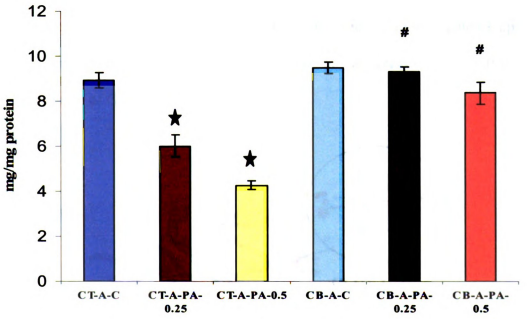


Fig 3.2 PA downregulates glucose uptake by cortical and not cerebellar astrocytes. The cortical and cerebellar astrocytes were treated for 24 hours with 0.25 mM of PA, or 0.5 mM PA or 4% BSA (control). PA treatment significantly decreased glucose uptake by cortical astrocytes and .did not affect the cerebellar astrocytes, in a concentration dependent manner. Data represent mean±S.D. of four independent experiments and the glucose uptake values are normalized based on the protein concentration determined by BCA Assay. Data represent mean±S.D. of six independent experiments. Student's t test was used to determine the differences between treatment groups (* $p < 0.05$ compared to the control, # $p<0.07$ compared within treatment groups.) *Images in this thesis are presented in color.*

Mechanism of Palmitate induced Glucose hypometabolism

AD brain is characterized by significant reductions in GLUT1 levels, and also the disease severity is associated with progressive decline in GLUT1 gene expression (Simpson et al 1994, Jacobs et al 1997). We hypothesized that the observed, palmitic acid (PA)-induced abnormal glucose metabolism may be due to the potential effect of PA on the level of astroglial glucose transporter (GLUT1). Consequently, the level of astroglial glucose transporter (GLUT1) was significantly down-regulated in PA-treated astroglia as compared to the untreated ones. Thus, the observed downregulation in glucose uptake by astroglia in presence of palmitic acid may be attributed to the PA-induced downregulation of GLUT1 levels in astroglia.

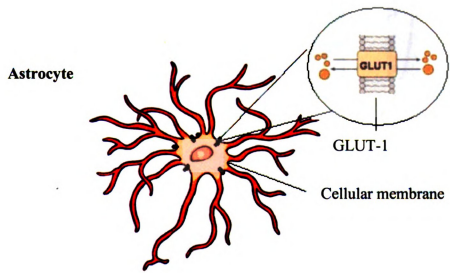


Fig 3.3 GLUT-1 proteins in cellular membranes. GLUT-1 are a member of transmembrane proteins specifically expressed in cortical astrocytes that facilitate the transport of glucose into the cell. *Images in this thesis are presented in color.*

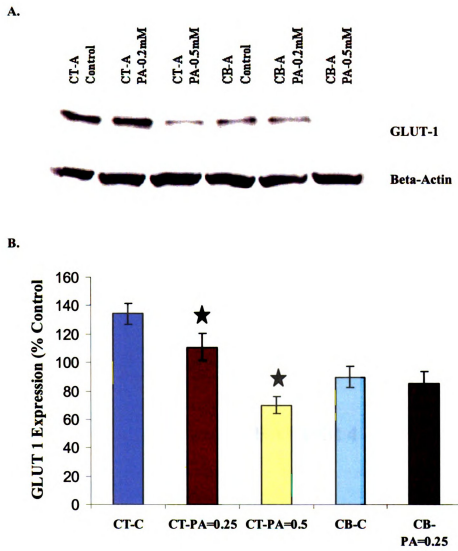


Fig 3.4 FFA induced GLUT-1 downregulation in cortical but not in cerebellar astrocytes. Astrocytes from the cortical and cerebellum were treated with 0.25 mM Palmitic Acid (PA) or 0.5 mM PA or 5% BSA (Control). A. Western Blot Analysis of GLUT1 expression using GLUT1 specific antibody in cortical and cerebellar astrocytes B. Histograms corresponding to GLUT1 blots represent quantification of the relevant bands' intensities. All the histograms were normalized based on the control data of each individual set of experiments. Data represent mean±S.D. of three independent experiments. *Images in this thesis are presented in color.*

As hypothesized, the cortical astrocytes show a PA dependent GLUT-1 downregulation which explains as how the cerebral glucose metabolism is regulated. Further studies could be undertaken to study the regulation of GLUT-1 expression by PA.

CHAPTER 4

4. Conclusions and future work

4.1 Conclusions

Saturated free fatty acids such as Stearic acid, and Palmitic acid has shown to induce tau hyperphosphorylation and BACE1 upregulation (two key events in progression of AD) in primary cortical neurons treated with the conditioned media from free fatty acid exposed primary cortical astrocytes. Primary astrocytes from cerebellum do not induce such protein abnormalities, which might explain as to why the cerebellum is relatively spared from neuronal degeneration in AD. Thus, the astrocytes have emerged as the cells that play an important role in neuronal metabolism irrespective of the region of the brain.

One of the key metabolites in free fatty acid metabolism are the ceramides and they have emerged as one of the intra-cellular molecules that could potentially act as a therapeutic drug target for AD. Hence, we focused on the effect of free fatty acid on the part of the brain that is least affected. It interesting to note that the levels of ceramide in cerebellum is low compared to the cortex and the hippocampus in AD brain. Our experiments show that the primary neurons irrespective of the region they originate from behave in a similar manner. We showed this in a hypothetical experiment in which the cerebellar neurons show tau hyperphosphorylation and BACE1 upregulation when treated with the conditioned media from free fatty acid treated cortical astrocytes. Thus, the basic difference in expressing the AD-like neuropathology is due to the difference in the astrocytic metabolism.

It was shown that glucose uptake by the cells is decreased in the cortical and not the cerebellar astrocytes. In addition, it was determined that the expression of ACS3- a key enzyme in free fatty acid metabolism was higher in the cortical astrocytes compared to the cerebellar astrocytes. The result of this is that the cortical astrocytes are much more metabolically active that the cerebellar astrocytes in metabolizing saturated free fatty acids. Hence, we could justify this regional difference in the neuronal/astrocytic behavior by the higher levels of the ACS3 in cortex.

We wanted to examine the effect of free fatty acids on the cerebellar astrocytes to ascertain that the cerebellar astrocytes indeed possess a different machinery to metabolize free fatty acid. It has been shown that the cortical astrocytes undergo apoptosis upon treatment with free fatty acids due to de novo synthesized ceramides. It has been shown for the first time that the cerebellar astrocytes do indeed proliferate upon treatment with palmitic acid. Thus lower levels of ceramides produced due to lower metabolic activity of the ACS3 causes the astrocytes to shift to a proliferative phase compared to the expected apoptotic phase of the cell fate as observed in cortical astrocytes.

4.2 Future directions to the study

4.2.1 Invivo and metabolic studies

Invivo studies could be undertaken to study the potential of the identified target-ceramides as a therapeutic agent. Since the cerebellar astrocytes are possessed with a different machinery to metabolize free fatty acids a metabolic model may be constructed on the neuron glial interaction in the cerebellum to determine the pathways that differ in the tow brain regions namely the cortex and cerebellum. It would more easy to identify drug targets if we consider the neuron-glial interaction from the two different regions and compare them step by step rather than analyze region specific interaction pathway.

4.2.2 Insulin signaling in response to FFAs in different regions

It has been shown that the cerebellum and cortex differ in even metabolizing glucose, which is the primary energy substrate. Insulin is a hormone that regulates blood glucose levels. Apart from being the primary agent in maintaining carbohydrate homeostasis, it has effects on fat metabolism and it changes the liver's activity in storing or releasing glucose and in processing blood lipids, and in other tissues such as fat and muscle. While some amount of literature is available on the effect of free fatty acids on insulin signaling in beta cells and myocytes, similar studies in neural cells are lacking. It is interesting to note that Type 2 Diabetes is a risk factor for AD. Taking all these ideas together, it would be worthwhile to study the effects of the different types of FFAs on insulin signaling in neural cells. These studies might determine the association between insulin signaling and the progression of AD.

4.2.3 Ceramide synthesis as regulatory machinery

Ceramide, a lipid molecule that increases the cellular oxidative state, has been shown to have be involved in several apoptotic patterns including trophic factor withdrawal and inflammation (Coroneos et al., 1995; Kyriakis and Avruch, 1996). One pathway of ceramide formation involves sphingomyelin hydrolysis and the other pathway (denovo synthesis) involves formation of ceramide by ceramide synthase from less complex molecules (Bose et al., 1995).

The implications for lower ceramide levels in cerebellum may actually be beneficial in terms of metabolic activity. Higher levels of ceramide synthesis in the cortex along with oxidative stress that further elevates the ceramide levels in AD may be responsible for the severe neuronal degeneration in cortex and hippocampus. Pharmacological studies involving inhibitors of ceramide synthesis could be undertaken to study the role of ceramide in causing neuronal degeneration in AD and further elucidate the role of ceramides in causing regional disparities in neuronal and astrocytic metabolism.

4.3 Shortcomings identified in the study

The study was mainly focused on assimilating the information that was obtained on a basis of an underlying hypothesis that the cerebellar region shows relatively less neuronal degeneration and lower levels of glucose metabolism compared to the cortex. Although, very few post-mortem studies have identified Amyloid beta plaques and fibrillary tangles in cerebellum and PET imaging studies have shown cerebellar glucose metabolism to be comparable to that of the cortical glucose metabolism, we could at least ascertain that in the in vitro primary culture studies cerebellar metabolism is different from the cortical metabolism. The study was done primarily on pure astrocytic and neuronal cultures. Usually a cell behaves completely differently when removed from its neighboring cells (also called a niche). In our study, a co-culture study (of neurons and astrocytes) might be more relevant in elucidating the involvement of astrocyte-neuron interaction in neural cell fate determination.

APPENDICES

APPENDIX A

QUANTIFYING THE PROTEIN LEVELS IN WESTERN BLOT

The 1-D electrophoresis gel is imaged and analyzed using the Quantity One software® (Bio-Rad Inc, USA). The analysis is done by determining the intensity of the blot obtained on exposure to X-Ray, by using a chemiluminescent substrate that detects Horseradish Peroxidase (HRP) on immunoblots.

Western Blotting with Actin antibody is used to confirm that equal proteins are run on each of the wells during the loading of the gel. Equal areas of the blots are compared for their relative intensities using the volume analysis tool in the software. Each of the blot intensity is normalized with their respective actin blot intensity run as a separate gel. Finally, all the blots are normalized with the control band intensity of that particular gel. An example has been discussed, to explain the analysis done throughout the study.

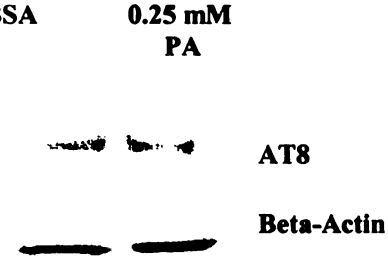


Fig a Direct treatment of cerebellar neurons with increasing concentration of palmitic acid. Primary rat cerebellar neurons were treated for 24 h with 0.25 μM of either palmitic acid (PA) or with 4% bovine serum albumin (BSA), carrier for FFAs (control). Cell lysates from fatty acid-treated and control cells were immunoblotted with AT8 antibody, which recognize phosphorylated tau. β-actin is shown as a marker for equal protein loading.

Using the volume analysis tool the respective intensities for the AT8 and the actin blot was obtained as shown in the tabulation. Each the condition, BSA (control) and PA treatment was normalized with respective actin values

	AT8 intensity	Actin intensity	Actin normal	% control
C	4594.37	7867.241	0.58398745	100
PA	4831.721	7486.841	0.64536178	110.5095

Since there was no change with respect to the control, we concluded that treatment of cerebellar neurons directly with palmitic acid caused no changes in protein levels of tau and BACE1.

APPENDIX B

PHASE CONTRAST IMAGES OF NEURONS AND ASTROCYTES FROM THE CEREBELLUM AND CORTEX OF THE RAT BRAIN

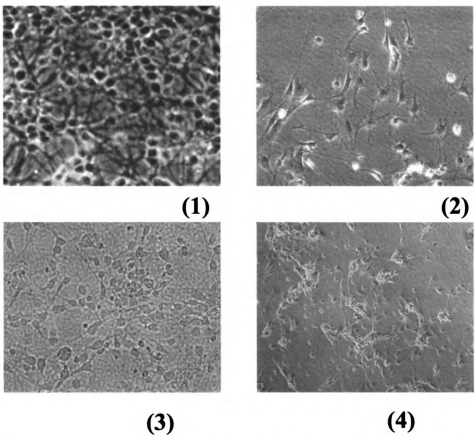


Fig b Phase contrast images of (1) Cerebellar neurons, (2) Cerebellar astrocytes, (3) Cortical neurons and (4) Cortical astrocytes

APPENDIX C

SUPPLEMENTARY IMAGES

Each of the experiments were done atleast thrice so that a good statistical analysis of the data presented could be obtained. The main section of the thesis contains representative blots for each of the protein level detection methods. This section puts together all the blots obtained during the course of the study undertaken.

1. DIRECT TREATMENT OF CEREBELLAR NEURONS WITH PALMITIC ACID

This experiment was done to confirm that cerebellar neurons like cortical neurons do not take up free fatty acids such as palmitic acid. We observed no changes in the protein levels of Tau and BACE1, which should be upregulated if the neurons actively metabolize free fatty acids.

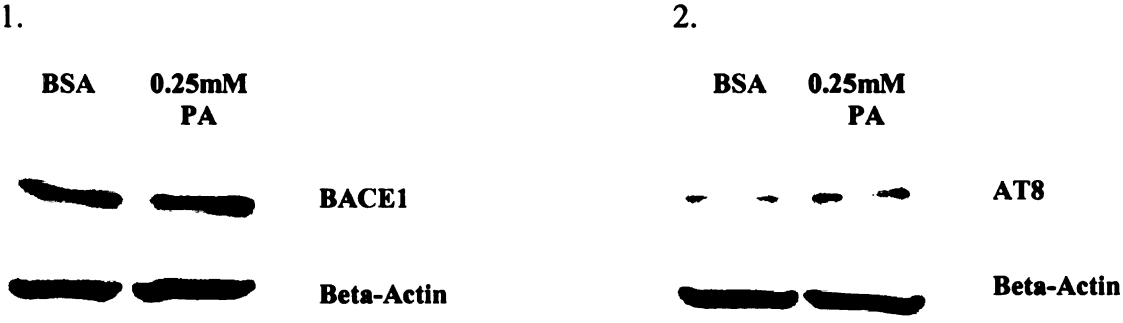


Fig c.1 Direct treatment of cerebellar neurons with palmitic acid. Primary rat cerebellar neurons were treated for 24 h with 0.25 μM of either palmitic acid (PA) or with 4% bovine serum albumin (BSA), carrier for FFAs (control). Cell lysates from fatty acid-treated and control cells were immunoblotted with (1)BACE1 and (2)AT8 antibody, which recognize phosphorylated tau. Beta-actin is shown as a marker for equal protein loading.

2. CROSS CELL TYPE EXPERIMENTS

A. TAU PHOSPHORYLATION OF CEREBELLAR NEURONS

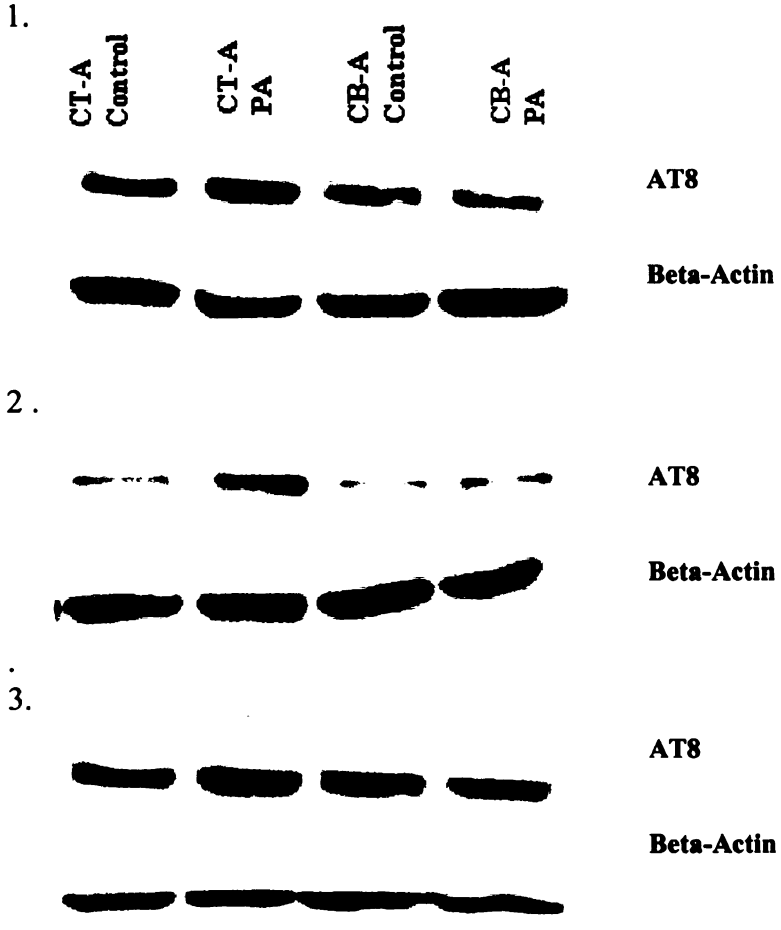


Fig c.2. FFA induced tau hyperphosphorylation in cerebellar neurons. Astrocytes from the cortical and cerebellum were treated with 0.2 uM Palmitic Acid (PA) or 4% BSA (Control). The conditioned media from the astrocytes were transferred to the cerebellar neurons (24 hours treatment). 1,2,3. Western Blot Analysis of hyper-phosphorylated tau using AT-8 antibody in cerebellar neurons. Beta-Actin was used a control for equal protein loading.

B. TAU PHOSPHORYLATION OF CEREBELLAR NEURONS

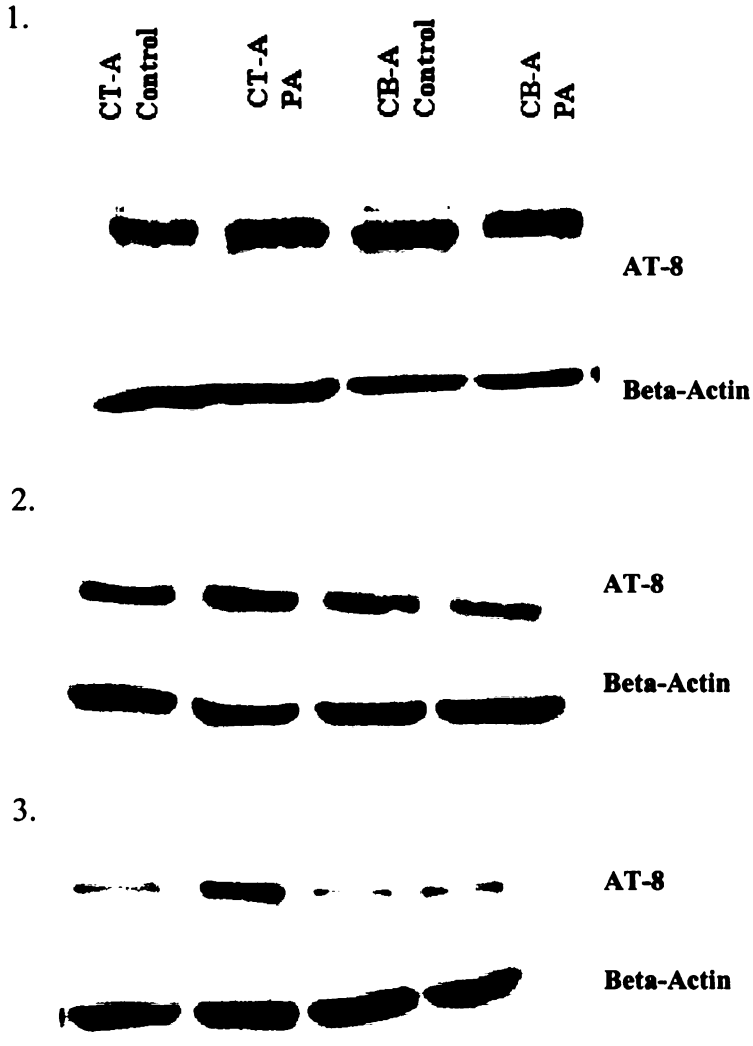


Fig c.3 FFA induced tau hyperphosphorylation in cortical neurons. Astrocytes from the cortical and cerebellum were treated with 0.2 uM Palmitic Acid (PA) or 4% BSA (Control). The conditioned media from the astrocytes were transferred to the cortical neurons (24 hours treatment). 1,2,3Western Blot Analysis of hyper-phosphorylated tau using AT-8 antibody in cortical neurons. Beta-Actin was used a control for equal protein loading.

C. BACE1 UPREGULATION IN CEREBELLAR NEURONS

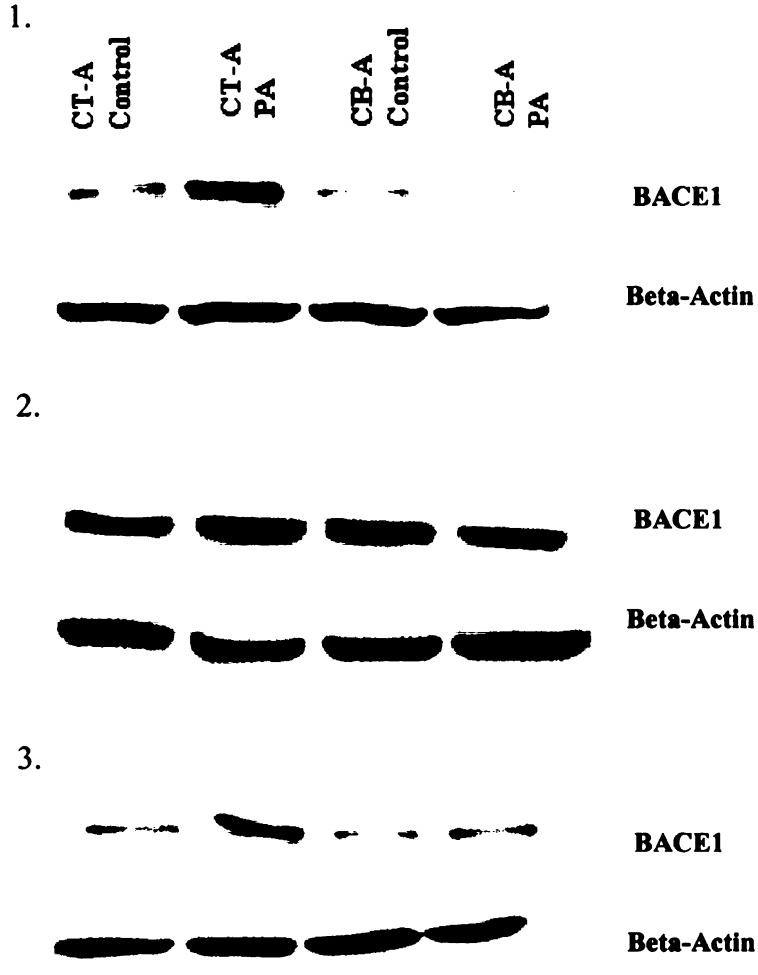
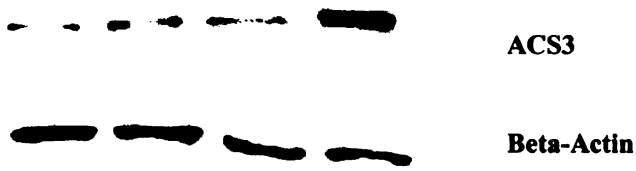


Fig c.4 FFA induced BACE1 upregulation in cerebellar neurons. Astrocytes from the cortical and cerebellum were treated with 0.2 uM Palmitic Acid (PA) or 4% BSA (Control). The conditioned media from the astrocytes were transferred to the cerebellar neurons (24 hours treatment). Western Blot Analysis of BACE1 using BACE1 antibody in cerebellar neurons.

3. ACS3 EXPRESSSION IN NEURONS AND ASTROCYTES OF THE CORTEX AND CEREBELLUM

1. CB-N CT-N CB-A CT-A



2.

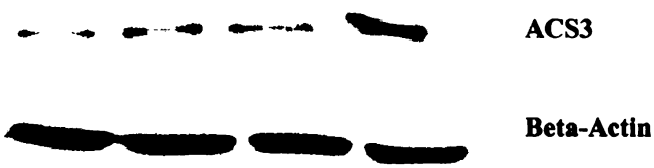


Fig c.5 Relative expression levels of ACS3 in cortical, cerebellar astrocytes and neurons. A. Western Blot Analysis of ACS3 using ACS3 specific antibody. Beta-Actin was used as a marker for equal protein loading.

4. GLUT-1 EXPRESSION

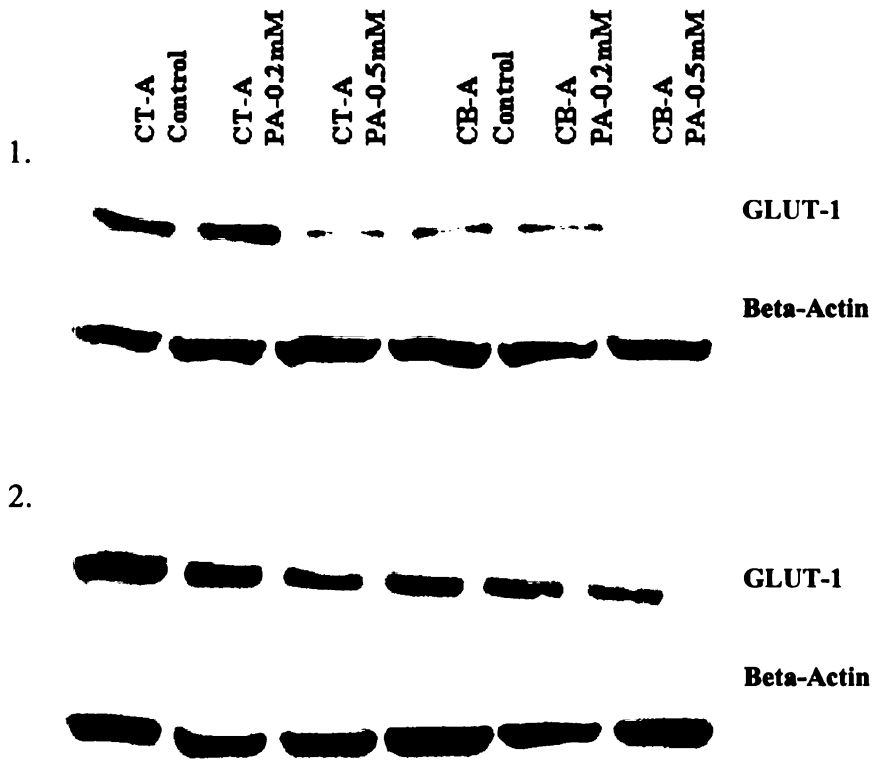


Fig c.6 FFA induced GLUT-1 downregulation in cortical but not in cerebellar astrocytes. Astrocytes from the cortical and cerebellum were treated with 0.2 mM Palmitic Acid (PA) or 0.5 mM PA or 5% BSA (Control). 1,2. Western Blot Analysis of GLUT1 expression using GLUT1 specific antibody in cortical and cerebellar astrocytes. Beta-Actin was used as a marker for equal protein loading.

APPENDIX D

CAVEATS IN MEASUREMENT OF REACTIVE OXYGEN SPECIES

In the study undertaken we have employed two different techniques to visualize the release of reactive oxygen species (ROS) namely Fluorescence Microscopy and Quantification of the fluorescence using a plate reader. Both the methods mentioned above employs a similar principle in visualizing the ROS released by the neurons on treatment with the media conditioned by the palmitic acid treated astrocytes for a given period of time. Detection of the intracellular ROS were done by staining with a dye 5-(6)-chloromethyl-2',7' dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, from Molecular Probes). Intracellular esterases cleave the ester group to form H_2DCFDA and is further converted to a membrane impermeable H_2DCF. The ROS released oxidizes the H_2DCF resulting in formation of 2,7-dichlorofluorescein (DCF) which is a fluorescent compound. The intensity of the fluorescence correlates with the ROS produced by the neurons.

We have used the 24-well plate for quantification purposes since it is easier to use one plate at a time for fluorescence readings and thereby eliminating errors due to cell count and conditions. At the same time, we had the problem of cell clustering especially in the case of cerebellar neurons when kept in the media for more than 24 hours, which is probably due to density differences. This also the reason as to why we do not observe any fluorescence in the microscopy images since they were taken using the cells plated on 6-well culture plates. Further imaging using Confocal Microscopy would have yielded much clearer and better images than the ones taken on the Fluorescence Microscope.

BIBLIOGRAPHY

- Abel Lajtha, Pak Chan, Handbook of Neurochemistry and Molecular Neurobiology, Edition 3.

- Alzheimer's Association Report 2007: www.alz.org/national/documents/Report_2007FactsAndFigures.pdf

- Alzheimer's Disease Education and Referral center: www.nia.nih.gov

- Arvanitakis Z., R.S. Wilson, J.L. Bienias, D.A. Evans, D.A. Bennett, Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function, Arch. Neurol 2004; 61:661–666.

- Bierer, L.M., Haroutunian, V., Gabriel, S., Knott, P.J., Carlin, L.S., Purohit, D.P., Perl, D.P., Schmeidler, J., Kanof, P. and Davis, K.L., Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits, Journal of neurochemistry 1995; 64:749-60.

- Blazquez C., I. Galve-Roperh and M. Guzman, De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase, FASEB J. 2000; 14:2315–2322

- Chandler L.J. ,H.Newsom, C. Sumners, F. Crews, Chronic ethanol exposure potentiates NMDA excitotoxicity in cerebral cortical neurons, J. Neurochem. 1993; 60:1578–1581.

- Camberos M.C., A.A. Perez, D.P. Udrisar, M.I. Wanderley, J.C. Cresto, ATP Inhibits Insulin-Degrading Enzyme Activity, Experimental Biology and Medicine 2001; 226:334-341.

- Chih CP, Lipton P, Roberts EL Jr: Do active cerebral neurons really use lactate rather than glucose? Trends Neurosic, 2001; 24:573–578.

- Craft, S.; Asthana, S.; Newcomer, J.W.; Tio-Matos, I.; Hunter, E.; Lofgreen, C; Raskind, M.; Wilkinson, C.W.; Veith, R.; Plymate, S.; Brodkin, K.; Gibson, L., Latendresse S.. Insulin-induced enhancement of memory in Alzheimer's disease is independent of glucose, Society for Neuroscience Abstracts, 1996; 22(2): 1177.

- Cryer PE: Glucose counters regulation in man (Review). Diabetes, 1981; 30:261–264.

- Cutler R.G., J. Kelly, K. Storie, W.A. Pedersen, A. Tammara, K. Hatanpaa, J.C. Troncoso, M.P. Mattson, Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease, Proc. Natl. Acad. Sci. U.S.A..,2004;101:2070–2075.

- Duval Carine, Anne-Valerie Cantero, Nathalie Auge, Laurence Mabile, Jean-Claude Thiers ,Anne Negre-Salvayre, Robert Salvayre, Proliferation and wound healing of vascular cells trigger the generation of extracellular reactive oxygen species and LDL oxidation, Free Radical Biology and Medicine; 35(12),2003;1589-1598.

- Fujino T, Kang MJ, Suzuki H, Iijima H, Yamamoto T, Molecular characterization and expression of rat acyl-CoA synthetase 3. J Biol Chem,1996; 271:16748-16752.

- Gargiulo Christina E., Sarah M. Stuhlsatz-Krouper, Jean E. Schaffer, Localization of adipocyte long-chain fatty acyl-CoA synthetase at the plasma membrane, Journal of Lipid Research, 1999; 40: 881-892.

- Ghosh B, Barbosa E , Singh I, Molecular cloning and sequencing of human palmitoyl-CoA ligase and its tissue specific expression. Mol Cell Biochem, 1995;151:77-81

- Gibson GE, Blass JP. Impaired synthesis of acetylcholine in brain accompanying mild hypoxia and hypoglycemia. J Neurochem 1976; 27:37–42.

- Goedert M., R. Jakes and E. Vanmechelen, Monoclonal antibody AT8 recognizes tau protein phosphorylated at both serine 202 and threonine 205, Neurosci. Lett. 1995; 189:167–170.

- Gong C.X., T.J. Singh, I. Grundke-Iqbal and K. Iqbal, Alzheimer's disease abnormally phosphorylated τ is dephosphorylated by protein phosphatase-2B, J. Neurochem. 1994; 62:803–806.

- Grant W.B, Dietary links to Alzheimer's disease: 1999 update, J. Alzheimer's Dis. 1999; 1:197–201

- Guo Z., L.A. Cupples, A. Kurz, S.H. Auerbach, L. Volicer, H. Chui, R.C. Green, A.D. Sadovnick, R. Duara, C. DeCarli, K. Johnson, R.C. Go, J.H. Growdon, J.L. Haines, W.A. Kukull, L.A. Farrer, Head injury and the risk of AD in the MIRAGE study, Neurology 2000; 54:1316–1323 .

- Hoyer, S., Brain glucose and energy metabolism abnormalities in sporadic Alzheimer disease. Causes and consequences: an update. Experimental Gerontology 2000;35, (9 10):1363-1372.

- Igal R. A., Wang P., Coleman R. A. Triacsin C blocks *de novo* synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into phospholipid: evidence for functionally separate pools of acyl-CoA. Biochem. J. 1997;324:529-534.

- Ishii K, Masahiro Sasaki, Hajime Kitagaki, Shigeru Yamaji, Setsu Sakamoto, Kant Matsuda, Etsuro MoriReduced ,Cerebellar glucose metabolism in Advanced Alzheimer's disease, The Journal of Nuclear Medicine 1997; 38 (6):925-928.

- Hellman J, Vannucci RC, Nardis EE: Blood-brain barrier permeability to lactic acid in the newborn dog: lactate as a cerebral energy fuel. Ped Res,1992;16:40–44.

- Jacobs, D. B., Mandelin, A. M., Wang, R., Bloom, A., Antuono, P. and Giordano, T., Effect of Alzheimer's disease on posttranscriptional regulation of GLUT1. Soc. Neurosci. Abstr., 1997; 23, 2218.

- Kang MJ, Fujino T, Sasano H, Minekura H, Yabuki N, Nagura H, Iijima H , Yamamoto T, A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis. Proc Natl Acad Sci USA, 1997 94: 2880-2884.

- Kim EK, Miller I, Aja S, Landree LE, Pinn M, McFadden J, Kuhajda FP, Moran TH, Ronnett GV: C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP activated protein kinase. J Biol Chem, 2004; 279:19970 –19976.

- Lehninger A.L, Nelson D.L, Cox M.M, Principles of biochemistry. New York: Worth Publishers, 1993.

- LeMay DR, Gehua L, Zelenock GB, D'Alecy LG.Insulin administration protects neurologic function in cerebral ischemia in rats, Stroke, 1988; 1411–1419.

- Levin-Allerhand J.A, C.E. Lominska, J.D. Smith, Increased Amyloid levels in APPSWE transgenic mice treated chronically with a physiological high-fat high-cholesterol diet, J. Nutr. Health Aging 2002; 6:315–319.

- Lipton P., Ischemic cell death in brain neurons, Physiol. Rev.1999; 79:1431–1568

- Magistretti PJ, Pellerin L: Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. Philos Trans R Soc Lond B Biol Sci,1999; 354:1155–1163.

- Manning, M.E. Ragozzino and P.E. Gold, Glucose enhancement of memory in patients with probable senile dementia of the Alzheimer's type, Neurobiol. Aging,1993;14:523–528.

- Mattson, M.P., Pathways towards and away from Alzheimer's disease, Nature 2004; 431:107.

- Meloni, I.; Muscettola, M.; Raynaud, M.; Longo, I.; Bruttini, M,Moizard, M.-P.; Gomot, M.; Chelly, J.; des Portes, V.; Fryns, J.-P.,Ropers, H.-H.; Magi, B.; Bellan, C.; Volpi, N.; Yntema, H. G.; Lewis, S. E.; Schaffer, J. E.; Renieri, A.: FACL4, encoding fatty acid-CoA ligase 4, is mutated in nonspecific X-linked mental retardation. Nature Genetics, 2002 ;30:436-440.

- Minekura H, Fujino T, Kang MJ, Fujita T, Endo Y Yamamoto THuman acyl-coenzyme A synthetase 3 cDNA and localization of its gene (ACS3) to chromosome band 2q34-q35,Genomics,1997;42:180-181.

- Monti L.D., C. Landoni, E. Setola, E. Galluccio, P. Lucotti, E.P. Sandoli, A. Origgi, G. Lucignani, P. Piatti, F. Fazio, Myocardial insulin resistance associated with chronic hypertriglyceridemia and increased FFA levels in Type 2 diabetic patients, Am. Journal. Physiol, 2004;287:H1225–H1231.

- Mosconi, L., Brain glucose metabolism in the early and specific diagnosis of Alzheimer's disease. European Journal of Nuclear Medicine and Molecular Imaging 2005; 32(4): 486-510.

- Munch, G.; Schinzel, R.; Loske, C.; Wong, A.; Durany, N.; Li, J. J.; Vlassara, H.; Smith, M. A.; Perry, G.; Riederer, P., Alzheimer's disease--synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. J Neural Transm 1998;105, (4-5): 439-61.

- Mutisya, E. M.; Bowling, A. C.; Beal, M. F., Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J Neurochem 1994; 63, (6): 2179-84.

- Ott, A., R.P. Stolk, F. van Harskamp et al.,Diabetes mellitus and the risk of dementia: the Rotterdam Study Neurology 1999;53: 1937–1942.

- Parker, W. D., Jr.; Mahr, N. J.; Filley, C. M.; Parks, J. K.; Hughes, D.; Young, D. A.; Cullum, C. M., Reduced platelet cytochrome c oxidase activity in Alzheimer's disease. Neurology 1994; 44, (6):1086-90.

- Patil S., C. Chan, Palmitic and Stearic Fatty acids induce Alzheimer like hyperphosphorylation of tau in primary rat cortical neurons, Neuro ScienceLetters 2005; 384: 288-293.

- 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, NeuroScience Letters 2006; 406 (1-2):55-59.

- Peter J. Nestor, Philip Scheltens, John R Hodges, Advances in the early detection of Alzheimer's disease, Nature Reviews Neuroscience 2005: S34–S41.

- Ragozzino ME, Unick KE, Gold PE. Hippocampal acetylcholine release during memory testing in rats: augmentation by glucose. Proc Natl Acad Sci USA 1996;93:4693–4698.

- Riboni L, Tettamanti G, Viani P.,Ceramide in primary astrocytes from cerebellum: metabolism and role in cell proliferation, Cerebellum ;2002;1(2):129-35.

- Ryan M., Geckle M., Why is learning and memory dysfunction in Type 2 diabetes limited to older adults?, Diabetes Metab. Res. Rev. ,2000;16:308–315.

- Sapolsky R, Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Archives of General Psychiatry, 2000;57, 925.

- Sauer H., B. Klimm, J. Hescheler and M. Wartenberg, 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.

- Schurr A, Payne RS, Miller JJ, Rigor BM: Brain lactate is an obligatory aerobic energy substrate for functional recovery after hypoxia: further in vitro validation. J Neurochem,1997; 69:423– 426.

- Schubert David, Glucose metabolism and Alzheimer's disease,Ageing Research Reviews, 2005;4(2),240-257.

- Shimabukuro Michio, Yan-Ting Zhou, Moshe Levi, Roger H. Unger, Fatty acid-induced Beta-cell apoptosis: A link between obesity and diabetes, Medical Sciences, 1998;95(5), 2498-2502.

- Simpson, I. A., Chundu, K. R., Davies-Hill, T., Honer, W. G. and Davies, P., Decreased concentrations of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. Ann. Neurol., 1994;5, 546-551.

- Smith D, Pernet A, Hallett WA, Bingham E, Marsden PK, Amiel SA: Lactate: a preferred fuel for human brain metabolism in vivo. J Cereb Blood Flow Metab, 2003; 23:658–664.

- Sorbi, S.; Bird, E. D.; Blass, J. P., Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain. Ann Neurol 1983; 13, (1):72-8.

- Suzuki H, Kawarabayasi Y, Kondo J, Abe T, Nishikawa K, Kimura S, Hashimoto T, Yamamoto T, Structure and regulation of rat long-chain acyl-CoA synthetase. J Biol Chem, 1990; 265: 8681-8685

- Suzuki H, Watanabe M, Fujino T and Yamamoto T, Multiple promoters in rat acyl-CoA synthetase gene mediate differential expression of multiple transcripts with 5'-end heterogeneity. J Biol Chem , 1995;270:9676-9682.

- Szutowicz A., Norman F. Harris, Paul A. Srere, Isaac L. Crawford, ATP-Citrate Lyase and Other Enzymes of Acetyl-CoA Metabolism in Fractions of Small and Large Synaptosomes from Rat Brain Hippocampus and Cerebellum, Journal of Neurochemistry, 1983; 41 (5):1502–1505.

- Tanaka T, Hosaka KT, Hoshimaru M and Numa S, Purification and properties of long-chain acyl-coenzyme A synthetase from rat liver. Eur J Biochem, 1979;98: 165-172

- Trenkner, E., Cerebellar cells in culture. In: Banker, G.,Goslin, K. (Eds.), Culturing Nerve Cells. MIT Press, Cambridge, 1999.

- Vassar R., B.D. Bennett, S. Babu-Khan, S. Kahn, E.A. Mendiaz, P.Denis, D.B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller, S. Edenson, J. Lile, M.A. Jarosinski, A.L. Biere, E. Curran, T. Burgess, J.C. Louis, F. Collins, J. Treanor, G. Rogers, M. Citron, Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE, Science 1999; 286:735–741.

- Watson, G.S. and Craft, The role of insulin resistance in the pathogenesis of Alzheimer's disease: Implications for treatment CNS. Drugs, 2003;17:27-45.

- Yamaguchi H, Mirai S, Morimatsu M, Shoji M, Nakazato Y, Diffuse type of senile plaques in the cerebellum of Alzheimer-type dementia demonstrated by beta protein immunostain. Ada Neuropathol 1989; 77:314-319.