UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Associated Effects of Alzheimer's Amyloid-β-Peptide and Disrupted Calcium Homeostasis on Reactive Astrogliosis

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Bioengineering

by

Siu Kei Chow

Committee in Charge:

Professor Gabriel Silva, Chair
Professor Nicholas Spitzer
Professor Amy Sung

2008

The Thesis of Siu Kei Chow is approved and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

Chair

University of California, San Diego

2008

DEDICATION

I would like to dedicate this thesis to my parents for their unconditional love and support. I would like to thank my brother Ray for his encouragement and faith. Thank you for all your love.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank everyone in the Silva Lab for providing such a friendly environment to work in. In particular, I would like to acknowledge Dr. Silva and Marie for their support, so that the experiments were conducted smoothly. I would like to give my special thanks to Diana, an excellent friend and mentor, for working together during the past three years. Her guidance and teaching was proved to be invaluable.

This thesis research was funded by the University of California, San Diego.

ABSTRACT OF THE THESIS

The Associated Effects of Alzheimer's Amyloid-β-Peptide and Disrupted Calcium Homeostasis on Reactive Astrogliosis

by

Siu Kei Chow

Master of Science in Bioengineering

University of California, San Diego, 2008

Professor Gabriel Silva, Chair

Alzheimer's Disease (AD) is the most devastating age-related neurodegenerative disease affecting more than 26 million people worldwide in 2006, whose neuropathological hallmarks include loss of neurons, deposition of neuritic plaques, and neurofibrillary tangles in brain regions. Amyloid-β-peptide (Aβ) and calcium dysregulation are suggested to be the possible causes for AD, while little evidence has shown the coupled effects of the two on astrocytes, especially in terms of reactive astrocytosis which is one of distinct features in AD. In the endeavor to scrutinize the associated effects of Alzheimer's Aβ and disrupted calcium homeostasis on reactive astrogliosis, we explored how Aβ act on reactive astrocytes modulated by $[Ca^{2+}]_{i,}$ which was illustrated by the protein expression of two reactive gliosis markers, S100B and

GFAP. The effect of Aβ on intracellular calcium oscillations in astrocytes was also studied. Quantification of protein expression level with time revealed that the expression of S100B induced by Aβ was calcium-dependent. GFAP expression level in Aβ condition was also modulated by $[Ca^{2+}]_i$ and in addition possibly by S100B in a concentration-dependent manner. In the calcium experiment, Aβ was found to increase intracellular calcium oscillations in astrocytes, while this augmentation in oscillations could be suppressed by thapsigargin and BAPTA-AM. More importantly, spontaneous intercellular calcium waves were first observed in and only in Aβ condition, in which the wave was presented in two forms, radial and unidirectional. The response to calcium modulation in Aβ model was suggested to be a type of astrocytic accommodations in AD.

CHAPTER 1. INTRODUCTION

1.1 Alzheimer's Disease and Amyloid-β-Peptide

Alzheimer's Disease (AD) is the most devastating age-related neurodegenerative disease affecting more than 26 million people worldwide in 2006 with the number of cases estimated to double every 20 years, and by 2050, 1 in 85 persons worldwide will suffer from this disease (Ferri et al., 2005; Brookmeyer et al., 2007). Apart from the distinct chronic progressive cognitive impairment in AD patients, some of the neuropathological hallmarks of AD include loss of neurons, degenerated synapses, deposition of neuritic plaques, and neurofibrillary tangles in brain regions, particularly the hippocampus and neocortex (Yankner, 1996; LaFerla, 2002). Drug treatments can be employed to slow down the progress of AD, but there is no cure still. AD is suggested to be a systemic disease affecting not only neurons but also other peripheral cells such as platelets and lymphocates (Baskin et al., 2001; Eckert et al., 2001; Evin et al., 2003). Genetic evidences have been showing polymorphisms in gene for sortilin 1 and dominantly inherited presenilin (PS1 and PS2) mutations cause early onset of AD (Rogaeva et al., 2007; Wolfe et al. 1999; Popescu et al., 2001). Mutations in presenilins are the primary causes of familial Alzheimer's Disease (FAD), in which presenilins are the integral components of a multiprotein protease complex, known as γ-secretase, to generate non-amyloidogenic P3 peptide or the APP carboxy-terminal intracellular domain (AICD) together with Amyloid-β-Peptide (Aβ) fragments of 40 or 42 residues (Fig. 1.1) (Hardy and Gwinn-Hardy, 1998; De Strooper et al., 1999, 1998; Levitan et al., 2001; Francis et al., 2002).

The etiology of AD is yet unclear, but there are three major hypotheses trying to reveal the cause of AD: i) the amyloid hypothesis; ii) the calcium hypothesis; and iii) the hyperphosphorylated tau protein hypothesis (Hardy and Selkoe, 2002; Khachaturian, 1994; Strittmatter et al., 1994; Pope et al., 1994). It is noticeable, though, the above hypotheses are not mutually exclusive, for Aβ influences the intracellular calcium content whereas calcium also modulates APP processing (Kawahara et al., 2000; Hensley et al., 1994; Mark et al., 1995; Buxbaum et al., 1994; Querfurth et al., 1997).

1.2 The Amyloid Hypothesis

The amyloid hypothesis suggests the accumulation of Aβ in the brain is the main cause of AD. Aβ results from a series of cleavages of amyloid precursor protein (APP), a type I membrane protein consisting of 695-770 amino acids, by groups of enzymes or enzyme complexes termed α-, β-, γ-secretase (LaFerla et al., 2007). AAP can undergo one of the two proteolytic pathways, in which the cleavage either precludes or generates Aβ. In the non-amyloidogenic pathway, APP cleavage is mediated by α-secretase. Since the cleavage site is within the aβ peptide sequence, it results in the generation of non-amyloidogenic carboxy-terminal fragment of APP (C83) (Buxbaum et al., 1998; Lammich et al. 1999). In the amyloidogenic case, β-secretase, such as aspartyl protease and a type I membrane protein, leads to the cleavage of APP without disturbing the aβ peptide sequence, thus eventually generates the amyloidogenic carboxy-terminal fragment (C99) (Sinha et al., 1999; Vassar et al.,

1999). The APP fragment resulted from α- or β-secretase cleavage is still embedded in the cytoplasmic membrane, which is subsequently cleaved by γ-secretase. γ-secretase is a mutlimeric enzyme complex consisting of PS1 or PS2, aph-1, nicastrin and pen-2, possessing a distinct feature that it is active within membrane layer and therefore capable of cleaving both C83 and C99 (Levitan et al., 2001; Francis et al., 2002; Yu et al., 2000). Processing of C83 produces a non-amyloidogenic P3 fragments, while that of C99 generates $A\beta_{1-40}$ and $A\beta_{1-42}$ at a ratio of 10:1. As some amount of Aβ is detected in healthy brain during post-mortem analysis, the cleavage of APP is regarded as a physiological event (Bojarski et al., 2008). APP is also upregulated in response to neural injury, in which APP can protect neurons against excitotoxic or ischemic insults by stabilizing the intracellular concentration ($[Ca^{2+}]_i$). Abnormal aggregated form of Aβ disrupts calcium homeostasis and increases the vulnerability of neurons to metabolic or excitotoxic injuries (Mattson et al., 1993). Different forms of Aβ causes different degree of neurotoxicity by distinct non-overlapping mechanism, which then leads to subsequent degeneration effects in AD (Pike et al., 1995; Deshpande et al., 2006). The exceptional Aβ accumulation in the brain may be attributable to the impaired clearance of Aβ under pathological condition.

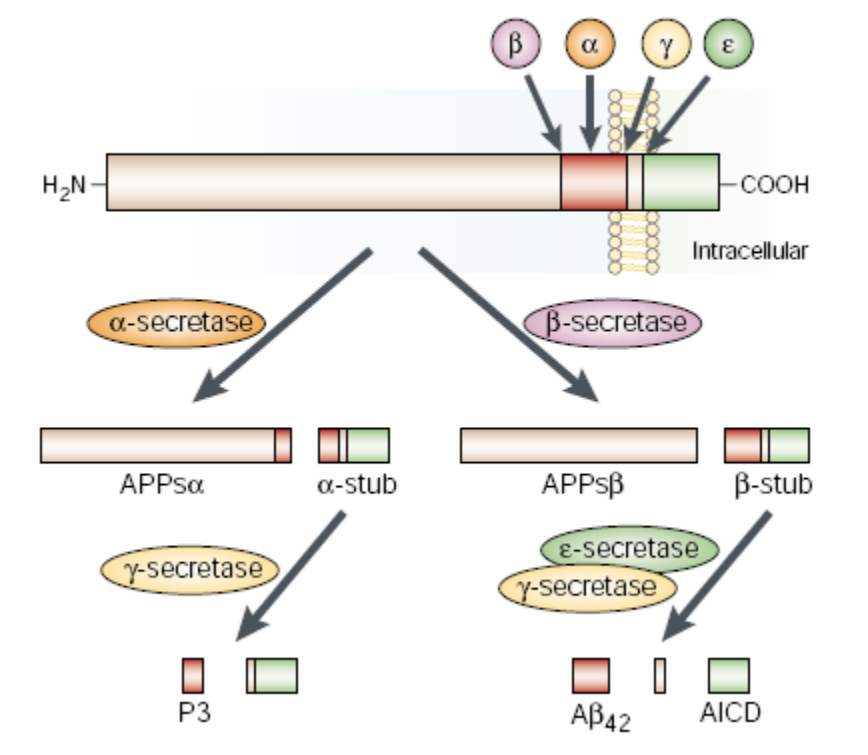

Fig. 1.1 Proteolysis of APP. APP cleavage yields P3 or Aβ in non-amyloidogenic and amyloidogenic pathway respectively. The occurrence of Aβ is due to the APP processing mediated by β-secretase, in which the amyloid peptide is not disturbed by the cleavage (LaFerla, 2002).

1.3 Calcium Homeostasis in Alzheimer

Calcium is universal as an intracellular messenger to control numerous cellular activities such as development, proliferation, protein expression, contraction, and secretion (Berridge et al., 2000). It is therefore plausible to hypothesize sustained disruption of intracellular calcium homeostasis can lead to neurodegenerative disorders including AD (Khachaturian, 1994). Upon stimulation, calcium is liberated from endoplasmic reticulum (ER) via two kinds of channels located on the ER membrane, the ryanodine receptors (RyR) and the inositol-1,4,5-triphosphate receptors (IP_3R). Small increase in $[Ca^{2+}]_i$ activates RyR, while IP_3R requires the presence of IP_3 to function. The intracellular calcium in ER is replenished by the activity of sarcoplasmic or endoplasmic reticulum Ca-ATPase (SERCA).The dynamics of intracellular calcium involving either channel plays an important role in its versatile cellular functions. The regulation of calcium release from ER is suggested to involve in Aβ toxicity and can contribute to Aβ-induced neuronal death (Ferreiro et al., 2004). Upon stimulations to IP_3R, mutations in PS1 and PS2 result in exaggerated cellular calcium signaling, revealing the significance of calcium homeostasis in AD pathogenesis (Cheung et al., 2008).

1.4 Reactive Astrogliosis in Alzheimer

Aβ toxicity and calcium dyshomeostasis in AD not only induce neuron loss and synaptic dysfunction, but also impact the adjacent glial cells, especially the astrocytes (Jorgensen et al., 1990). Reactive astrocytosis in AD is highly associated

with Aβ plaques in the molecular layer of cerebral cortex (Dickson et al., 1988; Mandybur and Chuirazzi, 1990; Wisniewski and Wegiel, 1991). These astrocytes are found at the periphery of Aβ plaques, enveloping the amyloid deposits with thick processes and fine processes deep into the lesion core (Mandybur and Chuirazzi, 1990; Sheng et al., 1994). Several cytokines and inflammatory mediators produced by reactive astrocytes may initiate or intensify the progression of neuropathology in AD. Nitric oxide (NO) is produced in astrocytes after stimulated with Aβ (Akama et al., 1998; Hensley et al., 1998; Johnstone et al., 1999). Physiological level of NO may favor synaptic efficacy by regulating the release of neurotransmitter, but excess NO causes excitotoxic brain injury (Garthwaite, 1991; Choi, 1994). Reactive astrocytes also secret proinflammatory cytokines, i.e. TNF-α and IL-1β, in Aβ-treated condition, while IL-1β in turn induces the production of IL-6 (Rossi and Bianchini, 1996; Gitter et al., 1995). These findings are further supported by the expression of IL-6 detected in the cerebral cortex from rapid autopsies of AD patients (Meda et al., 2001). Astrocytic TNF induces inflammation and degeneration in CNS of transgenic model (Akassoglou et al., 1997). Another hallmark of reactive astrocytes in AD is the upregulation of glial fibrillary acidic protein (GFAP) and S100B expressions (Mannoji et al., 1983, Crols et al., 1986; Ma et al., 1988; Griffin et al., 1989; Sheng et al., 1994). Currently there is limited evidence to illustrate the metabolic function of GFAP, but S100B, in contrast, plays versatile intracellular and extracellular roles (Donato, 1999, 2003).

1.5 S100B and GFAP in Alzheimer

S100B, formerly known as S100β, is one of the members in the S100 family, which has a molecular weight of 21 kDA existing as a homodimer consisting of two beta subunits. The two monomers possess a twofold axis of rotation and are linked together by disulphide bond (Barger et al., 1992; Drohat et al., 1996, 1998; Kilby et al., 1996). The S100B dimer appears highly stable with K_d < 500pM regarding the large amount of hydrophobic residues in the interface, suggesting most of the S100B within cell exists as dimer (Drohat et al., 1997). S100B is an Ca^{2+}-binding protein of the EF-hand type, whose function is calcium-dependent. Binding of calcium to S100B allows conformational change in S100B and the exposure of the target protein binding sites. These changes include the change in interhelical angle between helices III and IV which flank the Ca^{2+}-binding loop in the C-terminal half of S100B, the reorientation of helix III together with reorientation of the hinge region (Smith et al., 1997, 1998; Drohat et al., 1998). The C-terminal extension may also involve in the formation of target protein binding site in S100B (Drohat et al., 1996). S100B interacts with a large variety of target proteins by inhibiting their phosphorylation such as annexin II, GAP-43, GFAP, vimentin, and p53 (Hagiwara et al., 1988; Lin et al., 1994; Bianchi et al., 1996; Baudier et al., 1992). More importantly, the interaction between S100B and the microtubule (MT)-associated tau protein is critical in neurite development, whereas hyperphosphorylation of tau protein may contribute to the neurofibrillary tangles (Yu and Fraser, 2001). Both S100B and Aβ induce cell proliferation of astrocytes (Casal et al., 2004; Selinfreund et al., 1991). Elevated

S100B expression has been considered to be one of the characteristics in AD, found in earlier stages of AD, and the temporal lobe of AD patients (Griffin et al., 1989; Marshak et al., 1992; Peskind et al., 2001).

1.6 Scope of Thesis

Aβ and calcium dysregulation are suggested to be the possible causes for AD, in which complex mechanisms and pathways are involved. Calcium regulates numerous cellular activities, and its homeostasis for sure is a key factor to maintain normal metabolic functions. Dysregulation in $[Ca^{2+}]_i$ has been proven to affect glial and neuronal functions (Perea and Araque, 2005; Fiacco and McCarthy, 2006). The disturbance of intracellular calcium homeostasis has a proximal pathological role in neurodegeneration in AD, while Aβ forms neuritic plaques causing neuronal death (Smith et al., 2005; Carter and Lippa, 2001). Research in AD have been conducted extensively in neurons, but relevant studies in astrocytes have also brought in significant insights in understanding neuron-astrocyte and astrocyte-neuron interactions, the effect of reactive astrocytosis on neuron modulation in AD, and how S100B, an astrocytic protein, takes part in brain damage and neurodegeneration (Nedergaard, 1994; Pekny and Nilsson, 2005; Wang et al., 2006; Beattie et al., 2002; Rothermundt et al., 2003). There are studies stressing the significance of calcium and the effect of Aβ on glia in AD, but little evidence has shown the coupled effects of the two on astrocytes, especially in terms of reactive astrocytosis which is one of distinct features in AD.

It is the focus of this thesis to investigate the possible associated effects of Alzheimer's Amyloid-β-peptide and disrupted calcium homeostasis on reactive astrogliosis. The effect of Aβ on intracellular calcium oscillation in astrocytes is examined, for its remarkable role in communicating between neurons and astrocytes. Real-time imaging of calcium oscillation is executed to extract the initial effects of Aβ on astrocytes in intracellular calcium modulation. S100B and GFAP expressions are scrutinized in a time-dependent manner to reveal the combined influence of Aβ and calcium homeostasis.

CHAPTER 2. MATERIALS and METHODS

2.1 Astrocyte Cell Culture

Cortical cultures were prepared from postnatal day 1 Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) using methods modified from those described previously (Haughey and Mattson, 2003). Briefly, 1-day-old Sprague-Dawley rat pups were euthansized by anesthesia overdose. Their brains were removed, with the cerebral cortices dissected in sterile Krebs Hepes Buffer (KHB) (10mM HEPES, 4.2mM $NaHCO_3$, 10mM dextrose, 1.18 mM $MgSO_4 \cdot 2H_2O$, 1.18mM KH_2PO_4, 4.69mM KCl, 118mM NaCl, 1.29mM $CaCl_2$; pH 7.3). Cells were mechanically dissociated in KHB through a 70μm cell strainer (BD Biosciences, San Jose, CA) to remove viable neurons, and plated in uncoated culture dish containing Modified Eagle Medium (Cellgro/Fisher, Herndon, VA) with 10% heat-inactivated horse serum (Omega Scientific, Tarzana, CA), 1% Penicillin-Streptomycin (Gibco/Invitrogen, Carlsbad, CA), and 1% Glut-Max (Gibco/Invitrogen, Carlsbad, CA). Type 1 astrocytes were purified by the mechanical removal of less adherent cells. Culture medium was changed twice per week, and cultures were used within 2 passages. Astrocytes were removed from dishes by trypsinization and plated onto poly-D Lysine (PDL) coated 35mm glass bottom dishes (MakTek, Ashland, MA). Cells were allowed to grow to confluency prior to performing immunocytochemistry and calcium imaging studies.

2.2 GFAP and S100B Immunocytochemistry

Cultures grown on PDL-coated dishes were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes. After washing two times with PBS (5 mins each), cells were incubated in PBS containing 0.2% (v/v) Triton X-100. The cells were then washed twice again with PBS (5 mins each) before being incubated for 2 hrs with mouse monoclonal anti-S100B antibody (Sigma, S2657) or mouse anti-GFAP cocktail (BD Biosciences, 556330), diluted 1:100 and 1:400 respectively in PBS containing 10% FBS. After a double wash with PBS (5 mins each), goat anti-mouse IgG (whole molecule)-FITC (Sigma, F0257) was applied and incubated for 1 hr. The secondary antibodies were diluted 1:100 in PBS with 5% FBS prior to addition. Cells were finally washed two times with PBS and mounted with a cover slip in a drop of ProLong Gold antifade reagent counterstained with DAPI (Molecular Probes/Invitrogen, Carlsbad, CA). The cells were analyzed using an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Tokyo, Japan) using filter settings for FITC, TRITC, and DAPI. Images were taken with a customized LabVIEW program developed in the Silva Lab.

2.3 Immunocytochemistry Analysis

At least 3 sets of experiment were conducted per condition per time point, and 20 images were taken per set. Images were then analyzed by Image J employing the built-in Threshold Function. Images of negative control without primary antibody treatment were used to subtract the fluorescence background. The fluorescence

intensity of each ICC picture was then measured in Image J, while the area of fluorescence was normalized to the area occupied by the cell in the field of view to solve the problem of various cell numbers between samples.

2.4 Calcium Imaging

Cortical astrocyte cultures of ~90% confluency were washed twice with KHB and incubated with 8μM Fluo-4AM (Molecular Probes/Invitrogen, Carlsbad, CA) in KHB for 40 minutes at room temperature. Excess dye was removed by washing twice with KHB and an additional incubation of 20 minutes at room temperature was performed to equilibrate intracellular dye concentration and ensure complete intracellular esterification. In the intracellular calcium modulating experiments, pretreatment for 30 minutes of thapsigargin (Sigma, T9033) or BAPTA-AM (A1076) with a concentration of 10μM was executed. 5μM of Amyloid-β-peptide fragment 1-40 (Sigma, A1075) was used in the experiment. The time point of Aβ dosing was regarded as time 0 minute, at which the first calcium oscillation movie was taken. Real-time movie recording of calcium transient was acquired at 5 Hz using a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Each movie was 4.5 minutes long and were taken at every 15 minute intervals for 30 minutes. Visualization of Ca^{2+} indicator dye fluorescence was done using a 488 nm (FITC) filter on an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Tokyo, Japan). Images were acquired with a customized LabVIEW

program developed in the Silva Lab and processed in Image J and Matlab 7.0 (Mathworks, Natick, MA).

2.5 Quantification of Ca^{2+} Transients

Using ImageJ, an NIH funded open source morphometric application, the circle-select tool was modified to allow manual selection of individual cells on the *xy*-plane of each movie using circles of 7 pixels in diameter. Each cell was considered as an individual region of interest (ROIs). We traced all the cells from the frames in which they appeared brightest as a result of an oscillation event. The entire population of cells was traced so that the percentage of cells participating in calcium oscillation per condition per unit time could be investigated. Calcium imaging without disturbing the coordinate allows $[Ca^{2+}]_i$ tracing on the same groups of cell within the designated experimental time. An ImageJ plugin was used to calculate the average intensity for each ROI in each frame as well as the *x*-*y* coordinates of its area centroids. All of this data was organized in matrix format for post-processing analyses. A running-average filter of 3 frames was applied using a custom written Matlab routine to the data to reduce noise in the fluorescence signals. A low pass filter was employed to further smooth the oscillation noise. We thresholded data based on first derivative (positive indicates increasement) with a window size of n (10 in this experiment) frames. Sustained rise in calcium signal or activation was concluded when the first derivative values were all greater than zero for a width of 2*n. Total number of oscillations was

measured, and then normalized by the total number of cells in the movie. Frequency of oscillation was computed per cell per unit time.

CHAPTER 3. RESULTS and DISCUSSION

3.1 Effects of Aβ and Calcium Homeostasis on Astrocytic Morphology

Under control condition, astrocytes displayed the flatten and polygonal shape with few processes. The cytoskeleton labeling with GFAP indicated its relatively even distribution throughout the cell body (Fig. 3.1 A). In contrast, Aβ induced significant morphological change from the flat shape to stellate shape accompanied by increased number of extruding processes that were highly branched and filament-like (Fig. 3.1 B). While it does not affect viability, this morphological alteration induced by Aβ is one of the distinct features of reactive gliosis (Meske et al., 1998; Monnerie et al.,2005). Reactive astrocytes have an altered ability to support dendrite and axon growth, suggesting that it may take part in neuronal dystrophy in AD (Monnerie et al., 2005). The effects of Aβ on astrocyte morphologies increased with time as clearly illustrated by cells cultured under Aβ and Aβ+BAPTA-AM conditions (Fig. 3.3). Stellate cells were observed after 48 hours of incubation in Aβ, regardless of the intracellular calcium concentration. In Aβ conditions, treatment with thapsigargin and BAPTA-AM, whose functions are elevating and decreasing $[Ca^{2+}]_i$ respectively, have little effect on astrocytic morphological changes. Also, the two $[Ca^{2+}]_i$ modulators alone do not induce transformation to stellate shape. The critical role of Aβ on astrocytic morphology can be explained by the fact that the effect of Ndr, a nuclear serine/threonin protein kinase, on the regulation of cell morphology is stimulated by S100B, which is upregulated in the presence of Aβ (see below for details) (Millward

et al., 1998). The morphological studies revealed the important role of Aβ in reactive gliosis and its possible contribution to the pathology in AD.

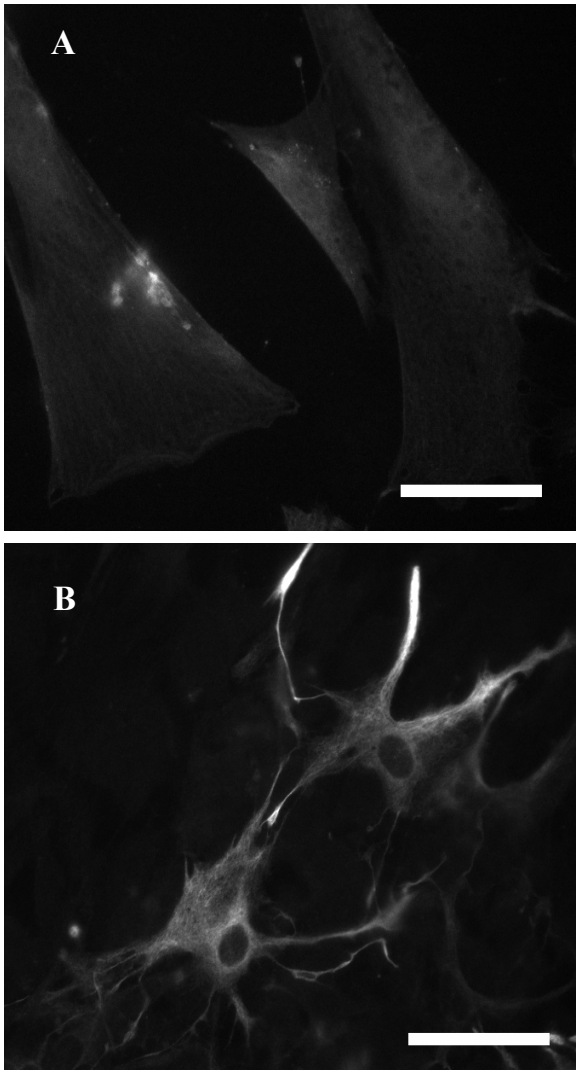

Fig. 3.1 Different morphologies of astrocytes labeled with GFAP. Under normal condition, the astrocyte expresses a flat shape with less branched processes. The cytoskeleton is more regularly distributed (A). In Aβ treated condition, the reactive astrocyte displays a stellate shape with a high number of protruding processes. Scale bar, 55μm.

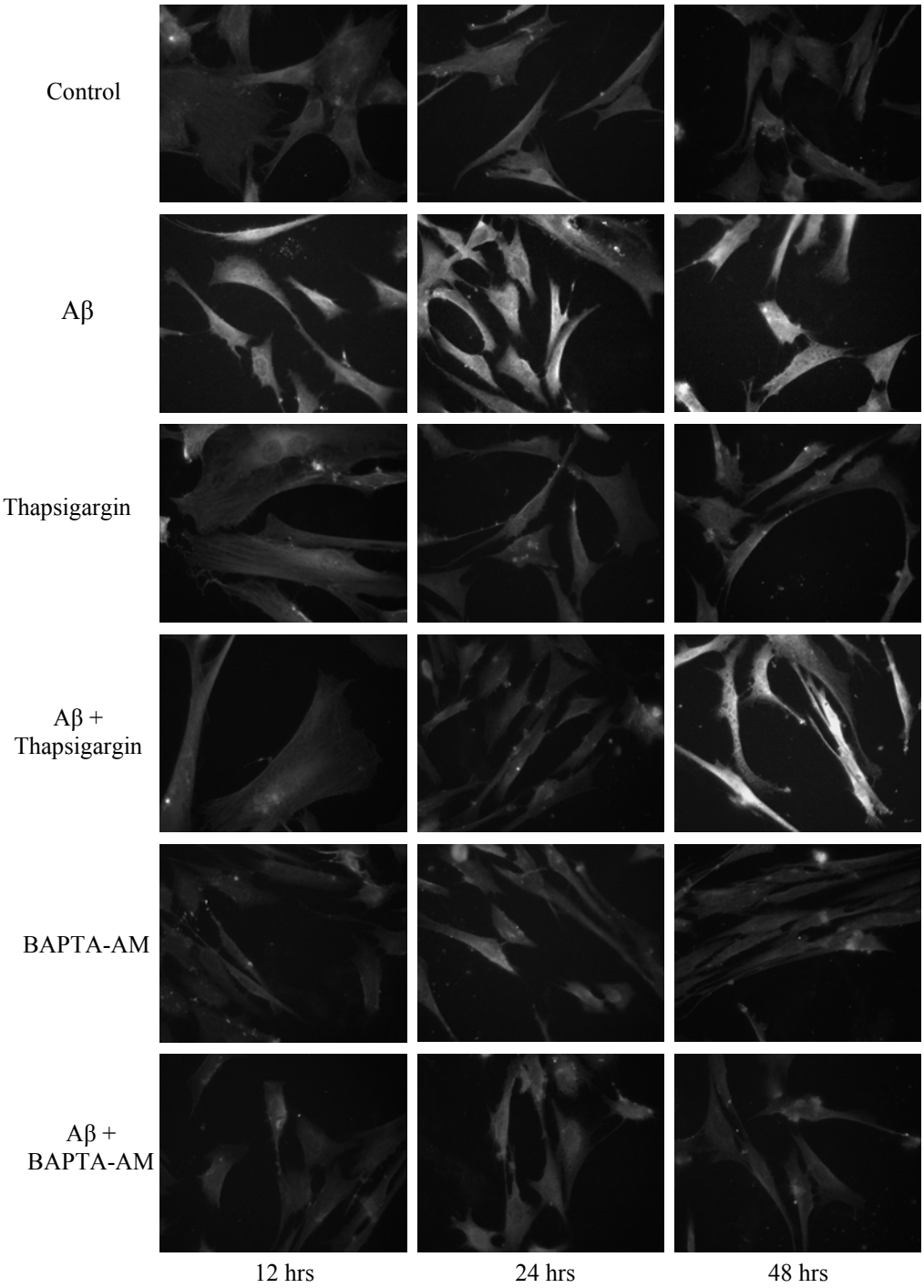

Fig. 3.2 GFAP expression in astrocytes. Different expression levels of S100B were observed under different conditions. Significant increased GFAP expressions were found at all time points of Aβ-treated condition and at 48 hrs of Aβ+Thapsigargin treated condition.

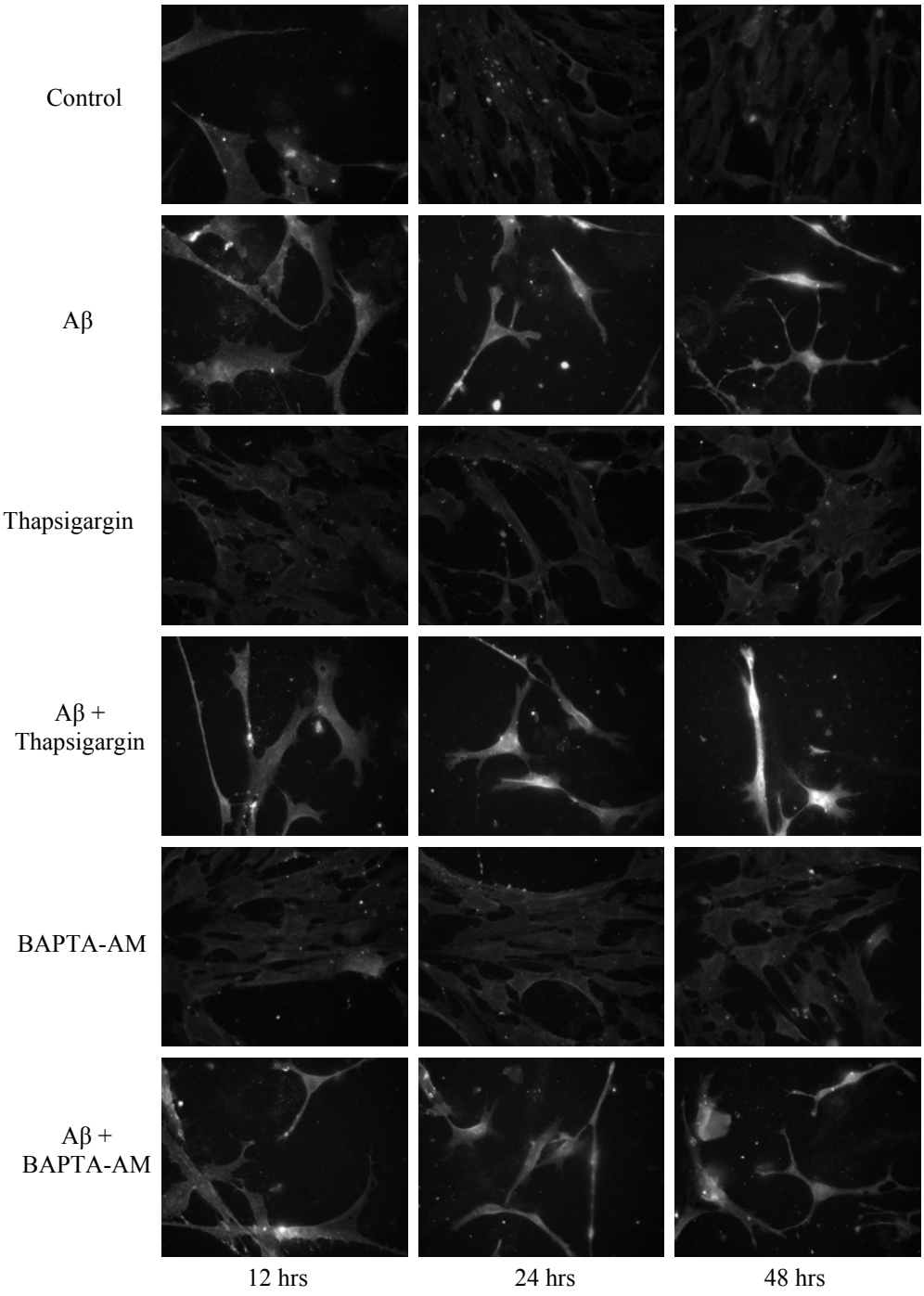

Fig. 3.3 S100B expression in astrocytes. Different expression levels of S100B were observed under different conditions. Morphological changes from flat shape to stellate shape became significant with increasing treatment time with Aβ.

3.2 Associated Effects of Aβ and Calcium Homeostasis on GFAP and S100B Expression

From histological studies, Aβ deposits are often surrounded by a wall of reactive astrocytes expressing increased levels of GFAP and S100B (Griffin et al., 1989; Jorgensen et al., 1990; Pike et al., 1995; Mrak and Griffinbc, 2001). Aβ has also been shown to upregulate GFAP and S100B expression in cultured astrocytes after 72 hours (Meske et al., 1998). It was revealed from our experiment that Aβ did increase GFAP and S100B expression and that the increased protein levels occurred as early as 12 hrs after dosing with Aβ. This early expression as a marker of reactive astrocytosis supports the fact that serum S100B level increases between 0 to 24 hours after severe cortical injury in rats (Rothoerl et al., 2000). For the Aβ-treated astrocytes, the protein expression levels of S100B and GFAP were fairly stable along the 48 hr experimental time (Fig. 3.2-3.5). However, when intracellular calcium was perturbed by either thapsigargin or BAPTA-AM in the presence of Aβ, the protein expression levels with time were significantly altered depending on the mode of intracellular calcium modulation. It was demonstrated from the experiment that mere application of thapsigargin or BAPTA-AM induced no effects on GFAP and S100B expression in the absence of Aβ. However, when thapsigargin was used to augment $[Ca^{2+}]_i$ in the presence of Aβ, S100B expression significantly increased after 24 hrs. In addition, there was a delay in the increase in GFAP expression under Aβ+thapsigargin condition as compared to Aβ alone. Furthermore, the GFAP increase, once initiated after the delay, was higher than that observed with only Aβ. Our observation on the dependence of Aβ-induced pathology on intracellular calcium is supported by

previous reports suggesting that GFAP-IR after CNS injury appears to be linked to altered intracellular calcium content (Lee et al., 2000). Previous studies have shown that S100B has inhibitory effect on microtubule assembly (Endo and Hidaka, 1983; Donato et al., 1985, 1987; Donato, 1988). S100 family members were suggested to inhibit GFAP assembly and stimulate the disassembly of preformed GFAP intermediate filaments upon elevation of $[Ca^{2+}]_i$ (Garbuglia et al., 1999a, 1999b; Ziegler et al., 1998; Bianchi et al., 1993). Decreased GFAP have been observed with increased S100B (Reali et al., 2005; Ohtaki et al., 2007). However, the function of S100B has also been suggested to be concentration dependent. Nanomolar concentration of S100B have been shown to stimulate proliferation of primary astrocytes and neuronal development while micromolar concentration can lead to production of proinflammatory cytokine IL-6 in fetal rat cortical cells and induces neuronal apoptosis (Selinfreund et al., 1991; Li et al., 2000; Wang et al., 1999). Transgenic mice with increased expression of S100B have increased GFAP expression and neurite proliferation, revealing that overexpression of S100B may lose its inhibitory effects and take on the opposite function of increasing GFAP expression. In addition to the concentration of S100B, S100-binding proteins, such as annexin VI and TRTK-12, can potentially block S100-GFAP interactions thereby inhibiting the ability of S100B to prevent GFAP polymerization into intermediate filaments (Bianchi et al., 1996; Garbuglia et al., 1998). The inhibition of S100B on GFAP assembly is intricately controlled by a combination of factors, the complexity of which is reflected from our experimental result. At 12 and 24 hrs, the expression of S100B was increased

in Aβ+thapsigargin condition where this augmentation of S100B, in a relatively low concentration, inhibited the assembly of GFAP causing the delay of GFAP expression. Later at 48 hrs, both GFAP and S100B were highly expressed, which may be attributed to the loss of the inhibitory function of S100B at higher concentration. It also may be due to other influences of S100-binding peptides, but to investigate this hypothesis, further experiments are necessary. In order to test if high concentration of S100B resulting from Aβ and perturbed calcium homeostasis increase GFAP levels, the thapsigargin supplement experiment was conducted where an additional dose of the drug was applied at 24 hrs (Fig. 3.6). The results show that further elevated $[Ca^{2+}]_i$ leads to even higher expression of S100B and GFAP. If S100B is suggested to inhibit GFAP in a linearly concentration-independent manner, GFAP level should be reduced. However, significantly increased GFAP level was reported. This piece of data strongly suggests S100B only inhibits GFAP in low concentration while it enhances GFAP at higher concentrations. Another interesting finding is that the level of S100B in cortical astrocytes detected via ICC in the presence of Aβ is calcium-dependent. At 24 and 48 hrs, Aβ+thapsigargin significantly increases S100B expression compared to just Aβ, while Aβ+BAPTA-AM reduces S100B expression. The effect of $[Ca^{2+}]_i$ modulation with Aβ on S100B level at 12 hrs is mild, suggesting that perturbation of calcium homeostasis needs certain period of time to take effect. To conclude, Aβ is the primary component to increase S100B and GFAP expression, while intracellular calcium homeostasis is a modulator of Aβ's action in astrogliosis.

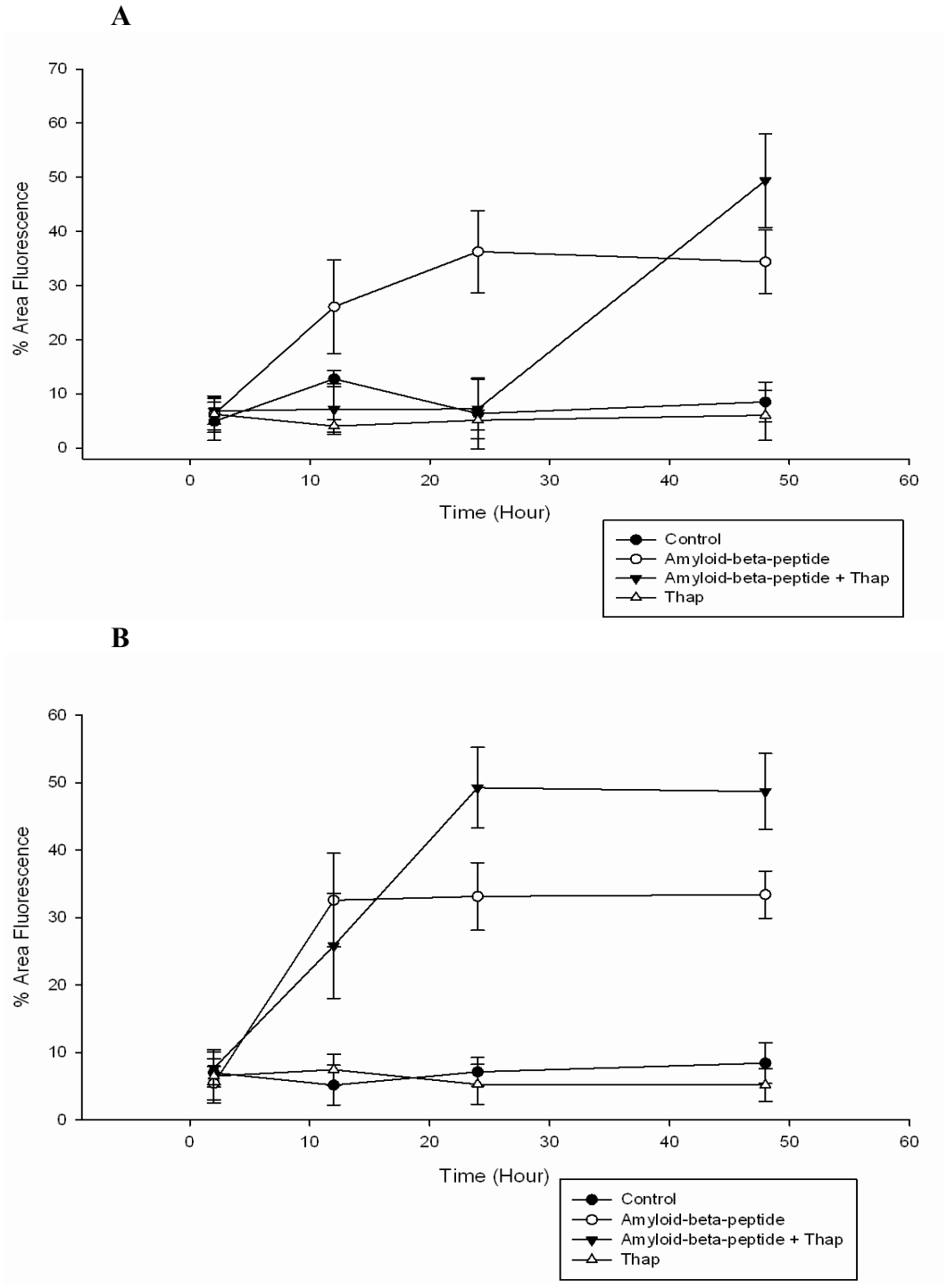

Fig. 3.4 GFAP and S100B expression level with time mediated by Thapsigargin. GFAP and S100B protein expressions with time were shown in A and B respectively. Aβ causes upregulation of the two proteins as early as 12 hrs. Disruption of calcium homeostasis by Thapsigargin displays effects on expression only in Aβ-treated conditions.

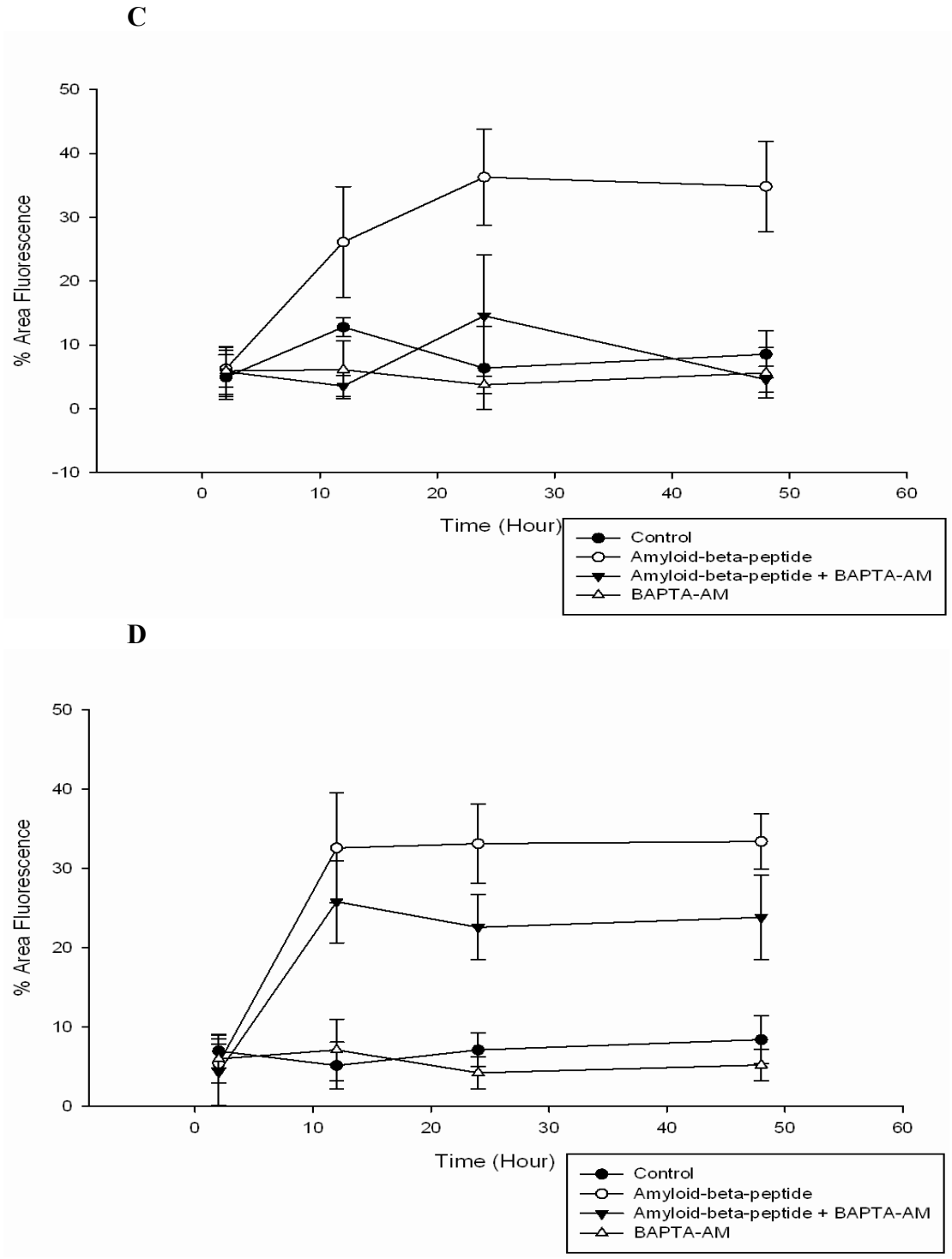

Fig. 3.5 GFAP and S100B expression level with time mediated by BAPTA-AM. GFAP and S100B protein expressions with time were shown in C and D respectively. Disruption of calcium homeostasis by BAPTA-AM with Aβ has a more significant effect on S100B than GFAP expression (Fig. 3.4 B and D versus Fig. 3.4 A and C).

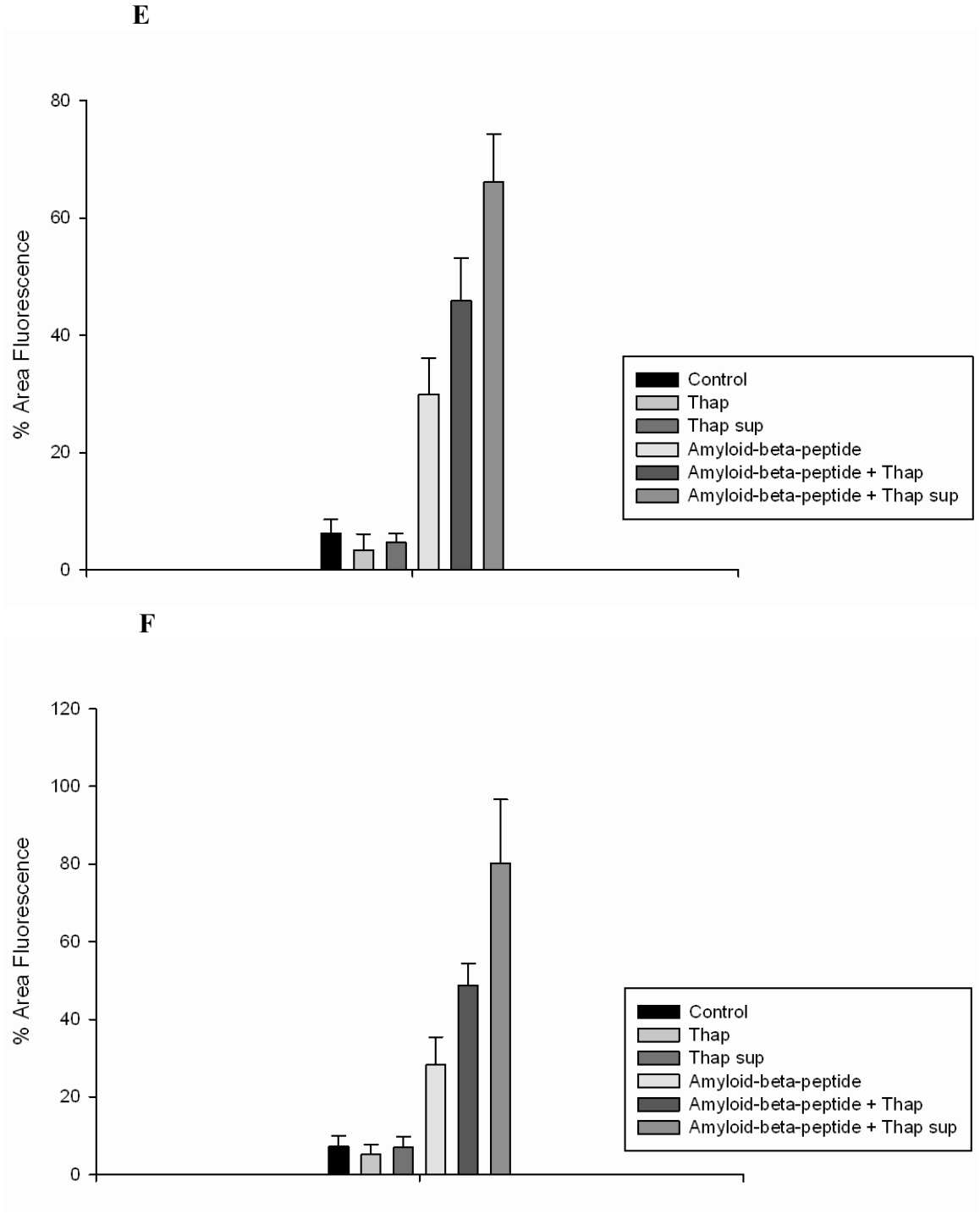

Fig. 3.6 GFAP and S100 expression level with Thapsigargin supplement. Expressions of GFAP and S100B at 48 hrs in Aβ-treated conditions were calcium-dependent (E and F respectively). Further elevation of $[Ca^{2+}]_i$ with Aβ induces a significant upregulation of GFAP and S100B protein expression.

3.3 Effect of Aβ on Intracellular Calcium Oscillations and Spontaneous Formation of Intercellular Calcium Waves

Intracellular calcium oscillation in astrocytes have been shown to persist for a period of 5-30 mins after chemical stimulation with glutamate, with frequencies ranging from 10 to 110 mHz (Cornell-Bell et al., 1990). Within a duration of 30 minutes, the oscillation frequencies of all conditions in our experiment lie within the physiological range as previously reported. The range of frequencies in controls, Aβ, Aβ+thapsigargin, and Aβ+BAPTA-AM in mHz are 21.80-43.85, 37.09-65.10, 13.88-18.11, and 20.24-46.74 respectively (Fig. 3.7). A slight reduction in the number of oscillations in consecutive recordings is noticed under both control and experimental conditions, which may be due to photobleaching as a result of repeated imaging sessions, each lasting 4.5 minutes, done under the same field of view for consistency. The viability of cells was shown to be robust by comparing the oscillation frequency per cell and the total number of cell participating between the movies of controls at 0 min and controls at 5 hrs after being kept in KHB in room temperature (Data not shown). Aβ causes significant increase in intracellular calcium oscillation of cortical astrocytes and this increase can be suppressed by pretreatment with thapsigargin and BAPTA-AM. While $[Ca^{2+}]_i$ stores in endoplasmic reticulum are replenished by the activity of SERCA, thapsigargin discharges $[Ca^{2+}]_i$ stores by specific inhibition of SERCA pumps, resulting the elevation of $[Ca^{2+}]_i$ and decreased calcium oscillation (Thastrup et al., 1990; Lytton et al., 1991). The inhibitory effects of thapsigargin suggest that mechanisms associated with intracellular release of calcium from endogenous intracellular calcium store, rather than ion fluxes across the plasma

membrane, contribute to the astrocytic oscillations (Johnston et al., 2006). BAPTA-AM chelates intracellular calcium thereby affecting oscillation. The effects of applying thapsigargin and BAPTA-AM are reflected in Fig. 3.7, showing that the two $[Ca^{2+}]_i$ modulators suppress the augmented calcium oscillation in Aβ-treated astrocytes.

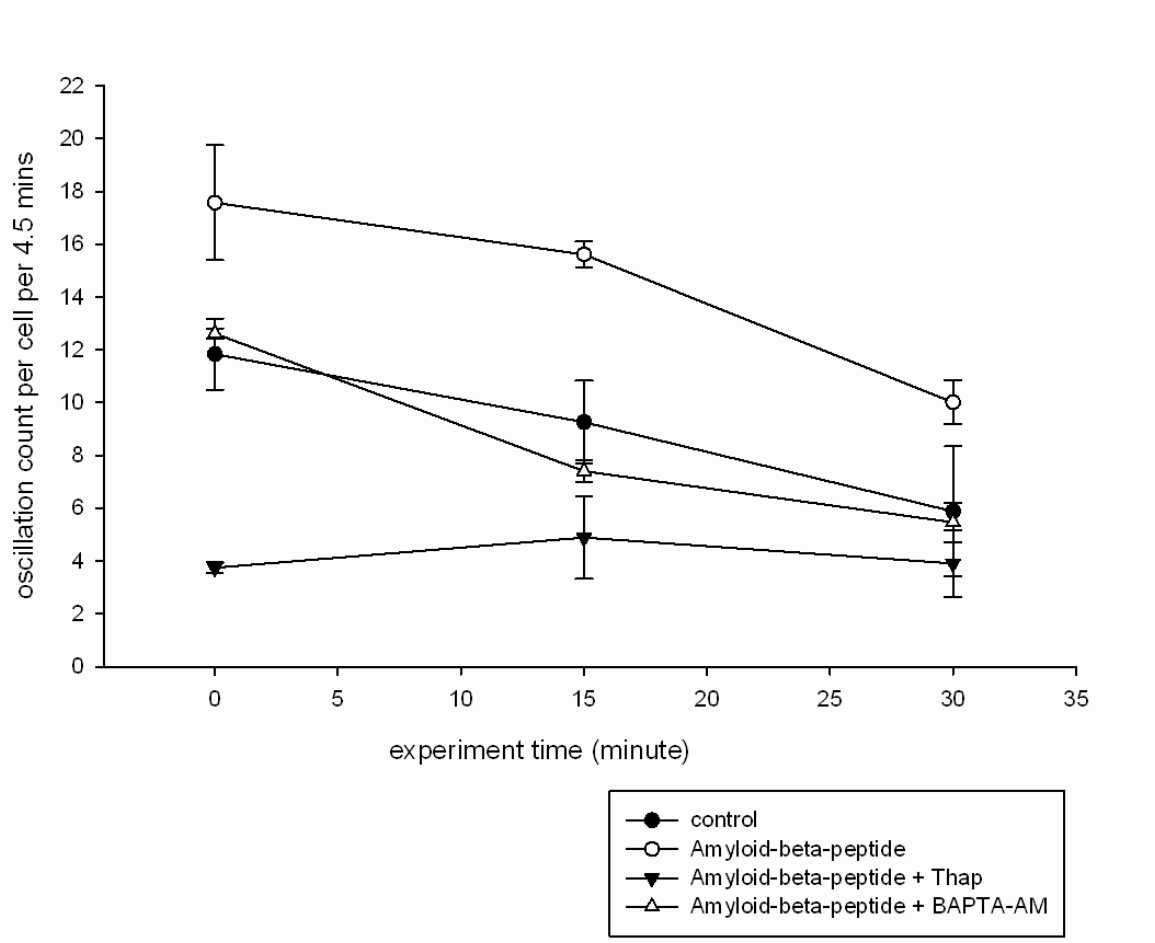

Fig. 3.7 Calcium oscillation count per astroyte per unit time. Aβ increases intracellular calcium oscillation in astrocytes. This oscillation augmentation can be suppressed by Thapsigargin and BAPTA-AM pretreatment.

Intercellular calcium wave in astrocytes can be induced by chemical stimuli such as ATP, glutamate, or mechanical stimulation (Fiacco and McCarthy, 2006). Spontaneous astrocytic calcium wave can be initiated by lowering extracellular calcium (Zanotti and Charles, 1997). Aβ has been shown to increase wave amplitude, distance, and velocity upon mechanical stimulation (Haughey and Mattson, 2003). Here, we report the presence of spontaneous calcium waves in Aβ-treated astrocytes. The calcium waves can propagate in various forms, i.e. radial and unidirectional (Fig. 3.8, 3.9). The traveling distance of radial and unidirectional waves is 141.1 ± 30.9 μm and 160.9 ± 22.5 μm respectively (Mean ± SD). These values agree with previous studies which reported the traveling distance of calcium wave in glial cells to be several hundred micrometers (Cornell-Bell et al., 1990; Newman and Zahs, 1997). The velocity of the radial and unidirectional waves are 6.03 ± 1.60 μm/sec and 4.66 ± 0.48 μm/sec respectively (Mean ± SD), laying within the previously reported value of less than 50 μm/sec (Haydon, 2001; Newman, 2003). In fact, pharmacologically and mechanically-induced intercellular calcium wave in cultured astrocytes has a mean of velocity of 18 μm/sec; electrically stimulated brain slice preparations has a mean wave velocity of 15 μm/sec (Scemes and Giaume, 2006, reference therein). Aβ enhanced the velocity of mechanically stimulated calcium waves from 10 μm/sec in controls to 38 μm/sec in Aβ-treated astrocytes (Haughey and Mattson, 2003). Taken together, these reports and our observation suggest that the velocity of astrocytic calcium wave can vary depending on the methods and conditions that it is produced, i.e. spontaneous wave versus stimulus-induced wave. From our data, the velocities of spontaneous

waves are smaller than the reported values in stimulus-induced waves, suggesting that potential calcium waves under physiological conditions may actually travel at a lower speed. The discrepancy in velocity of intercellular waves can also be attributable to the strength and type of stimuli. The traveling distance of wave is also highly variable. For example, intercellular calcium wave spread to twice as many cortical and hippocampal astrocytes as between astrocytes from the hypothalamus and brain stem (Blomstrand et al., 1999). Similarly, mechanically induced calcium waves in cultured telencephalic astrocytes spread radially to an area of 450 μm^2 while waves traveling between cultured diencephalic astrocytes spread unevenly to an area of 130 μm^2 (Peters et al., 2005). From our experiment, the two forms of intercellular calcium waves, i.e. unidirectional and radial, suggested the existence of plastic functional circuits between astrocytes, as calcium elevations may spread to some but not all adjacent astrocytes (Sul et al., 2004). Intercellular calcium waves take highly specific pathways instead of going for the shortest distance to target cells. It reveals the importance to understand the structure and connectivity in glial and neuronal networks in order to fully understand their physiological significance in neuropathology.

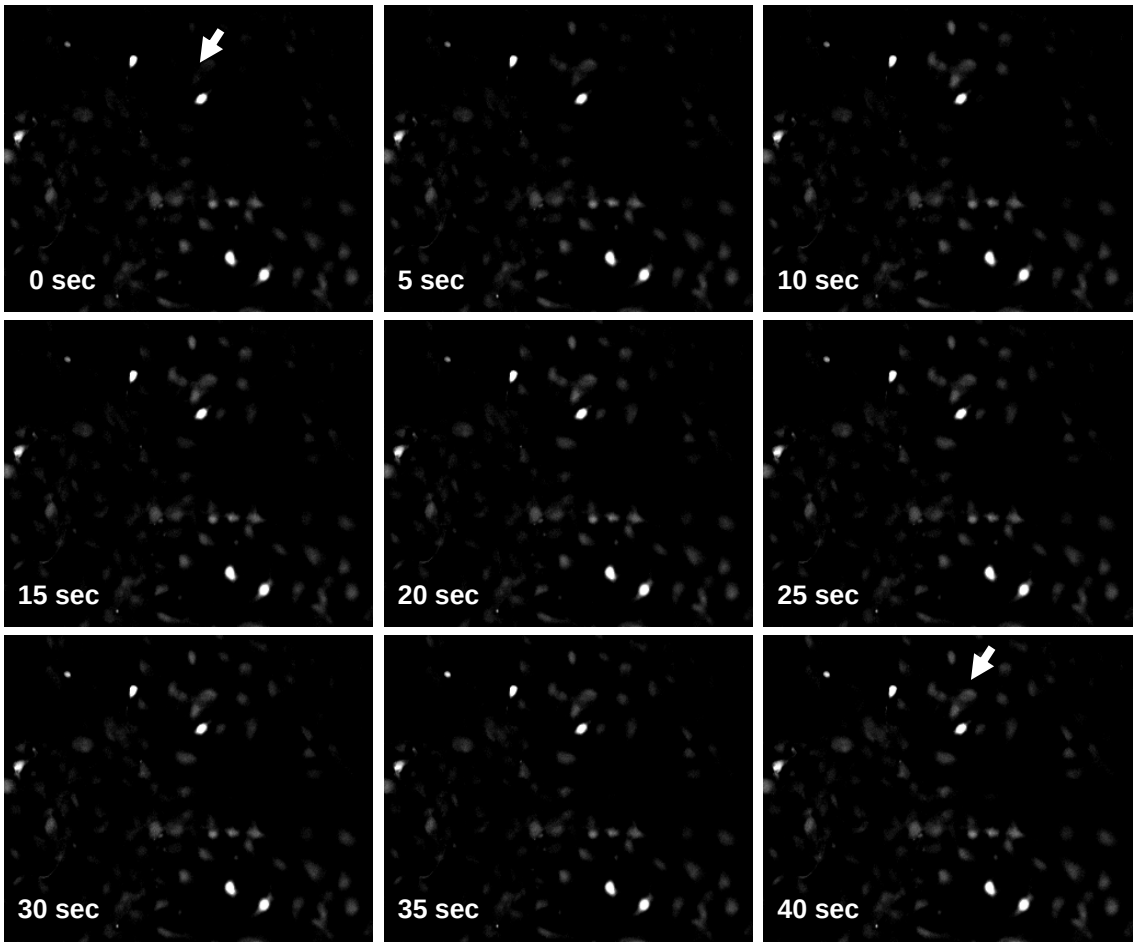

Fig. 3.8 Radial intercellular calcium wave in astrocytes. Spontaneous intercellular calcium wave was observed in Aβ-treated condition. White arrow indicates the initiation point of the wave.

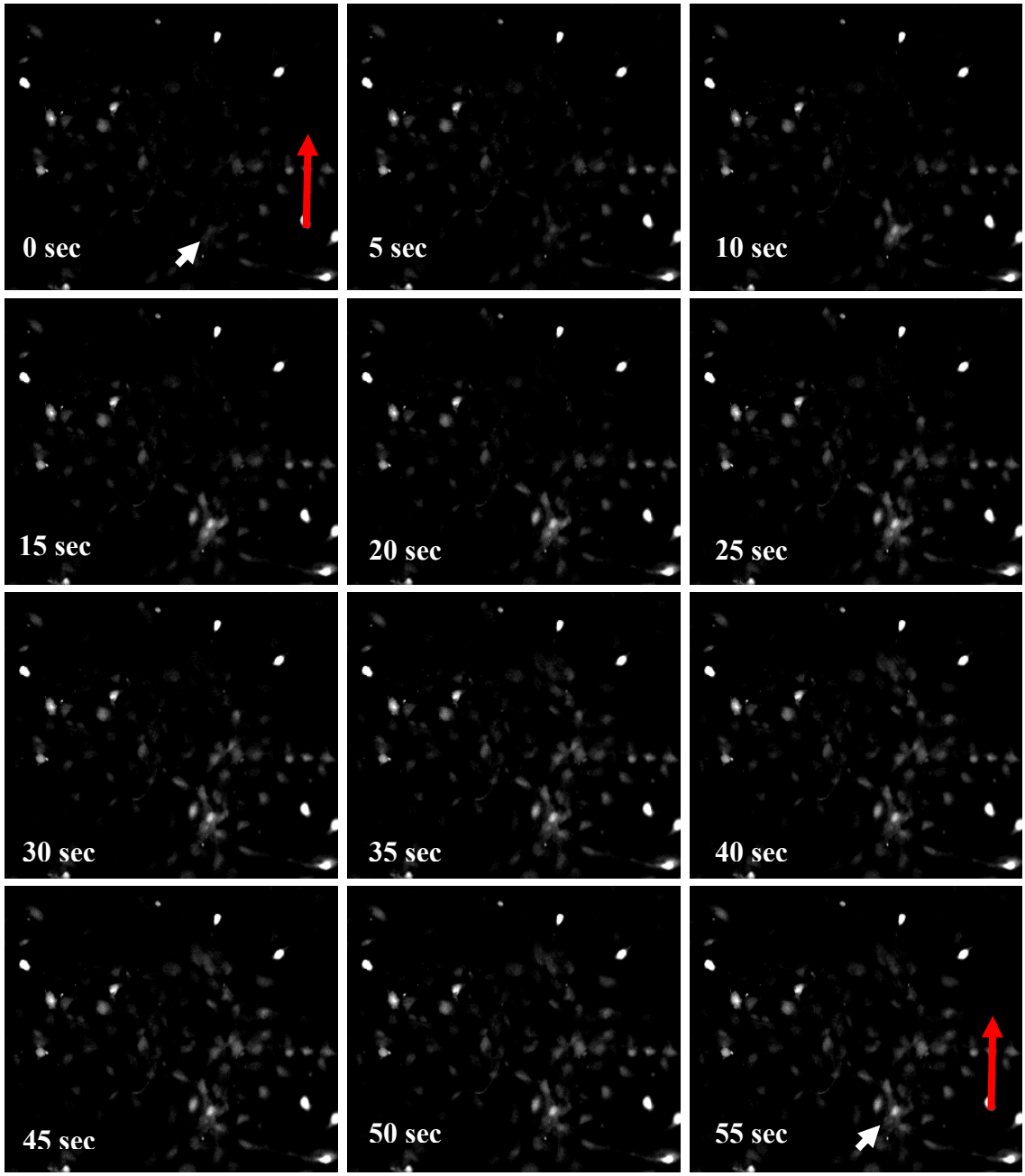

Fig. 3.9 Unidirectional intercellular calcium wave in astrocytes. Spontaneous intercellular calcium wave was observed in Aβ-treated condition. White arrow indicates the initiation point of the wave; red arrow indicates the direction of traveling wave.

Intracellular calcium oscillation and intercellular calcium wave are recognized as modes of signaling in astrocytes where they play an important role in modulating the activity and survival of neurons (Charles et al., 1991; Bezzi et al., 2001; Blanc et al., 1998; Verkhratsky and kettenmann, 1996). Spontaneous calcium oscillations are observed in the neocortex (Aguado et al., 2002; Peters et al., 2003). Stimulation of neuronal afferents triggers periodic $[Ca^{2+}]_i$ oscillations in astrocytes which represents a highly plastic signaling system underlying the bidirectional communication between neurons and astrocytes (Pasti et al., 1997). It takes part in the development and maturation of neural network by releasing glutamate on adjacent neuronal processes, thereby manipulating the excitability of the neighboring cells and synaptic transmission (Kang et al., 1998; Nett et al., 2002; Parri et al., 2001, Zhang et al., 2004). Glutamate has been known as a key molecule in neuron-astrocyte and astrocyte-neuron signaling where the release of glutamate by astrocyte is calcium-dependent (Wang et al., 2006; Angulo et al., 2004; Parpura et al., 1994). Increased $[Ca^{2+}]_i$ in astrocytes is required for its release of glutamate (Araque et al., 1998a, 1998b). It is thought that the administration of Aβ, a peptide capable of forming Ca^{2+}-permeable channels in artificial membranes and likely intact neuronal membranes as well, can disrupt the intracellular calcium homeostasis (Arispe et al., 1993a, 1993b). Fig. 3.7 illustrates the immediate effect of Aβ on calcium oscillation in astrocytes, in which Aβ increased the oscillation frequency of the cells. From the movies taken, it was clear that the frequency of oscillation in Aβ-treated astrocytes was significantly higher than the controls while accompanied with a slight decrease in amplitude. The

greater effect of Aβ on the calcium oscillatory frequency suggests that it is a frequency rather than an amplitude dependent mode of communication where the information to be transferred regarding the nature and properties of the initial stimulus, i.e. the intensity, is preserved and converted into a modified frequency of oscillations (Woods et al., 1986; Jacob 1990). Aβ elevates $[Ca^{2+}]_i$ in both neurons and astrocytes with increased sensitivity to glutamate and calcium ionophore neurotoxicity (Mattson and Rydel, 1992; Stix and Reiser, 1998). It thereby induces a change in the mode of calcium signaling from intracellular oscillation to intercellular calcium wave. This is also supported by previous studies showing that glutamate at lower concentration (below 1μM) causes asynchronous and localized intracellular oscillation in astrocytes while at higher concentration (10-100 μM) intercellular calcium waves propagating over long distances (up to several hundred micrometers) can be observed (Cornell-Bell et al., 1990; Newman and Zahs, 1997). The phenomenon was attributed to the increased release of glutamate from astrocytes due to the presence of Aβ that elevates $[Ca^{2+}]_i$, together with the enhanced sensitivity to glutamate caused by Aβ. An alternate explanation is that the increased secretion of cytokines from astrocytes induced in Aβ enhances glutamate release thereby causing the occurrence of calcium wave (Ida et al., 2008). Our observation that spontaneously forming calcium waves are present only in Aβ-treated astrocyte cultures provides invaluable insights into the connection between the effect of Aβ and calcium homeostasis with reactive astrocytosis. Different modes of calcium signaling would likely carry out different metabolic functions under various conditions. In this case, we observed pure calcium oscillations in controls

versus a switch to a hybrid of calcium wave and oscillation in Aβ-treated condition. While much detail in the down stream consequences of these two types of calcium signaling is still unknown, it is certain that both modes of signaling are critical in connecting neurons and astrocytes (Dani et al., 1992; Pasti et al., 1997). Further investigation on calcium dynamics is needed in order to reveal its physiological significance, especially in neuropathological models.

CHAPTER 4. CONCLUSIONS and FUTURE DIRECTIONS

In order to scrutinize the possible associated effect of Alzheimer's Amyloid-β-peptide and disrupted calcium homeostasis on reactive astrogliosis, we explored how Aβ act on reactive astrocytes modulated by $[Ca^{2+}]_{i,}$ which was illustrated by the protein expression of two reactive gliosis markers, S100B and GFAP. The expression of S100B induced by Aβ was found to be calcium-dependent. GFAP expression level in Aβ condition was also modulated by $[Ca^{2+}]_i$ and in addition possibly by S100B in a concentration-dependent manner. Aβ is the primary component to increase S100B and GFAP expression, while calcium homeostasis acts as a modulator on Aβ's activity.

In the calcium experiment, Aβ was found to increase intracellular calcium oscillations in astrocytes. This augmentation of oscillation could be suppressed by calcium modulations with thapsigargin and BAPTA-AM. The oscillation frequencies in all conditions laid within the physiological range, suggesting astrocytes modulate calcium signaling in order to compensate the changing need of metabolic activity under various conditions. More importantly, spontaneous intercellular calcium waves were first observed in and only in Aβ condition, in which the wave was presented in two forms, radial and unidirectional. Both the traveling distance and velocity laid within the physiological range, while the wave velocity was noticed to be smaller than those in stimulus-induced wave as reported before. The highly specific pathway of calcium waves suggested the plastic function circuits between astrocytes. This altered

response in calcium signaling in Aβ model was suggested to be a type of astrocytic accommodations in AD.

The interactions between Aβ, calcium homeostasis, and reactive astrocytosis are complex as expected, yet underlying the possible causes of the most devastating neurodegenerative disease and its corresponding therapy. Further investigations in astrocytes on GFAP and S100B expressions with respect to calcium signaling, physiological significance of calcium oscillation and wave, mechanism of the calcium signaling transformation, and how Aβ plays the central roles in connecting all three aspects will reveal the astrocyte-specific pathologies and lead to the progress of targeted therapies for pathways in which reactive astrocytes participate.

REFERENCES

Aguado, F., J. F. Espinosa-Parrilla, et al. (2002). "Neuronal activity regulates correlated network properties of spontaneous calcium transients in astrocytes in situ." J Neurosci **22**(21): 9430-44.

Akama, K. T., C. Albanese, et al. (1998). "Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism." Proc Natl Acad Sci U S A **95**(10): 5795-800.

Akassoglou, K., L. Probert, et al. (1997). "Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice." J Immunol **158**(1): 438-45.

Angulo, M. C., A. S. Kozlov, et al. (2004). "Glutamate released from glial cells synchronizes neuronal activity in the hippocampus." J Neurosci **24**(31): 6920-7.

Araque, A., V. Parpura, et al. (1998). "Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons." Eur J Neurosci **10**(6): 2129-42.

Araque, A., R. P. Sanzgiri, et al. (1998). "Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons." J Neurosci **18**(17): 6822-9.

Arcuino, G., J. H. Lin, et al. (2002). "Intercellular calcium signaling mediated by point-source burst release of ATP." Proc Natl Acad Sci U S A **99**(15): 9840-5.

Arispe, N., H. B. Pollard, et al. (1993). "Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1-40)] in bilayer membranes." Proc Natl Acad Sci U S A **90**(22): 10573-7.

Arispe, N., E. Rojas, et al. (1993). "Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum." Proc Natl Acad Sci U S A **90**(2): 567-71.

Barger, S. W., S. R. Wolchok, et al. (1992). "Disulfide-linked S100 beta dimers and signal transduction." Biochim Biophys Acta **1160**(1): 105-12.

Baskin, F., R. N. Rosenberg, et al. (2001). "Platelet APP isoform ratios in asymptomatic young adults expressing an AD-related presenilin-1 mutation." J Neurol Sci **183**(1): 85-8.

Baudier, J., C. Delphin, et al. (1992). "Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein." Proc Natl Acad Sci U S A **89**(23): 11627-31.

Beattie, E. C., D. Stellwagen, et al. (2002). "Control of synaptic strength by glial TNFalpha." Science **295**(5563): 2282-5.

Berridge, M. J., P. Lipp, et al. (2000). "The versatility and universality of calcium signalling." Nat Rev Mol Cell Biol **1**(1): 11-21.

Bezzi, P., M. Domercq, et al. (2001). "Neuron-astrocyte cross-talk during synaptic transmission: physiological and neuropathological implications." Prog Brain Res **132**: 255-65.

Bezzi, P., V. Gundersen, et al. (2004). "Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate." Nat Neurosci **7**(6): 613-20.

Bianchi, R., M. Garbuglia, et al. (1996). "S-100 (alpha and beta) binding peptide (TRTK-12) blocks S-100/GFAP interaction: identification of a putative S-100 target epitope within the head domain of GFAP." Biochim Biophys Acta **1313**(3): 258-67.

Bianchi, R., I. Giambanco, et al. (1993). "S-100 protein, but not calmodulin, binds to the glial fibrillary acidic protein and inhibits its polymerization in a Ca(2+)-dependent manner." J Biol Chem **268**(17): 12669-74.

Blanc, E. M., A. J. Bruce-Keller, et al. (1998). "Astrocytic gap junctional communication decreases neuronal vulnerability to oxidative stress-induced disruption of Ca2+ homeostasis and cell death." J Neurochem **70**(3): 958-70.

Blomstrand, F., N. D. Aberg, et al. (1999). "Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions." Neuroscience **92**(1): 255-65.

Bojarski, L., J. Herms, et al. (2008). "Calcium dysregulation in Alzheimer's disease." Neurochem Int **52**(4-5): 621-33.

Brookmeyer, R., E. Johnson, et al. (2007). "Forecasting the global burden of Alzheimer's disease." Alzheimer's and Dementia **3**(3): 186-191.

Buxbaum, J. D., K. N. Liu, et al. (1998). "Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor." J Biol Chem **273**(43): 27765-7.

Buxbaum, J. D., A. A. Ruefli, et al. (1994). "Calcium regulates processing of the Alzheimer amyloid protein precursor in a protein kinase C-independent manner." Proc Natl Acad Sci U S A **91**(10): 4489-93.

Carter, J. and C. F. Lippa (2001). "Beta-amyloid, neuronal death and Alzheimer's disease." Curr Mol Med **1**(6): 733-7.

Casal, C., J. Serratosa, et al. (2004). "Effects of beta-AP peptides on activation of the transcription factor NF-kappaB and in cell proliferation in glial cell cultures." Neurosci Res **48**(3): 315-23.

Charles, A. C., J. E. Merrill, et al. (1991). "Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate." Neuron **6**(6): 983-92.

Cheung, K. H., D. Shineman, et al. (2008). "Mechanism of Ca2+ disruption in Alzheimer's disease by presenilin regulation of InsP(3) receptor channel gating." Neuron **58**(6): 871-83.

Choi, D. W. (1994). "Glutamate receptors and the induction of excitotoxic neuronal death." Prog Brain Res **100**: 47-51.

Cornell-Bell, A. H., S. M. Finkbeiner, et al. (1990). "Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling." Science **247**(4941): 470-3.

Crols, R., J. Saerens, et al. (1986). "Increased GFAp levels in CSF as a marker of organicity in patients with Alzheimer's disease and other types of irreversible chronic organic brain syndrome." J Neurol **233**(3): 157-60.

Dani, J. W., A. Chernjavsky, et al. (1992). "Neuronal activity triggers calcium waves in hippocampal astrocyte networks." Neuron **8**(3): 429-40.

De Strooper, B., W. Annaert, et al. (1999). "A presenilin-1-dependent [gamma]-secretase-like protease mediates release of Notch intracellular domain." **398**(6727): 518-522.

De Strooper, B., P. Saftig, et al. (1998). "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein." **391**(6665): 387-390.

Deshpande, A., E. Mina, et al. (2006). "Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons." J Neurosci **26**(22): 6011-8.

Dickson, D. W., J. Farlo, et al. (1988). "Alzheimer's disease. A double-labeling immunohistochemical study of senile plaques." Am J Pathol **132**(1): 86-101.

Donato, R. (1988). "Calcium-independent, pH-regulated effects of S-100 proteins on assembly-disassembly of brain microtubule protein in vitro." J Biol Chem **263**(1): 106-10.

Donato, R. (1999). "Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type." Biochim Biophys Acta **1450**(3): 191-231.

Donato, R. (2003). "Intracellular and extracellular roles of S100 proteins." Microsc Res Tech **60**(6): 540-51.

Donato, R., F. Battaglia, et al. (1987). "Characteristics of the effect of S-100 proteins on the assembly-disassembly of brain microtubule proteins at alkaline pH in vitro." Cell Calcium **8**(4): 299-313.

Donato, R., T. Isobe, et al. (1985). "S-100 proteins and microtubules: analysis of the effects of rat brain S-100 (S-100b) and ox brain S-100a0, S-100a and S-100b on microtubule assembly-disassembly." FEBS Lett **186**(1): 65-9.

Drohat, A. C., J. C. Amburgey, et al. (1996). "Solution structure of rat apo-S100B(beta beta) as determined by NMR spectroscopy." Biochemistry **35**(36): 11577-88.

Drohat, A. C., D. M. Baldisseri, et al. (1998). "Solution structure of calcium-bound rat S100B(betabeta) as determined by nuclear magnetic resonance spectroscopy." Biochemistry **37**(9): 2729-40.

Drohat, A. C., E. Nenortas, et al. (1997). "Oligomerization state of S100B at nanomolar concentration determined by large-zone analytical gel filtration chromatography." Protein Sci **6**(7): 1577-82.

Eckert, A., K. Schindowski, et al. (2001). "Alzheimer's disease-like alterations in peripheral cells from presenilin-1 transgenic mice." Neurobiol Dis **8**(2): 331-42.

Endo, T. and H. Hidaka (1983). "Effect of S-100 protein on microtubule assembly-disassembly." FEBS Lett **161**(2): 235-8.

Evin, G., A. Zhu, et al. (2003). "Proteolytic processing of the Alzheimer's disease amyloid precursor protein in brain and platelets." J Neurosci Res **74**(3): 386-92.

Ferreiro, E., C. R. Oliveira, et al. (2004). "Involvement of endoplasmic reticulum Ca2+ release through ryanodine and inositol 1,4,5-triphosphate receptors in the neurotoxic effects induced by the amyloid-beta peptide." J Neurosci Res **76**(6): 872-80.

Ferri, C. P., M. Prince, et al. (2005). "Global prevalence of dementia: a Delphi consensus study." The Lancet **366**(9503): 2112-2117.

Fiacco, T. A. and K. D. McCarthy (2006). "Astrocyte calcium elevations: properties, propagation, and effects on brain signaling." Glia **54**(7): 676-90.

Francis, R., G. McGrath, et al. (2002). "aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation." Dev Cell **3**(1): 85-97.

Garbuglia, M., M. Verzini, et al. (1998). "Annexin VI binds S100A1 and S100B and blocks the ability of S100A1 and S100B to inhibit desmin and GFAP assemblies into intermediate filaments." Cell Calcium **24**(3): 177-91.

Garbuglia, M., M. Verzini, et al. (1999). "Role of the C-terminal extension in the interaction of S100A1 with GFAP, tubulin, the S100A1- and S100B-inhibitory peptide, TRTK-12, and a peptide derived from p53, and the S100A1 inhibitory effect on GFAP polymerization." Biochem Biophys Res Commun **254**(1): 36-41.

Garbuglia, M., M. Verzini, et al. (1999). "The calcium-modulated proteins, S100A1 and S100B, as potential regulators of the dynamics of type III intermediate filaments." Braz J Med Biol Res **32**(10): 1177-85.

Garthwaite, J. (1991). "Glutamate, nitric oxide and cell-cell signalling in the nervous system." Trends Neurosci **14**(2): 60-7.

Gitter, B. D., L. M. Cox, et al. (1995). "Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells." Proc Natl Acad Sci U S A **92**(23): 10738-41.

Griffin, W. S., L. C. Stanley, et al. (1989). "Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease." Proc Natl Acad Sci U S A **86**(19): 7611-5.

Hagiwara, M., M. Ochiai, et al. (1988). "Modulation of tyrosine phosphorylation of p36 and other substrates by the S-100 protein." J Biol Chem **263**(13): 6438-41.

Hardy, J. and K. Gwinn-Hardy (1998). "Genetic classification of primary neurodegenerative disease." Science **282**(5391): 1075-9.

Hardy, J. and D. J. Selkoe (2002). "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics." Science **297**(5580): 353-6.

Haughey, N. J. and M. P. Mattson (2003). "Alzheimer's amyloid beta-peptide enhances ATP/gap junction-mediated calcium-wave propagation in astrocytes." Neuromolecular Med **3**(3): 173-80.

Haydon, P. G. (2001). "GLIA: listening and talking to the synapse." Nat Rev Neurosci **2**(3): 185-93.

Hensley, K., J. M. Carney, et al. (1994). "A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease." Proc Natl Acad Sci U S A **91**(8): 3270-4.

Hensley, K., M. L. Maidt, et al. (1998). "Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation." J Neurosci **18**(20): 8126-32.

Ida, T., M. Hara, et al. (2008). "Cytokine-induced enhancement of calcium-dependent glutamate release from astrocytes mediated by nitric oxide." Neuroscience Letters **432**(3): 232-236.

Jacob, R. (1990). "Calcium oscillations in electrically non-excitable cells." Biochim Biophys Acta **1052**(3): 427-38.

Johnston, J. M., P. Burnett, et al. (2006). "Calcium oscillations in type-1 astrocytes, the effect of a presenilin 1 (PS1) mutation." Neurosci Lett **395**(2): 159-64.

Johnstone, M., A. J. Gearing, et al. (1999). "A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced." J Neuroimmunol **93**(1-2): 182-93.

Jorgensen, O. S., B. W. Brooksbank, et al. (1990). "Neuronal plasticity and astrocytic reaction in Down syndrome and Alzheimer disease." J Neurol Sci **98**(1): 63-79.

Kang, J., L. Jiang, et al. (1998). "Astrocyte-mediated potentiation of inhibitory synaptic transmission." Nat Neurosci **1**(8): 683-92.

Kawahara, M., Y. Kuroda, et al. (2000). "Alzheimer's beta-amyloid, human islet amylin, and prion protein fragment evoke intracellular free calcium elevations by a common mechanism in a hypothalamic GnRH neuronal cell line." J Biol Chem **275**(19): 14077-83.

Khachaturian, Z. S. (1994). "Calcium hypothesis of Alzheimer's disease and brain aging." Ann N Y Acad Sci **747**: 1-11.

Kilby, P. M., L. J. Van Eldik, et al. (1996). "The solution structure of the bovine S100B protein dimer in the calcium-free state." Structure **4**(9): 1041-52.

Kilby, P. M., L. J. Van Eldik, et al. (1997). "Identification of the binding site on S100B protein for the actin capping protein CapZ." Protein Sci **6**(12): 2494-503.

LaFerla, F. M. (2002). "Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease." Nat Rev Neurosci **3**(11): 862-72.

Lammich, S., E. Kojro, et al. (1999). "Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease." Proc Natl Acad Sci U S A **96**(7): 3922-7.

Lee, Y. B., S. Du, et al. (2000). "Rapid increase in immunoreactivity to GFAP in astrocytes in vitro induced by acidic pH is mediated by calcium influx and calpain I." Brain Res **864**(2): 220-9.

Levitan, D., J. Lee, et al. (2001). "PS1 N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling." Proc Natl Acad Sci U S A **98**(21): 12186-90.

Li, Y., S. W. Barger, et al. (2000). "S100beta induction of the proinflammatory cytokine interleukin-6 in neurons." J Neurochem **74**(1): 143-50.

Lin, H., R. Bhatia, et al. (2001). "Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology." Faseb J **15**(13): 2433-44.

Lin, L. H., L. J. Van Eldik, et al. (1994). "Inhibition of protein kinase C- and casein kinase II-mediated phosphorylation of GAP-43 by S100 beta." Brain Res Mol Brain Res **25**(3-4): 297-304.

Lytton, J., M. Westlin, et al. (1991). "Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps." J Biol Chem **266**(26): 17067-71.

Ma, K. C., Z. R. Ye, et al. (1988). "Glial fibrillary acidic protein immunohistochemical study of Alzheimer I & II astrogliosis in Wilson's disease." Acta Neurol Scand **78**(4): 290-6.

Mandybur, T. I. and C. C. Chuirazzi (1990). "Astrocytes and the plaques of Alzheimer's disease." Neurology **40**(4): 635-9.

Mark, R. J., K. Hensley, et al. (1995). "Amyloid beta-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca2+ homeostasis and cell death." J Neurosci **15**(9): 6239-49.

Marshak, D. R., S. A. Pesce, et al. (1992). "Increased S100 beta neurotrophic activity in Alzheimer's disease temporal lobe." Neurobiol Aging **13**(1): 1-7.

Mattson, M. P., S. W. Barger, et al. (1993). "beta-Amyloid precursor protein metabolites and loss of neuronal Ca2+ homeostasis in Alzheimer's disease." Trends Neurosci **16**(10): 409-14.

Mattson, M. P. and R. E. Rydel (1992). "beta-Amyloid precursor protein and Alzheimer's disease: the peptide plot thickens." Neurobiol Aging **13**(5): 617-21.

Meda, L., P. Baron, et al. (2001). "Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins." Neurobiol Aging **22**(6): 885-93.

Meske, V., U. Hamker, et al. (1998). "The effects of beta/A4-amyloid and its fragments on calcium homeostasis, glial fibrillary acidic protein and S100beta staining, morphology and survival of cultured hippocampal astrocytes." Neuroscience **85**(4): 1151-60.

Millward, T. A., C. W. Heizmann, et al. (1998). "Calcium regulation of Ndr protein kinase mediated by S100 calcium-binding proteins." Embo J **17**(20): 5913-22.

Molnar, M. L., K. Stefansson, et al. (1984). "Distribution of S-100 protein and glial fibrillary acidic protein in normal and gliotic human retina." Exp Eye Res **38**(1): 27-34.

Monnerie, H., S. Esquenazi, et al. (2005). "Beta-amyloid-induced reactive astrocytes display altered ability to support dendrite and axon growth from mouse cerebral cortical neurons in vitro." Neurol Res **27**(5): 525-32.

Mrak, R. E. and W. S. Griffinbc (2001). "The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease." Neurobiol Aging **22**(6): 915-22.

Nedergaard, M. (1994). "Direct signaling from astrocytes to neurons in cultures of mammalian brain cells." Science **263**(5154): 1768-71.

Nett, W. J., S. H. Oloff, et al. (2002). "Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity." J Neurophysiol **87**(1): 528-37.

Newman, E. A. (2003). "New roles for astrocytes: regulation of synaptic transmission." Trends Neurosci **26**(10): 536-42.

Newman, E. A. and K. R. Zahs (1997). "Calcium waves in retinal glial cells." Science **275**(5301): 844-7.

Ohtaki, N., W. Kamitani, et al. (2007). "Downregulation of an astrocyte-derived inflammatory protein, S100B, reduces vascular inflammatory responses in brains persistently infected with Borna disease virus." J Virol **81**(11): 5940-8.

Parpura, V., T. A. Basarsky, et al. (1994). "Glutamate-mediated astrocyte-neuron signalling." Nature **369**(6483): 744-7.

Parri, H. R., T. M. Gould, et al. (2001). "Spontaneous astrocytic Ca2+ oscillations in situ drive NMDAR-mediated neuronal excitation." Nat Neurosci **4**(8): 803-12.

Pasti, L., A. Volterra, et al. (1997). "Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ." J Neurosci **17**(20): 7817-30.

Pekny, M. and M. Nilsson (2005). "Astrocyte activation and reactive gliosis." Glia **50**(4): 427-34.

Perea, G. and A. Araque (2005). "Glial calcium signaling and neuron-glia communication." Cell Calcium Frontiers in calcium signalling **38**(3-4): 375-382.

Peskind, E. R., W. S. Griffin, et al. (2001). "Cerebrospinal fluid S100B is elevated in the earlier stages of Alzheimer's disease." Neurochem Int **39**(5-6): 409-13.

Peters, O., C. G. Schipke, et al. (2003). "Different mechanisms promote astrocyte Ca2+ waves and spreading depression in the mouse neocortex." J Neurosci **23**(30): 9888-96.

Pike, C. J., A. J. Walencewicz-Wasserman, et al. (1995). "Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25-35 region to aggregation and neurotoxicity." J Neurochem **64**(1): 253-65.

Pope, W. B., M. P. Lambert, et al. (1994). "Microtubule-associated protein tau is hyperphosphorylated during mitosis in the human neuroblastoma cell line SH-SY5Y." Exp Neurol **126**(2): 185-94.

Popescu, B. O., A. Cedazo-Minguez, et al. (2001). "Caspase cleavage of exon 9 deleted presenilin-1 is an early event in apoptosis induced by calcium ionophore A 23187 in SH-SY5Y neuroblastoma cells." J Neurosci Res **66**(1): 122-34.

Putney, J. W., Jr. (2003). "Capacitative calcium entry in the nervous system." Cell Calcium **34**(4-5): 339-44.

Querfurth, H. W., J. Jiang, et al. (1997). "Caffeine stimulates amyloid beta-peptide release from beta-amyloid precursor protein-transfected HEK293 cells." J Neurochem **69**(4): 1580-91.

Reali, C., F. Scintu, et al. (2005). "S100b counteracts effects of the neurotoxicant trimethyltin on astrocytes and microglia." J Neurosci Res **81**(5): 677-86.

Reeves, R. H., J. Yao, et al. (1994). "Astrocytosis and axonal proliferation in the hippocampus of S100b transgenic mice." Proc Natl Acad Sci U S A **91**(12): 5359-63.

Rogaeva, E., Y. Meng, et al. (2007). "The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease." Nat Genet **39**(2): 168-77.

Rossi, F. and E. Bianchini (1996). "Synergistic induction of nitric oxide by beta-amyloid and cytokines in astrocytes." Biochem Biophys Res Commun **225**(2): 474-8.

Rothermundt, M., M. Peters, et al. (2003). "S100B in brain damage and neurodegeneration." Microsc Res Tech **60**(6): 614-32.

Rothoerl, R. D., A. Brawanski, et al. (2000). "S-100B protein serum levels after controlled cortical impact injury in the rat." Acta Neurochir (Wien) **142**(2): 199-203.

Scemes, E. and C. Giaume (2006). "Astrocyte calcium waves: what they are and what they do." Glia **54**(7): 716-25.

Selinfreund, R. H., S. W. Barger, et al. (1991). "Neurotrophic protein S100 beta stimulates glial cell proliferation." Proc Natl Acad Sci U S A **88**(9): 3554-8.

Selinfreund, R. H., S. W. Barger, et al. (1990). "Antisense inhibition of glial S100 beta production results in alterations in cell morphology, cytoskeletal organization, and cell proliferation." J Cell Biol **111**(5 Pt 1): 2021-8.

Sheng, J. G., R. E. Mrak, et al. (1994). "S100 beta protein expression in Alzheimer disease: potential role in the pathogenesis of neuritic plaques." J Neurosci Res **39**(4): 398-404.

Sinha, S., J. P. Anderson, et al. (1999). "Purification and cloning of amyloid precursor protein beta-secretase from human brain." Nature **402**(6761): 537-40.

Smith, I. F., K. N. Green, et al. (2005). "Calcium dysregulation in Alzheimer's disease: recent advances gained from genetically modified animals." Cell Calcium **38**(3-4): 427-37.

Smith, S. P. and G. S. Shaw (1997). "Assignment and secondary structure of calcium-bound human S100B." J Biomol NMR **10**(1): 77-88.

Smith, S. P. and G. S. Shaw (1998). "A novel calcium-sensitive switch revealed by the structure of human S100B in the calcium-bound form." Structure **6**(2): 211-22.

Stix, B. and G. Reiser (1998). "Beta-amyloid peptide 25-35 regulates basal and hormone-stimulated Ca2+ levels in cultured rat astrocytes." Neurosci Lett **243**(1-3): 121-4.

Strittmatter, W. J., K. H. Weisgraber, et al. (1994). "Hypothesis: microtubule instability and paired helical filament formation in the Alzheimer disease brain are related to apolipoprotein E genotype." Exp Neurol **125**(2): 163-71; discussion 172-4.

Sul, J. Y., G. Orosz, et al. (2004). "Astrocytic Connectivity in the Hippocampus." Neuron Glia Biol **1**(1): 3-11.

Thastrup, O., P. J. Cullen, et al. (1990). "Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase." Proc Natl Acad Sci U S A **87**(7): 2466-70.

Vassar, R., B. D. Bennett, et al. (1999). "Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE." Science **286**(5440): 735-41.

Verkhratsky, A. and H. Kettenmann (1996). "Calcium signalling in glial cells." Trends Neurosci **19**(8): 346-52.

Wang, S., L. E. Rosengren, et al. (1999). "Bcl-2 expression regulates cell sensitivity to S100beta-mediated apoptosis." Brain Res Mol Brain Res **70**(1): 167-76.

Wang, X., N. Lou, et al. (2006). "Astrocytic Ca2+ signaling evoked by sensory stimulation in vivo." **9**(6): 816-823.

Wisniewski, H. M. and J. Wegiel (1991). "Spatial relationships between astrocytes and classical plaque components." Neurobiol Aging **12**(5): 593-600.

Wolfe, M. S., W. Xia, et al. (1999). "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity." Nature **398**(6727): 513-7.

Woods, N. M., K. S. R. Cuthbertson, et al. (1986). "Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes." **319**(6054): 600-602.

Yankner, B. A. (1996). "Mechanisms of neuronal degeneration in Alzheimer's disease." Neuron **16**(5): 921-32.

Yu, G., M. Nishimura, et al. (2000). "Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing." Nature **407**(6800): 48-54.

Yu, W. H. and P. E. Fraser (2001). "S100beta interaction with tau is promoted by zinc and inhibited by hyperphosphorylation in Alzheimer's disease." J Neurosci **21**(7): 2240-6.

Zanotti, S. and A. Charles (1997). "Extracellular calcium sensing by glial cells: low extracellular calcium induces intracellular calcium release and intercellular signaling." J Neurochem **69**(2): 594-602.

Zhang, Q., T. Pangrsic, et al. (2004). "Fusion-related release of glutamate from astrocytes." J Biol Chem **279**(13): 12724-33.

Ziegler, D. R., C. E. Innocente, et al. (1998). "The S100B protein inhibits phosphorylation of GFAP and vimentin in a cytoskeletal fraction from immature rat hippocampus." Neurochem Res **23**(10): 1259-63.