MECHANISMS OF PALMITATE INDUCED AMYLOIDOGENESIS IN ALZHEIMER'S DISEASE MEDIATED BY ASTROCYTES

By

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ABSTRACT

MECHANISMS OF PALMITATE INDUCED AMYLOIDOGENESIS IN ALZHEIMER'S DISEASE MEDIATED BY ASTROCYTES

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Alzheimer's disease is a devastating, irreversible and progressive neurodegenerative disease with unknown etiology and no effective cure. A dietary factor, saturated fatty acid palmitate, induces amyloidogenesis in primary rat neurons mediated by astrocytes, however, the molecular mechanisms by which conditioned media from palmitate-treated astrocytes upregulates amyloid beta level by BACE1 and γ-secretase in neurons are unknown. The present study demonstrates that upregulated serine palmitoyltransferase in the astrocytes increased ceramide levels, leading to the release of cytokines, namely TNFα and IL-1β, which mediated the activation of neutral and acidic sphingomyelinase (SMase) to propagate the deleterious effects of palmitate. Activated SMases elevated ceramide levels and in turn enhanced BACE1 level in the neurons. Thus, this study suggests that tight regulation of ceramide production may be an important therapeutic approach to modulating BACE1 level. Moreover, this study identified a signaling pathway in which the condition media from palmitate-treated astrocytes rapidly elevates calcium level in the neurons, and subsequently calpain activity, a calcium-dependent protease, resulting in enhanced p25/Cdk5 activity, and in turn the phosphorylation and activation of STAT3, a transcription factor. STAT3 transcriptionally regulates the expression of both BACE1 and presenilin-1, a catalytic subunit of γ-secretase, in primary neurons, suggesting that STAT3 is an important potential therapeutic target to control AD pathogenesis.

Inflammatory response has been strongly implicated in numerous diseases, including AD, and IL-1β has been indicated in the pathogenesis of AD. Elevated IL-1β in conditioned media released by palmitate-treated astrocytes activates the SMases-ceramide-BACE1 pathway in primary neurons, enhances calcium level, and triggers the calcium-calpain-Cdk5/p25-STAT3 signaling cascade, leading to the upregulation of BACE1 and presenilin-1 in neurons. However, little is known about the molecular mechanisms that initiate the generation of IL-1β in astrocytes upon palmitate treatment. Here we investigated and identified that palmitate induced the activation of the IPAF-ASC inflammasome in astrocytes leading to the maturation of IL-1β, thereby indicating that not only pathogen-related factors can activate IPAF-ASC inflammasome. The expression of IPAF was found to be regulated by transcription factor CREB. Moreover, downregulating IPAF in astrocytes decreased IL-1β secretion from the astrocytes and reduced the generation of amyloid β42 by primary neurons. Furthermore, the expression levels of IPAF and ASC were found significantly elevated in a subgroup of sporadic AD patients, suggesting the involvement of the IPAF-ASC inflammasome in the inflammatory response associated with AD, and a potential role of IPAF-ASC as a therapeutic target to control AD.

DEDICATED TO MY FAMILY

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ActD	Actinomycin D
AD	Alzheimer's disease
APH-1	Anterior pharynx-defective 1
APP	Amyloid beta precursor protein
ASC	Apoptosis-associated speck-like protein containing a CARD
ATase	Acetyltransferases
Aβ	Amyloid beta
BACE1	Beta site amyloid beta precursor protein cleaving enzyme 1
BSA	Bovine serum albumin
C99	Carboxy-terminal fragment of APP
Cdk 5	Cycline-dependent kinase 5
CM-B	Bovine serum albumin-treated astrocyte conditioned media
CM-P	Conditioned media from palmitate-treated astrocytes
CNS	Central nervous system
CREB	cAMP response element binding protein
DAMPs	Damage associated molecular patterns
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FFA	Free fatty acid
FI	Forskolin and IBMX
GFAP	Glial fibrillary acidic protein

GSK3β	Glycogen synthase kinase 3β
IBMX	Isobutylmethylxanthine
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-18	Interleukin-18
IL-33	Interleukin-33
IPAF	ice protease-activating factor, also known as NLRC4
JNK	c-Jun N-terminal kinase
LCS	L-cycloserine
LDH	Lactate dehydrogenase
MCI	Mild cognitive impairment
MMSE	Mini mental state examination
mRNA	Messenger RNA
Naip 5	NLR family, apoptosis inhibitory protein 5
NFκB	Nuclear factor kappa B
NFATc4	Nuclear factor of activated T-cells, cytoplasmic 4
NFTs	Neurofibrillary tangles
NLR protein 1	Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) protein 1
NLR protein 3	Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) protein 3
NLRC4	CARD domain-containing protein 4, also called
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs

PA	Palmitate
PAMPs	Pathogen associated molecular patterns
PEN-2	Presenilin enhancer 2
PKA	Protein kinase A
PKR	Double-stranded RNA-dependent protein kinase
ROS	Reactive oxygen species
SM	Sphingomyelin
SMase	Sphingomyelinase
SPT	Serine palmitoyltransferase
STAT3	Signal transducer and activator of transcription 3
TNF-α	Tumor necrosis factor-α

CHAPTER 1. INTRODUCTION

Sections 1.1 to 1.6 are integrated into a review paper which is in review.

Li Liu and Christina Chan, The role of inflammasome in Alzheimer's disease (In review)

Abstract

Alzheimer's disease (AD) is a chronic, progressive and irreversible neurodegenerative disease with clinical characteristics of memory loss, dementia and cognitive impairment. Although the pathophysiologic mechanism is not fully understood, inflammation has been shown to play a critical role in the pathogenesis of AD. Inflammation in the central nervous system (CNS) is characterized by the activation of glial cells and release of proinflammatory cytokines and chemokines. Accumulating evidence demonstrates that inflammasomes, which cleave precursors of interleukin-1β (IL-1β) and IL-18 to generate their active forms, play an important role in the inflammatory response in the CNS and in AD pathogenesis. Therefore, modulating inflammasome complex assembly and activation could be a potential strategy for suppressing inflammation in the CNS. This review aims to provide insight into the role of inflammasomes in the CNS, with respect to the pathogenesis of AD, and may provide possible clues for devising novel therapeutic strategies.

Keywords: Alzheimer's disease, Inflammasome, Inflammation, Astrocytes, Micrioglia, Interleukins

1.1 Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disease, characterized by extracellular deposition of Aβ beta (Aβ) plaques and intracellular accumulation of hyper-

phosphorylated tau protein and neurofibrillary tangles (NFTs) [1]. Studies in AD patients demonstrate a significant reduction in the size of specific brain regions through the loss of neurons and synapses as compared with healthy subjects [2, 3]. The early stages of AD manifest in mild cognitive decline, with progressive memory loss and impairment of other intellectual abilities as the disease progresses, and at the late stages the patients suffering from AD alter their personality and lose their bodily functions [1]. Currently, AD affects more than 38 million people worldwide and every four seconds a new patient is diagnosed with AD [4]. The number of AD patients is expected to double every 20 years and is expected to reach 115 million in 2050 [5]. As the baby boom generation ages, it is anticipated that the number of AD cases will rise dramatically. However, effective treatment of AD remains a challenge, although numerous hypotheses of possible mechanisms have been put forth.

To date, there are several competing hypotheses that attempt to explain the cause of AD, such as the cholinergic hypothesis, Aβ hypothesis, tau hypothesis and inflammation hypothesis. Cholinergic deficiency is the oldest hypothesis, but has not been widely accepted due to unsuccessful clinical and experimental results [6, 7]. The Aβ and tau hypotheses postulate that Aβ deposits or tau protein abnormalities are the fundamental causes of the disease. Nevertheless, Aβ plaques and neurofibrillary tangles are not sufficient in explaining all the features of AD, since abnormal levels of Aβ plaques have been found among normal healthy elderly individuals [8]. In addition, highly expressed Aβ or tau protein in animal models of AD do not show significant neurodegenerative changes [9]. Furthermore, clinical trials with immune-therapeutics to reduce Aβ level in the brain did not improve cognitive function in AD patients, although evidence suggests clearance of the Aβ plaques [10]. These findings suggest that other factors might also be involved in the pathogenesis of AD. Inflammation has been proposed as a key

effector of AD, and several features of AD, such as microglial activation, reactive astrocytes and elevated cytokine expression, have all been observed in AD patients, leading to an inflammatory hypothesis. Additionally, epidemiological evidence suggests that non-steroidal anti-inflammatory drugs (NSAIDs) could delay onset and reduce severity of AD symptoms, although several studies failed to confirm this [11]. More recently, analysis of human AD samples revealed highly expressed inflammatory cytokines during the early stages of AD [12], and genome-wide studies showed an upregulation of inflammatory genes, indicating a potential role of inflammation in AD [12, 13].

Considering the importance of inflammation in the pathogenesis of AD, several recent review articles have discussed how inflammatory molecules, signaling pathways and processes could be involved in the pathogenesis of AD [9, 14-17]. In this review, we focus on specific inflammasomes identified in the central nervous system (CNS), in particular the brain, their potential as therapeutics, and the cytokines released by these inflammasomes with emphasis on environmental factors, i.e. fatty acids, that trigger the generation of cytokines through inflammasomes.

1.2 Inflammatory process in AD

Inflammation is a complex cellular and molecular defense mechanism in response to diverse stimuli, including stress, injury and infection. In the brain, an upregulation in inflammatory signaling (i.e. neuroinflammation) is characterized by the activation of astrocytes and microglia, and the release of proinflammatory mediators. Neuroinflammation is involved in neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, psychiatric, as well as AD [12, 18-20]. Mounting evidence

indicates an upregulation in the inflammatory molecules and activated glial cells surrounding the senile plaques in brains of AD patients and AD transgenic animal models [21-23]. Concomitantly, a downregulation in anti-inflammatory molecules are observed in post mortem brains of AD patients [24]. These findings implicate an involvement of inflammatory responses in the pathogenesis of AD. In the CNS, activated astrocytes and microglia are major sources of inflammatory molecules, such as cytokines, chemokines, neurotransmitters, reactive oxygen species, and nitric oxide (NO) [25]. The released cytokines, in particular IL-1β, IL-6 and TNFα (tumor necrosis factor α), are major effectors of the neuroinflammatory signals [26, 27], and can affect neurophysiologic mechanisms regarding cognition and memory [28, 29]. Cytokines could establish a feedback loop, to activate additional astrocytes and microglia and lead to further generation of inflammatory molecules. In addition, the secreted inflammatory molecules also recruit other cells such as monocytes and lymphocytes to cross the blood brain barrier to enhance neuroinflammation in the CNS [30].

1.2.1 Astrocytes and AD

Astrocytes are the most abundant non-neuronal cells in the CNS, constituting about 20-50% of the human brain volume, much higher than microglia [31]. Astrocytes have multiple functions, such as regulation of extracellular ions and energy reserves, clearance and metabolism of neurotransmitters, and facilitating the maintenance of normal neuronal functions in the CNS [32, 33]. Astrocytes can be activated by numerous factors, including pathogens, lipopolysaccharide, oxidative stress [34], free saturated fatty acids [17] as well as Aβ [35]. The activation of astrocytes is believed to last longer than that of microglia, enabling a prolonged engagement of astrocytes in the neuroinflammatory response [14].

Reactive astrocytes as opposed to quiescent astrocytes, can produce cytokines such as interleukins, TNFα and interferon γ (IFN- γ), etc. [17]. They can also generate low amounts of Aβ, in addition to neurons, the major producer of Aβ [14, 16]. Cytokines, IFN-γ along with TNFα or IL-1β, have been demonstrated to induce the generation of Aβ in primary human astrocytes and astrocytoma cells [16]. Proinflammatory molecules secreted by reactive astrocytes can elevate the expression of secretases in neurons, enhancing the production of Aβ [36, 37], and activating microglia to further produce inflammatory factors [38].

1.2.2 Microglia and AD

Microglia constitute around 20% of the total glial cells in the brain, and 10% of the cells in the CNS [39, 40]. They have various functions in the CNS, including phagocytosis and inducing cytotoxicity [41]. Microglia can be activated by many factors including Aβ. Activated microglia have phagocytic ability by migrating to damaged cells and clearing debris. Like astrocytes, they can also generate inflammatory molecules such as cytokines, chemokines, reactive oxygen and nitrogen species.

Activated microglia colocalize in areas of heavy Aβ concentration [42], and preferentially associate with certain types of Aβ plaques, such as fibrillar Aβ [43]. Aβ binds to microglia and induces phagocytosis or expression of proinflammatory molecules [44, 45], and this activation is dependent on the load of Aβ [46]. In an earlier study, upon depletion of microglial cells in animal models the Aβ level increased thereby suggesting microglial cells play a role in the clearance of Aβ by phagocytosis [47, 48]. However, more recent work confirmed a detrimental role of microglia in AD, which depended upon the degree of activation of the microglial cells. They suggest that with moderate activation by light Aβ concentration microglia

have strong phagocytic ability to clear Aβ, while heavily activated microglia by high levels of Aβ increase the production of proinflammatory molecules, such as IL-1β and TNFα, and trigger neuronal damage and coincide with a reduced ability to clear Aβ [9, 49]. The lower clearance is due to a significant decrease in the expression of Aβ binding scavenger receptors and Aβ degrading enzymes in microglia [9, 49]. Therefore, the exact function of microglia in AD, whether it is beneficial or detrimental, may depend on the activation state of the microglia.

1.3 Inflammatory cytokine molecules in AD

Elevated inflammatory cytokines, TNFα, IFNγ, and interleukins, have been found in the brains of Alzheimer's patients, in particular near the Aβ plaques, and also in AD transgenic animals [9]. Cytokines were also detected in the peripheral blood and cerebrospinal fluid of AD patients [40, 50, 51]. Proinflammatory cytokines have multiple functions, including stimulation or inhibition of cell proliferation, differentiation, apoptosis, and inducing inflammation [40]. In the CNS, proinflammatory cytokines induce the release of a number of other proinflammatory molecules from the same or different cell types, amplifying the cytokine effects. Although these studies do not demonstrate whether inflammation is an initiator, contributor, or side effect in the pathophysiological changes associated with AD [12, 14], it nevertheless is involved since cytokines have been shown to regulate Aβ plaque deposition and BACE1 expression in AD transgenic mice [52]. Interleukins, in particular IL-1β and IL-18 are upregulated in AD brain, and overexpression of IL-1β or IL-18 are critical for onset of the inflammatory process [40], and both mediate the expression of a vast array of inflammatory genes [53]. IL-1β and IL-18 are

synthesized as inactive precursors, proIL-1β and proIL-18, respectively, and inflammasomes are required for their maturation.

1.3.1 Inflammasome

Inflammasome, the platform for proIL-1β and proIL-18 processing, is an intracellular multiprotein complex activated by pathogen associated molecular patterns (PAMPs) as a host-defense reaction, or by damage associated molecular patterns (DAMPs) as a self-defense mechanism for danger signals [54]. Factors that activate inflammasomes include microbial proteins, crystalline urea, RNA, Alum, ATP, potassium efflux, fatty acids, and Aβ, and most recently, degraded mitochondrial DNA was identified as a DAMP [17, 55, 56]. To date, the most well-characterized inflammasomes are NLRP1 (nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) protein 1), NLRP3 (NOD-like receptor protein 3) and NLRC4 (CARD domain-containing protein 4, also called IPAF (ICE-protease activating factor)) inflammasomes [57]. Inflammasomes consist of several factors, and the basic components of inflammasomes include a NOD-like receptor recognizing danger signals or ligands, and procaspase-1 which is the protease that cleaves the precursor of the proinflammatory molecules to form their mature form [58]. In addition to these basic components, other factors could also be involved in the assembly or activity of inflammasomes depending on the cell type and stimulus. Adaptor protein, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), recruits the effector protein procaspase-1 to the inflammasome complex, and is an essential component of NLRP1 and NLRP3 inflammasomes, but not for NLRC4 inflammasome [57, 58]. The composition of NLRC4 inflammasome is more complex than the other two inflammasomes. Under certain but not all conditions, ASC [57] or Naip5 (NLR family, apoptosis

inhibitory protein 5) [59] is required for NLRC4 inflammasome activation. Overall the assembly and activation of inflammasomes are cell-type and stimulus specific [57, 58].

1.3.2 Proinflammatory cytokine: Interleukin-1β

IL-1β is expressed in many types of cells including macrophages, monocytes and neurtrophils, while in the CNS IL-1β can be released from astrocytes, microglia and neurons [60]. IL-1β induces diverse signaling which is cell-type specific [61]. For example, in glial cells, IL-1β activates NFκB signaling leading to cytokine production. In contrast in neurons, IL-1β activates the MAPK-p38 signaling cascade to increase the secreted fragment of APP cleaved by BACE1, enhancing the ability to form Aβ [54, 61, 62].

1.3.2.1 IL-1β relationship to AD

IL-1β plays a key role in the onset and development of diverse diseases, including neurodegenerative diseases such as AD [60]. The IL-1β gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2 [63]. Elevated IL-1β has been observed in serum, cerebrospinal fluid and brain of AD patients as well as in other forms of dementia [64-66]. Elevated levels also were reported in cerebrospinal fluid and brain parenchyma of both humans and rodents shortly after traumatic brain injury, which is an independent risk factor of AD [67-69]. IL-1β can activate other cell types, in particular astrocytes and microglia, to further induce cytokine release, such as IL-1β, IL-6 and IL-18, as well as inducible nitric oxide synthase activity to produce NO, a free radical, leading to neurotoxicity [40, 70]. IL-1β secreted from astrocytes have been shown to enhance the production of APP and Aβ from neurons [14, 16, 71]. IL-1β also increased the level of S100B released from astroglioma cell line and plaque-associated activated astrocytes in primary cortex to promote the synthesis of APP production in

neurons [14, 72]. Injecting IL-1β into the cerebral hemisphere upregulated the level of APP and amyloidogenesis in the brain tissue [73]. Furthermore, several studies demonstrated that IL-1β can induce the phosphorylation of tau protein and mediate the formation of neurofibrillary tangles through the MAPK-p38 pathway [54, 74]. Blocking IL-1β signaling altered the inflammatory responses of the brain, rescued cognition, attenuated tau pathology, and reduced fibrillar Aβ level in AD mice [75]. Conversely, knockout of IL-1β receptor antagonist in mice increased the neuronal damage induced by Aβ [76]. These studies implicate a proinflammatory role of IL-1β in the progression of AD pathogenesis.

1.3.2.2 Inflammasomes involved in IL-1β maturation in the CNS

In the CNS the production of IL-1β by inflammasomes is well-characterized as compared to other interleukins, with NLRP1, NLRP3 and NLRC4 inflammasome studied thus far as the predominant ones involved in generating IL-1β [77]. NLRP1 inflammasome are present in neurons, but not in astrocytes, oligodendrocytes, and microglia [78-80]. Spinal cord injury can activate NLRP1 inflammasome to produce IL-1β in mouse spinal cord neurons [78]. Decreasing NLRP1inflammasome activity reduced the production of IL-1β in neurons [81].

In vivo and cell studies demonstrate that fibrillar Aβ activates NLRP3 inflammasome, composed of NLRP3 receptor, ASC and caspase-1, to produce IL-1β in microglia [82]. Phagocytosis and subsequent lysosomal damage triggered by Aβ initiated the activation of NLRP3 inflammasome in the microglia [82]. In support, a recent study in APP/PS1 mice confirmed that NLRP3 inflammasome contributed to the AD pathology [83]. Deficiency of the NLRP3 gene played a protective role on memory and behavior, and reduced Aβ deposition [83].

Similarly, inhibiting NLRP3 inflammasome reduced neuritic plaque burden in AD transgenic mice [84].

Palmitate, a fatty acid, activated NLRC4 inflammasome in the primary astrocytes leading to the release of IL-1β [85]. Thus far NLRC4 inflammasome is known to be triggered by *Legionella pneumophila* in primary microglial cells [86].

1. 3.3 Proinflammatory cytokine: Interleukin-18

Interleukin-18 (IL-18), belonging to the IL-1 superfamily, is constitutively expressed in several cell types, and the active form of IL-18 is generated by cleavage of precursor, proIL-18. IL-18 has several similarities in their properties with IL-1β, such as, an inactive precursor, is activated by pathogen and danger associated factors, involves inflammasome, and induces similar signaling events [87, 88]. However, there are considerable differences between the two cytokines, such as their expression levels, regulation and action [87, 88]. In normal brain tissue, IL-18 is constitutively and highly expressed, whereas IL-1β is expressed at a very low level [89]. IL-1β is significantly increased within a very short time after ischaemia, but the upregulation of IL-18 took much longer in peripheral immune cells [90], suggesting different regulation of the two cytokines. IL-1β activates NFκB and p38 signaling pathways [54, 61, 74] while IL-18 binds to the receptor IL-18R and initiates several signaling events through the activation of NFκB (Nuclear factor kappa B), STAT3 (Signal transducer and activator of transcription 3) and NFATc4 (Nuclear factor of activated T-cells, cytoplasmic 4) [91, 92]. Moreover, it activates both Fas and Fas-L promoter activities, and thus has been suggested to be an apoptosis inducer and initiator of pathogenesis of atherosclerosis and cardiovascular diseases [93]. IL-18 can modulate neuronal excitability [94], and inhibit long term-potentiation, a form a neuronal plasticity considered to underlie learning and memory [95].

1. 3.3.1 IL-18 relationship to AD

IL-18 is believed to play an important role in various diseases, in particular AD. The IL-18 gene is located in the 11q22.2-22.3 region close to the dompamine receptor D2 locus, near chromosome 11, a chromosomal region of interested in AD defined by genome studies and suggested as a linkage area for AD pathology in familial AD [96]. Moreover, IL-18 promoter polymorphism has been shown to increase the risk of developing sporadic late onset AD in Han Chinese and Italian populations [97, 98]. In the CNS, IL-18 can be produced by astrocytes, microglia and ependymal cells, and was also detected in neurons [99-101]. The mRNA and protein levels of IL-18 are increased significantly in astrocytes, microglia and neurons that co-localized with Aβ plaques in the brains of AD patients [102]. IL-18 is significantly elevated in the plasma of mild cognitively impaired and AD patients [103, 104]. Moreover, a significant increased in IL-18 was observed in stimulated mononuclear cells or macrophages of peripheral blood from AD patients [50, 51], as well as in the blood of patients with ischemic heart disease, type-2 diabetes, and obesity, which are risk factors for AD [91]. These results support that IL-18 is involved in AD disease pathology. In mild AD patients, IL-18 level is significantly increased, but is slightly lowered in moderate AD patients, albeit still upregulated, whereas no significant upregulation is observed in severe AD patients as compared to age-matched control subjects [105]. This gradual decline in immune response in AD indicates that the immune response, in particular IL-18, could be an initiator of AD pathogenesis. Studies have demonstrated that IL-18 triggers an elevation in the protein levels of APP, BACE1, the N-terminal fragment of presenilin-1 and presenilin enhancer 2 which are components of the γ-secretase complex [91], suggesting IL-18 accelerates Aβ genesis. In addition, IL-18 increased the expression of glycogen synthase kinase 3β (GSK-3β) and cyclin dependent kinase 5 (Cdk5), which are involved in the

hyperphosphorylation of the tau protein [101]. These studies suggest an important role of IL-18 in AD.

1.3.3.2 Inflammasomes involved in IL-18 maturation in the CNS

Like IL-1β, in most cases, the mature secretable form of IL-18 is generated by caspase-1 through the activation of inflammasome. In contrast, the mature secretable form of IL-18 also can be derived from various extracellular enzymes including protease 3, serine protease, elastase and cathepsin G [106-108]. Maturation of IL-18 and IL-1β could be regulated by the same type of inflammasome. For example, down-regulation of NLRP1 in macrophages triggered by Cordyceps sinensis mycelium reduced both IL-18 and IL-1β levels [88]. The release of IL-18 and IL-1β could also be regulated by different inflammasomes even though they are in the same type of cells and exposed to the same stimuli. A recent report showed that IL-18 and IL-1β are secreted from primed murine dendritic cells in response to Listeria protein p60, but inhibition of NLRP3 reduced the production of IL-1β, but did not impair IL-18 secretion [55]. This suggests that the maturation of IL-18 and IL-1β is regulated condition-specifically by different signaling mechanisms upon exposure to similar stimulation.

Spinal cord injury caused IL-18 and IL-1β release from neuronal cells through the activation of NLRP1 inflammasome, composed of receptor NLRP1, adaptor protein ASC and caspase-1 [78]. ASC neutralization reduced the upregulation in IL-18 and IL-1β levels [78]. Spinal cord injury elevates extracellular ATP levels during neuroinflammation, which may act on purinergic receptors to trigger the activation of inflammasome [81]. However, upon further study of purinergic receptor $P2X_4$ knockout mice with spinal cord injury, the production of IL-1β

in neurons reduced but not of IL-18 as compared with wild-type mice [81]. This further supports the differential regulation of IL-18 and IL-1β expression.

Microglia also can release IL-18 and IL-1β through the activation of NLRC4 inflammsome upon stimulation by *Legionella pneumophila*, a flagellated bacterium [86]. The NLRC4 inflammasome activated by *L. pneumophila* consists of the NLRC4 receptor, caspase-1 and Naip5, the latter has been identified as a receptor that directly binds flagellin within the cells. In addition, the adaptor protein ASC also participated in modulating the NLRC4 inflammasome by directing caspase-1 activity to cleave the precursor proteins of IL-1β and IL-18 [86].

Diverse factors can upregulate IL-18 level in the CNS. Kainic acid has been show to induce IL-18 production from microglia [109]. Stress, a risk factor of AD, has been confirmed to elevate IL-18 synthesis and promote the maturation of IL-18 [110]. Aging, another risk factor of AD, have been found to activate NLRP1 inflammasome and upregulate IL-18 and IL-1β levels in the hippocampus of aged mice [111]. Currently information on the type and composition of the inflammasomes involved in the maturation of IL-18 in the CNS are largely uncharacterized. Further studies are required to reveal the role of IL-18 in AD pathogenesis.

1.3.4 Proinflammatory cytokines: Interleukin-33

Interleukin-33 (IL-33), a newer member of the IL-1 superfamily, is a dual function cytokine, containing both chromatin-associated function and proinflammatory function [112]. IL-33 is constitutively expressed in many types of cells [113]. Pathogen-associated patterns can induce IL-33 production in astrocytes [114]. In resting cells, IL-33 is mainly in the nucleus [115], and has been suggested to interact with chromatin and affect chromatin compaction [116]. A recent report showed that nuclear IL-33 sequesters NFκB through direct binding, leading to dampen NFκB-stimulated gene activation [117]. During tissue injury or trauma, nuclear IL-33 is

released to the extracellular space [118] to exert its function by binding to the receptor IL-33R presented on various cell types. IL-33R is a heterodimer of IL-1 receptor-like 1 (IL-1RL1, also called ST2) and IL-1 receptor accessory protein (IL-1RAcp). Signaling cascades triggered by IL-33R is similar to those induced by IL-1β and IL-18 [112]. In the CNS, IL-33 has been found to be expressed only in astrocytes while the receptor of IL-33 has been found on microglia and astrocytes [118]. IL-33 is intracellularly localized in the nucleus of resting astrocytes, and secreted by reactive astrocytes. Extracellularly IL-33 acts as a cytokine to trigger autocrine or paracrine inflammatory responses in glial cells [119]. For example, IL-33 induces the production of cytokines and chemokines in microglia [118], and triggers the release of TNFα, IL-1β and NO from astrocytes through phosphorylated ERK [119]. Additionally, IL-33 augments the release of TNFα from astrocytes triggered by glia maturation factor [119].

1.3.4.1 IL-33 relationship to AD

Abnormal expression of IL-33 is associated with many diseases, and currently the pathophysiological role of IL-33 in CNS, in particular its role in the pathogenesis of AD is being investigated [118-121]. The IL-33 gene is located on chromosome 9p24, a chromosomal region of interested in AD defined by genome studies [120, 121]. Single nucleotide polymorphism within IL-33 was shown to be associated with risk of AD in both Caucasian and Chinese populations [120, 122]. A genetic study showed that IL-33 expression level is lower in AD brain as compared with control subjects, and overexpression of IL-33 in cells reduced the production of Aβ40 [122], suggesting a protective role of IL-33. This differs from other proinflammatory cytokines such as TNFα and IL-1β, which contribute to neuronal damage and neurodegenerative diseases. Therefore, further studies are needed to clarify the exact role of IL-33 in neurological disorders.

1.3.4.2 Inflammasomes involved in IL-33 maturation in the CNS

IL-33, like IL-1β and IL-18, has a precursor form, proIL-33, but, unlike IL-1β and IL-18, proIL-33 has a nuclear localization sequence and localizes in the nucleus to exert unique biological function [115, 123]. Upon stimulation, the secretion of IL-33 depends on the activation of inflammasome, but the specific type of inflammasome has not been elucidated [124]. Surprisingly, further study has indicated that the cleavage of proIL-33 by caspase-1 results in the inactivation of IL-33, rather than activation [125]. In addition to cleavage by caspase-1 mediated by inflammasome, other proteases like calpain and caspase-3 could also participate in the cleavage of IL-33 leading to its inactivated form [126]. Further studies are needed to elucidate how the secreted, active IL-33 is generated.

1.4 Fatty acids involvement in inflammasome activation and pathogenesis of AD

Environmental factors, in particular, saturated fatty acids have been reported to induce the production of proinflammatory cytokines, such as IL-1β, IL-6 and TNFα, the major effectors of neuroinflammatory cascade, in particular from astrocytes and microglia [17, 127, 128]. Palmitate, the most common saturated fatty acid in the diet [129], triggers the production of cytokines, in particular IL-1β, from many cell types, including astrocytes [17, 130]. Recently we identified that palmitate activates NLRC4 inflammasome in primary astrocytes to release IL-1β, and ASC participates in the activation of NLRC4 inflammasome [85]. Reducing NLRC4 or ASC levels in palmitate (PA)-treated astrocytes significantly reduced IL-1β production [85]. In addition, NLRC4 and ASC levels are upregulated in the brains of AD patients [85], suggesting a possible role of NLRC4 inflammasome in AD pathogenesis. In support, palmitate induces IL-1β release from HepG2 (hepatocellular carcinoma cells), and knockdown of NLRC4 inhibits the

palmitate induced inflammation and cytokine release [130, 131]. Fatty acids can also cause the release of IL-1β from microglia, but the specific inflammasome that regulates this process in microglia has not been identified [127]. These studies highlighted the role of saturated fatty acids in the production of IL-1β by inflammasomes, i.e. NLRC4.

Fatty acids are known to cross the blood-brain barrier through passive diffusion [132, 133], and diets high in saturated fats increase brain uptake of fatty acids from the plasma [134, 135]. It is possible that increase uptake of fatty acids by the brain from the plasma [135] could lead to enhanced cytokine release and inflammatory response in the brain. Fatty acid metabolism has been suggested as a risk factor for the development of AD [136, 137], and epidemiological studies suggest that consumption of saturated fatty acids increase while unsaturated fatty acids decrease the risk of AD [138-140]. AD brains showed high fatty acid content as compared with normal healthy subjects [141]. Elevated saturated free fatty acids (FFAs) have been found in the plasma of patients with diabetes mellitus, hypertension and obesity, which are risk factors for developing AD [142, 143]. Traumatic brain injury, an independent risk factor for AD, showed increased levels of palmitate and stearate in the brain [144, 145]. Many *in vivo* studies reported that high fat diet induces Aβ deposition and memory deficits in APP transgenic mice [146-150]. Concomitanlty, our group demonstrated that palmitate can trigger the upregulation of AD-like characteristics, such as elevated BACE1, Aβ, and hyperphosphorylated tau levels in neurons, mediated by conditioned media from astrocytes cultured in palmitate [17, 151, 152]. A subsequent study reported that cytokines in the conditioned media, in particular IL-1β, secreted by palmitate-treated astrocytes, contributed to the upregulation of BACE1 and Aβ42 levels in primary neurons [17, 153]. However, palmitate does not directly induce the AD-like changes in neurons [152, 154], which could be attributed to the lower ability of neurons to take up and

metabolize long chain fatty acids [155, 156]. In contrast, astrocytes readily metabolize fatty acids, and furthermore peripheral administration of fatty acids was found to accumulate primarily in astrocytes [157, 158].

Palmitate and its metabolites, such as ceramides, regulate many gene expression and immunological pathways [35, 159-165]. Serine palmitoyltransferase (SPT), the rate-limiting enzyme that synthesizes ceramide, is upregulated in palmitate-treated astrocytes. Downregulating SPT to decrease ceramide levels mitigated the increase in IL-1β and TNFα secreted by the astrocytes into the cultured media [17, 166], suggesting that ceramide is involved in the cytokine production. Neutralizing TNFα and IL-1β in the conditioned media significantly reduced the upregulation of BACE1 in the neurons [17]. Moreover, our group and others have found the levels of SPT are upregulated in human AD brains as compared to age-matched controls [164, 166]. We confirmed that silencing SPT reduced while overexpressing SPT increased Aβ expression in primary mouse astrocytes expressing the human Aβ precursor protein [166]. *In vivo* animal studies demonstrated that inhibiting SPT decreased Aβ and tau hyperphosphorylation in an AD mouse model [167]. Taken together, the aforementioned studies suggest an involvement of saturated fatty acids in neuroinflammation mediated by glial cells. Due to the quantity, location, and function of astrocytes, they are likely major players in the induction of inflammation in the CNS generated by saturated fatty acids.

1.5 Therapeutic for inflammasome-mediated inflammation

Interleukins, in particular, IL-1β and IL-18, have been implicated in neuronal damage in chronic and progressive neuronal diseases, such as AD, Parkinson's disease, Huntington's disease, and Amyotrophic lateral sclerosis. Several approaches, described below, have been

evaluated for their ability to reduce the deleterious effects of excessive inflammatory cytokine production and signaling. Secreted extracellular domains of soluble IL-1 receptor and IL-1 receptor antagonist (IL-1Ra) were shown to mitigate IL-1β signaling by binding to IL-1β [168, 169]. Similarly, the IL-1 receptor II acts as a decoy receptor to inhibit IL-1β signaling [170]. Constitutively expressing IL-18 binding protein, which binds to IL-18, prevented the binding of IL-18 to its receptor and regulated IL-18 signaling [171]. Consequently, therapeutic approaches that have been developed to mimick the actions of these intrinsic factors. To date, blocking IL-1 signaling is the most effective therapy in many autoinflammatory disorders such as familial cold autoinflammatory syndrome, Muckle-Wells syndrome, chronic infantile neurologic cutaneous and articular syndrome, and TNF-receptor associated periodic syndrome [172]. Cytokine traps such as rIL-Ra, which recognizes the effects of IL-1β, are being tested in clinical trials for several autoinflammatory disorders [173, 174]. In support, inhibitors of IL-1 are effective in treating cryopyrin‑associated periodic syndromes (CAPS) and gout [175, 176]. Although inhibitors of IL-1β have been used to systemically treat inflammation [174], this type of therapy has not been reported for treating CNS inflammation. Non-steroidal anti-inflammatory drugs (NASIDs), used to suppress inflammation [177, 178], appeared to reduce the risk of developing AD after 2-3 years of treatment based on cognitive decline if taken prior to age 65 [48, 177]. This is controversial, since there are other reports of their increasing the risk of developing AD [179]. The diverse outcomes could be the result of different conditions, such as the age of the patients on treatment, dose of the drugs, duration of the treatment, and the severity of the neurodegeneration [178]. Nevertheless there are promising NSAIDs and combination of NSAIDs currently in clinical trials [180].

Inflammasome, required for the maturation of IL-1β and IL-18, is composed of a receptor such as NLPR1, NLPR3 and NLRC4, and caspase-1, and sometimes ASC. Therefore, targeting any of these components, such as the receptor, caspase-1 or ASC, could prevent the assembly and activation of the inflammasome, reducing IL-1β and IL-18 generation. In support, knockout of NLRP3 and caspase-1 have been shown to suppress amyloidogenesis and neuropathology, and improve cognition in AD transgenic mice [83]. Consequently, designing agents that control the activation or assembly of inflammasome could provide a promising approach to tackling neuroinflammasome. It was reported that a neutralizing antibody against NLRP1 was able to reduce cytokine levels after throboembolic stroke in mice, thereby reducing the detrimental consequences of postischemic inflammation [181]. The administration of anti-ASC neutralizing antibodies has been used in spinal cord injury in rats, which showed reduced caspase-1 activation and IL-1β and IL-18 levels, leading to better recovery after spinal cord trauma [78]. A tetrapeptide recognition sequence has been uncovered for caspase-1, WEHD (Trp-Glu-His-Asp), which can be inhibited by a compound, N-Acetyl-WEHD-aldehyde [169, 182]. Thus far clinical trials of this promising application have not been reported. Therapies for treating inflammasome-mediated inflammation have not been fully investigated and would be an interesting area for future exploration.

1.6 Conclusion

To date, studies on the role of inflammasomes in regulating the maturation of interleukins have enhanced our understanding of inflammation in diseases, including AD. Blocking or neutralizing IL-1β reduced cognitive impairment and decreased AD-like pathological changes in AD mouse models [75, 183]. Similarly, knockout of IL-1β receptor antagonist in mice increased

the neuronal damage induced by Aβ [76]. These findings support a role of inflammasome in the pathogenesis of AD, however, the detailed mechanisms of inflammasome assembly and activation in the CNS is still emerging. AD is a complex neurodegenerative disease with an unclear etiology and thus far, there are no proven treatments to prevent or slow its progression. Evidence suggests that environmental factors, e.g. saturated fatty acids, can initiate the activation of glial cells to release inflammatory molecules including IL-1β and IL-18 [17, 85, 127]. IL-1β and IL-18 can further induce the activation of glial cells in the CNS to secret more inflammatory molecules, creating a feedback loop to generate more proinflamatory molecules and resulting in increased amyloidogenesis and neurofibrillary tangles (Figure 1). Therefore, studying interleukin generation through the inhibition of inflammasomes assembly and activation could prove crucial to gaining a better understanding of the role of inflammasomes in AD, and providing novel therapeutics for AD.

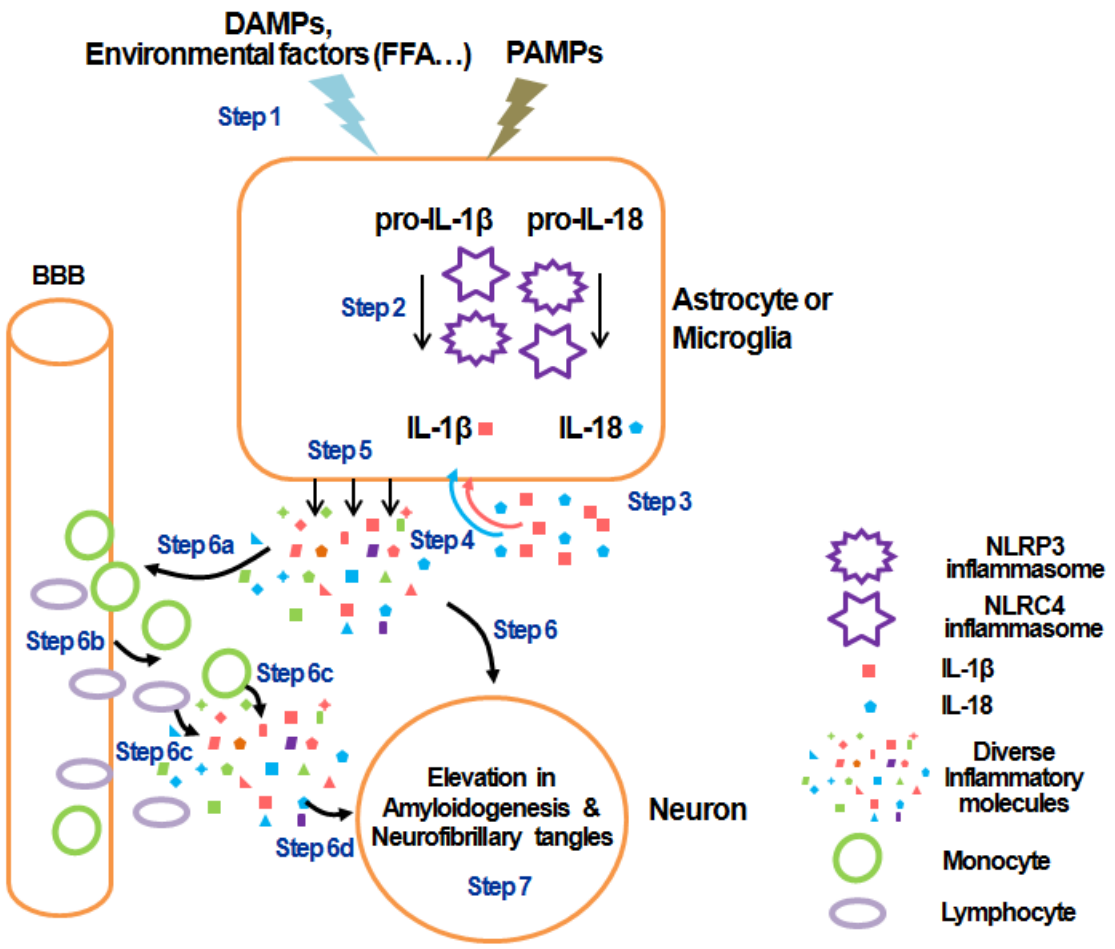

Figure 1.1 Scheme of inflammasome-mediated inflammatory response. Damage associated molecular patterns (DAMPs), environmental factors such as elevated free fatty acids (FFA), or pathogen associated molecular patterns (PAMPs) initiate the activation of inflammasomes in astrocytes or microglial cells to release inflammatory molecules, IL-1β and IL-18 (Step1-3). IL-1β and IL-18 can further activate more astrocytes or microglial cells in the CNS to secret more diverse inflammatory molecules (Step4, 5). The inflammatory molecules induce the elevation of amyloidogenesis and neurofibrillary tangles in neurons (Step6, 7). Concomitantly, the diverse inflammatory molecules also recruit other cells such as monocytes and lymphocytes to cross the

Figure 1.1 (cont'd) blood brain barrier (BBB) and to release more diverse inflammatory molecules, resulting in an increase in amyloidogenesis and neurofibrillary tangles in the neurons (Step6a-7). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

1.7 Specific aims of the current study

AD, a neurodegenerative brain disorder, is a leading cause of dementia in the adult population, and eventually leads to death. Currently there are 38 million AD patients worldwide, and every 4 seconds a new person is diagnosed with AD [5, 184]. Due to the aging population, the number of AD patients is predicted to reach to 115 million worldwide by 2050, and the average survival time of patients diagnosed with AD is 4.5 years [5, 184]. However, there is no way to prevent, cure or even slow its progression, and the etiology of AD remains unclear. Many *in vivo* and *in vitro* studies indicated that an environmental factor, fatty acid, is a risk factor of AD [17, 148-152, 154, 167]. Previous publications from our laboratory showed that palmitate (PA), the most abundant saturated fatty acid in the western diet, induced AD-like changes in primary rat neurons mediated by astrocytes [152, 154]. However, the molecular mechanism(s) leading to amyloidogenesis in primary neurons mediated by palmitate-treated astrocytes is unclear. The investigation of this subject will provide insights into the mechanism by which dietary fats may be contributing to the development of AD, and help find novel therapeutic targets to mitigate the physical and mental pain of AD patients. Therapeutic drugs potentially developed based on these mechanisms could help to decrease the death rate, lengthen the life span, and reduce the payment of AD patients and the financial burden to society. Therefore, in

the present study, three specific aims have been designed to elucidate the mechanism(s) involved in the induction of amyloidogenesis in primary neurons.

Specific aim 1 (Chapter 2): To characterize sphingomyelinase (SMase) and ceramide signaling pathway involved in the regulation of BACE1 in primary neurons.

Amyloid beta (Aβ), a hallmark of AD, is generated from amyloid precursor protein (APP) by sequential cleavage by BACE1 (β-site amyloid precursor protein-cleaving enzyme 1) and γ-secretase. The upregulation of BACE1, the rate-limiting protease in generating Aβ, was detected in AD patients' brains, AD transgenic mice, and primary neurons cultured in CM-P (conditioned media from palmitate-treated astrocytes). Ceramide, a product of both SMase and Serine palmitoyltransferase (SPT), has been shown to regulate BACE1 to promote Aβ biogenesis [165, 185]. A previous study in our laboratory reported that the increased ceramide levels were generated from *de novo* synthesis by SPT in primary rat astrocytes cultured in PA, and inhibiting SPT in astrocytes significantly reduced the AD-like changes in primary neuron cultured in CM-P [151]. Preliminary data showed an upregulation of ceramide levels in neurons cultured in CM-P. Since studies indicate that neurons have a low capacity to take up ceramides and long chain fatty acids, such as PA [155, 186], the rise in ceramide levels in the neurons treated with CM-P is likely being generated intracellularly. But inhibiting SPT in neurons does not significantly reduce the upregulation of ceramide levels. Therefore, we hypothesize that the increased ceramide levels in the neurons upon CM-P treatment are generated by SMases, leading to the rise of BACE1 level. Proinflammatory cytokines can activate SMases, and astrocytes are known to secret cytokines [187, 188]. Therefore, we hypothesize that PA induces the secretion of cytokines from astrocytes, and the secreted cytokines in the CM-P activate SMases in the

neurons. The next part of this aim is to determine which molecular factors in the CM-P activate SMases in neurons. This aim is discussed in Chapter 2.

Specific aim 2 (Chapter 3): To investigate a potential signaling pathway that transcriptionally regulates BACE1 and presenilin-1 in primary neurons treated with CM-P.

Increased Aβ level was reported in primary neurons upon treating with CM-P [151], and we demonstrate the mRNA levels of BACE1 and presenilin-1, a catalytic subunit of γ-secretase, increase in primary neurons treated with CM-P. pSTAT3 (signal transducer and activator of transcription) was shown to regulate BACE1 in neuroblastoma cells [189], and the regulation of BACE1 expression is cell-type and stimulus-specific [189, 190]. We confirmed that pSTAT3 could be a potential putative transcription factor of presenilin-1 by performing promoter analysis. pSTAT3 can be activated by cyclin-dependent kinase 5 (Cdk5)/p25 complex [189]. Increased p25, an activator of Cdk5 and the cleavage product of calpain, was found in the AD patient brain [191] and in primary neurons cultured in CM-P [154]. Mounting evidences indicate that increased calpain level, the calcium-dependent protease [192], and calcium dysregulation are involved in AD pathogenesis [193-195]. We observe elevated calcium level in neurons upon CM-P treatment. Collectively, we hypothesize a potential signaling pathway that CM-P elevates intracellular calcium levels and results in upregulated calpain activity. The increased calpain leads to STAT3 phosphorylation via cdk5 activation. STAT3 phosphorylation finally upregulates the mRNA levels of both BACE1 and presenilin-1. Therefore, Chapter 3 strives to identify this potential signaling pathway.

Specific aim 3 (Chapter 4): To identify the molecular mechanism involved in the production of IL-1β in primary astrocytes cultured with PA.

Many *in vivo* and *in vitro* studies suggested that IL-1β plays a role in the pathogenesis of AD [75, 76, 183]. However, the molecular mechanism initiating the generation of IL-1β in the central nervous system is poorly understood. PA triggers the generation of cytokines from primary astrocytes into the cultured media, in particular IL-1β [17]. IL-1β in CM-P is involved in the activation of SMases, leading to the upregulation of BACE1 in primary neurons cultured in CM-P [17]. We observe increased levels of both precursor IL-1β (pro-IL-1β) and mature IL-1β produced by astrocytes treated with PA. The multiprotein complex, inflammasome, is required for the cleavage of pro-IL-1β to form the mature pro-IL-1β. The assembly and activation of inflammasome is cell-type and stimulus-specific. However, the molecular mechanism(s) regarding the type and composition of inflammasome in primary astrocytes treated with PA is unclear. Therefore, Chapter 4 of this dissertation seeks the answers to these questions.

Finally, Chapter 5 presents the concluding remarks and identifies potential future studies that can be derived from this research.

CHAPTER 2. PALMITATE-ACTIVATED ASTROCYTES VIA SPT INCREASE BACE1 IN PRIMARY NEURONS BY SMASES

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Abstract

Astrocytes play a critical role in neurodegenerative diseases, including Alzheimer's disease (AD). Previously, we showed that saturated free-fatty acid, palmitate (PA), upregulates BACE1 level and amyloidogenesis in primary rat neurons mediated by astrocytes. However, the molecular mechanisms by which conditioned media from PA-treated astrocytes upregulates BACE1 level in neurons are unknown. This study demonstrates that serine palmitoyltransferase (SPT) in the astrocytes increases ceramide levels, which enhances the release of cytokines that mediate the activation of neural and acidic sphingomyelinase (N-SMase and A-SMase) in the neurons, to propagate the deleterious effects of palmitate, i.e. BACE1 upregulation. In support of the relevance of SPT in AD, our lab recently measure and found SPT levels to be significantly upregulated in AD brains as compared to controls [166]. Cytokines, namely TNFα and IL-1β, released into the conditioned media of PA-treated astrocytes activate N-SMase and A-SMase in the neurons. Neutralizing the cytokines in the PA-treated astrocyte conditioned media reduced BACE1 upregulation. However, inhibiting SPT in the astrocytes decreased the levels of both TNFα and IL-1β in the conditioned media, which in turn reduced the SMase activities and BACE1 level in primary neurons. Thus, our results suggest that the activation of the astrocytes

by PA is mediated by SPT, and the activated astrocytes increases BACE1 level in the neurons, the latter is mediate by the SMases.

Keywords: Alzheimer's disease, Fatty acid, Sphingomyelinase, Serine palmitoyltransferase, Ceramide, TNFα, IL-1β

2.1 Introduction

Alzheimer's disease (AD) is the most common dementia, affecting over 35.6 million people worldwide, with a total cost in the US of approximately $604 billion in 2010 [196]. Up to now, the etiology of AD remains enigmatic and numerous hypotheses of possible mechanisms have been put forth, of which the amyloid hypothesis is the most widely accepted. The amyloid cascade hypothesis posits that amyloid beta (Aβ) plays an early role and triggers a cascade of events which leads to neurodegeneration [197, 198]. The protein and activity levels of BACE1 (β-site amyloid precursor protein-cleaving enzyme 1), the rate-limiting protease involved in generating Aβ, have been found significantly elevated in AD brain [199]. BACE1 expression is constitutively expressed and inducible. Its regulation is complex and cell type-dependent [200].

Astrocytes, the most abundant glial cells in the central nervous system (CNS), are highly responsive to environmental changes. The activation of astrocytes has been attributed to the pathogenesis of several neurodegenerative diseases, including AD [201]. In further support, Aβ plaques are surrounded by activated astrocytes in human AD brains and in transgenic AD mouse brains [188]. These activated cells secrete proinflammatory molecules, such as tumor necrosis factor alpha (TNFα) and interleukin-1beta (IL-1β), IL-6, and nitric oxide [202]. Anti-inflammatory drugs have been suggested to potentially reduce the risk of AD [203]. However,

the molecular mechanism by which activated astrocytes enhances Aβ levels [204] and facilitates neuronal loss [35] is unclear.

Diet, an environmental factor, has been suggested to play a potential role in AD [136]. Further, studies showed that the AD brain is characterized by high fatty acid content as compared to the healthy subjects [141]. Epidemiological findings suggest that consumption of saturated free fatty acids (FFAs) may increase while unsaturated FFA may reduce the risk of AD [205]. *In vivo* animal studies further support this hypothesis, namely, mice fed a high fat and 1% cholesterol diet accelerated AD-like pathophysiological changes in their brains [146, 147]. Our group also confirmed that PA induces AD-like changes in the primary rat neurons mediated by ceramide generated through the SPT pathway in the primary rat astrocytes [151, 152, 154]. More recently we demonstrated that SPT levels are elevated in AD brain, and suppressing SPT reduced the Aβ expression, while overexpressing SPT increased Aβ expression in primary mice astrocytes expressing the human APP Swedish mutation [166]. We found previously that direct treatment of primary neurons with PA had no effect on the levels of BACE1 and Aβ [152]. This may be attributed to the low ability of neurons to take up and metabolize long chain fatty acids [155, 156]. However, conditioned media from PA-treated astrocytes (CM-P) significantly increased the levels of BACE1 and Aβ in the primary rat cortical neurons. The *de novo* synthesis of ceramides produced by the astrocytes upon treatment with PA mediated the increased levels of BACE1 and Aβ in the primary neurons [151, 152, 154]. However, the mechanisms leading to these changes in the primary neurons and mediated by the CM-P is unclear.

Recently, it was demonstrated that Aβ activates human astrocytes, which in turn released soluble neurotoxic substances, i.e. nitric oxide that killed the primary human neurons mediated by N-SMase. Knockdown of N-SMase decreased the ceramide level, which in turn, reduced the

activation of astrocytes and neuronal loss [35]. Ceramide, a product of both SMase and SPT, is three times higher in AD brain as compared to their age-matched controls [206, 207]. Reducing ceramide levels decreased Aβ levels in neuroblastoma cells [208], suggesting a potential role of ceramide in AD pathology. Indeed Puglielli et al. found that ceramide stabilizes BACE1 posttranslationally to promote Aβ biogenesis in human neuroglioma cells [165, 185].

Many studies have found associations between Aβ, SMase and ceramide [209-211]. For example, sphingomyelin (SM) levels are reduced while SMase levels are elevated in AD brain, and the upregulated SMase level correlated with increased Aβ and hyperphosphorylated tau levels [207]. The Aβ peptide has been shown, in turn, to induce apoptosis of both primary neurons and glial cells through the SMase-ceramide pathway [35, 212].

In the present study, we set out to elucidate the mechanism by which CM-P up-regulates BACE1 level in primary neurons and establish that an environmental factor, namely elevated saturated PA, can initiate the ceramide-amyloid cascade. We demonstrate that ceramide levels are up-regulated in primary rat neurons upon culture with CM-P. Ceramide can be produced through either the *de novo* or the SMase pathways. Evidence in the literature support that inhibiting *de novo* ceramide synthesis decreases the Aβ production and adding ceramide exogenously increases Aβ production (Puglielli et al., 2003; Patil et al., 2007). More recently, we showed that ceramide and SPT expression levels are elevated in a subgroup of sporadic AD patients [166]. In this study we demonstrate how increased levels of ceramides in the astrocytes upon culture with PA leads to the upregulation of BACE1 in the primary neurons, mediated by the SMases-ceramide pathway. The elevated level of ceramides in the PA cultured astrocytes enhanced the secreted levels of TNFα and IL-1β, which upregulated the activity of the SMases in primary neurons.

2.2 Materials and methods

2.2.1 Isolation and culture of primary rat neurons and astrocytes

All procedures were performed according to guidelines approved by the Institutional Animal Care and Use Committee at Michigan State University. Cortices from postnatal day 0 Sprague Dawley rats were used for neuronal culture according to the published methods [213]. Briefly, the brain regions were dissected, and the tissues were digested with papain (10 units/ml; Worthington, NJ, USA) and DNaseI (100units/ml; Roche, IN, USA) at 37 ^{o}C for 30 min and washed with Neurobasal A medium (all reagents are from Invitrogen, Carlsbad, CA, USA, unless otherwise specified). The mechanically separated cells were plated on poly-L-lysine (PLL, 50g/ml; Cultrex, Gaithersburg, MD, USA) coated plates at 2.5X10^{5} cells/cm^{2}. Neurons were maintained in neurobasal A medium with B27, 0.5 mM glutamine, and 1X penicillin/streptomycin (P/S). The medium was changed every 2 days. The primary neurons were cultured 3-4 days prior to treatment. The purity of neurons was >90% as determined by βIII tubulin immunostaining and flow cytometry.

Primary rat cortical astrocytes were isolated from newborn pups (P0-2). Cerebral cortices were removed, digested with papain and DNaseI for 30min at 37 ^{o}C, and washed with DMEM/F12. Approximately 4X10^{4}cells/cm^{2} were seeded on PLL coated plates and the cells were maintained in DMEM/F12 plus 10% fetal bovine serum and 1X P/S. The cultured medium was changed every 2-3 days. Cells were allowed to grow to confluence prior to treatment. The purity of the astrocytes monolayers were >90% as determined by GFAP immunoreactivity and flow cytometry.

2.2.2 Astrocyte conditioned media (CM) preparation

At 24 hr prior to treatment, the media for the primary neurons and astrocytes were changed to neuronal cell culture medium [DMEM 10313 supplemented with 10% horse serum, 10mM HEPE (Sigma, St. Louis, MO, USA), 2mM glutamine, and 1X P/S]. Astroctyes, cultured to 70-80% confluence, were treated with neuronal media containing 0.2mM PA (Sigma) plus BSA (fatty acid-free bovine serum albumin) (Millipore, Billerica, MA, USA) as a carrier protein, or BSA as control, or PA plus BSA and 2mM LCS (L-cysteine, SPT inhibitor) (Sigma). The PA/BSA molar ration was at 3:1 [214]. After 12 hr of incubation, the astrocytes conditioned media (CM-B, or CM-P, or CM-P+LCS) were used to treat neurons or used in combination with antibodies to neutralize the cytokines.

2.2.3 Quantitative real time polymerase chain reaction

mRNA was extracted using the RNA extraction kit (Qiagen, Valencia, CA, USA), then mRNA was reverse transcribed into cDNA using the cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The following primer sets (Operon, Huntsville, AL, USA) were used for PCR: N-SMase (5'-ccggatgcacactacttcagaa-3',5'-ggattgggtgtctggagaaca-3'), A-SMase(5'-tttcccgagccctgtaga-3',5'-atctgacccacgccaatg-3'), actin (5'-ctcttccagccttccttcct-3', 5'-aatgcctgggtacatggtg-3'), IL-1β (5'-gcatccagcttcaaatctc-3', 5'-ggtgctgatgtaccagttg-3'), TNFα (5'-ctactgaacttcggggtgatcggtc-3',5'-ctggtatgaagtggcaaatcggct-3'), INFγ (5'-agagcctcctcttggatatctgg-3',5'-gcttccttaggctagattctggtg-3'). Amplifications of the cDNA templates were detected by SYBR Green Supermix (Bio-Rad) using Real-Time PCR Detection System (Bio-Rad). The cycle threshold values were determined by the MyIQ software.

2.2.4 Western blot

Whole cell extracts lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer were assayed for protein concentrations by Bradford assay (Bio-Rad). 15-30 μg protein samples

were separated by 10% Tris–HCl gel and transferred to nitrocellulose membrane. Membranes were then blocked with 5% milk or 5% BSA in 0.05% Tween 20-TBS (Tris buffered saline) (USB corporation, Fremont, CA, USA) for 1 hr and incubated with primary antibodies, BACE1 (Abcam, Cambridge, MA, USA) and actin (Sigma) overnight at 4℃. Next day, anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific, Asheville, NC, USA) was added and the blots were incubated for 1 hr at room temperature. The blots were washed three times with 0.05% Tween 20-TBS, and then visualized by SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

Secreted cytokines in the astrocyte supernatants were analyzed by an ELISA kit (R&D system, Minneapolis, MN, USA). The sensitivity of the assay was 5pg/ml for TNFα and IL-1β. Optical densities were measured by Spectra MAX Plus384 plate reader at 450nm wavelength. Each sample was assayed in triplicates and three independent reactions were performed. All readings were normalized to the total cell protein content (Bradford assay), and then the data were normalized to the control (BSA treatment).

2.2.6 Neutralization of cytokine bioactivity

The concentration of TNFα and IL-1β recombinant proteins (R&D system) used for neutralization were optimized based on the concentration of TNFα and IL-1β in the CM-P and the neutralization $dose_{50}$ of recombinant proteins. Neutralization was performed as described in manufacturer's information sheet. Briefly, the conditioned media was collected and incubated with recombinant proteins for 2 hr at 37 ^{o}C prior to treating the cells.

2.2.7 Sphingomyelinase assay

Cells were lyzed with the neutral (100 mM Thris-HCl, 1mM $MgCl_2$, pH 7.4) or acid lysis buffer (50mM sodium acetate, pH 5.0), to measure N-SMase or A-SMase activities, respectively, along with protease inhibitor cocktail (Sigma). SMase activities were measured using Amlpex Red Sphingomyelinase Assay Kit (Invitrogen) as described in the manufacturer's protocol. The total protein level in the cells was determined by Bradford method. The SMase activity levels were normalized to the total cell protein content, and then normalized to the control (CM-B treatment).

2.2.8 Measurement of ceramide

Primary neurons were treated with astrocyte conditioned media for the indicated time. The cells were washed twice with PBS, and the lipid was extracted as described in [215]. The ceramide levels were detected by mass spectrometry using an AB 3200 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA, USA). Multiple reaction monitoring (MRM) for quantitative LC/MS/MS was performed. C16 and C24 ceramide (Avanti, Alabaster, AL, USA) were used as standards. The total protein levels were measured by the Bradford method. The total ceramide levels were normalized to the protein level, and then normalized to the control (CM-B treatment).

2.2.9 Statistical analysis

All experiments were performed at least three times, and representative results are shown. Statistical analysis were performed by an unpaired, two tail student t-test. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

2.3 Results

2.3.1 Ceramide level is up-regulated in primary neurons with CM-P treatment

Previously we showed that conditioned media from palimtate-treated astrocytes (CM-P) upregulated the BACE1 level and amyloidogenesis in primary rat neurons. The upregulation was mediated by the increase in ceramides that were generated through the *de novo* ceramide synthesis pathway (SPT) in the astrocytes [151]. It is known that ceramide can stabilize BACE1 to promote Aβ generation [165]. Therefore, we examined the ceramide level in primary neurons upon CM-P treatment, and found it increased dramatically and peaked at 12hr (Figure 2.1A). L-cycloserine (L-CS), a specific inhibitor of the rate-limiting enzyme SPT, was used to pre-treat the primary astrocytes. This was followed by co-treatment of the astrocytes with PA plus L-CS, denoted as CM-P+LCS. The CM-P+LCS media was subsequently used to treat the primary neurons. Upon inhibiting SPT in the astrocytes, the increased ceramide level in the neurons cultured in PA-treated astrocyte conditioned media (namely, CM-P+LCS) was reduced (Figure 2.1B). Note that the direct pre-treatment of the neurons with L-CS follow by culturing in the PA-treated astrocyte conditioned media concomitantly with L-CS (namely, CM-P-LCS) did not reduce the ceramide levels (Figure 2.1C). These results raised the question of whether the ceramide, produced by the astrocytes upon treatment with PA, was released into the supernatant, i.e. conditioned media (CM-P), and subsequently crossed the membrane of the neurons.

A recent study demonstrated that Aβ can significantly increase the ceramide level within primary human astrocytes, however the extracellular ceramide levels in the cultured media was undetectable [35]. This suggests that the high concentration of intracellular ceramide level in the astrocytes was not likely released into the supernatant. Furthermore, numerous studies indicate that neurons have a very low capacity to take up ceramides and long chain fatty acids, such as PA [155, 156]. Thus the rise in ceramide levels in the neurons treated with CM-P is likely generated intracellularly. Since ceramides can be produced by either SMases or SPT, we first

tested whether L-CS could inhibit SPT to reduce ceramide levels in the CM-P treated neurons. To assess this possibility, primary rat neurons were treated with 2mM L-CS for 2hrs, followed by co-treatment with 2 mM L-CS and the CM-P from the astrocytes and found this treatment with L-CS did not significantly reduce the ceramide levels in the neurons (Figure 2.1C). Therefore, the *de novo* ceramide synthesis pathway in the neurons is not likely responsible for the rise in neuronal ceramide level upon CM-P treatment. Next, given that SMases are also major pathways for generating ceramides, we investigated whether the increased ceramide levels in the neurons are due to A-SMase and N-SMase, the major enzymes that hydrolyze SM.

2.3.2 SMases are responsible for the increased ceramide in primary rat neurons upon CM-P treatment

The mRNA (Figure 2.2 A, B) and activity (Figure 2.2 C, D) levels of A-SMase and N-SMase increased significantly in primary neurons upon CM-P treatment. Inhibiting SPT with L-CS in the astrocytes significantly reduced the mRNA (Figure 2.2 A, B), and activity (Figure 2.2 C, D) levels of the SMases and ceramide (Figure 2.1B) in the neurons. This raised the possibility that the SMases in the neurons could be responsible for the ceramide rise. Nevertheless the SPT in the astrocytes initiated the path towards this upregulation of SMase expression and activity levels in the neurons.

To confirm that the increased ceramide levels in the neurons are due to the SMases, we inhibited A-SMase and N-SMase with specific inhibitors, 15uM desipramine (Sigma) and 25uM GW4869 (Cayman Chemical, Ann Arbor, MI, USA), respectively, individually and in combination. The ceramide levels in the CM-P treated primary neurons decreased significantly upon SMase inhibition (Figure 2.3). Therefore the ceramide rise in the CM-P treated primary neurons is due to the SMases rather than the *de novo* synthesis pathway of SPT. Further, it

indirectly confirms that the ceramide produced by the astrocytes are not likely being taken up by the neurons. Therefore, other soluble products released into the CM-P media from the astrocytes upon PA treatment are responsible for upregulating the SMase activities in the neurons. SMase activity can be induced by many different stimuli, i.e. UV or ionizing irradiation, heat shock, nerve-growth factor, Fas, TNFα or IL-1β [187].

2.3.3 Palmitate induces the secretion of proinflammatory cytokines from primary rat astrocytes and neutralizing the cytokines decreases the SMase activity

Astrocytes are known to secret proinflammatory molecules, such as TNFα, IL-1β, IL-6, interferon (IFN)-γ and nitric oxide (NO) [188]. Our lab demonstrated that PA does not induce the production of IL-6 in astrocytes upon PA treatment [216]. A recent report showed that PA induces the production of TNFα and IL-1β in macrophages through the *de novo* ceramide synthesis pathway [217]. Further, an *in vivo* study established that IFN-γ and TNFα regulate Aβ plaque deposition and BACE1 expression in a transgenic AD mouse model [52]. Therefore we measured the mRNA levels of TNFα and IL-1β and found them, most notably IL-1β, to be significantly upregulated in the astrocytes treated with 0.2mM PA (Figure 2.4 A). Correspondingly, the protein levels of TNFα and IL-1β were also elevated (Figure 2.4 B). However INF-γ was not detected (data not shown). Patil et al. demonstrated that PA increased ceramide levels in astrocytes through the *de novo* ceramide synthesis pathway [151]. To determine if SPT in the astrocytes induces TNFα and IL-1β generation upon PA treatment, we inhibited SPT with L-CS and found the levels of TNFα and IL-1β secreted by the astrocytes decreased (Figure 2.4 B). Therefore, ceramide is involved in the production of cytokines in the primary astrocytes. These cytokines, in turn, activate the SMases in the neurons.

To determine if these cytokines are involved in mediating SMase activities in the neurons, anti-rat TNFα and IL-1β antibodies, were used to neutralize TNFα and IL-1β in the CM-P media, prior to transferring the CM-P to the primary neurons. The A-SMase and N-SMase activities decreased significantly in neurons treated with CM-P that had the cytokines, TNFα and IL-1β, neutralized (Figure 2.5). Therefore the upregulated SMase activities in the neurons are mediated in part by TNFα and IL-1β. In support, SMase activities in various cell types, including neurons, were reported to be activated by cytokines, i.e. TNFα and IL-1β [218, 219].

2.3.4 Upregulated BACE1 in primary neurons upon CM-P treatment is related to SMase-ceramide pathway in the primary neurons

Previously, we demonstrated that neurons treated with CM-P increased their BACE1 protein level, which decreased upon inhibiting SPT in the astrocytes [151]. CM-P up-regulated the BACE1 protein level in the neurons in a time-dependent manner (Figure 2.6). Ceramide has been reported to stabilize the BACE1 protein and increase Aβ generation [165]. Inhibiting SPT reduced ceramide levels in the astrocytes, which in turn decreased neuronal ceramide generation upon CM-P treatment (Figure 2.1B). Since the ceramide increase in the CM-P treated neurons are due to the SMases, we pre-treated the neurons with respective A-SMase and N-SMase inhibitors, GW4869 and desipramine, individually and in combination, followed by co-treatment of the neurons with the inhibitors and CM-P media for 12hr. The BACE1 level decreased in the CM-P cultured neurons pre- and co-treated with the inhibitors as compared to the CM-P cultured neurons without the inhibitor treatment (Figure 2.7). Therefore, the ceramide produced in the neurons by the SMases enhanced the BACE1 protein level in the CM-P treated neurons. A recent report showed that ceramide can increase the expression level of acetyltransferases' (ATase1 and ATase2) and the latter posttranslationally regulates BACE1 by stabilizing the protein [185].

Therefore, we measured the ATase levels in the CM-P treated neurons, and found the mRNA levels of ATase1 and ATase2 are significantly increased (Figure A.1). Thus ATases may be involved in upregulating the BACE1 level in neurons treated with CM-P.

Since TNFα and IL-1β activate the SMases to increase ceramide production in the neurons, they should indirectly impact the BACE1 level. Neutralizing TNFα and IL-1β in the CM-P with their respective antibodies, prior to transferring the CM-P to the primary neurons for 12 hr significantly decreased the BACE1 levels (Figure 2.8). Therefore cytokines, namely TNFα and IL-1β, released by the palmitate-treated astrocytes, activate the SMase-ceramide pathway in the neurons to thereby upregulate the BACE1 levels.

2.4 Discussion

Alzheimer's disease is a complex, devastating neurodegenerative disorder that results from multiple environmental and genetic factors. Epidemiological studies suggest that saturated FFAs may increase the risk of AD [205]. Feeding animals with high fat diets leads to cognitive impairment and accelerated AD-like pathology [220]. Evidence from our group suggest that PA does not directly induce AD-like pathogenesis in primary rat neurons, whereas CM-P media does, therefore implicating PA nevertheless participates in the Aβ biogenesis in primary neurons [152, 154]. In further support, previous studies demonstrate that the increased production of ceramide by the astrocytes through SPT played a critical role, and inhibiting SPT in the astrocytes attenuated the AD-like changes in primary neurons [151]. However, the mechanism by which CM-P induced AD changes in the neurons is unknown. To address this, we investigated a possible mechanism and found the neuronal ceramide level was significantly enhanced upon CM-P treatment, mediated by SMases rather than SPT. This is consistent with studies that have

shown that the SMases levels are upregulated upon treating with amyloid peptides to increase the ceramide levels and neuronal apoptosis [211]. Nevertheless SPT upregulation in the astrocytes is involved in upregulating the SMase activities in the neurons.

It is not surprising that activated astrocytes play a role in the AD-like pathological changes in primary neurons, given the strong neuroinflammatory response in AD brains, and that amyloid plaques are surrounded by activated astrocytes and microglia [188]. Recent studies showed that overexpressing molecules from activated astrocytes in AD transgenic mouse model increased inflammation in the brain and exacerbated AD pathology [221]. Activated astrocytes can release inflammatory molecules, such as cytokines, reactive oxygen species and nitric oxide (NO) [34]. Many have reported that SMase activity can be activated by cytokines, i.e. TNFα and IL-1β, in several different cell types, including neurons [218, 219]. To link astrocytes and neurons under pathological conditions, i.e. elevated FFA (i.e. PA) levels, we set out to determine which molecules released from the astrocytes upon PA treatment are involved in increasing BACE1 level and amyloidogenesis in the neurons. We found that TNFα and IL-1β are secreted by astrocytes, and these cytokines upregulate the SMase activities. Neutralizing TNFα, IL-1β, or both in the CM-P media decreased both the A-SMase and N-SMase activities in the neurons upon CM-P treatment. This attenuated the upregulation in the BACE1 level, thereby indicating that TNFα and IL-1β released upon *de novo* synthesis of ceramides in the astrocytes induced an upregulation in the SMase activities in the neurons. In addition to cytokines, NO may account for the residual increase in SMase levels. Recently, a transwell study further implicated a connection between activated astrocytes and neurons in AD pathogenesis and found that NO released from the astrocytes activated the SMase-ceramide pathway to cause neuronal cell loss [35].

Numerous studies have shown that SMases play important roles in the induction of apoptosis upon treatment with Aβ in the neurons as well as other cells in the CNS [211]. Most of the prior cell studies have focused on a single cell type, i.e. neurons, and did not incorporate the interactions mediated by soluble factors between neurons and astrocytes. Here we demonstrate for the first time that upregulated SMase activities in the neurons are due directly to the activation of the *de novo* ceramide synthesis (namely, SPT) in the astrocytes upon PA treatment. SPT is the first and rate-limiting enzyme in *de novo* synthesis of ceramide from PA. The ceramide produced in the astrocytes released soluble factors into the CM-P to modulate the AD-like pathophysiology in the neurons. Thus SPT may play an initiating role in the AD-like pathology and the neuronal damage that results from elevated levels of saturated fatty acids arising from environmental factors, i.e. diet or head trauma.

Many studies showed that perturbed sphingomyelin metabolism is an important event in neurodegeneration, elevating ceramide levels and changing the structural and functional plasticity of the neurons [161]. Studies in AD brain suggest that the expression of several genes controlling the synthesis of ceramide are upregulated [160], e.g. increased SMase activities (He et al., 2008) and SPT protein levels [207]. Ceramide has been shown to stabilize BACE1 and promote Aβ biogenesis [165], including post-translational regulation of BACE1 by enhancing the acetyltransferase (ATase) activity [185]. Similarly, we also found the mRNA levels of ATases are upregulated in primary neurons cultured with CM-P (Figure A.1). Ceramide also can increase the level of the activated form of the double stranded RNA-activated protein kinase, p-PKR [222]. Elevated levels of the active form of the eukaryotic translation initiation factor 2 (eIF2α), a downstream target of p-PKR, can increase the translation of BACE1 to promote amyloidogenesis [223, 224]. We found that p-PKR level was significantly increased in primary

neurons treated with CM-P (Figure A.2). These events also could contribute to increased BACE1 level in primary neurons treated with CM-P.

Based on our findings and the published results, we proposed the following sequence of events on how elevated level of PA may initiate the Aβ cascade leading to the pathophysiology of AD (Figure 2.9). The astrocytes in the brain metabolize PA to ceramide through the *de novo* ceramide synthesis pathway (SPT), which initiates the release of soluble molecules, i.e. cytokines, from the astrocytes. In turn, these soluble molecules activate the SMase-ceramide pathway to increase ceramide levels in the neurons. The ceramide produced in the neurons could post-translationally upregulate the BACE1 levels in the neurons to enhance Aβ production [165, 185]. The upregulated extracellular Aβ level, in turn, could act on both the astrocytes and the neurons to further enhance the intracellular ceramide levels [35]. This would continue to reinforce the ceramide-Aβ-ceramide cascade. Finally, although the cell studies do not truly recapitulate the *in vivo* situation of AD pathogenesis, nevertheless the literature information coupled with our results suggest that tight regulation of ceramide production may be an important therapeutic approach to modulating BACE1 level.

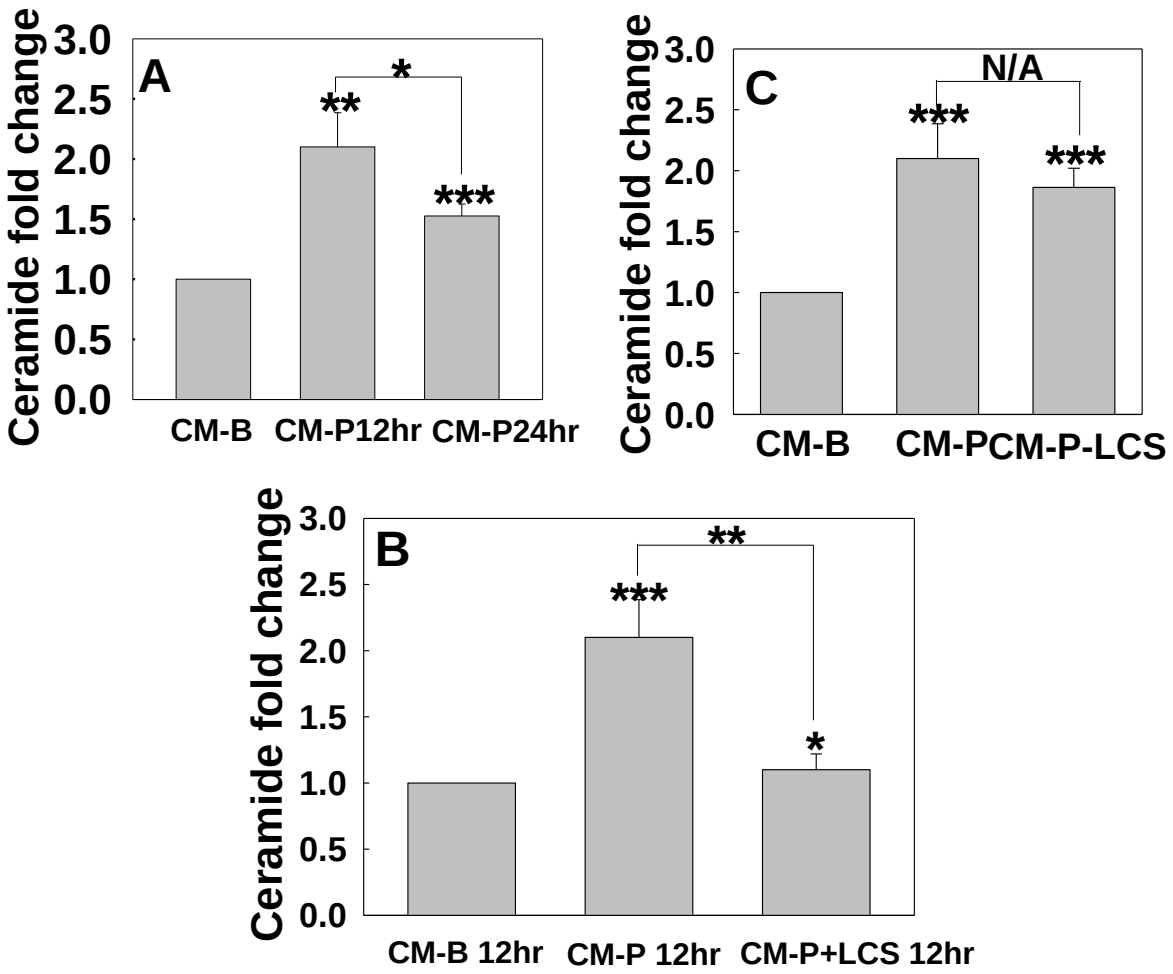


Figure 2.1 Changes in ceramide levels in primary rat neurons. Primary rat astrocytes were cultured with BSA (B) or palmitate (P) or palmitate plus L-CS (P+LCS) (LCS: SPT inhibitor) for 12hr. The conditioned media (CM-B, CM-P, or CM-P+LCS, respectively) were subsequently used to treat primary rat neurons for 12 or 24hr. Ceramide was detected by LC/MS/MS and normalized to CM-B (ctrl) (n=3) (A, B). (C) Primary rat neurons were treated with CM-B, CM-P, or pre and co-treated with L-CS (CM-P-LCS) for 12hr. Ceramide was detected by LC/MS/MS and normalized to CM-B (ctrl) (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

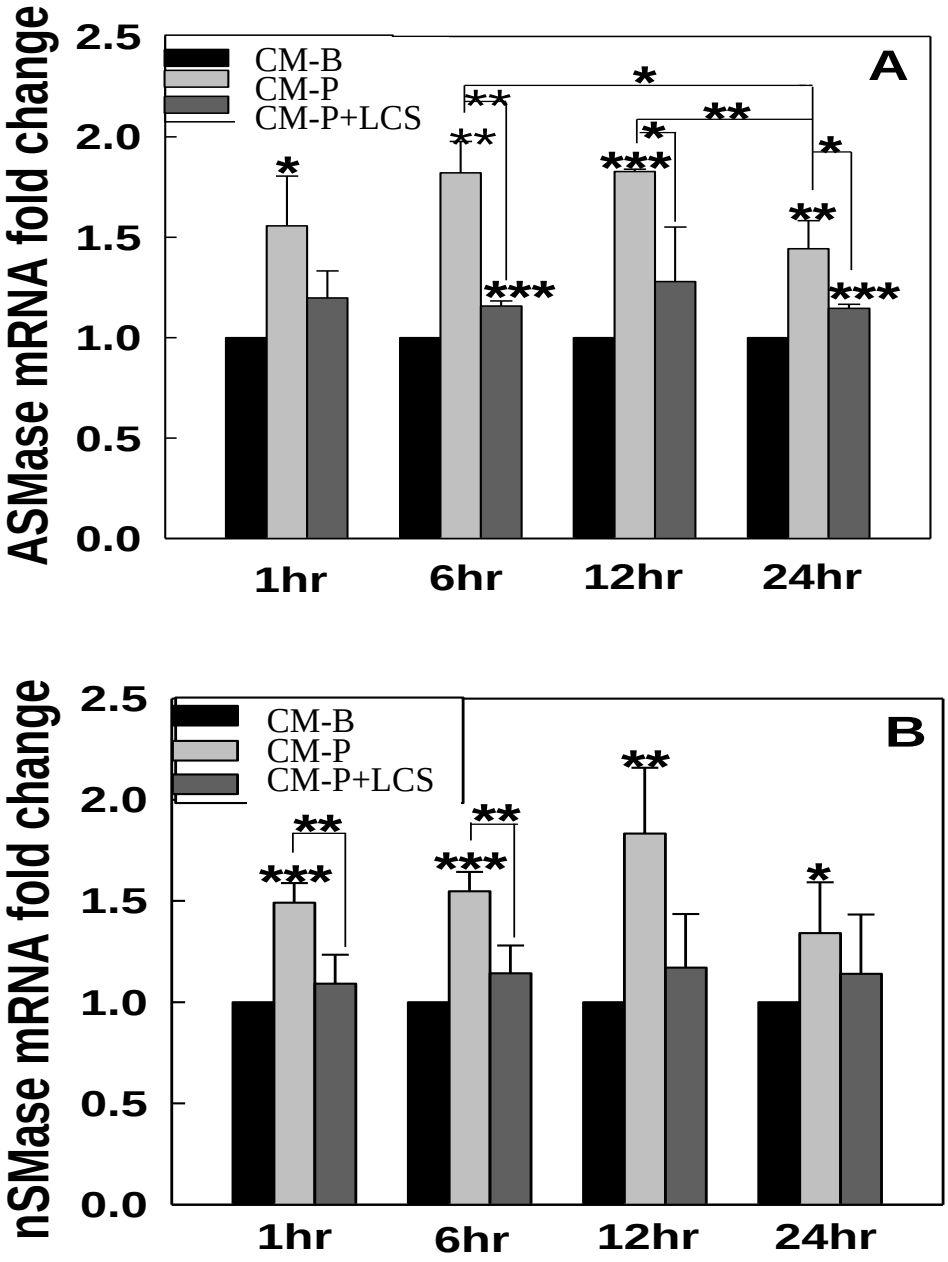
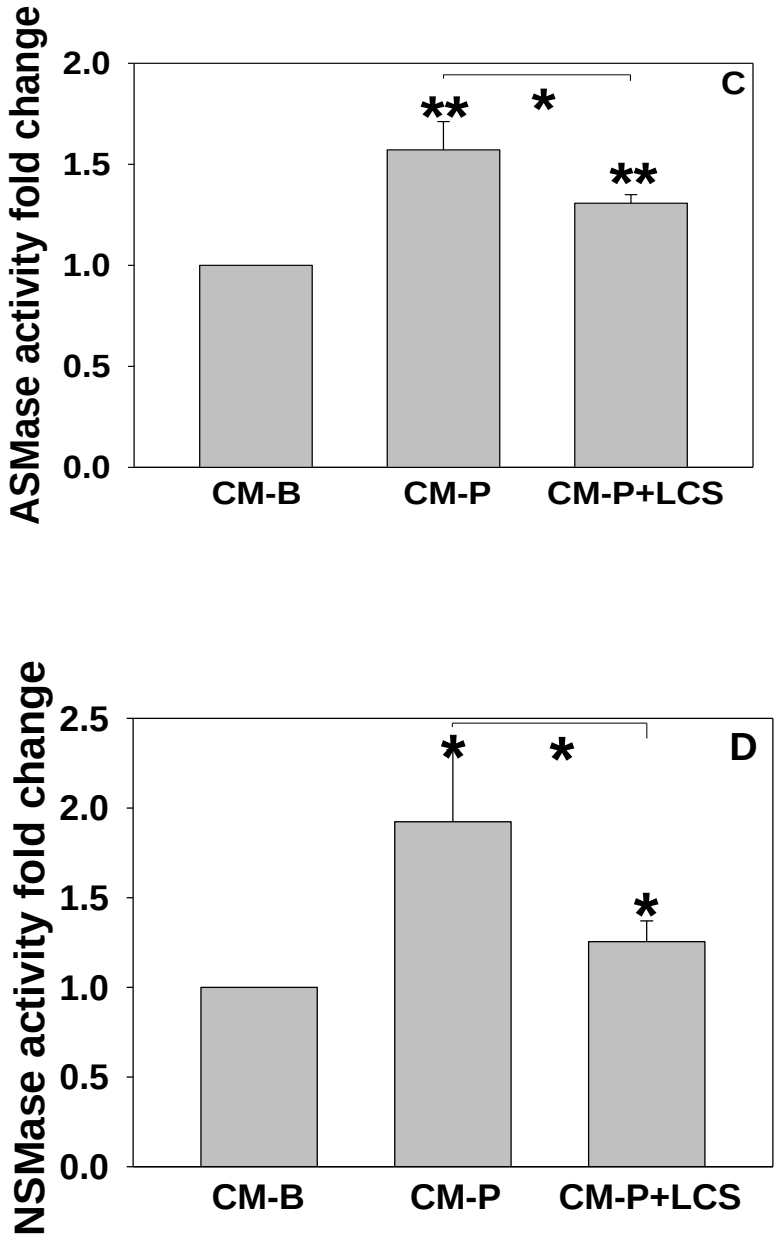


Figure 2.2 Effect of CM on the SMase level in the neurons. (A, B) The conditioned media, CM-B, CM-P or CM-P+LCS, were used to treat neurons for 1, 6, 12 and 24hr and their mRNA levels were measured by real-time PCR. The mRNA of A-SMase (A) and N-SMase (B) were normalized to actin, then normalized to CM-B (ctrl) at each time point (n=3) . A-SMase (C) and N-SMase (D) activities in neurons cultured in CM-B (ctrl), CM-P or CM-P+LCS for 12hr (n=3).

Figure 2.2 (cont'd) *: p<0.05, **: p<0.01, ***: p<0.001. A line indicates comparison between the two bars connected by the line.



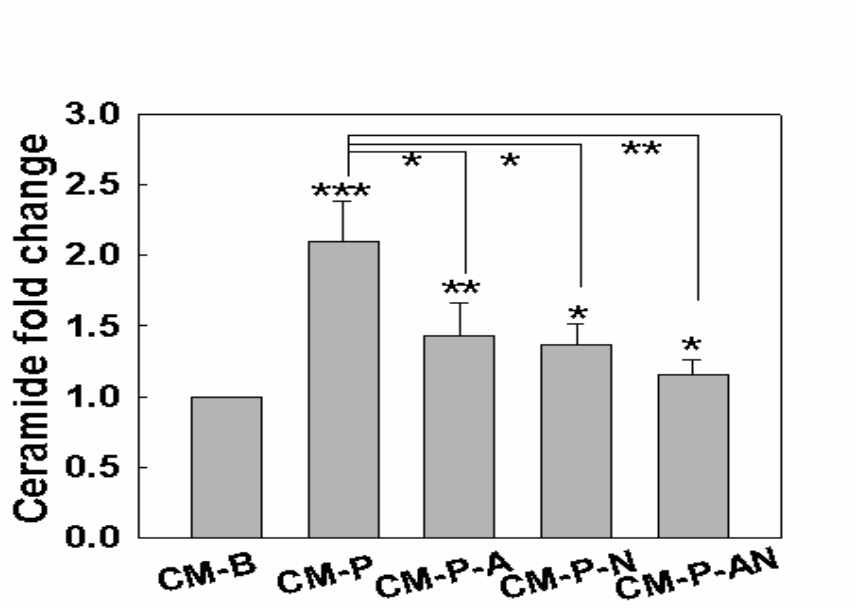


Figure 2.3 Effect of inhibiting SMase activities on the ceramide levels in the neurons. Ceramide levels of primary rat neurons cultured in CM-B (ctrl), CM-P, CM-P plus desipramine (A-SMase inhibitor, CM-P-A), CM-P plus GW4869 (N-SMase inhibitor, CM-P-N), or CM-P plus desipramine and GW4869 (CM-P-AN) for 12hr. Ceramide was detected by LC/MS/MS (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

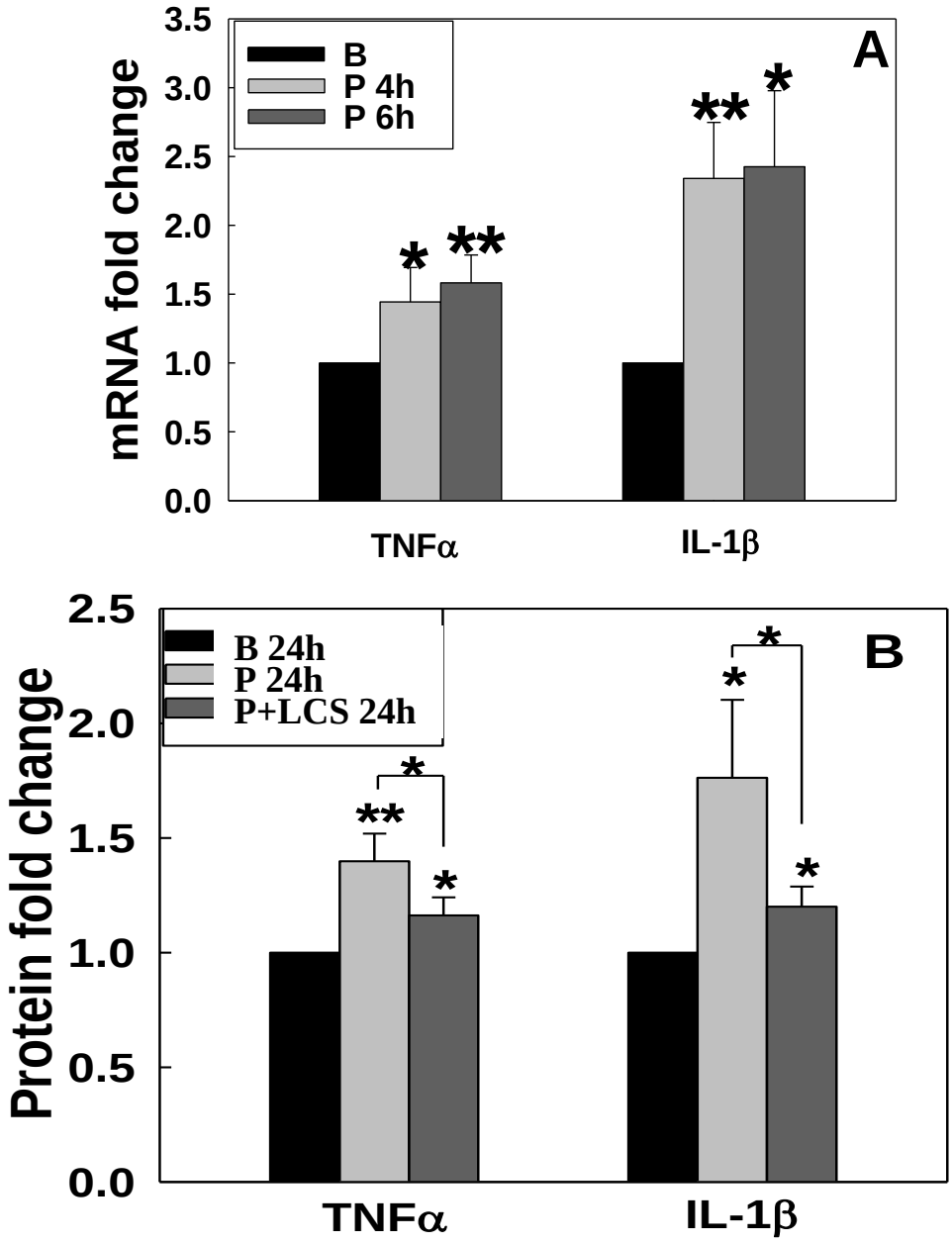


Figure 2.4 TNFα and IL-1β levels of astrocytes upon PA treatment. Astrocytes were treated with BSA (B), palmitate (P) or palmitate plus L-CS (P+LCS) for indicated time. (A) mRNA levels of TNFα and IL-1β were detected by real-time PCR (n=3). (B) Protein fold-change of TNFα and IL-1β. The protein levels of cytokines in the supernatant were measured by ELISA (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

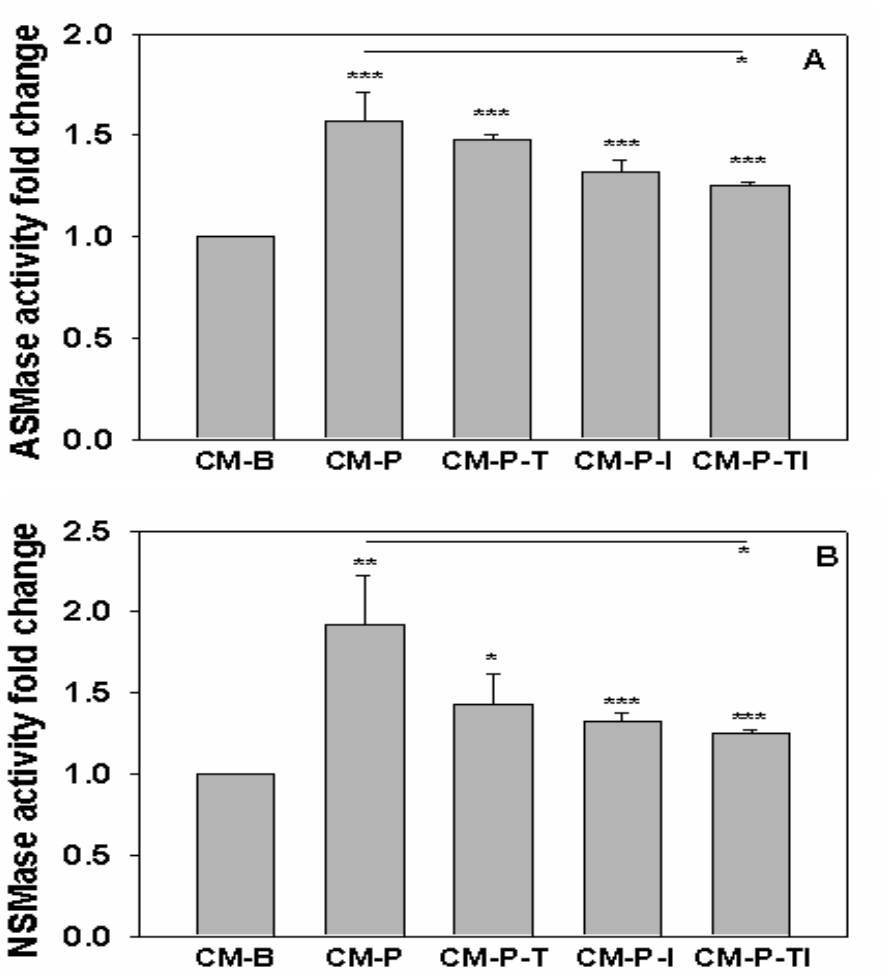


Figure 2.5 Neutralizing TNFα and IL-1β decrease SMase activity. A-SMase (A) or N-SMase (B) activities in neurons upon treatment with CM-B (ctrl), CM-P, CM-P-T (TNFα in CM-P media was neutralized with TNFα antibody), CM-P-I (IL-1β in CM-P media was neutralized with TNFα antibody), CM-P-I (IL-1β in CM-P media was neutralized with IL-1β antibody), or CM-P-TI (TNFα and IL-1β were neutralized in CM-P media) treatment for 12hr (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

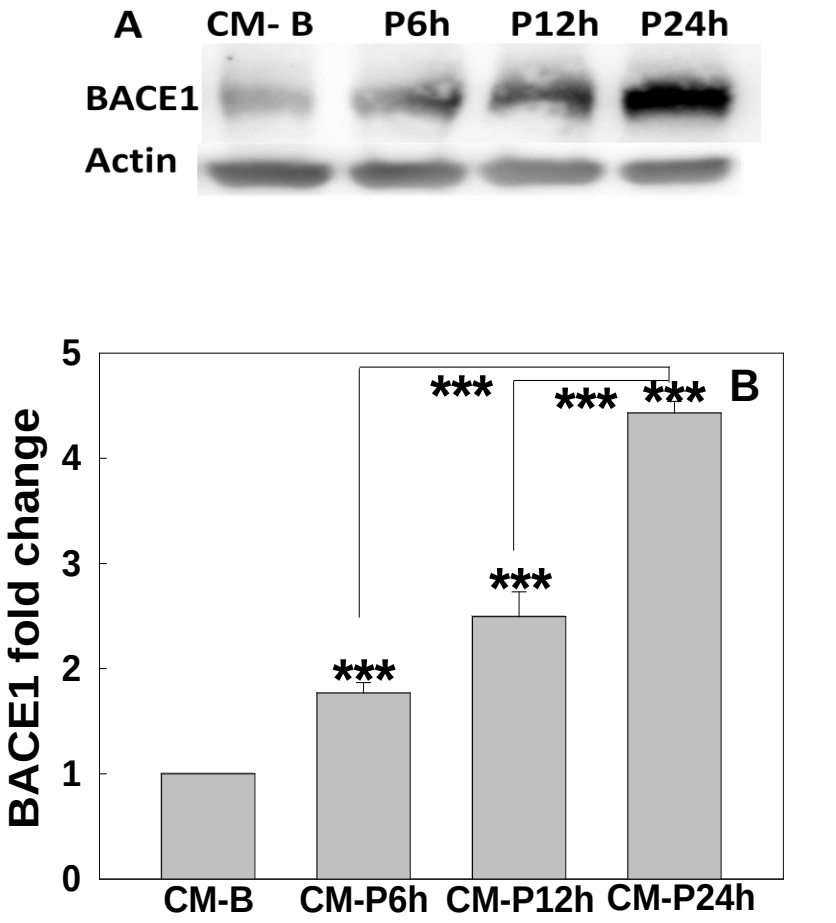


Figure 2.6 Effect of CM-P on BACE1 level in the neurons. Primary rat neurons were cultured with CM-B (ctrl) or CM-P for 6, 12 and 24hr. (A) Representative western blot result of BACE1 protein. (B) Quantification of western blot results (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

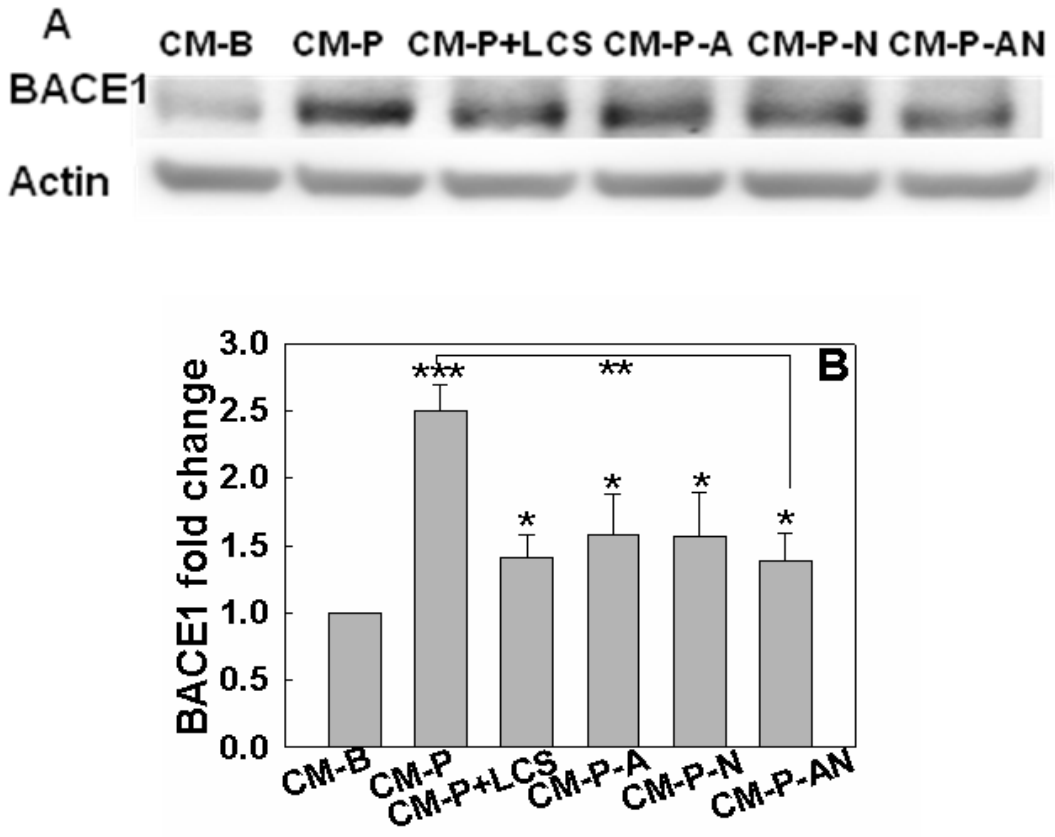


Figure 2.7 Effect of inhibiting SMase on the BACE1 level in neurons upon CM treatment. (A) Representative western blot result of BACE1 protein levels in primary rat neurons cultured in CM-B (ctrl), CM-P, CM-P plus desipramine (A-SMase inhibitor, CM-P-A), CM-P plus GW4869 (N-SMase inhibitor, CM-P-N), or CM-P plus desipramine and GW4869 (CM-P-AN) for 12hr (n=3). (B) Quantification of western blot results (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

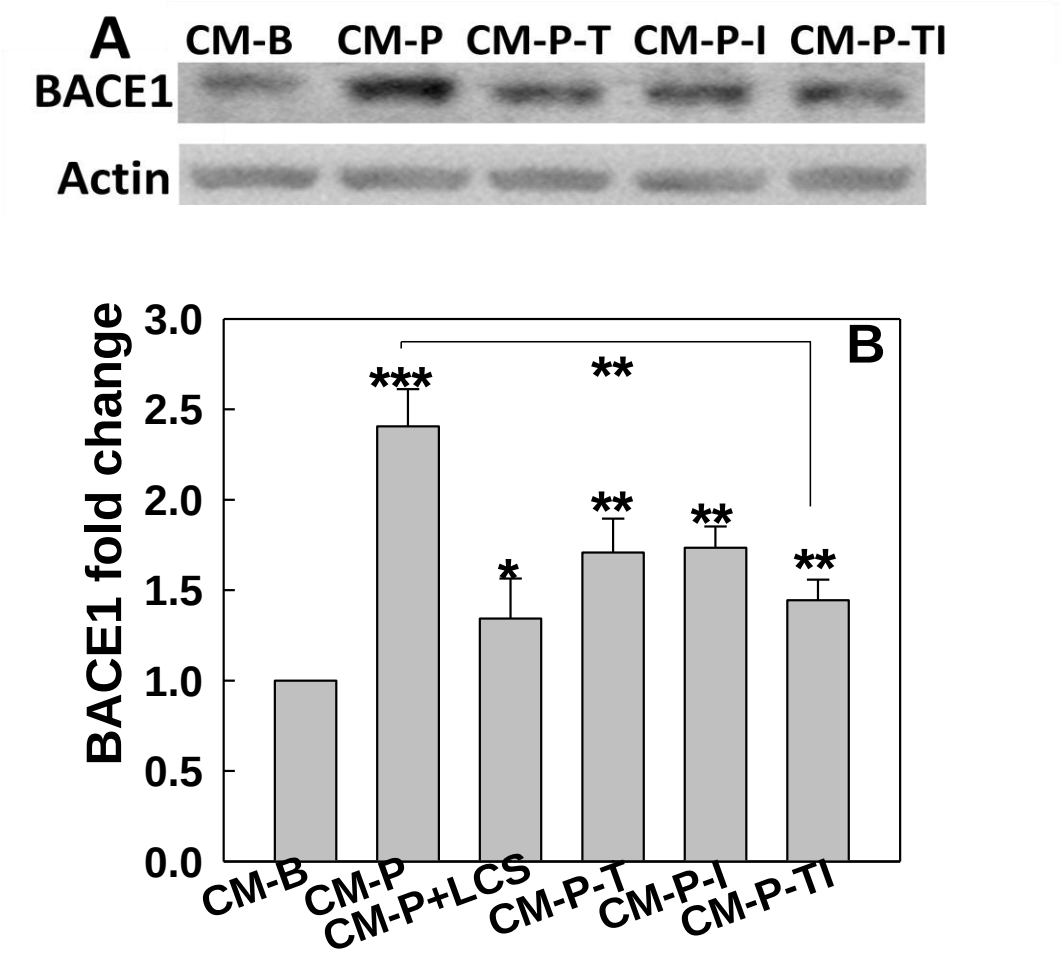


Figure 2.8 Neutralizing TNFα and IL-1β decrease BACE1 in neurons. (A) BACE1 protein levels in neurons upon treatment with CM-B (ctrl), CM-P, CM-P-T (TNFα in CM-P media was neutralized with TNFα antibody), CM-P-I (IL-1β in CM-P media was neutralized with IL-1β antibody), or CM-P-TI (TNFα and IL-1β in CM-P media were neutralized) treatment for 12hr (n=3). (B) Quantification of western blot results (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

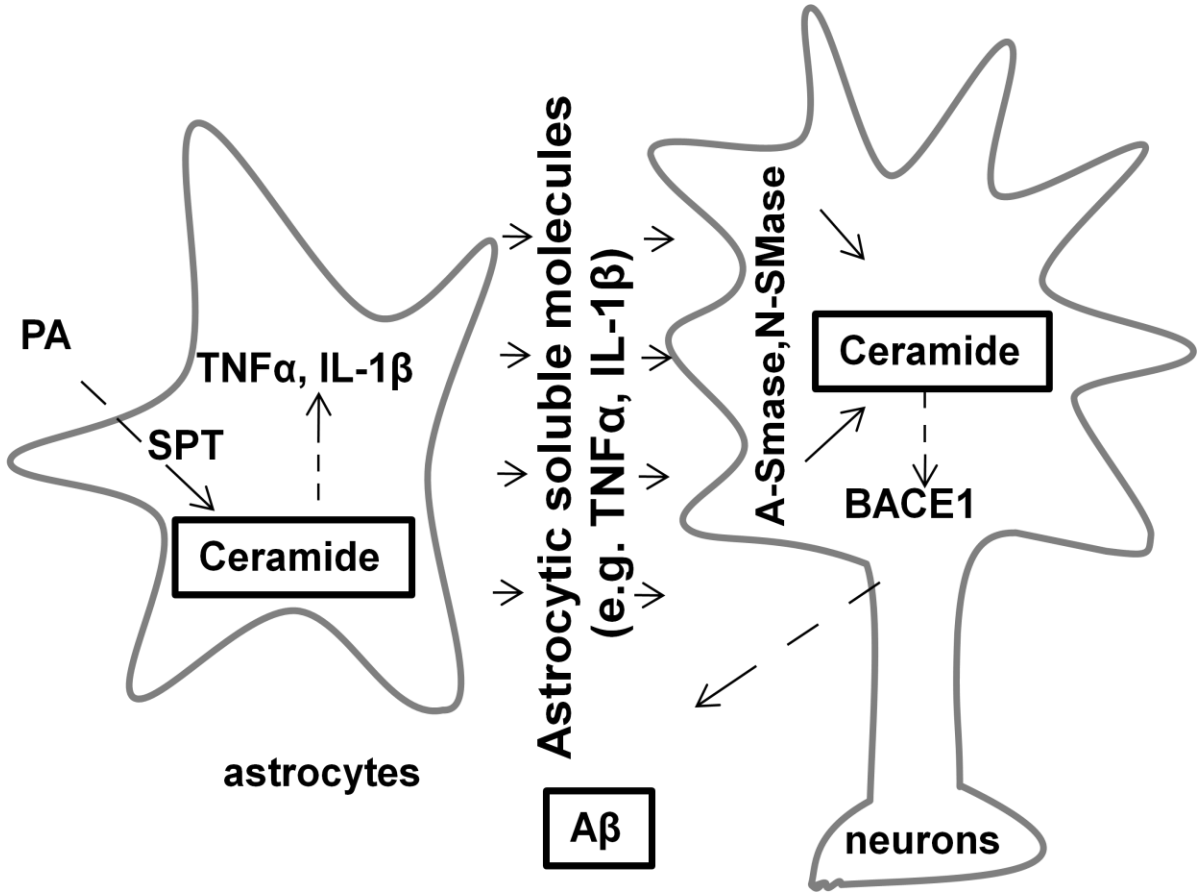


Figure 2.9 Proposed cellular mechanism by which palmitic acid metabolism induces amyloidogenesis in primary neurons mediated by astrocytes. The astrocytes in the brain metabolize PA to generate ceramides through the *de novo* ceramide synthesis pathway by SPT, which initiates the release of soluble molecules, i.e. TNFα and IL-1β, from the astrocytes. In turn, these soluble molecules activate the SMase-ceramide pathway in the neurons to increase ceramide levels. Upregulated ceramide increase BACE1 level and enhance Aβ production [165, 185]. The increased extracellular Aβ level may act on both the astrocytes and the neurons to enhance the intracellular ceramide levels. This ceramide-Aβ-ceramide cascade creates a continuing cycle to further induce cell death [35, 225].

CHAPTER 3. PALMITATE INDUCES TRANSCRIPTIONAL REGULATION OF BACE1 AND PRESENILIN BY STAT3 IN NEURONS MEDIATED BY ASTROCYTES

This work has been published in Experimental Neurology:

Li Liu, Rebecca Martin, Garrett Kohler and Christina Chan, Palmitate induces transcriptional regulation of BACE1 and presenilin by STAT3 in neurons mediated by astrocytes, Experimental Neurology (2013)248:482-490

Abstract

Deregulation of calcium has been implicated in neurodegenerative diseases, including Alzheimer's disease (AD). Previously, we showed that saturated free-fatty acid, palmitate, causes AD-like changes in primary cortical neurons mediated by astrocytes. However, the molecular mechanisms by which conditioned media from astrocytes cultured in palmitate induces AD-like changes in neurons are unknown. This study demonstrates that this condition media from astrocytes elevates calcium level in the neurons, which subsequently increases calpain activity, a calcium-dependent protease, leading to enhance p25/Cdk5 activity and phosphorylation and activation of the STAT3 (signal transducer and activator of transcription) transcription factor. Inhibiting calpain or Cdk5 significantly reduces the upregulation in nuclear level of pSTAT3, which we found to transcriptionally regulate both BACE1 and presenilin-1, the latter is a catalytic subunit of γ-secretase. Decreasing pSTAT3 levels reduced the mRNA levels of both BACE1 and presenilin-1 to near control levels. These data demonstrate a signal pathway leading to the activation of STAT3, and the generation of the amyloid peptide. Thus, our results suggest that STAT3 is an important potential therapeutic target of AD pathogenesis.

Keywords: Alzheimer's disease, fatty acids, calcium, STAT3, BACE1, presenilin-1

3.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, characterized by the deposition of amyloid β (Aβ) peptide generated through cleavage by BACE1 (β-site amyloid precursor protein cleaving enzyme) and γ-secretase. AD begins with mild memory deficits and ultimately results in total loss of cognition and executive functions. One in 85 people worldwide are predicted to be affected with AD by year 2050 [4]. The etiology of AD is unknown and no effective cures currently exist [226]. Calcium signaling is involved in numerous neuronal functions, and the dysregulation of neuronal calcium homeostasis is a hallmark of AD pathology and found in both AD patients and in AD animal models [226], impairs neuronal activity, accelerates Aβ formation [227] and is linked to the neurodegenerative process in AD [228]. Nevertheless, the mechanism by which abnormal level of calcium leads to amyloidogenesis is unclear.

Calpain, a calcium-dependent protease that can be triggered by abnormally high calcium levels, is over-activated during early stages of the AD process [193, 194, 229]. Pharmacological inhibitors of calpain improve cognition and reduce amyloid plaque load in AD transgenic mice model, implicating their potential therapeutic application for AD [230]. P25, the proteolytic cleavage product from p35 generated by calpain, activates cyclin-dependent kinase 5 (Cdk5) and was reported to be upregulated in human AD brain [191]. Cdk5, a proline-directed serine/threonine kinase, has diverse functions in the central nervous system, including neuronal migration, synaptic plasticity and cognition [231, 232]. In support of its role in the pathogenesis of AD, the activity of Cdk5 is upregulated in AD patient's brain [189]. Activation of Cdk5 increases while inhibiting Cdk5 activity attenuates Aβ generation [233].

Aβ peptide is generated from sequential proteolytic cleavages of the Aβ precursor protein by BACE1 and γ-secretase [199, 234]. The expression of BACE1 is cell-type and stimuli-specific [235-237], and is tightly regulated at the transcriptional level [200, 238, 239]. The promoter region of BACE1 contains many putative transcription factor binding sites that are conserved among different species, including rat, mouse and human [200, 239, 240]. Several putative transcription factors of BACE1, such as SP1 and Yin Yang 1, have been confirmed in neuroblastoma cells [237, 241]. Similarly, p25/Cdk5 has been reported to phosphorylate and activate the transcription factor, signal transducer and activator of transcription 3 (STAT3), to positively regulate the transcription of the BACE1 gene, also, in neuroblastoma cells [189].

γ-secretase consists of presenilin, nicastrin, APH-1 (anterior pharynx-defective 1) and PEN-2 (presenilin enhancer 2). The catalytic core of γ-secretase consists of presenilin-1 which is essential for the activity of γ-secretase. The expression of the presenilin-1 gene increases during aging and during brain injury [242]. The expression of presenilin-1 can be regulated by the Ets transcription factor [243], and further p53 has been shown to be involved in the regulation of presenilin-1 expression [243], while inhibiting c-Jun N-terminal kinase (JNK) represses presenilin-1 expression [244].

The AD brain is characterized by high fatty acid content, and significant increases in fatty acids from brain trauma have been suggested as a risk factor for AD [136, 245]. In animal models, the consumption of saturated fatty acids increases the pathophysiological changes associated with AD [205]. Previously our group confirmed that saturated fatty acids, e.g. palmitate, induced AD-like changes in primary rat neurons mediated by conditioned media from astrocytes, although it was not determined whether palmitate directly increases BACE1 and hyperphosphorylation of tau in the primary neurons [17, 151]. Further, the conditioned media

from astrocytes treated with palmitate increased sphingomyelinase activities and enhanced the stability of BACE1, to propagate the deleterious effects of palmitate in primary neurons [17]. The process, however, by which Aβ is generated in primary neurons cultured in conditioned media from palmitate-treated astrocytes, is unclear. Here we show that the conditioned media from these astrocytes rapidly increases the calcium levels, enhances the activity of calpain and of Cdk5 through p25 (the cleavage product of calpain) and in turn activates STAT3 in the neurons. Further we found that the STAT3 transcription factor could regulate the expression of both BACE1 and presenilin-1, a core subunit of γ-secretase, thereby supporting pSTAT3 as a potential therapeutic target.

3.2 Materials and methods

3.2.1 Isolation and culture of primary rat astrocytes and neurons

All procedures in the cell isolation were approved by the Institutional Animal Care and Use Committee at Michigan State University. Primary cortical astrocytes were isolated from postnatal day 0-2 newborn Sprague-Dawley rats as previously described [17]. Briefly, the cortices were digested with papain (10units/ml; Worthington, NJ, USA) and DNaseI (100units/ml; Roche, IN, USA) for 30min at 37^{o}C, and washed with DMEM/F12 (Invitrogen, Carlsbad, CA, USA). The cells were seeded on poly-L-lysine (PLL, 50g/ml; Cultrex, Gaithersburg, MD, USA) coated plate for 1hr and the media was changed to fresh complete DMEM/F12 media (DMEM/F12 supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100U/mL penicillin (Invitrogen)) to remove dead cells. The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ℃, the cultured medium changed every 3

days. The purity of the astrocytes were >90% [17], and ready for use in experiments when the cells reached around 80% confluence.

Cortices from postnatal day 0 Sprague Dawley rats were used for the neuronal culture. Briefly, the cortical tissues were digested with papain and DNaseI for 30min at 37^{o}C, and washed with Neurobasal A medium (Invitrogen) three times. The cells were seeded on PLL coated plates for 1hr and the media was changed to fresh complete cell culture media (neurobasalA medium with B27, 0.5mM glutamine, and 100μg/mL streptomycin and 100U/mL penicillin). The purity of the neurons was >90% [17] and used for experiments after 3 or 4 days of culture.

3.2.2 Astrocyte conditioned media (CM) and Materials

Neuronal cell culture medium (DMEM 10313 supplemented with 10% horse serum, 10mM HEPES, 2mM glutamine, 100μg/mL streptomycin and 100U/mL penicillin) was used to culture primary neurons and astrocytes for 24hr prior to treatment. BSA (fatty acid-free bovine serum albumin) (Millipore, Billerica, MA, USA), or 0.2mM PA (Sigma, St. Louis, MO, USA) plus BSA as a carrier protein (molar ration is 3:1) was used to incubate the astrocytes for 12hr. This astrocyte conditioned media (CM-B or CM-P) were used to culture the neurons.

Roscovitine (Cat# R7772, Sigma) and butyrolactone-1 (Cat# BML-CC210, Enzo life sci, Farmingdale, NY, USA) are specific inhibitors of Cdk5 and were used at concentrations of 10μM. Calpeptin (Cat# 03-34-0051, Calbiochem, Billerica, MA, USA) and PD150606 (Cat#CAS 659-22-3, Santa Cruz, Dallas, Texas, USA) are specific inhibitors of calpain and were used at 20μM and 100μM, respectively. Cell permeable STAT3 inhibitor (Calbiochem 573095) was used to inhibit the dimerization of STAT3 at 235μM. STAT3 inhibitor VI (Calbiochem

573102) was used to inhibit STAT3 activity at 100 μM. ActinomycinD (ActD) (Cat#9415, Sigma) was used to inhibit transcription at a concentration of 1 μg/ml.

3.2.3 Total mRNA Extraction and Quantitative real time PCR

Primary neurons were incubated for 12hr with conditioned media from astrocytes. Then the cells were lysed and total mRNA from the cells was extracted using the RNeasy Plus kit (QiaGen, Valencia, CA, USA) according to the manufacturer's instructions and the total mRNA was reverse transcribed into cDNA using the cDNA synthesis kit (BioRad, Hercules, CA, USA) as described [246]. The following primer sets (Operon, Huntsville, AL, USA) were used for PCR: actin (5'-ctcttccagccttccttcct-3'and 5'-aatgcctgggtacatggtg-3'), BACE1 (5'-aatcagtccttccgcatcac-3' and 5'-ggctcgatcaaagaccacat-3'), presenilin-1 (5'-ggtacccaaaaaccccaagt-3'and 5'-agtgaggatgctcccagaaa-3'), Quantitative real-time PCR was performed using iQSYBR Green Supermix and Real-Time PCR Detection System (BioRad). The cycle threshold values were determined by the MyIQ software. Three independent experiments were performed for statistical analysis.

3.2.4 Western blot

Primary neurons were cultured in conditioned media at the indicated time in the figures. Then the cells were lysed and protein concentrations from whole cell extracts were determined by Bradford assay (BioRad). 15-30μg of protein samples were used for Western blot analysis as previously described [17, 247, 248] using specific antibodies for BACE1 (Cat#ab2077, Abcam, Cambridge, MA, USA), pSTAT3 (Cat#9131, Cell signaling, Danvers, MA, USA), spectrin (Cat# MAB1622, Millipore, Billerica, MA, USA), Histone H1 (Cat#ab11079, Abcam, Cambridge, MA, USA), C99 (Cat#2136, QED Bioscience, San Diego, CA, USA), and beta-actin (Cat# NB600-501H, Novus Biologicals, Littleton, CO, USA). Anti-mouse and anti-rabbit HRP-conjugated

secondary antibodies were purchased from Thermo Scientific (Cat#31430, 31460, Asheville, NC, USA). The blots were visualized by Super Signal West Femto maximum sensitivity substrate (Thermo Scientific).

3.2.5 Nuclear extracts

Primary rat neurons were cultured in conditioned media at the indicated time in the figures. Then the cells were lysed and nuclear extraction was performed according to the protocol described in [249]. Briefly, cells were suspended in buffer A (10mM HEPES (pH=8.0), 1.5mM MgCl2, 10mM KCl, protease and phosphatase inhibitor cocktail) and kept on ice for 15 minutes. The cells were lysed with a 25-gauge 5/8 inch needle and the nuclear pellets were collected by centrifugation. Nuclear pellets were re-suspended in buffer C (20mM HEPES (pH=8.0), 1.5mM MgCl2, 25% (v/v) glycerol, 420mM NaCl, 0.2mM EDTA (pH=8.0), protease and phosphatase inhibitor cocktails) and incubated on ice for 30min, then spun down to obtain the nuclear extracts.

3.2.6 Calpain activity assay

Calpain activity in primary neurons cultured for 30min in conditioned media from astrocytes was detected by a calpain activity assay kit according to the manufacturer's instructions (Cat#K240, BioVision, Milpitas, CA, USA). Briefly, cells were lysed in lysis buffer for 20 min on ice, and the lysates were incubated with the calpain substrate, fluorogenic peptide (Ac-LLY-AFC), and reaction buffer for 1hr at 37^{o}C in the dark. Upon the cleavage of the substrate, the fluorogenic portion produces fluorescence at a wavelength of 505nm and excitation at 400nm. The fluorescence emission was measured by Spectra MAX GEMINI EM plate reader. All readings were normalized to the protein levels obtained by Bradford assay.

3.2.7 Enzyme-linked immunosorbent assay (ELISA) and Aβ_{42} assay

Primary rat neurons were cultured for 12hr in conditioned media from astrocytes. The cultured media was used for the ELISA assay. The level of $A\beta_{42}$ was detected by $A\beta_{42}$ ELISA kit (Invitrogen) according to the manufacture's instruction. Optical densities were measured at 450nm wavelength by Spectra MAX Plus384 plate reader. Each sample concentration was calculated based on a standard curve of $A\beta_{42}$ standards. All readings were normalized to the total protein levels determined by Bradford's assay, and the data then were normalized to the control. Three independent experiments were performed for statistical analysis.

3.2.8 Calcium imaging

Calcium imaging was performed according to the protocol described in [249]. Briefly, neurons were cultured in 4-well chambered cover-glass (Thermo Fisher Scientific). 4μM non-ratiometric dye Fluo-4 (Invitrogen) was added to the cultured media for 30min at 37^{o}C. Excess dye was removed by washing with PBS, and the cells were incubated with complete neuronal media and placed in a chamber on a 37^{o}C stage of an Olympus FluoView 1000. Images were captured and fluorescence intensity is represented by a spectral table (warmer colors represent higher intensity whereas cooler colors represent lower intensity). The completed neuronal media were replaced with CM-P or CM-B, and incubate for 20min prior to imaging.

Quantitative analysis of calcium was measured with Fluo-4. Briefly, neurons were cultured in 12-well plates. 4μM non-ratiometric dye Fluo-4 was added to the cultured media for 30min at 37^{o}C, excess dye was removed by washing with PBS, and neurons were incubated with complete neuronal media. The fluorescence signal (F0) was detected by Spectra MAX GEMINI EM plate reader at 520nm and excitation at 480nm. Completed neuronal media were replaced with CM-P or CM-B, and incubate for 20min. The fluorescence signal (F) was measured again.

Changes in the fluorescence intensity of the Ca^{2+} signal are represented as F/F0. Three or more independent experiments were performed.

3.2.9 Statistical analysis

All experiments were performed at least three times, and representative results are shown. Statistical analysis was performed using an unpaired, two tail student t-test * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

3.3 Results

3.3.1 Abnormal calcium and calpain levels in primary neurons treated with CM-P

Condition media from astrocytes cultured in palmitate or control media (BSA) is herein denoted as CM-P or CM-B, respectively. We reported previously that primary neurons cultured in CM-P developed AD-like changes [151]. Upregulated levels of calcium have been implicated in neurodegenerative diseases including AD. We observe the calcium level is upregulated in neuronal cells incubated in CM-P (Figure 3.1). Elevated levels of calcium could enhance calcium-dependent protein activities that trigger signaling cascades, such as calpain, a calcium-dependent protease. Mounting evidences indicate that abnormal activation of calpain is involved in AD pathogenesis [193, 194]. To validate whether the activity of calpain is upregulated in neurons cultured in conditioned media from astrocytes, the activity of calpain was measured and the results show a significant increase in calpain activity in the primary neurons (Figure 3.2A). This increased calpain activity is likely due to elevated calcium levels. Calpain activity comes from two main isoforms of calpain in the cells, μ-calpain and m-calpain. The primary difference between the two isoforms is the amount of calcium required for activation. μ-calpain can be activated by micromolar concentration of Ca^{2+}, while m-calpain is activated by millimolar

concentration of Ca^{2+}. We detected m-calpain protein level in primary neurons cultured for 30mins with condition media from astrocytes. The western blot results show that m-calpain protein levels are increased significantly in primary neurons cultured in CM-P (Figure 3.2B). In further support, to monitor the activity of calpain in neurons treated with CM-P, spectrin, an intracellular substrate of calpain that can be cleaved by calpain to indicate elevated calpain activity, was detected by western blot analysis. A cleavage fragment of around 150kDa from the 220kDa spectrin protein was detected in primary neurons cultured for 30 min in CM-P (Figure 3.2C, D), suggesting increased calpain activity. To determine if spectrin is cleaved by calpain, two specific calpain inhibitors, calpeptin (20 μM) and PD150606 (100 μM) were used individually to pre- and co-treat the primary neurons cultured in CM-P, and found to decrease significantly the 150kDa spectrin fragment (Figure 3.2C, D), indicating that CM-P increased the activity of calpain in primary cortical neurons.

3.3.2 CM-P elevates C99 and BACE1 levels, and calpain is involved in APP processing in neurons

We previously reported that BACE1 protein levels increased in a time-dependent manner in primary neurons cultured in CM-P [17]. BACE1 initiates the production of Aβ by cleaving the Aβ precursor protein (APP) to generate a membrane bound carboxy-terminal fragment (C99). A higher level of C99 indicates higher BACE1 activity. Incubating the primary neurons in CM-P significantly elevated, while inhibiting calpain with specific inhibitors, calpeptin at 20 μM, or PD150606 at 100 μM significantly reduced, the accumulation of C99 (Figure 3.3). This suggests that calpain could be involved in regulating C99 level by modulating BACE1 activity.

To determine if the increase in BACE1 mRNA level is transcriptionally regulated, a transcriptional inhibitor, actinomycinD (ActD, 1 μg/ml) was incubated with the neurons cultured

in CM-P. Transcriptional inhibition with ActD significantly decreased the upregulation in BACE1 mRNA (Figure 3.4A) and protein levels (Figure 3.4B) suggesting BACE1 is regulated at the transcriptional level in primary neurons cultured in CM-P.

3.3.3 pSTAT3 is involved in the transcriptional regulation of BACE1

The transcription factor STAT3 is known to regulate numerous genes [250], including BACE1 expression in neuroblastoma cells [189]. Nevertheless, the regulation of BACE1 expression is cell-type and stimuli-specific [189, 190]. To determine if STAT3 could regulate BACE1 in primary cortical neurons treated with CM-P, nuclear level of pSTAT3 was measured after 1hr of culture.

Cytoplasmic STAT3 is phosphorylated at the serine 727 site by kinases [251, 252], and the phosphorylation of this site induces its dimer formation, which causes auto-phosphorylation at multiple tyrosine sites, including tyrosine 705, leading to translocation of STAT3 to the nucleus [250, 253]. Therefore, pSTAT(Y705) was measured in the nucleus and found to increase (Figure 3.5A). In contrast, treating primary neurons directly with palmitate did not change the pSTAT3 level (Figure 3.5B). To determine if the upregulated pSTAT3 level could regulate BACE1, STAT3 inhibitor at 235 μM, and STAT3 inhibitor VI at 100 μM were used individually to pre- and co-treated the primary neurons for 12hr in CM-P. Western blot results showed that phosphorylated STAT3 levels were significantly reduced (Figure 3.5C, D). Upon inhibition, the mRNA and protein levels of BACE1 reduced significantly as compared to the control-treated neurons (CM-B) (Figure 3.5E, F and G), suggesting that pSTAT3 could transcriptionally regulate BACE1 expression.

3.3.4 Elevated calpain and p25/Cdk5 leads to phosphorylation of STAT3

pSTAT3 is elevated in neurons cultured in CM-P, and this elevated pSTAT3 level correlates with increased BACE1 accumulation (Figure 3.5). p25/Cdk5 is known to activate STAT3 [189] and p25, an activator of Cdk5, increases significantly in primary neurons cultured in CM-P [154]. To confirm whether the elevated pSTAT3 level is due to p25/Cdk5, specific inhibitors of Cdk5, roscovitine (10μM) or butyrolactone-1(10μM), was used to pre- and co-treated the primary neurons cultured in CM-P for 12hr. The protein level of pSTAT3 and the mRNA level of BACE1 reduced significantly upon inhibition of Cdk5, (Figure 3.6). This suggests that p25/Cdk5 is involved in upregulating pSTAT3 level. The activator of Cdk5, p25, is cleaved from p35 by calpain [192]. Since the activity of calpain is upregulated in neurons treated with CM-P (Figure 3.2), we propose a potential signaling pathway in which the upregulated calpain activity in neurons treated with CM-P enhances p25 production, which elevates Cdk5 activity and in turn pSTAT3 level. To evaluate whether the increased pSTAT3 level is due to upregulated calpain activity, primary neurons were pre- and co-treated with calpeptin (20μM) or PD150606 (100μM), specific calpain inhibitors, along with CM-P for 12hr. The calpain inhibitors significantly decreased pSTAT3 as well as BACE1 mRNA levels (Figure 3.6). These findings support that both the increased calpain and p25/Cdk5 activities are involved in the upregulation of pSTAT3 activity and BACE1 mRNA level in primary neurons treated with CM-P. The upregulated calcium level in primary neurons (Figure 3.1) could enhance calpain activity, and induce the signaling cascade of calcium-calpain-p25/Cdk5-pSTAT3 to transcriptionally regulate BACE1 in primary neurons treated with CM-P.

3.3.5 pSTAT3 could regulate the transcription of γ-secretase

We found the increased C99 level corresponded with the elevated BACE1 mRNA and protein levels in neurons treated with CM-P. In addition, we also observe increased Aβ42 level

in primary cortical neurons cultured in CM-P (Figure 3.7), which is consistent with the increased mRNA presenilin-1 levels observed (Figure 3.8), the latter is a catalytic subunit of γ-secretase that cleaves C99 to generate amyloid peptide. Thus we proposed that STAT3 could regulate presenilin-1. We analyzed the promoter region of presenilin-1 with JASPAR and TRANSFAC databases, and found that STAT3 could be a potential putative transcription factor of presenilin-1. Inhibiting pSTAT3 significantly reduces the mRNA level of presenilin-1 in neurons cultured in CM-P, suggesting that pSTAT3 could regulate presenilin-1 (Figure 3.8).

3.4 Discussion

Astrocytes are the most abundant cells in the central nervous system. Mounting evidence suggest that local inflammatory responses are mediated by astrocytes and that these astrocytes are not simply passive supportive cells, but contribute to the pathological processes of neurodegenerative diseases [254] and precede neuronal alterations and behavioral impairment in the progression of AD [255]. Epidemiological studies suggest that saturated free fatty acids may increase the risk of AD [205]. To date, the level of palmitate in AD brain has not been reported. However, traumatic brain injury, considered an independent risk factor for AD, is characterized by elevated levels of palmitate in the brain [144], with palmitate increasing from ~60 to 180 μM [145]. Further supporting reports demonstrated that in traumatic brain injury, free fatty acid (FFA) levels increase significantly in the cerebrospinal fluid (CSF), and are recognized as markers of brain injury. In particular, the concentration of palmitate almost doubled as compared to control, above 1100 μg/L in the CSF of traumatic brain injury versus around 600 μg/L in control [256-258]. FFAs in plasma can cross the blood-brain barrier [132, 133], and high fat diets increase the uptake of fatty acids by the brain from the plasma [134, 135]. The concentration of

FFAs in normal human plasma *in vivo* generally ranges between 0.3-1.0mM [259-261]. In the brain, astrocytes readily take up and metabolize fatty acids. In fact peripheral administration of radio-labeled saturated fatty acid was found to accumulate primarily in astrocytes [157, 158]. Previously our group showed that palmitate does not directly induce AD-like changes in primary cortical neurons, whereas, it does upregulate Aβ levels in primary neurons mediated by astrocytes [17, 151]. Furthermore, the condition media from palmitate-treated astrocytes increased ceramide levels in neurons through sphingomyelinase, which could stabilize BACE1 and promote Aβ biogenesis [17, 165, 262]. Aβ peptide accumulation derived from BACE1 and γ-secretase is a well-established pathological hallmark of AD patient brains. Here we demonstrate a role of STAT3 in transcriptionally regulating not only the expression of BACE1, but also the expression of presenilin-1, a core protein of the γ-secretase complex in primary cortical neurons cultured in CM-P. Inhibiting pSTAT3 reduced the upregulation in the mRNA levels of both BACE1 and presenilin-1 in neurons cultured in CM-P. Thus, suggesting that reducing the activity of STAT3 might decrease the amyloidogenic processing, and be a possible therapeutic target for AD pathogenesis. Although we found pSTAT3 could regulate the expression of BACE1 and presenilin-1, it does not preclude the possibility that pSTAT3 could be mediating the expression of BACE1 and presenilin-1 through other proteins. STAT3 can be phosphorylated by Cdk5 kinase, and our results suggest that calpain, a calcium-dependent protease, could increase Cdk5 activity mediated by p25. We observed that inhibiting calpain and Cdk5 reduced the upregulated pSTAT3 levels in the nucleus, suggesting that p25/Cdk5 and calpain could be upstream components in the signaling cascade to activate STAT3.

Deregulation of calcium-mediated signaling has been implicated in many neurodegenerative diseases including AD [263]. Calcium generates diverse intracellular signals

involved in a variety of cellular functions. The concentration of cytosolic calcium is tightly regulated by the balance between calcium influx and efflux, and by the exchange of calcium with the endoplasmic reticulum (ER) and mitochondria. Several reports have indicated that elevated calcium promotes amyloidogenesis and Aβ aggregation. In turn, Aβ can increase cytosolic levels of calcium by forming voltage-independent cation channels in the cell membrane, causing calcium influx and degeneration of the neurites [193, 264, 265]. Aβ can also stimulate the release of ER calcium into the cytoplasm [265]. In addition, factors, such as aging, ceramide and cytokines have been shown to disrupt brain calcium homoeostasis [266-268]. Our previous study showed that the ceramide levels increased significantly in primary cortical neurons cultured in CM-P [17]. Furthermore, palmitate triggered cytokine secretion from the astrocytes into the CM-P, in particular IL-1β and TNFα [17]. The upregulation in the levels of cytokines and intracellular ceramides could trigger deregulation of calcium homeostasis in the neurons cultured in CM-P. Indeed here we observed the calcium level is upregulated in primary cortical neurons cultured in CM-P.

Abnormally high calcium levels can over-activate calpain, a calcium-dependent protease [229]. In the central nervous system, upregulated calpain activity has been linked to a number of diseases, including AD [229]. Furthermore, selective calpain inhibitors have been shown to mitigate the AD-like changes and cognitive decline in animal model [229]. Aβ can activate calpain to enhance the accumulation of p25 [195] by cleaving p35 to p25, to form the p25/Cdk5 complex [195]. P25/Cdk5 has many substrates which have been shown to be involved in AD pathogenesis. For example, p25/Cdk5 can phosphorylate APP, and BACE1 preferentially cleaves phosphorylated APP to increase Aβ production [269], and could also phosphorylate BACE1 and tau, leading to altered BACE1 trafficking or sorting with the former [270, 271], and tau

hyperphosphorylation with the latter [189]. Elevated p25/Cdk5 has also been reported in animal model of ischemia, and ischemia has been shown to increase amyloidogenesis and is considered a risk factor for AD [272, 273]. Both calpain and p25/Cdk5 play multiple roles in the pathogenesis of AD and have been suggested to be potential therapeutic targets of AD. In this study, calpain activity is increased significantly, and our group previously showed p25 level is upregulated in primary cortical neurons incubated in CM-P [151]. Inhibiting Cdk5 and calpain significantly reduces pSTAT3 levels in primary cortical neurons cultured in CM-P. Thus, it further supports the role of Cdk5 and calpain in AD pathogenesis.

In conclusion, our previous studies showed that the conditioned media from astrocytes cultured in palmitate increased the AD-like changes in primary neurons [151]. Therefore, to further elucidate the mechanism by which CM-P induces AD-like changes, and based on our findings and previously published results, we propose the following mechanism (Figure 3.9). In summary, conditioned media from palmitate-treated astrocytes elevates the calcium level in primary cortical neurons, to increase calpain activity, a calcium-dependent protease, which subsequently enhances p25/Cdk5 activity. STAT3 is a substrate of p25/Cdk5 and upon phosphorylation becomes activated and translocates into the nucleus. Elevated pSTAT3 level in the nucleus could transcriptionally upregulate both BACE1 and presenilin-1, directly or indirectly, enhancing the production of Aβ. Elevated Aβ level could further trigger neurons to disrupt calcium homeostasis [193, 264, 265] and reinforce a calcium-Aβ-calcium cascade, and exacerbate the AD pathology. In this signaling cascade STAT3 regulates both BACE1 and presenilin-1, thereby suggesting that STAT3 could be a potential therapeutic target for AD.

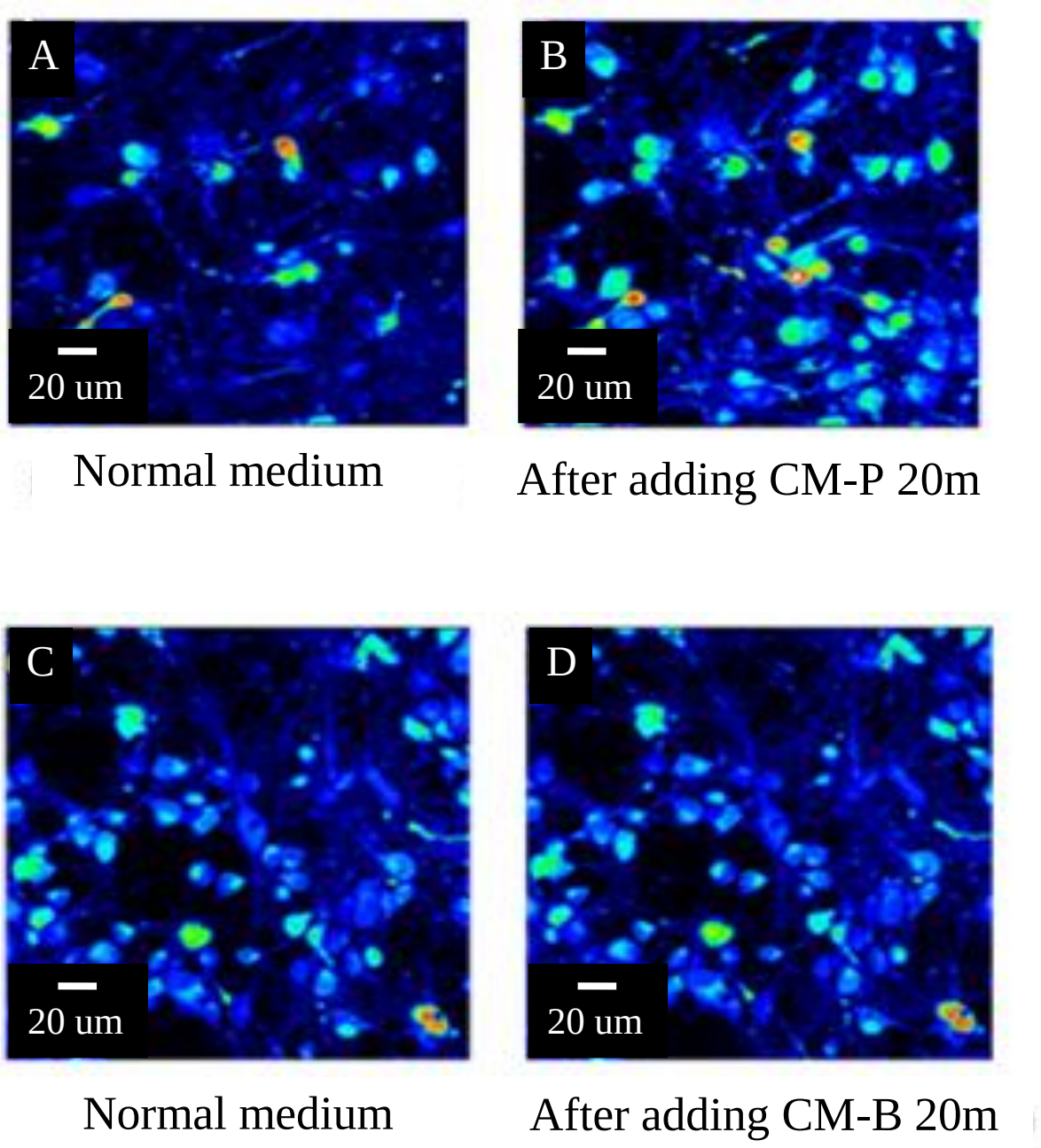
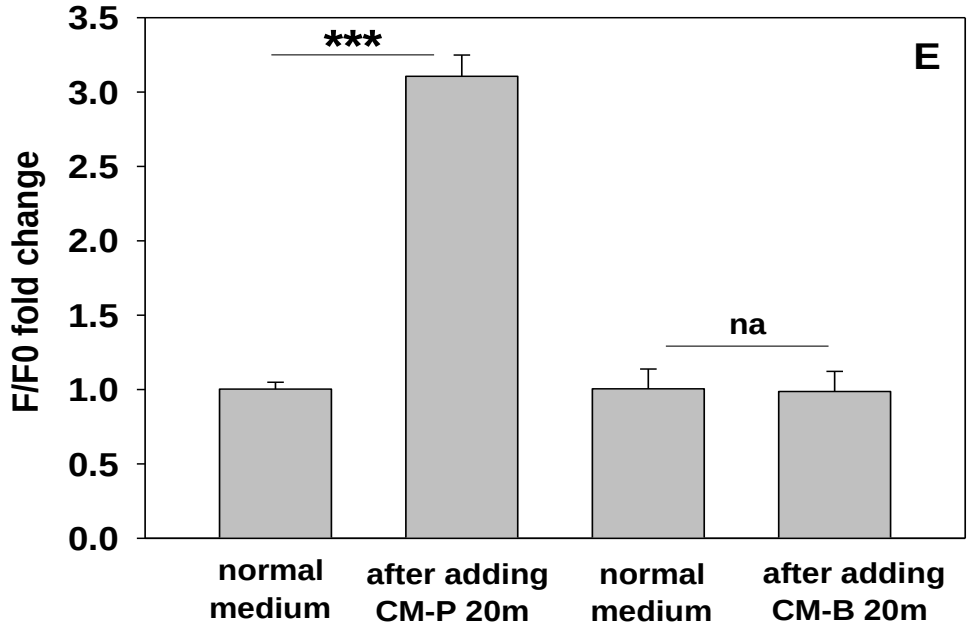


Figure 3.1 Calcium imaging of neurons in response to conditioned media from astrocytes.

Figure 3.1 (con'd) (A) Representative fluorescence image of primary neurons cultured with normal cell culture media, after capturing the image, the normal cell cultured media was replaced with conditioned media from astrocytes cultured in palmitate (CM-P) for 20min, and the image of the same field of view as (A) was captured and presented in (B). (C) Representative image of primary neurons cultured with normal cell culture media, after capturing the image, the normal cell cultured media was replaced with control conditioned media from astrocytes cultured in DMEM/BSA (CM-B) for 20min, then the image of the same field of view as (C) was captured and presented in (D). Fluorescence images represented by a spectral table; the warmer colors indicate the higher fluorescence intensities and cooler colors indicate the lower fluorescence intensities. (E)Relative signal intensity (F/F0) with Fluo-4 loaded neurons cultured with conditioned media from astrocytes. The fluorescence intensity was measured with a plate reader. ***p<0.001. A line indicates a comparison between the 2 bars connected by the line.



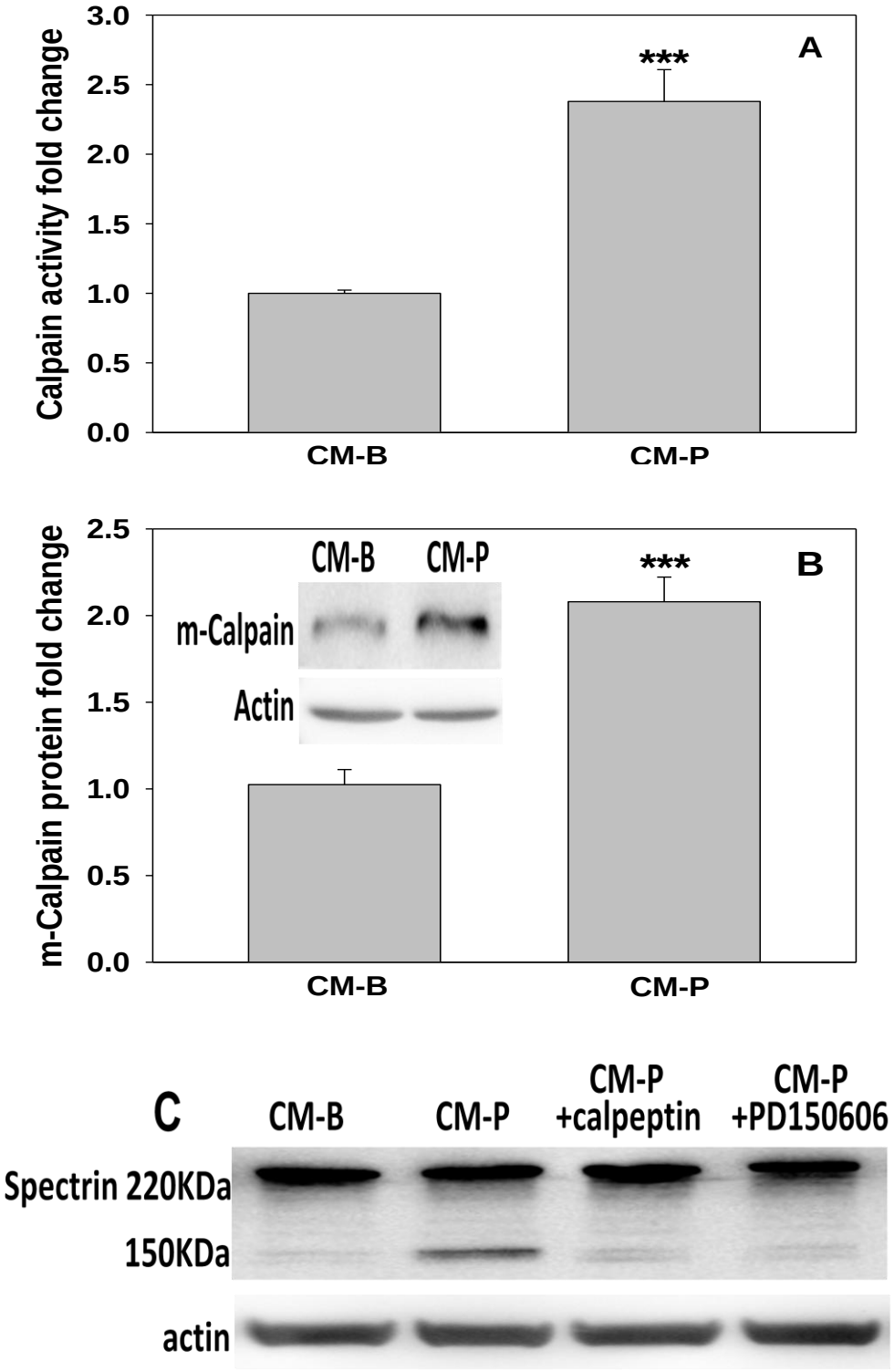
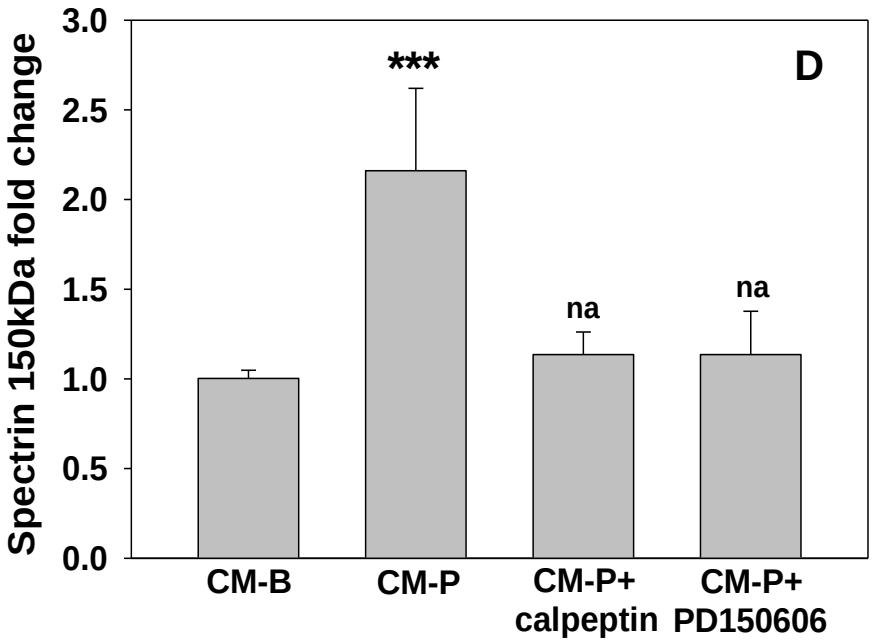


Figure 3.2 Calpain activity increases in primary neurons. (A) Primary neurons were

Figure 3.2 (cont'd) incubated for 30min in conditioned media from astrocytes cultured in DMEM/BSA (CM-B) or in palmitate (CM-P) and the activity of calpain was measured. (B) m-calpain level in primary neurons. Primary neurons were incubated with CM-B (control) or CM-P for 30min and the cells were lysed. m-calpain levels were detected by western blot. Actin was used as a loading control. (C, D) Spectrin levels in neurons. Primary neurons were incubated with CM-B (control) or CM-P for 30min. Calpeptin (20μM) or PD150606 (100μM), specific inhibitors of calpain, was used to pre- and co-treated the neurons with CM-P for 30min individually. Then the cells were lysed and representative western blot results of spectrin are shown in (C). The results were normalized to control (CM-B). *p<0.05, **p<0.01, ***p<0.001.



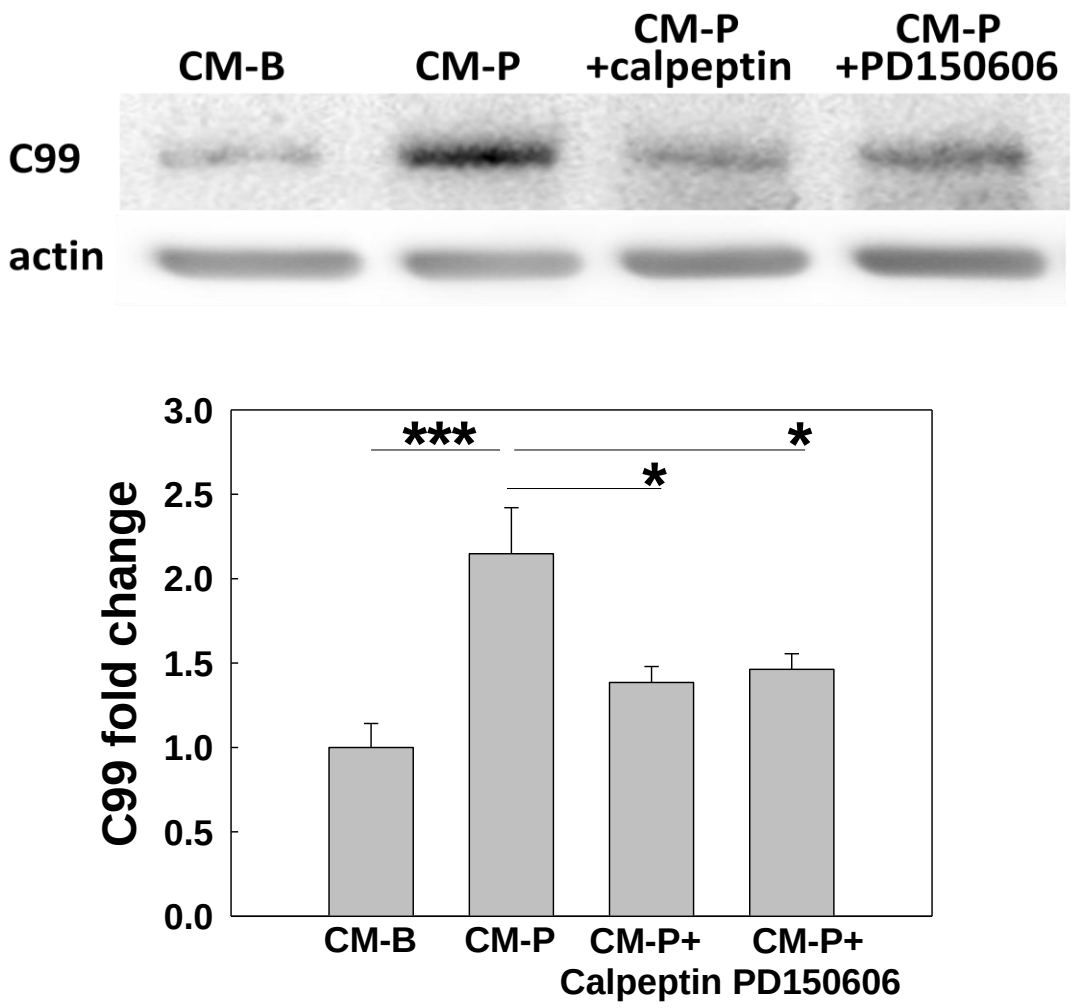


Figure 3.3 C99 level in neurons. Neurons were treated with conditioned media (CM-B or CM-P) for 12hr. Calpeptin (20 μM) or PD150606 (100 μM), specific inhibitors of calpain, was used to pre- and co-treated the neurons with CM-P (CM-P+Calpeptin) or (CM-P+PD150606) for 12hr. Then the cells were lysed and representative western blot result of C99 is shown (n=3). *p<0.05, **p<0.01, ***p<0.001. A line indicates comparison between the 2 bars connected by the line.

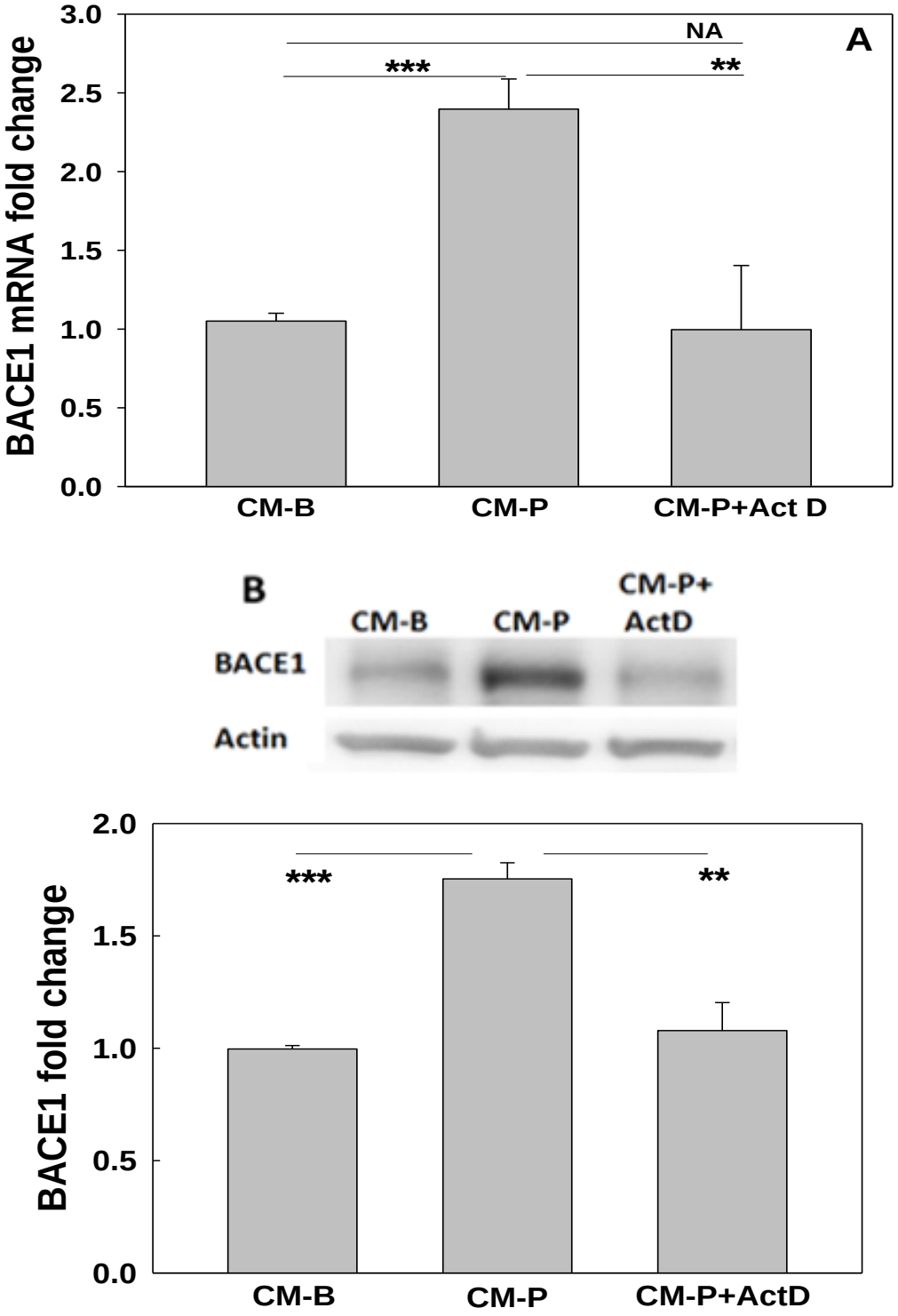


Figure 3.4 The expression of BACE1 is regulated at the transcriptional level in conditioned media from astrocytes. (A) The mRNA expression of BACE1 in neurons treated with CM-B

Figure 3.4 (cont'd) (control) or CM-P, or CM-P plus actinomycinD (ActD) for 12hr. Then the mRNA was extracted and mRNA levels were detected by real-time PCR. (B) Representative western blot results of BACE1 level in control cells (CM-B), in cells treated with CM-P, in cells treated with CM-P in the presence of ActD (1μg/ml) for 12hr (n=3). *$p<0.05$, **$p<0.01$, ***$p<0.001$. A line indicates comparison between the 2 bars connected by the line.

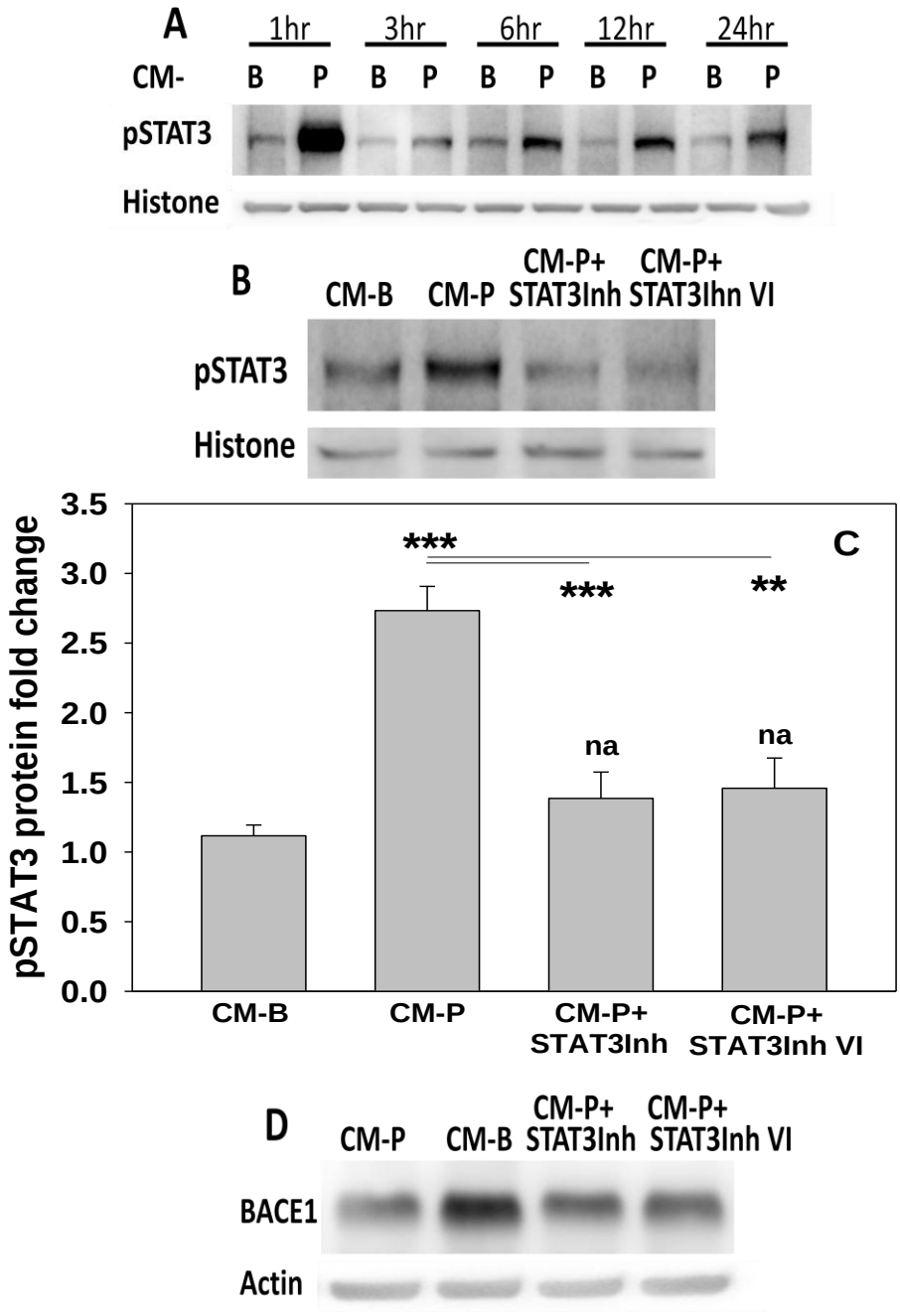


Figure 3.5 STAT3 is involved in regulating BACE1. (A) pSTAT3 levels in the nucleus of primary neurons incubated in control (CM-B) or CM-P for the indicated time. Histone was used as loading control for the nuclear extracts. (B) pSTAT3 levels in the nucleus of primary neurons cultured in BSA (control) or 0.2mM palmitate (PA) for 12hr. Then the cells were lysed and western blot for pSTAT3 was performed. Histone was used as loading control for the nuclear

Figure 3.5 (cont'd) extracts. (C, D) pSTAT3 levels in the nucleus. Primary neurons treated with control (CM-B), CM-P or CM-P plus specific inhibitors of STAT3 (STAT3Inh at 235μM or STAT3Inh VI at 100μM) for 12hr. Then the cells were lysed and pSTAT3 levels were measured by western blot and histone was used as loading control for the nuclear extracts. (D, E, F) BACE1 mRNA or protein levels in primary neurons treated with control (CM-B), CM-P or CM-P plus specific inhibitors of STAT3 (STAT3Inh at 235μM or STAT3Inh VI at 100μM) for 12hr. Then the cells were lysed and BACE1 protein levels were measured by western blotting of whole cell lysates with actin used as a loading control. The mRNA of BACE1 was detected by real-time PCR. The results were normalized to control (CM-B) and * or na (not significant) above the bar indicates the significance. A line indicates a comparison between the 2 bars connected by the line. (n=3). *p<0.05, **p<0.01, ***p<0.001.

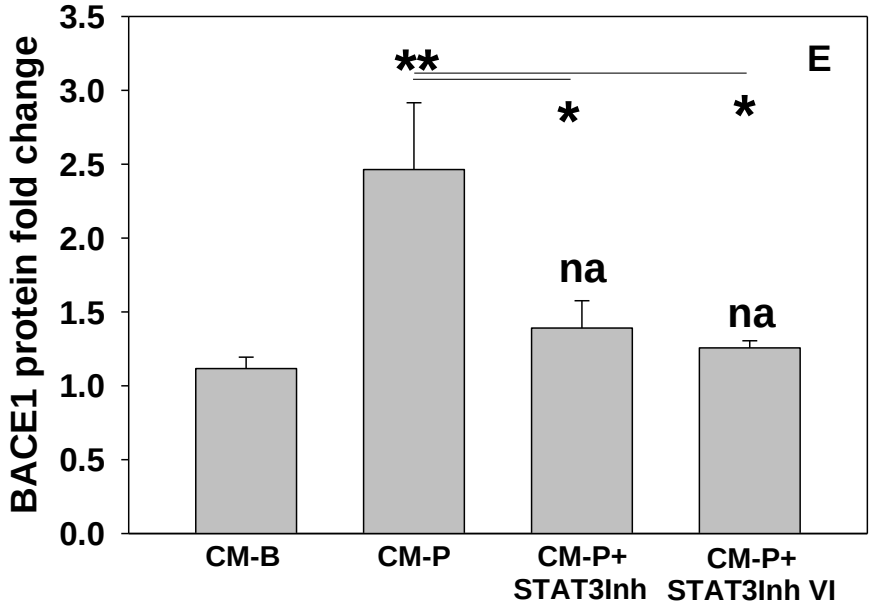
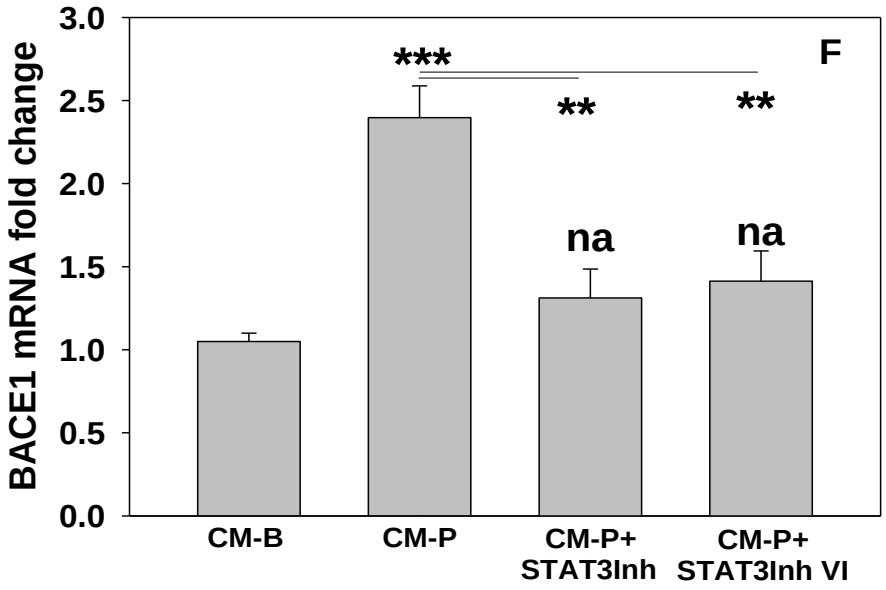


Figure 3.5 (cont'd)



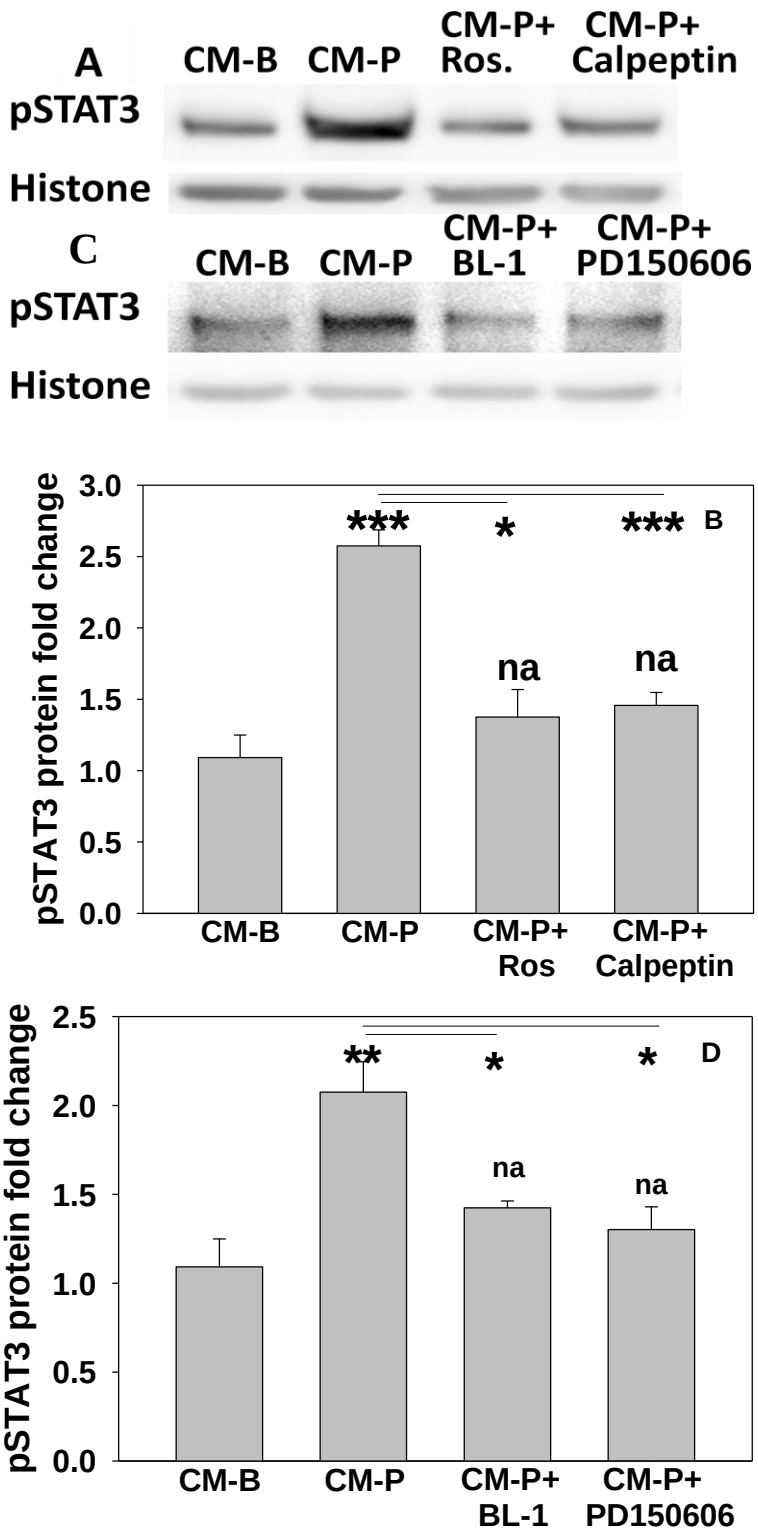
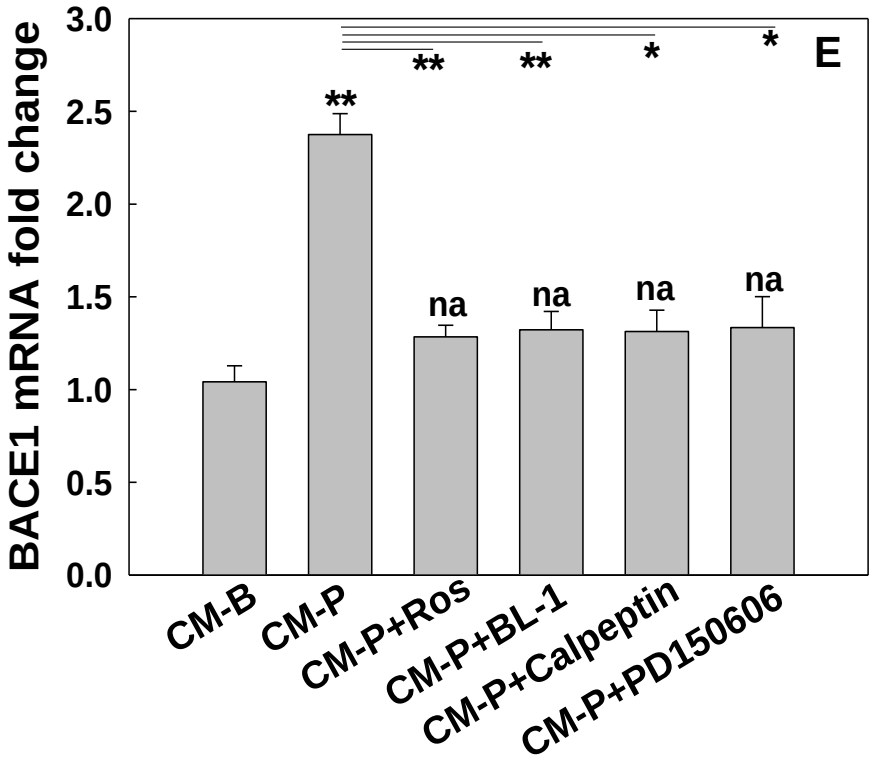


Figure 3.6 Elevated calpain and p25/Cdk5 activity correlated with STAT3 levels and

Figure 3.6 (con'd) BACE1 mRNA level. Neurons were incubated with conditioned media, CM-B (control), CM-P, CM-P plus specific inhibitors of Cdk5, Roscovitine at 10μM or butyrolactone-1 at 10μM (CM-P+Ros or CM-P+BL-1, respectively), or CM-P plus specific inhibitors of calpain, calpeptin at 20μM or PD150606 at 100μM (CM-P+calpeptin or CM-P+PD150606, respectively) for 12hr. Then the cells were lysed. (A-D) pSTAT3 levels in the nucleus of primary neurons. Histone is a loading control for the nuclear extracts. (E) BACE1 mRNA fold change. The results were normalized to control (CM-B), and * or na (not significant) above the bar indicates the significance. A line indicates comparison between the 2 bars connected by the line. (n=3). *p<0.05, **p<0.01, ***p<0.001.



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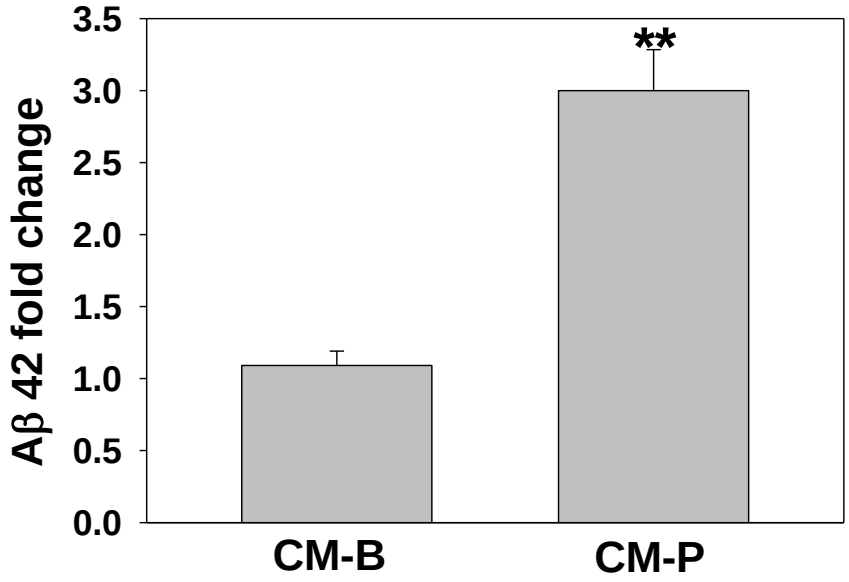


Figure 3.7 Aβ42 fold change. Primary neurons were treated with conditioned media CM-B (control) or CM-P for 12hr. Then the cells were lysed and Aβ42 levels were detected by ELISA (n=3). **p<0.01.

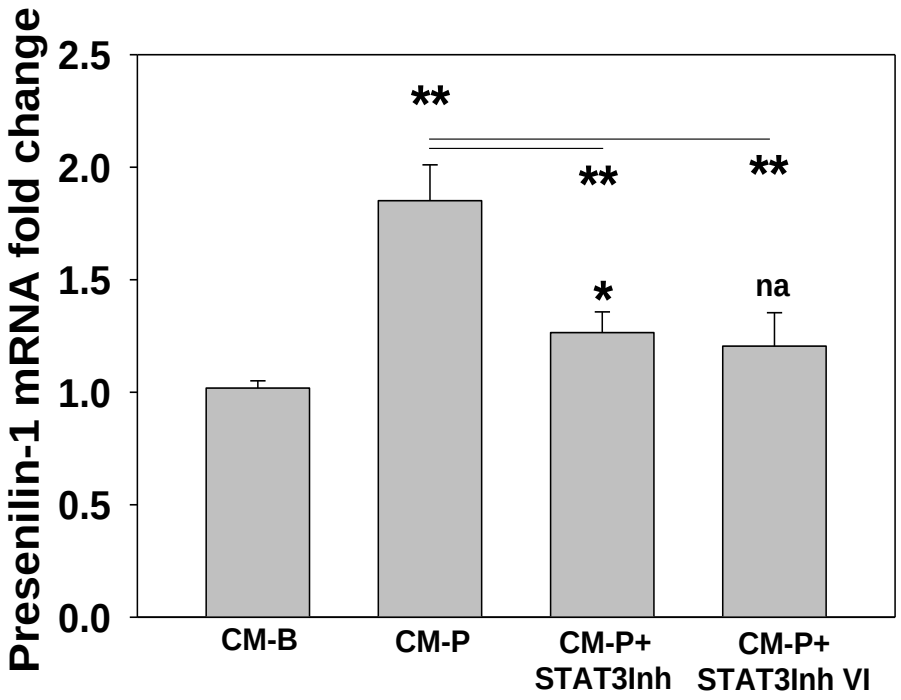


Figure 3.8 Presenilin-1 expression in neurons. Primary neurons were cultured in conditioned media CM-B (control), CM-P or CM-P plus STAT3 inhibitor or STAT3 inhibitor VI (CM-P+STAT3Inh at 235 μM, CM-P+STAT3Inh VI at 100 μM, respectively) for 12hr. Then the cells were lysed and the mRNA level of presenilin-1 was monitored by real-time PCR. The results were normalized to control (CM-B), and * or na (not significant) above the bar indicates the significance. A line indicates comparison between the 2 bars connected by the line. (n=3). *p<0.05, **p<0.01, ***p<0.001.

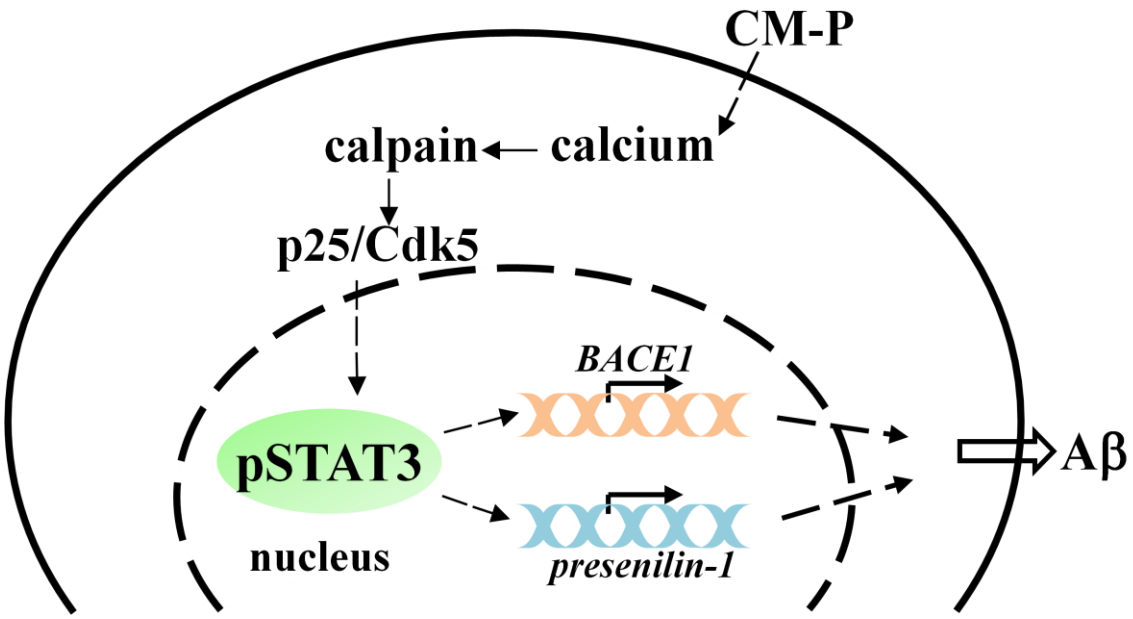


Figure 3.9 Scheme of neuronal responses upon CM-P treatment. CM-P elevates calcium level in primary neurons. Elevated calcium increases calpain activity, a calcium dependent protease, which in turn enhances p25/Cdk5 activity. STAT3 is phosphorylated by p25/Cdk5 and translocates into the nucleus. Elevated pSTAT3 in the nucleus transcriptionally upregulates both BACE1 and presenilin-1 mRNA, which could further enhance the production of Aβ.

CHAPTER 4. IPAF INFLAMMASOME IS INVOLVED IN IL-1B PRODUCTION FROM ASTROCYTES, INDUCED BY PALMITATE; IMPLICATIONS FOR ALZHEIMER'S DISEASE

This work is in press:

Li Liu and Christina Chan, IPAF inflammasome is involved in IL-1β production from astrocytes, induced by palmitate; implications for Alzheimer's Disease, Neurobiology of Aging, (in press)

Abstract

The inflammatory response has been strongly implicated in the pathogenesis of numerous diseases, including Alzheimer's disease (AD). However, little is known about the molecular mechanisms initiating the generation of inflammatory molecules in the central nervous system, such as interleukine-1β (IL-1β). Previously we identified that palmitate (PA) can induce primary astrocytes to produce cytokines, causing AD-like changes in primary neurons. Here we investigated and identified that PA induced the activation of IPAF-ASC inflammasome in astrocytes leading to the maturation of IL-1β, thereby implicating not only pathogen-related factors can activate IPAF-ASC inflammasome. Moreover, downregulating IPAF (which was found to be regulated by CREB) in astrocytes through silencing to decrease IL-1β secretion from the astrocytes reduced the generation of amyloid β42 by primary neurons. Furthermore, the expression levels of IPAF and ASC were found significantly elevated in a subgroup of sporadic AD patients; suggesting an involvement of IPAF-ASC inflammasome in the inflammatory response associated with AD, and thus could be a potential therapeutic target for AD.

Keywords: Alzheimer's disease, Fatty acid, inflammasome, IL-1β, IPAF

4.1 Introduction

Interleukin-1β (IL-1β) is a major proinflammatory cytokine that initiates and amplifies diverse cellular responses. Elevated IL-1β has been linked to diseases, such as type 2 diabetes, certain cancers, central nervous system (CNS) dysfunction and dementia [274, 275]. Systemic inhibition of inflammation has been suggested to improve type 2 diabetes, enhance neuroprotection during brain injury, and delay the onset of Alzheimer's disease (AD) [276-279]. Blocking or neutralizing IL-1β reduced cognitive impairment and decreased AD-like pathological changes in AD mouse models [75, 183]. Similarly, knockout of IL-1β receptor antagonist in mice increased the neuronal damage induced by Aβ [76]. These findings suggest that IL-1β could be involved in AD pathogenesis.

IL-1β is produced by various types of cells, including astrocytes [17, 280, 281]. Astrocytes are resident brain cells that play crucial roles in synaptic formation and function, as well as neuronal cell survival and death. Upon activation of astrocytes by diverse factors, chemokines and cytokines are released, including IL-1β [280, 282, 283]. As major contributors of IL-1β production in the CNS, activated astrocytes are involved in the pathogenesis of many neurodegenerative diseases, including AD [280]. Elevated IL-1β levels have been detected in astrocytes of brains of AD patients and AD animal models [82, 284, 285]. The inflammatory mediators secreted from activated astrocytes alter normal function of neurons and microglial cells, and trigger neurotoxicity, which further exacerbates the neurodegenerative pathology [286].

IL-1β is derived from an inactive precursor, pro-IL-1β, by a multiprotein complex, inflammasome [287]. Inflammasomes generally consist of an nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) member and pro-caspase-1. Upon activation, caspase-1 cleaves pro-IL-1β resulting in the maturation of IL-1β. The composition of each inflammasome

differs and is cell-type and stimulus-specific. The NLRP1 (NLR protein 1) and NLRP3 (NLR protein 3) inflammasome can be activated by pathogenic or danger-associated molecules [288]. ASC (apoptosis-associated speck-like protein containing a CARD), the adaptor protein, is required for the activation of NLRP1 and NLRP3 inflammasomes [288]. The IPAF inflammasome (ice protease-activating factor, also known as NLRC4) has been demonstrated to play a role in host defense mechanism against pathogen-associated molecules [58, 86]. Although the ACS adaptor protein is not essential for the activation of the IPAF inflammasome, under certain conditions ASC is required for IPAF inflammasome activation [57].

We previously reported that palmitate (PA) induced IL-1β maturation and secretion from primary rat astrocytes, which contributed to AD-like changes in primary rat neurons [17]. PA is the most abundant saturated fatty acid in the diet. Studies have shown that high fat diet induces Aβ deposition and memory deficits in APP transgenic mice [148-150]. PA and its metabolites, such as ceramides, regulate many gene expression and immunological pathways [159]. Elevated ceramide levels have been consistently reported in AD patients [164, 166, 206, 289]. Ceramide can be synthesized from serine and palmitoyl-CoA by serine palmitoyltransferase (SPT), the rate-limiting enzyme of *de novo* ceramide synthesis [290, 291]. We found that the effects of high fat diets, in particular PA, on AD-like pathology are mediated by ceramides through SPT [17, 151, 166, 292]. We reported that high fat diet containing 60% of the total fat as PA significantly increased ceramide and SPT levels in AD mouse model as compared with those fed a control chow diet and the elevated ceramide and SPT levels mediated the increase in Aβ levels [166, 292]. Inhibiting SPT in a high fat fed AD mouse model through subcutaneous administration of L-cylcoserine, an inhibitor of SPT, significantly down-regulated cortical $A\beta_{42}$ and hyperphosphorlated tau levels [166, 292].

To elucidate how PA, through ceramides, increased Aβ levels, we performed cell studies. We demonstrated that an upregulation of BACE1 and hyperphosphorylation of tau in primary neurons is mediated through conditioned media from astrocytes cultured with PA [152, 154]. Interestingly, culturing primary neurons directly with PA did not induce these AD-like pathologies [152, 154]. We determined that PA induced the release of cytokines, in particular IL-1β and TNFα, from the astrocytes into the media (i.e. conditioned media). In support saturated fatty acid PA also has been shown to induce pro-inflammatory cytokine release from microglia cells [127]. Inhibiting SPT in the astrocytes to mitigate the elevated ceramide levels significantly reduced IL-1β release from the astrocytes cultured with PA. Congruently, neutralizing IL-1β and TNFα in the astrocyte conditioned media significantly reduced the upregulation of BACE1 in the primary neurons cultured in the neutralized astrocyte conditioned media [17]. Taken together, the evidence suggest a potential connection exists between PA and AD pathogenesis mediate by ceramides and the generation of cytokines, thus we conducted experiments in this study to uncover the molecular mechanism by which PA triggers the production of IL-1β from primary astrocytes. We demonstrate that PA activates the IPAF inflammasome in primary rat astrocytes to induce the maturation of IL-1β. This is the first report in which the IPAF inflammasome is induced by factors other than pathogen-associated molecules. Moreover, the expression of IPAF in the astrocytes upon exposure to PA is regulated by the transcription factor CREB. Downregulating IPAF in primary rat astrocytes significantly reduced the $A\beta_{42}$ levels produced by the primary neurons cultured in conditioned media from PA-treated astrocytes. Finally, the levels of IPAF and ASC were found significantly upregulated in a subgroup of sporadic AD patient brains.

4.2 Materials and methods

4.2.1 Human samples

AD and control neocortical brain samples were obtained from the University of Kentucky Alzheimer's disease center tissue bank (ADC). Diagnoses were confirmed by neurologists, neuropathologists, and neuropsychologists in the ADC clinic. Most samples have been obtained in less than 4hr postmortem interval, and the age for the patients ranged from 88-99 years. Table 4.1 lists the reference number, gender, Braak stage, Mini Mental State Examination scores, frontal neuritic plaque numbers, neurofibrillary tangle numbers, and ApoE genotype of the individuals. The information was provided by the ADC.

4.2.2 Isolation and culture of primary rat astrocytes and neurons, and astrocyte conditioned media (CM)

All procedures in the cell isolation were approved by the Institutional Animal Care and Use Committee at Michigan State University. Primary cortical astrocytes were isolated from postnatal day 0-2 newborn Sprague-Dawley rats as previously described [17, 151]. Approximately $4x10^4$ cells/cm^2 were seeded on poly-L-lysine (PLL, Cultrex, Gaithersburg, MD, USA) coated plates. Primary astrocytes were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100U/mL penicillin (Invitrogen, Carlsbad, CA, USA). When the astrocytes reach around 60-70% confluence, the astrocyte medium was removed and subsequently cultured in cortical media for 24hr (see below). The purity of the astrocytes monolayers were >90% as determined by GFAP immunoreactivity and flow cytometry (Figure B.1).

Cortices from postnatal day 0 Sprague Dawley rats were used for neuronal culture. The primary neurons were plated on PLL coated plates at $2.5X10^5$cells/cm^2. The primary neurons

were cultured in neurobasalA medium with B27, 0.5mM glutamine, and PS for 3-4 days after isolation. Then the neurobasalA/B27 media was removed and the neurons were cultured in cortical media (see below) for 24hr prior to treatment (i.e. conditioned media from the astrocytes). The purity of the neurons was >90% as determined by βIII tubulin immunostaining and flow cytometry (Figure B.1).

Cortical medium (DMEM 10313 supplemented with 10% horse serum, 10mM HEPE, 2mM glutamine, 100μg/mL streptomycin and 100U/mL penicillin, (Invitrogen)) was used to culture primary neurons and primary astrocytes for 24hr prior to treatment, i.e. conditioned media from astroyctes for treating primary neurons or PA for treating astrocytes. BSA (fatty acid-free bovine serum albumin) (Millipore, Billerica, MA, USA), or 0.4mM PA (Sigma, St. Louis, MO, USA) plus BSA as a carrier protein (molar ration is 3:1) was added to the cortical medium and used to incubate the astrocytes for 12hr. This astrocyte conditioned media (CM-B or CM-P) were used subsequently to treat primary neurons for 12hr.

Forskolin and isobutylmethylxanthine (IBMX) (Sigma) at concentrations of 10μM and 100μM, respectively, were used to increase the CREB level.

4.2.3 Total mRNA Extraction and Quantitative real time PCR

Total mRNA from cells was extracted using the RNeasy Plus kit (QiaGen, Valencia, CA, USA) according to the manufacturer's instructions. Total mRNA was extracted from human brain neocortices using TRIzol and RNeasy Plus kit. The total mRNA was reverse transcribed into cDNA using the cDNA synthesis kit (BioRad, Hercules, CA, USA) as previously described [246, 249]. The following primer sets (Operon, Huntsville, AL, USA) were used for PCR: rat actin (5'-ctcttccagccttccttcct-3'and 5'-aatgcctgggtacatggtg-3'), rat pro-IL-1β (5'-gcatccagcttcaaatctc-3' and 5'-ggtgctgatgtaccagttg-3'), rat pro-caspase-1 (5'-

gacaagatcctgagggcaaa-3'and 5'-ggtctcgtgccttttccata-3'), rat IPAF (5'-gcgaaacctgaagaagatgc-3' and 5'-aacgctcagcttgaccaaat-3'), rat ASC (5'-gcaatgtgctgactgaagga-3'and 5'-tgttccaggtctgtcaccaa-3'), rat CREB (5'-tgttcaagctgcctctggt-3'and 5'-tctttcgtgctgcttcttca-3'), human actin (5'-tggacttcgagcaagagatg-3' and 5'- aggaaggaaggctggaagag-3'), human IPAF (5'-agcttgctgaaggcttgttgct-3' and 5'-tcacccatctggattgcaca-3'). Quantitative real-time PCR was performed using iQSYBR Green Supermix and Real-Time PCR Detection System (BioRad). The reaction program is: cycle1 (1x) 95^{o}C, 15min; cycle 2 (40x) step1: 94^{o}C, 15sec, step2: 57^{o}C, 30sec, step3: 70^{o}C, 30sec; cycle 3 (1x) 72^{o}C, 7min; cycle 4 (80x) 55^{o}C, 10min. The cycle threshold values were determined by the MyIQ software.

4.2.4 Western blot

Whole cell extracts from cells and from human brain neocoritices (homogenized) were measured for protein concentrations by Bradford assay. Protein samples 15-30μg were subjected to Western analysis as previously described [247, 248] using pro-IL-1β antibody (Biovision, Milpitas, CA, USA), caspase-1 (Abcam, Cambridge, MA, USA), IPAF (Santa Cruz biotech, Dallas, Texas, USA), ASC (Novus Biologicals, Littleton, CO, USA), CREB and pCREB (Cell signaling, Danvers, MA, USA), beta-actin and TBP (TATA binding protein) (Sigma). Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Thermo Scientific, and donkey anti-goat IgG-HRP was purchased from Santa Cruz biotech. The blots were visualized by SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific, Asheville, NC, USA).

4.2.5 Enzyme-linked immunosorbent assay (ELISA) and $A\beta_{42}$ assay

The levels of IL-1β in the astrocyte cultured supernatants from various treatments were analyzed by an ELISA kit (R&D system, Minneapolis, MN, USA) according to the

manufacturer's instructions. The sensitivity of the assay was 5pg/ml for IL-1β. $A\beta_{42}$ levels were detected by $A\beta_{42}$ ELISA kit (Invitrogen). Optical densities were measured by Spectra MAX Plus384 plate reader. Each sample concentration was calculated based on a standard curve of IL-1β or $A\beta_{42}$ standards. All readings were normalized to the total protein levels determined by Bradford's assay, and the data then was normalized to the control.

4.2.6 Endotoxin assay and Measurement of cytotoxicity

To detect for possible contamination of PA with lipopolysaccaride, the endotoxin assay was performed using ToxinSensor, an endotoxin detection system, following the manufacturer's instructions (GenScript, Piscataway, NJ, USA). The endotoxin level in PA-BSA mixture was less than 0.022ng/ml. which is far below the concentration required to induce astrocytic activation [293, 294].

The cytotoxicity of the PA treatment was determined by intracellular and secreted lactate dehydrogenase (LDH assay) according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). LDH released into the medium and retained in the cells were denoted as LDH_m and LDH_c, respectively. Percentage of release was calculated with the following equation: LDH release $\%=LDH_m/(LDH_m+LDH_c)*100$

4.2.7 Caspase -1 fluorometric activity assay and Caspase-1 inhibition

Caspase-1 activity in astrocytes was determined using a Caspase-1 FLICA kit (Immunochemistry Technologies, Bloomington, MN, USA) according to the manufacturer's instructions. Briefly, the astrocytes were incubated with BSA or 0.4mM PA for 12hr. FAM-FLICA Caspase-1 reagent, FAM-YVAD-FMK, was reconstituted with DMSO, and was further diluted with PBS to a concentration of 150μM. 150μM FAM-FLICA Caspase-1 reagent was added to the cultured media at a final concentration of 5μM. The cells were incubated with

FAM-FLICA Caspase-1 reagent for 1hr at 37℃. The cells bearing active caspase-1 coupled to FLICA showed green. Fluorescence images were taken by confocal microscope Olympus FluoView 1000, and the green fluorescence signal was detected by Spectra MAX GEMINI EM plate reader. All readings were normalized to the protein levels obtained by Bradford assay. Caspase-1 activity in astrocytes was inhibited using either the general caspase inhibitor Z-VAD (R&D system) or the specific inhibitor Z-YVAD to caspase-1 (Biovision).

4.2.8 Transfection

SiTENOME SMARTpool siRNAs targeting ASC, SPT, IPAF, CREB and CASP1 were purchased from Dharmacon (Pittsburgh PA, USA), and scramble siRNA was purchased from Ambion. In brief, for one well in 6 well plate, 200-250pmol siRNA was diluted to 250μl Opti-MEM I reduced serum medium without serum (Invitrogen), 5μl lipofectamine RNAiMAX was added to 250μl Opti-MEM. Diluted siRNA and diluted RNAiMAX were combined, mixed gently and incubated for 10-20min at room temperature to form siRNA-RNAiMAX complex. When the confluence of the astrocytes reaches 70-80%, the astrocyte culture medium was changed to 2ml of astrocyte culture medium without antibiotics. The siRNA-RNAiMAX complexes 500μl were added to the 2ml of astrocyte culture medium without antibiotics. The final concentration of the siRNA in the medium was 80-100nM and the media containing siRNA-RNAiMAX complexes was used to treat the astrocytes for 18-24 hr. After silencing, either PA or BSA was used to culture the astrocytes for 12 hrs.

The empty vector pCMV and the constitutively active form CREB (VP16-CREB) were used for overexpression. Briefly, the cells were transfected with 0.8μg pCMV or VP16-CREB using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. Media were

changed after 6hr and the cells were incubated in fresh astrocyte media for up to 24hr prior to treatment with PA.

4.2.9 Statistical analysis

All experiments were performed at least three times, and representative results are shown. Statistical analysis was performed by an unpaired, two tail student t-test (and ANOVA), and Mann-Whitney tests was used for human brain samples. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

4.3 Results

4.3.1 Both pro-IL-1β and the mature form of IL-1β increase in astrocytes upon PA treatment

IL-1β can be produced by monocytes, macrophages, dendritic cells and astrocytes upon stimulation by diverse factors [32, 295]. PA can induce IL-1β production from several types of cells, including macrophages and microglia [127]. Fatty acid concentration in normal human plasma *in vivo* generally ranges between 0.3-1.0 mM [259-261]. We demonstrated that 0.2mM PA can induce IL-1β secretion from primary rat astrocytes [17]. Here we confirm that IL-1β secreted from astrocytes increases in a dose-dependent manner with PA concentration as compared with the control (BSA) (Figure 4.1A). To determine whether PA promoted cell death and cytotoxicity, primary astrocytes were incubated with different concentrations of PA for 12hr and LDH release was measured. At concentrations of 0.2 and 0.4mM PA cell death was not induced while at 0.7mM concentrations of PA cell death increased significantly (Figure B.2), therefore 0.2 and 0.4mM PA concentrations were used in subsequent experiments. In addition to the mature form of IL-1β, the mRNA and protein levels of precursor IL-1β (pro-IL-1β) increased

in the astrocytes upon culture with 0.4 mM PA and treatment time (6 and 12hr) (Figure 4.1B-D). Previously we reported that PA increased ceramide levels in primary astrocytes through SPT [151], the rate-limiting enzyme for *de novo* ceramide synthesis pathway [290, 291]. Inhibiting SPT to reduce ceramide levels by specific inhibitor L-cycloserine significantly reduced the upregulation in IL-1β [17], suggesting that SPT is involved in the generation of IL-1β in primary astrocytes treated with PA. Here we further demonstrated that transient silencing of SPT by siRNA significantly reduced secreted IL-1β from astrocytes cultured with PA (Figure 4.1E, F).

4.3.2 Upregulation in caspase-1 is involved in the release of IL-1β

The processing of pro-IL-1β usually requires cleavage by caspase-1, thus the activity of caspase-1 is critical to the inflammatory response. To determine if caspase-1 is involved in the maturation of IL-1β in astrocytes treated with PA, confocal microscopy and caspase-1 fluorometric activity assay were performed and established that the level of activated caspase-1 was elevated (Figure 4.2A, B). This is further supported by western blot analysis which showed increased caspase-1 as well as pro-caspase-1 mRNA and protein levels in astrocytes upon PA treatment (Figure. 4.2 C-F). Primary astrocytes pre- and co-treated, for 30min and 12hr, respectively, with the general caspase-1 inhibitor Z-VAD or specific caspase-1 inhibitor Z-YVAD, along with PA, significantly reduced the IL-1β secreted (Figure 4.2 G, H). To further confirm the inhibition results, pro-caspase-1 was transiently silenced in astrocytes by siRNA (Figure 4.2 I-K), and the pro-caspase-1 silenced astrocytes were subsequently incubated with PA. Silencing caspase-1 reduced the IL-1β secreted by the PA-cultured astrocytes as compared with the PA-cultured astrocytes with scramble siRNA (Figure 4.2 L). Taken together these results are consistent with caspase-1 involvement in the generation of IL-1β from astrocytes cultured with PA.

4.3.3 PA activates IPAF inflammasome to produce mature IL-1β

Inflammasomes are multiprotein complexes that recruit pro-caspase-1 and trigger the activation of caspase-1. Activation of cytoplasmic receptors of inflammasomes, which are initiators of inflammasome assembly, is cell-type and stimulus-dependent. In the CNS, NLRP1 (the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family protein 1) was reported to be expressed in neurons but was not detected in astrocytes [78]. A recent study uncovered IPAF inflammasome in microglial cells [86]. To date, the involvement of inflammasome receptors in astrocytes treated with PA has not been reported. Since PA can induce IL-1β secretion from astrocytes [17], we evaluated whether the other two receptors, IPAF and NLRP3, are involved in the processing of IL-1β in primary rat astrocytes. Notably, we found PA increased the mRNA level of IPAF significantly (Figure 4.3 A). To further confirm the involvement of IPAF in the production of IL-1β by astrocytes, IPAF was transiently silenced with siRNA (Figure 4.3 B, C, D) and the upregulation of IL-1β was abolished in the astrocytes cultured in PA (Figure 4.3 E). In contrast, silencing NLRP3 in primary astrocytes did not reduce the upregulation of IL-1β upon cultured with PA (Figure 4.3 F). These results suggest that IPAF, expressed in primary rat astrocytes, is activated by PA, and could recruit pro-caspase-1 and activate caspase-1 to lead to subsequent maturation and release of IL-1β.

4.3.4 ASC is important for the activation of IPAF inflammasome

ASC (apoptosis-associated speck-like protein containing a CARD) is an adaptor protein required for the activation of the NLRP1 and NLRP3 inflammasomes, whereas the involvement of ASC in the activation of IPAF inflammasome is stimulus dependent [296]. Upon exposure to bacteria, such as *Salmonella typhimurium*, *Shigella flexneri* or *Pseudomonas aeruginosa*, ASC is required for activation of the IPAF inflammasome [58], but not for IPAF activation in response

to *Legionella pneumophila* [297]. We found the levels of ASC protein increased dramatically in astrocytes cultured with PA for 6 and 12hr (Figure 4.4 A, B). To determine if ASC is required for the activation of IPAF inflammasome in astrocytes cultured in PA, ASC was transiently silenced in astrocytes (Figure 4.4 C-E) prior to PA treatment for 12hr. This abolished the upregulation of IL-1β in the astrocytes cultured in PA (Figure 4.4 F) implying that ASC is required for the activation of the IPAF inflammasome. In addition to ASC, Naip5 is sometimes needed for IPAF activation, depending on the stimulus [59, 296]. Upon transient silencing of Naip5 in primary astrocytes, as confirmed by a decrease in both its mRNA and protein levels, the ELISA analysis indicated, however, that the level of IL-1β was not reduced significantly in the astrocytes cultured in PA (Figure B.3). These results suggest that ASC, but not Naip 5 (NLR family, apoptosis inhibitory protein 5), is required for the activation of IPAF in primary astrocytes treated with PA. Currently the mechanisms by which ASC and Naip5 are involved in the activation of IPAF are unknown.

4.3.5 IPAF inflammasome regulates $A\beta_{42}$ level in primary neurons

Many *in vivo* and *in vitro* studies suggest that inflammation is involved in the pathogenesis of AD. Specifically, IL-1β has been shown to induce tau hyperphosphorylation and enhance neurofibrillary tangles [54, 74]. We previously also reported that conditioned media from primary astrocytes cultured in PA contained elevated IL-1β, which induced an upregulation in the levels of tau hyperphosphorylation and $A\beta_{42}$ in primary rat neurons [151]. This study investigates whether the increase in the level of IPAF inflammasome in astrocytes cultured in PA affects the level of Aβ produced by primary rat cortical neurons treated with this astrocyte conditioned media. IPAF was silenced in primary rat astrocytes for 20hr, and the astrocytes were subsequently treated with either BSA or PA for 12hr, and then the astrocyte conditioned media

was used to culture primary neurons for 12hr. A significant decrease in the level of extracellular $A\beta_{42}$ was detected in primary neurons cultured in conditioned media from PA-treated astrocytes with IPAF silenced as compared with PA-treated astrocytes without IPAF silenced (Figure 4.5). This suggests that IL-1β generated from IPAF inflammasomes in the astrocytes is involved in enhancing the level of $A\beta_{42}$ in the neurons.

4.3.6 IPAF and ASC levels are elevated in sporadic AD patients

It was shown that the level of IL-1β in the brains of AD patients is significantly elevated [298, 299]. Since the IPAF inflammasome in the astrocytes, induced by PA, is involved in upregulating $A\beta_{42}$ levels in primary rat neurons through the astrocyte conditioned media, we measured the level of IPAF in the frontal brain cortices of a subgroup of sporadic AD patients and found both the mRNA and protein levels of IPAF were increased significantly as compared with the controls (Figure 4.6 A, D and F, Table 4.1). Furthermore, the mRNA and protein levels of ASC were also significantly elevated (Figure 4.6 B, D and F) while Naip5 did not changed significantly in this subgroup of sporadic AD brains as compared with normal controls (Figure B.4). These results raise the possibility that the IPAF inflammasome could be involved in the inflammatory response associated with AD. Another inflammasomes, NLRP3, was also evaluated and an increase in the mRNA and protein levels of NLRP3 was not detected in this subgroup of sporadic AD brains (Figure 4.6 C, E and F). Similarly in a recent report, elevated NLRP3 level was not detected in AD patient brains, nevertheless Aβ was able to activate NLRP3 to release cytokines in a transgenic animal model [83, 299], suggesting that NLRP3 could still play a role in the inflammatory reactions involved in AD pathogenesis.

4.3.7 CREB regulates IPAF expression in astrocyte treated with PA

The mRNA level of IPAF is significantly increased, more than 5 times, in primary rat astrocytes treated with PA for 6hr (Figure 4.3 A), and is also upregulated in a subgroup of sporadic AD brains (Figure 4.6 A). Thus far, the regulation of IPAF has not been extensively studied. The transcription factor, AP1, has been reported to regulate IPAF in human leukemia cells treated with tumor necrosis factor α [300] . Furthermore several other transcription factors have been predicted, including CREB (cAMP response element-binding protein), Stat (Signal Transducer and Activator of Transcription), and E2F [300], however, there has been no report on the regulation of IPAF in astrocytes. Therefore, we analyzed the promoter region of IPAF using JASPAR and TRANSFAC databases, and found that CREB could be a putative transcription factor of IPAF (Figure B.5). pCREB was detected by immunoblotting and the results showed an upregulation of pCREB in astrocytes treated with PA (Figure 4.7 A, B). Forskolin and isobutylmethylxanthine (FI), well studied chemicals to increase CREB level, were used to treat primary astrocytes for 6hr. mRNA analysis indicates the level of IPAF increased 5-fold in the FI-treated over the untreated astrocytes (controls) (Figure 4.7 C). To further confirm that CREB could be involved in the regulation of IPAF transcription, transient silencing of CREB and overexpression of a constitutively active form of CREB (VP16-CREB) were performed (Figure 4.7 D-F). Overexpression of the constitutively active CREB significantly increased IPAF mRNA level while silencing CREB significantly reduced the IPAF transcript (Figure 4.7 G). Similarly silencing CREB in the astrocytes prior to culturing with PA significantly reduced, while overexpressing CREB in the astrocytes enhanced the mRNA level of IPAF upon treatment with PA (Figure 4.7 H), suggesting that CREB could regulate IPAF expression in primary rat astrocytes. To further monitor the effect of CREB on IL-1β expression in astrocytes cultured in PA, CREB was silenced or overexpressed in primary astrocytes, and the astrocytes were

subsequently incubated in PA for 12hr. Silencing CREB significantly reduced the upregulation of IL-1β, while overexpressing CREB further enhanced the IL-1β secreted by astrocytes cultured with PA (Figure 4.7 I, J), suggesting that CREB could regulate IL-1β expression in PA-treated astrocytes mediated by IPAF.

4.4 Discussion

We identify that the IPAF inflammasome is involved in mediating the effects of astrocytes in determining the outcome of primary neurons in response to environmental factors, i.e. fatty acids. IL-1β expression is regulated at both the transcriptional and posttranscriptional proteolytic processing levels. Namely, it requires two signals, one to induce the expression of pro-IL-1β and the other to activate the inflammasome [281]. We found that PA alone can activate both signals; increase the level of the precursor IL-1β protein and activate the IPAF inflammasome, leading to the maturation of IL-1β in primary rat astrocytes. The regulation of pro-IL-1β expression by transcription factor NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) is well established [301], and NFκB is known to be activated by fatty acids, such as PA or high fat diet [127]. In support, we observed that NFκB expression in the astrocytes is increased upon PA treatment (Figure B.6) and inhibiting NFκB significantly reduced the mRNA level of pro-IL-1β (Figure B.7). This indicates that NFκB could be involved in the regulation of pro-IL-1β expression in PA-treated astrocytes. Furthermore, treating primary astrocytes with PA elevates ASC expression in the primary astrocytes (Figure 4.4). The regulation of ASC expression is not as well studied, and an analysis of the promoter region of ASC identified that NFκB could be a potential regulator of ASC. Indeed inhibiting NFκB reduced the upregulation of ASC in astrocytes cultured in PA (Figure B.6), suggesting that

NFκB could be involved in the regulation of both ASC and pro-IL-1β expression in PA-treated astrocytes. In addition to pro-IL-1β and ASC, the mRNA level of IPAF also increased in PA-treated astrocytes (Figure 4.3). Thus far, the regulation of IPAF has not been extensively studied. Several transcription factors, CREB, Stat and E2F have been predicted to potentially bind the human IPAF promoter region [300]. Our results suggest that CREB could transcriptionally regulate rat IPAF expression (Figure 4.7). Although the activation of IPAF inflammsome is not well understood, elevated levels of IPAF and ASC raise the possibility of enhanced assembly and activation of the IPAF inflammasome.

The activation of inflammasomes is critical for the maturation of IL-1β. NLRP1 and NLRP3 inflammasomes can be activated by diverse factors, involving both pathogen-associated molecular patterns, such as microbial and viral components, as well as danger-associated molecular factors, such as ATP, Aβ, and fibronectin [288]. Prior studies of IPAF inflammasome showed that bacterial and viral pathogenic factors are involved [86, 302], while our study demonstrates that non-pathogen associated factor, fatty acids, namely PA, also can activate IPAF inflammasome. To date, the signals and mechanisms leading to the activation of IPAF inflammasome in astroctyes are unclear. Aβ activates NLRP3 inflammasome in microglial cells through the phagocytosis-lysosome pathway [58]. A recent study in mice reported that the double-stranded RNA-dependent protein kinase (PKR) physically interacts with the inflammasome, including IPAF, to regulate inflammasome activity [303], and the phosphorylation of IPAF is critical for the activation of inflammasome [304]. Thus diverse mechanisms may be involved in the activation of inflammasomes.

Many types of inflammasomes have been found in the CNS [80]. NRLP1 has been shown to be expressed in neurons, while NLRP3 and IPAF have been detected in microglial cells [86,

288]. We found the IPAF inflammamsome is also expressed in primary astrocytes (Figure 4.3), suggesting that in the CNS the same type of inflammasome could be expressed in different cell types and a single cell type could express different inflammasomes. Astrocytes, acting as sentinels, and highly reactive to the microenvironment in their support of neurons and the blood-brain barrier, are important contributors to the release of IL-1β [287]. However, the composition of the inflammasome expressed in PA-treated astrocytes had not been determined prior to this study. The compositions of NLRP1 and NLRP3 inflammasomes require ASC. For example, in spinal cord injury ASC was shown to be required in the activation of NLRP1 inflammasome in the neurons of rat spinal cord [78, 79]. However, the composition of IPAF is cell-type and stimulus-specific, i.e. under some but not all conditions ASC is required for its activation [57]. In addition to ASC, it was reported that activation of the IPAF inflammasome could require Naip5 [58]. In this study, we found that ASC, but not Naip 5, is required for the activation of the IPAF inflammasome in the astrocytes treated with PA. Currently the molecular mechanism and function of ASC and Naip5 in the activation of IPAF inflammasome remains unclear, although ASC has been suggested to stabilize or facilitate caspase-1 recruitment [57].

Inflammation is an important characteristic of AD and has been implicated in the etiology of this neurodegenerative disease [305]. Specifically, elevated IL-1β level has been reported in plasma, brains and cerebrospinal fluid of patients with AD and mild cognitive impairment. Blocking IL-1β rescued cognition, reduced Aβ and attenuated tau pathology in AD transgenic mice [75]. In addition, the IL-1 receptor antagonist knock-out mice studies exhibited increased mortality and neuroinflammation to Aβ-induced neuropathology [76]. We previously showed PA induced AD-like pathological changes, such as BACE1, Aβ and hyperphosphorylated tau, in primary neurons mediated by the conditioned media from astrocytes cultured in PA, in particular

we confirmed the involvement of IL-1β [17, 151]. Silencing IPAF in the astrocytes reduced IL-1β secretion, which significantly diminished $A\beta_{42}$ level in the neurons cultured with the conditioned media from PA-treated astrocytes (Figure 4.5). We further confirmed the mRNA and protein levels of IPAF and ASC (but not Naip5) are upregulated in a subgroup of sporadic AD patient's brains (Figure 4.6, Figure B.4). In this study, IPAF inflammasome was detected in primary rat astrocytes cultured with PA. However recently, IPAF inflammasome, along with Naip 5, were reported in microglial cells to be involved in IL-1β production [86]. This suggests that multiple cell types in the CNS have the ability to express the same type of inflammasome. Thus, the upregulated IPAF level in human AD brains (Figure 4.6) could arise from astrocytes or microglial cells. Aβ was reported to trigger the activation of NLRP3 inflammasome in bone marrow derived dendritic and microglia cells [288]. Although an elevated level of NLRP3 was not detected in our subgroup of AD patient brains, nevertheless, NLRP3 could still play a role in the inflammatory reaction in AD pathogenesis triggered by other factors, such as Aβ [83]. Taken together, the results suggest that IPAF could be involved in the inflammatory response in AD.

Saturated fatty acids have been shown to induce cytokine release from several cell types [127]. Diets rich in fat and brain trauma increase the levels of fatty acids [245], such as PA, in the brain. Since fatty acids can freely cross the blood-brain barrier, they are readily taken up by astrocytes, the major cell type in the brain that metabolizes fatty acids. Our results suggest that PA activates IPAF inflammasome in primary astrocytes to releases IL-1β, which in turn increases $A\beta_{42}$ production in primary neurons. PA has been reported to induce IL-1β production in microglia [127], which raises the possibility that fatty acids could contribute to IL-1β production in the brain. IL-1β could further activate other cell types to release inflammatory molecules, e.g. chemotactic and neurotoxic molecules, to initiate and further propagate neuronal

dysfunction and eventual neuronal death. In the CNS, there are many inflammasomes that could be activated in the different cell types. For examples, Aβ activates NLRP3-ASC inflammasome in microglia, brain trauma induces the activation of NLRP1-ASC inflammasome in neurons [78], and we found that PA activates IPAF-ASC inflammasome in astrocytes and furthermore is upregulated in a subgroup of AD brains. Regardless of the complexity and which inflammasome is involved, ASC and caspase-1 are concomitantly implicated in the production of IL-1β in the CNS [78, 79, 306]. In support, neutralization of ASC spared the tissue and improved function in rat neurons exposed to inflammation induced by NLRP1-ASC inflammasome [78]. Taken together this suggests that IPAF, ASC and caspase-1 could be potential therapeutic targets to decrease inflammation in sporadic AD, as well as other CNS inflammatory diseases.

Group	Ref#	Age	Sex	PMI	Braak Stage	Front NP count	NFT counts	ApoE alleles	MMSE
Control	1132	95	F	3.5	0	3.0	0	3/4	26
Control	1159	86	F	3.5	0	0.0	0	3/3	28
Control	1165	92	M	3.3	0	10.4	0	3/3	28
Control	1206	94	F	2.3	2	0.0	0.4	3/3	29
Control	1221	81	M	2.8	2	5.6	0	3/3	27
AD	1013	80	M	2.5	5	5.8	1		
AD	1098	81	F	2.8	5	6.2	2	3/3	15
AD	1174	96	M	3.5	5	19.2	4	2/3	22
AD	1201	94	M	2.0	5	6.0	0.2	3/3	22
AD	1215	91	F	3.0	5	15.2	7.2	3/3	13

Table 4.1 Patient information. Reference number (Ref#), age, gender, Braak stage, postmortem interval (PMI), front neuritic plaque (NP) count, neurofibrillary tangle (NFT) count, ApoE genotype and Mini Mental State Examination (MMSE) score were provided by the UK ADC.

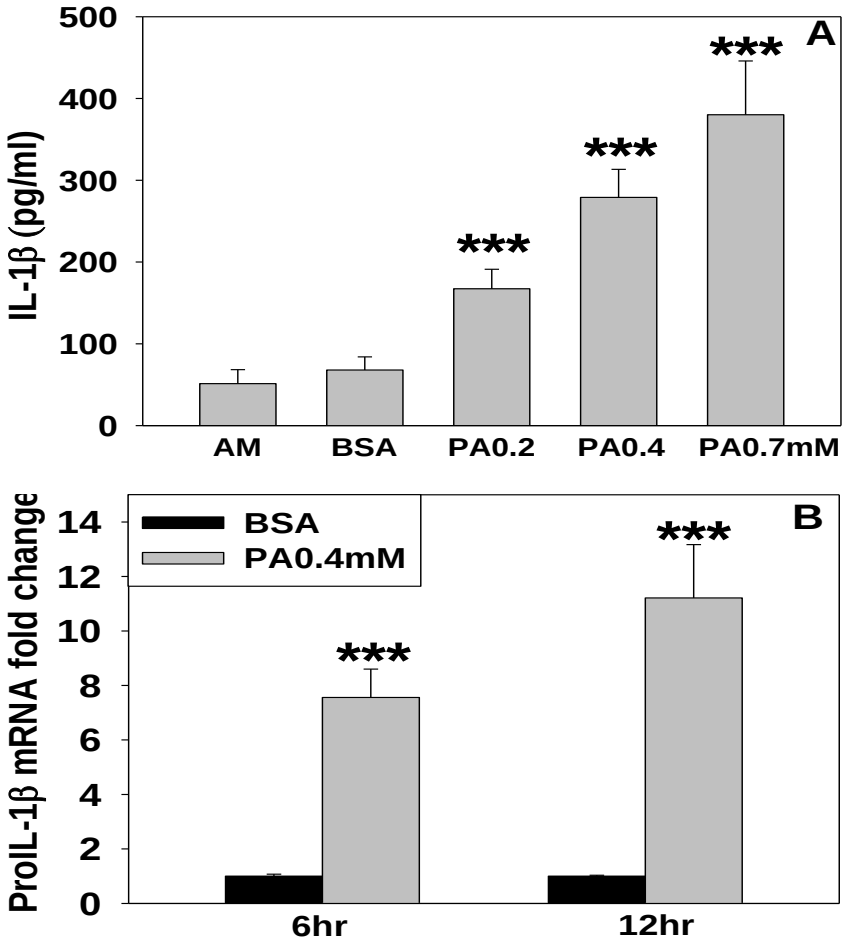
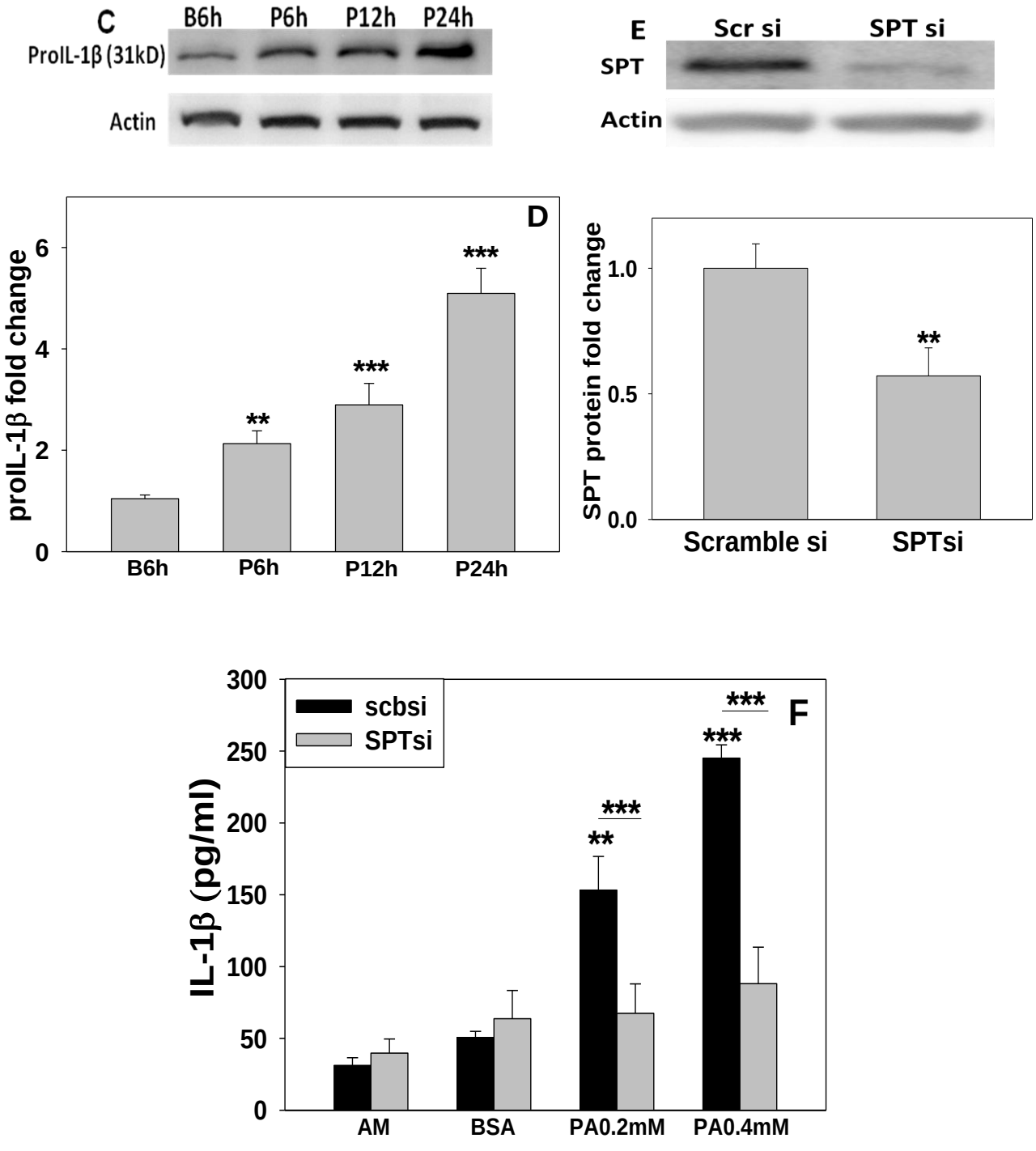


Figure 4.1 Pro-IL-1β and IL-1β levels produced by astrocytes with PA treatment. (A) Primary rat astrocytes were treated with regular astrocyte cultured medium (AM), or BSA (ctrl) or PA at the indicated concentration for 12hr. The mature form of IL-1β in cultured media was detected by ELISA assay (n≥3). (B, C, D) The mRNA and protein levels of pro-IL-1β in astrocytes treated with BSA (ctrl) or 0.4mM PA were detected by quantitative real-time PCR and western blot analysis, respectively (n≥3). (E and F) Silencing SPT decreased IL-1β level. (E) Representative western blot result and quantification of western blots of SPT levels in primary astrocytes treated with scramble siRNA (Scbsi) or SPT siRNA (SPTsi) for 24hr. (F) IL-1β protein expression level. Wild-type astrocytes or astrocytes silenced with scramble siRNA or SPT siRNA cultured with astrocyte medium (AM), BSA or PA for 12hr. IL-1β was detected by

Figure 4.1 (cont'd) ELISA and normalized to astrocytes treated with BSA (ctrl) (n=3). **: p<0.01, ***: p<0.001. A line indicates comparison between the two bars connected by the line.



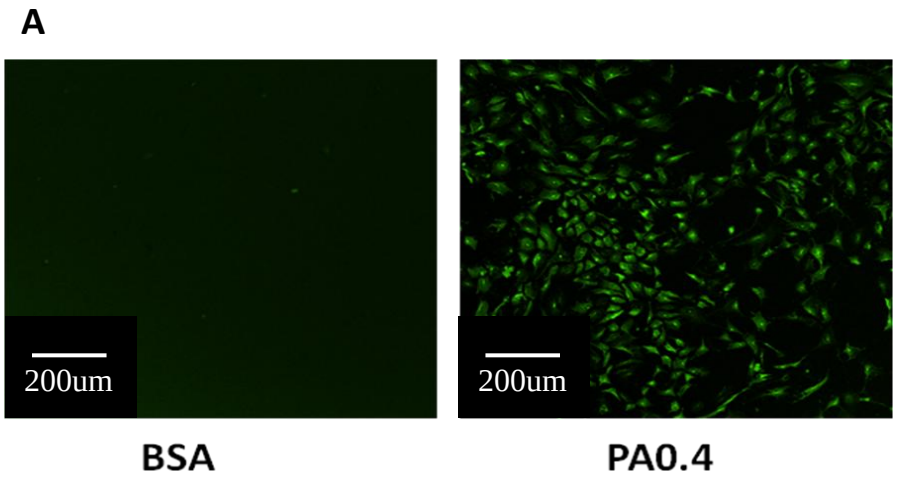


Figure 4.2 The upregulated caspase-1 increased IL-1β level. The primary rat astrocytes were treated with BSA or 0.4mM PA for 12hr and the caspase-1 was staining with FAM-YVAD-FMK (green fluorescence). (A) Images taken by confocal microscope. (B) Fuorescence fold change and the fluorescent signal detected by fluorescent plate reader. (C, D) Primary astrocytes treated with BSA or PA for the indicated time. The mRNA of pro-caspase-1 (ProCASP1) was monitored by real-time PCR and normalized to astrocytes treated with BSA (ctrl), and the protein expression was determined by immunoblot. Quantification of western blot results shown in (E, F). (G, H) Astrocytes pre- and co-treated with inhibitor ZVAD or ZYVAD and with PA for 12hr at the indicated concentration. The expression of IL-1β was monitored by ELISA and normalized to astrocytes treated with BSA (ctrl). (I, J) Astrocytes cultured with scramble siRNA (scbsi) or siRNA targeting CASP1 (CASPsi) for 24hr. Representative western blot results of CASP1 and the quantification of western blot results are shown (n=3). (K) The mRNA level of silenced CASP1 confirmed by real-time PCR (n=3). (L) The mature form of IL-1β in wild-type or silenced astrocytes. The astrocytes were silenced with scramble siRNA (scbsi) or CASP1 siRNA for 24hr, or wild-type astrocytes were treated with astrocyte medium (AM), BSA (ctrl), or PA

Figure 4.2 (cont'd) for 12hr. ELISA was performed to detect IL-1β level and the results were normalized to astrocytes treated with BSA (ctrl) (n=3). NA: not available. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.

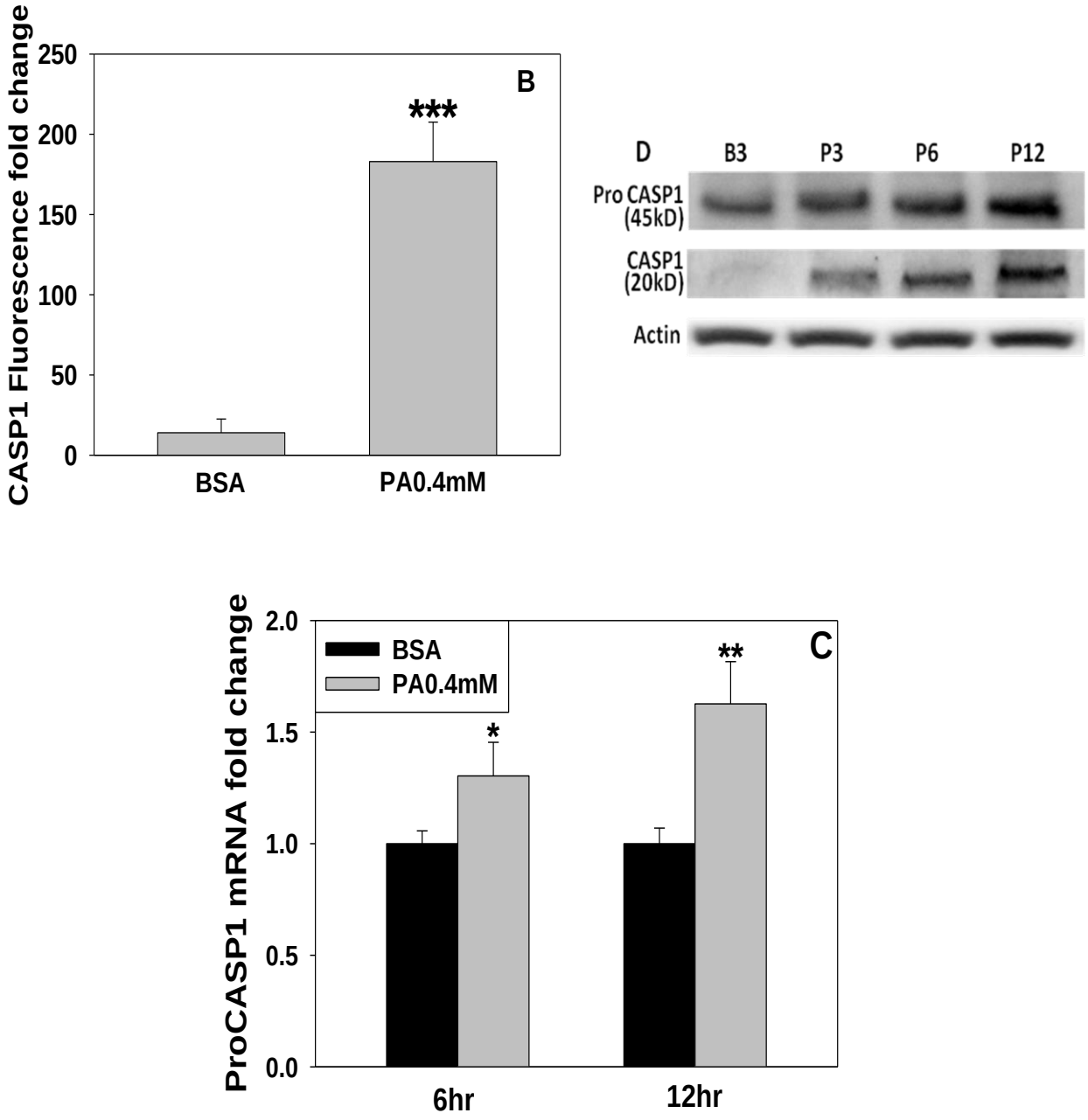
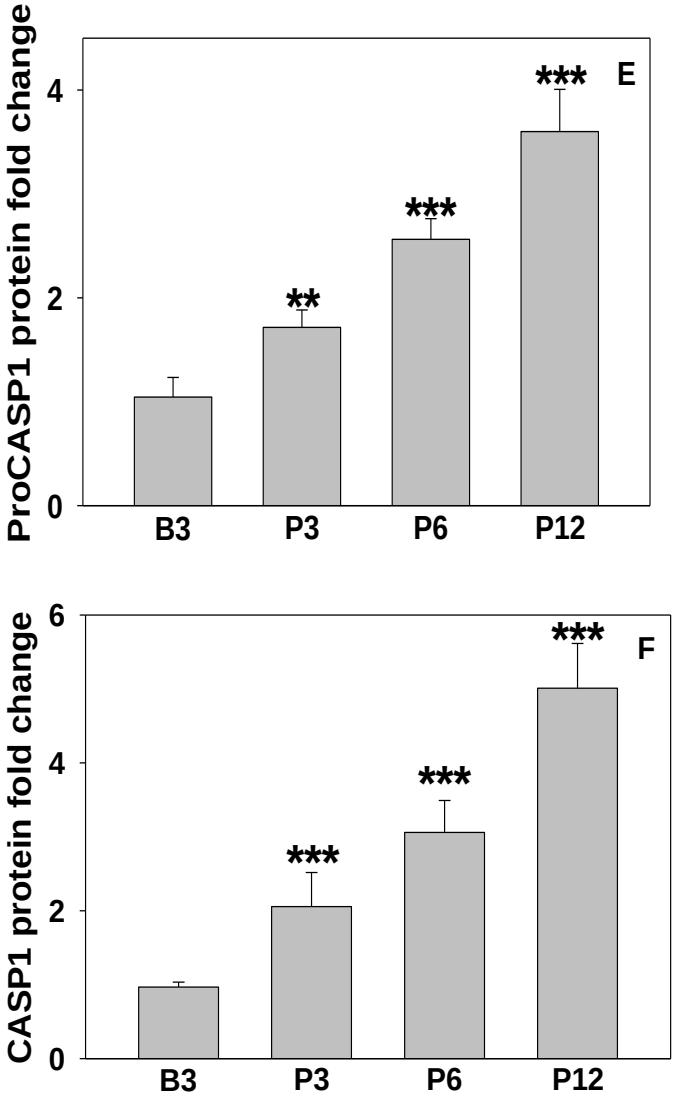


Figure 4.2 (cont'd)



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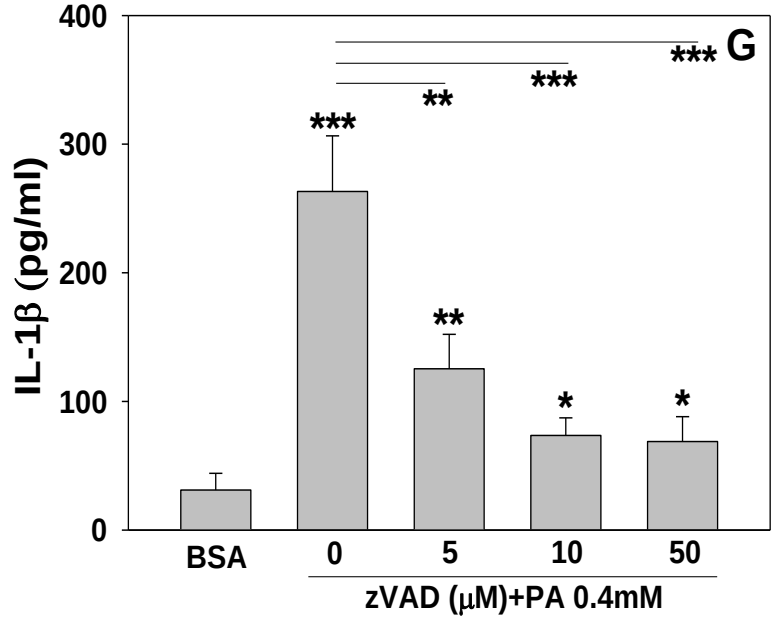

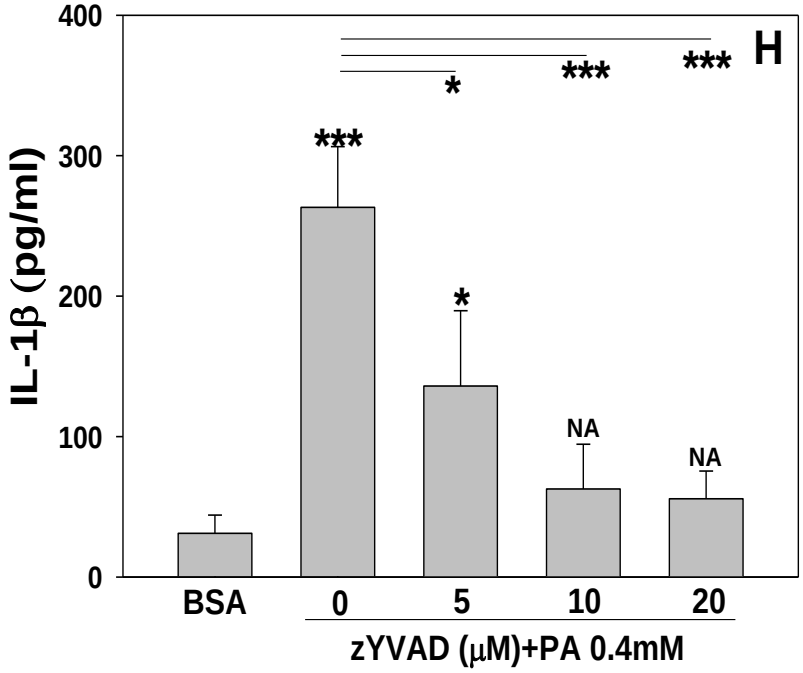

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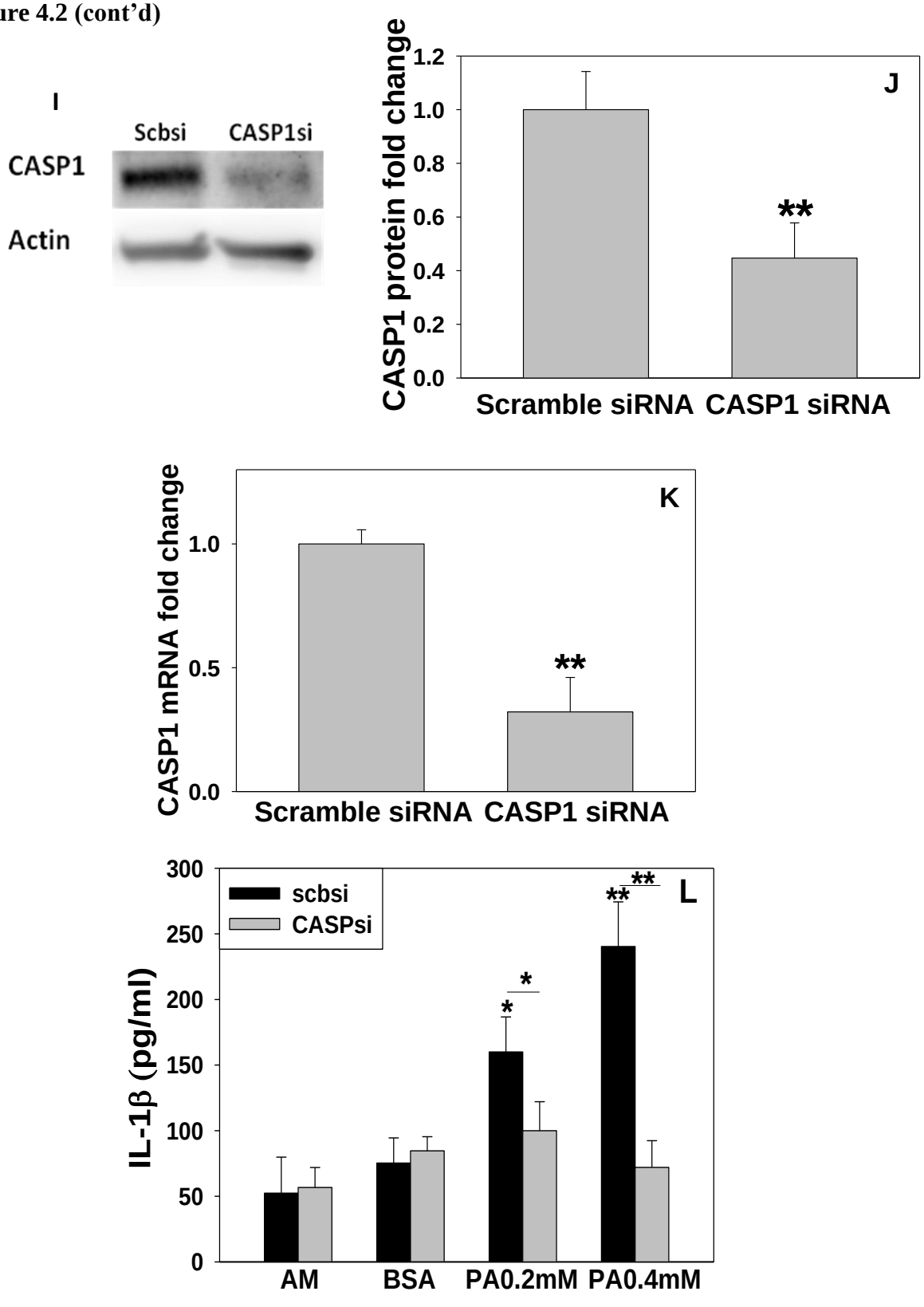


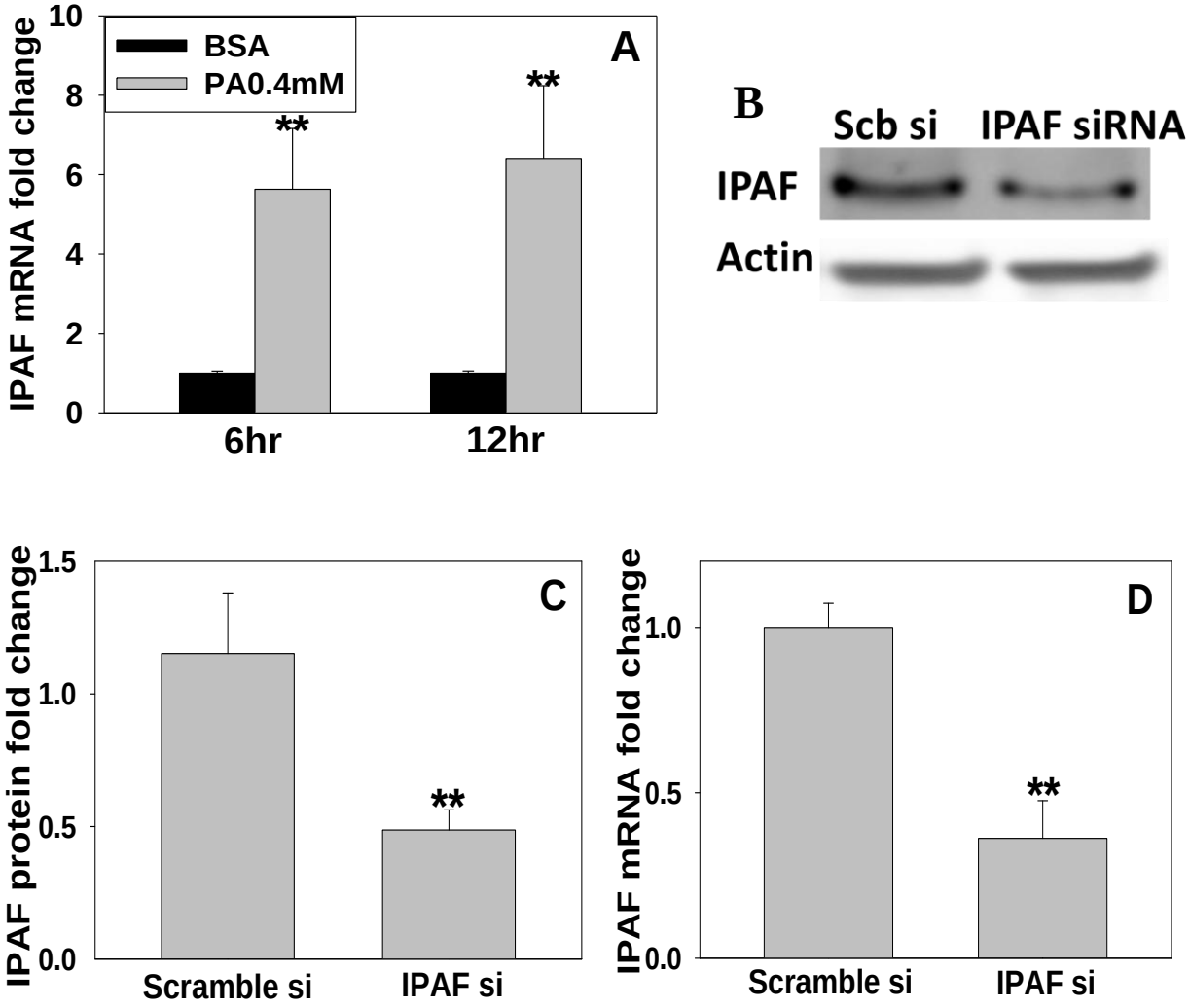
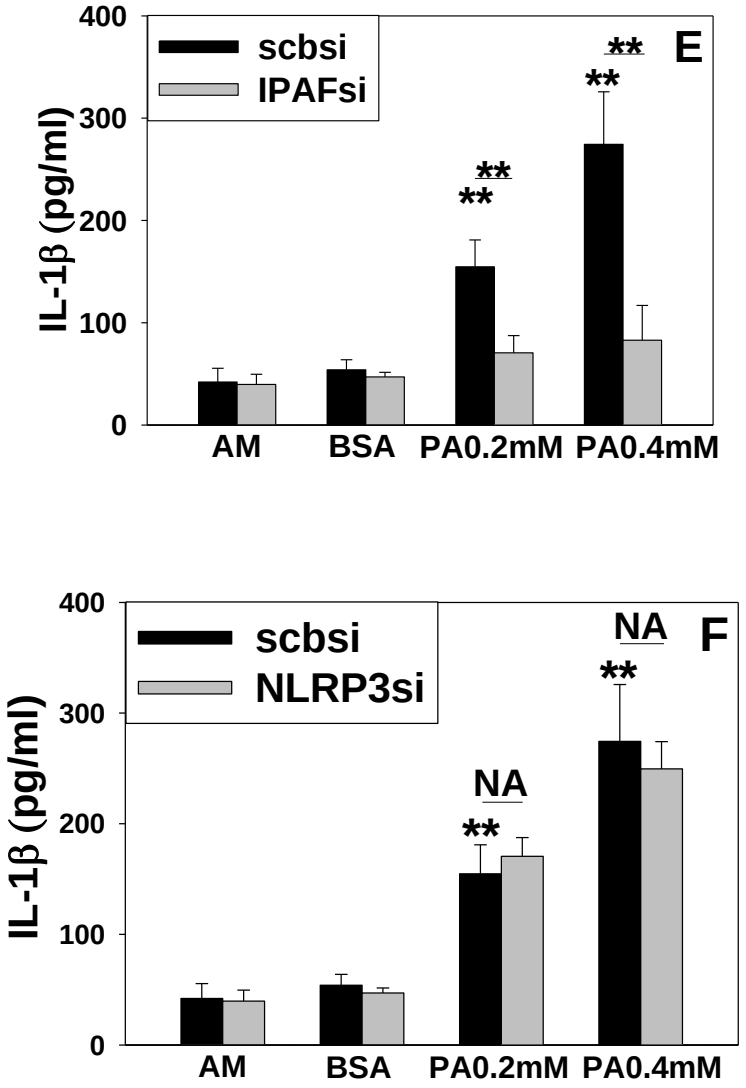


Figure 4.3 IPAF is involved in IL-1β maturation. (A) The mRNA expression of IPAF in astrocytes treated with BSA or PA for 6 or 12hr. The mRNA levels in astrocytes treated with PA and were normalized to astrocytes treated with BSA (n=3). (B, C, D) mRNA and protein levels of silenced IPAF. Astrocytes were cultured with scramble siRNA (scbsi) or siRNA targeting IPAF (IPAF siRNA) for 20hr. The IPAF level was detected by immunoblot and real-time PCR (n=3). (E, F) IL-1β expression levels. Wild-type astrocytes or astrocytes silenced with scramble

Figure 4.3 (cont'd) siRNA or IPAF siRNA or NLRP3 siRNA were cultured for 12hr with astrocyte medium (AM), BSA, or PA. ELISA was performed for IL-1β level and the expression levels of IL-1β were normalized to BSA treated wild-type or silenced astrocytes (n≥3). NA: not available. **: p<0.01. A line indicates comparison between the two bars connected by the line.



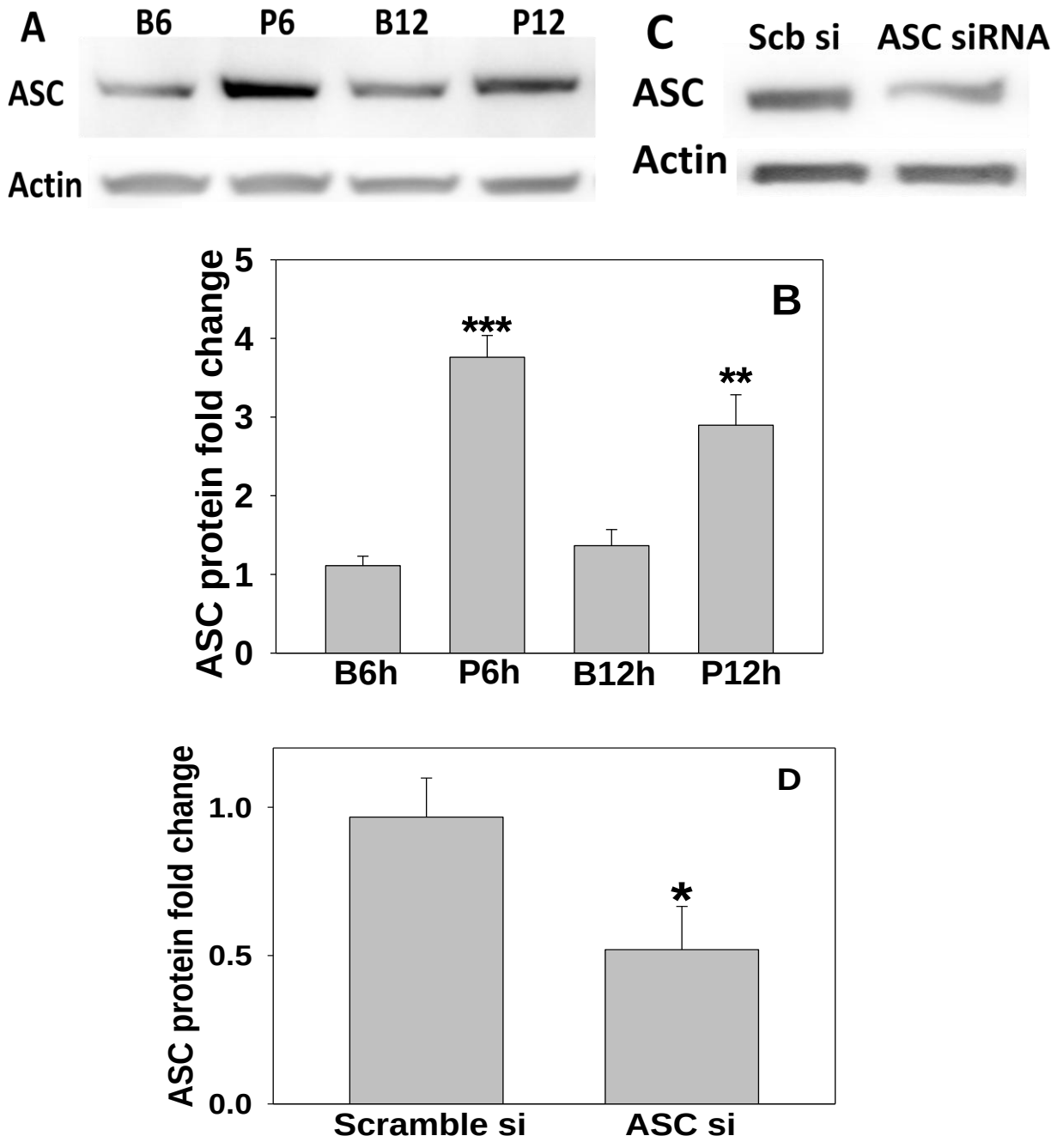
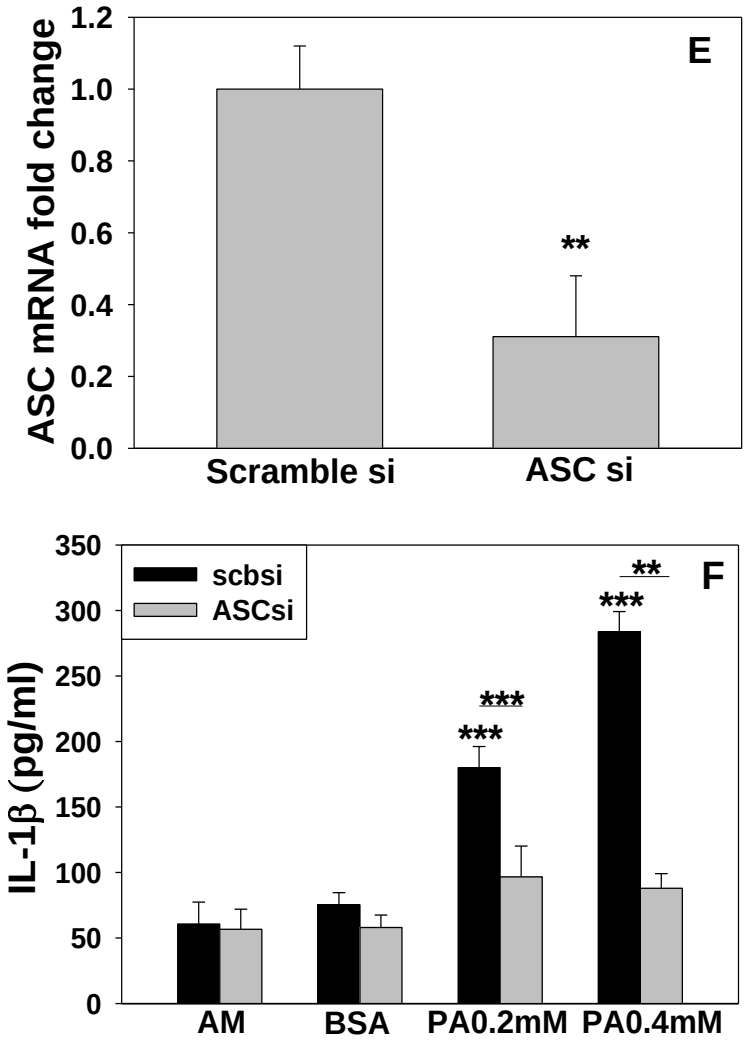


Figure 4.4 ASC is involved in IL-1β production. (A-E) ASC expression levels. (A, B) Representative western blot results of ASC protein levels in astrocytes treated with PA for the

Figure 4.4 (cont'd) indicated time. Quantification of ASC western blots (n=3). (C, D) Representative western blot results of ASC protein levels in astrocytes cultured with scramble siRNA or siRNA targeting ASC for 20hr. Quantification of ASC immunoblots. (E) mRNA level of ASC detected by real-time PCR (n=3). (F) IL-1β expression levels. Wild-type astrocytes or astrocytes silenced with scramble siRNA (scbsi) or ASC siRNA (ASCsi) were cultured with astrocyte medium (AM), BSA or PA for 12hr. IL-1β was detected by ELISA and was normalized to astrocytes treated with BSA (ctrl) (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. A line indicates comparison between the two bars connected by the line.



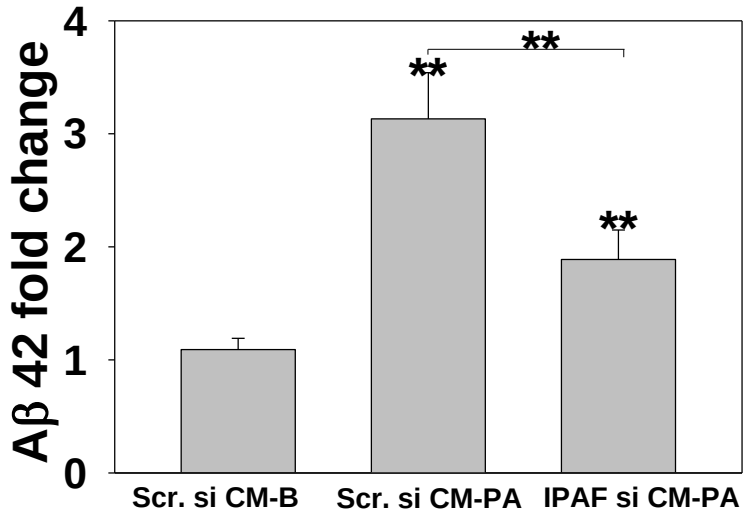


Figure 4.5 IPAF silencing in PA-treated astrocytes reduces neuronal Aβ42 levels. Primary astrocytes treated with scramble siRNA or IPAF siRNA for 20hr. After silencing, either BSA or 0.4 mM PA was used to culture the astrocytes for 12hr. Then the astrocyte conditioned medium: scramble siRNA-BSA (Scr.si CM-B), scramble siRNA-PA (Scr.si CM-PA), or IPAF siRNA-PA (IPAF si CM-PA) was transferred to treat primary rat neurons for 12hr. The supernatant was collected and the $A\beta_{42}$ levels were monitored by ELISA (n=3). **: $p<0.01$. A line indicates comparison between the two bars connected by the line.

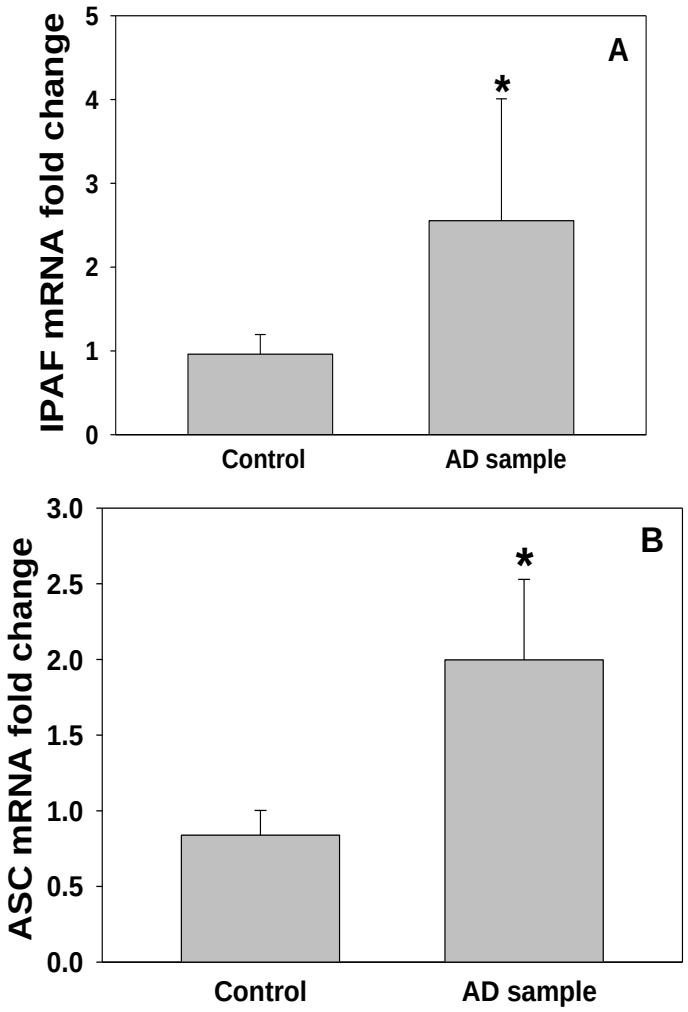
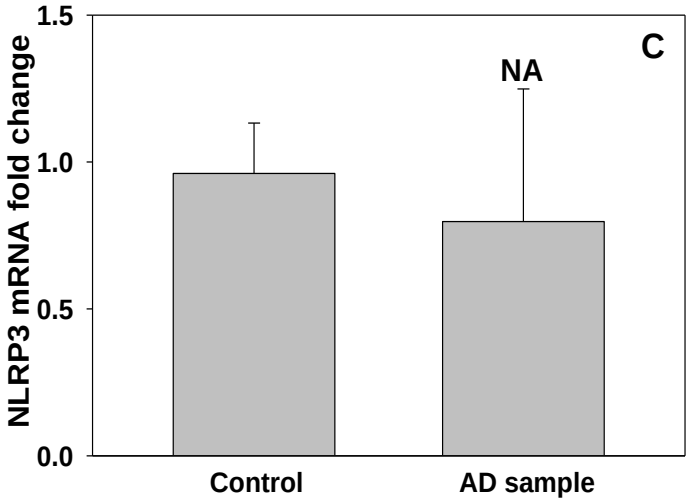
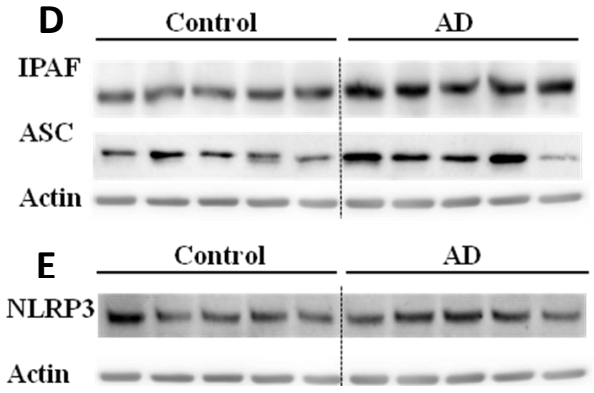
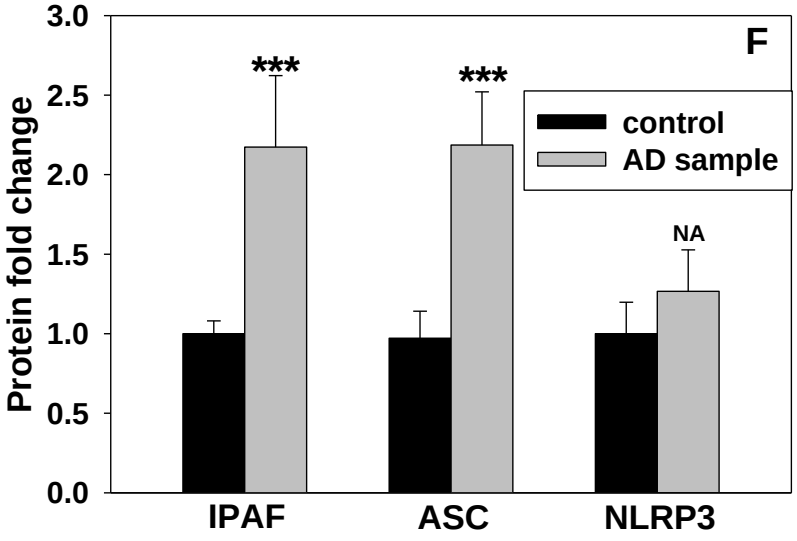


Figure 4.6 IPAF and ASC are upregulated in sporadic AD brain. The neocortical brain samples were analyzed for IPAF, ASC and NLRP3. (A-C) The mRNA levels of IPAF, ASC and NLRP3 in neocotrical brain samples. The mRNA levels were measured by real-time PCR. (D, E) The protein levels of IPAF, ASC and NLRP3 in neocotrical brain samples. The protein levels were detected by western blot and (F) shows the quantification of the western blots (n=5) NA: not available. *: $p<0.05$, ***:$p<0.001$.

Figure 4.6 (cont'd)







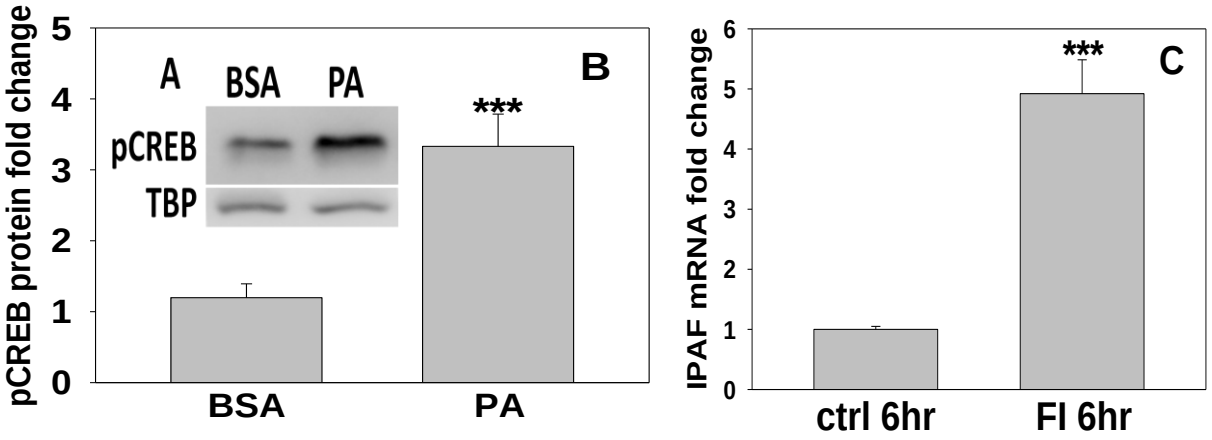
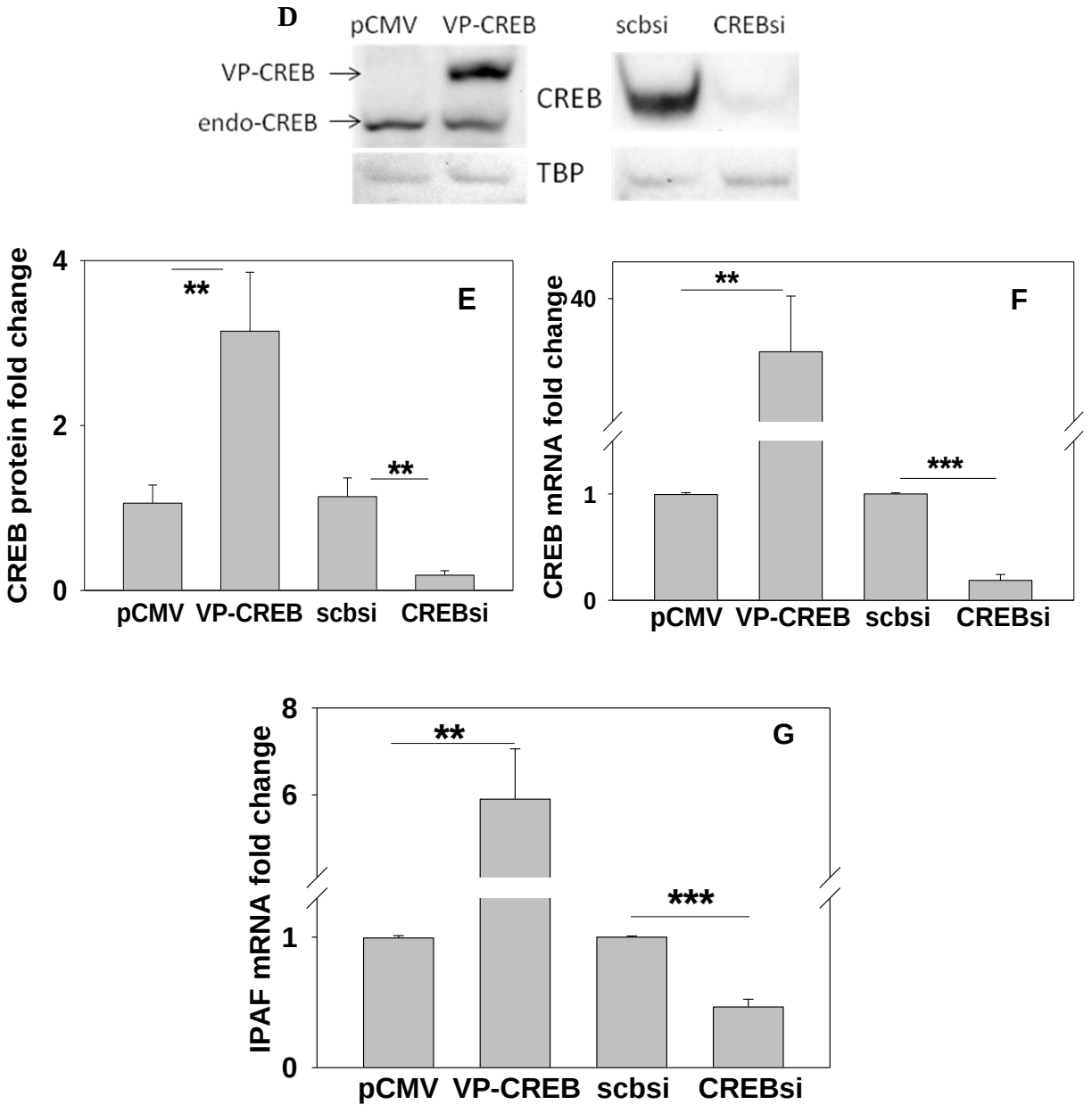


Figure 4.7 CREB regulates IPAF expression in astrocytes (A) pCREB protein levels in primary rat astrocytes upon treatment with PA for 12hr, and quantified in (B). TBP, TATA binding protein, was used as loading control. (C) Astrocytes incubated with forskolin and isobutylmethylxanthine (FI) for 6hr and mRNA of IPAF detected by real-time PCR. (D) Representative western blot results of silencing and overexpression of CREB in primary astrocytes. TBP was used as a loading control for the western blots. CREB overexpression was performed by transfection of the constitutively active VP16-CREB and silencing was achieved by CREB siRNA. pCMV and scramble siRNA were used as control. Endogenous CREB is denoted as endo-CREB. (E) Quantification of western blot. mRNA fold changes of (F) CREB and (G) IPAF levels in astrocytes overexpressing or silencing CREB. (H) Astrocytes treated with pCMV, VP16-CREB, scramble siRNA (scbsi), or CREB siRNA, followed by treatment with PA or BSA (ctrl) for 12hr. IPAF mRNA was detected by real-time PCR. (I) IL-1β expression level of astrocytes silenced with scramble siRNA or CREB siRNA. The cells were cultured for 12hr with astrocyte medium (AM), BSA or PA. ELISA was performed for IL-1β level and the expression levels were normalized to BSA treated wild-type or silenced astrocytes. (J) IL-1β expression level of astrocytes overexpressed with pCMV or VP16-CREB. The cells were

Figure 4.7 (cont'd) cultured for 12hr with astrocyte medium (AM), BSA or PA. ELISA was performed for IL-1β level and expression levels were normalized to BSA treated wild-type or silenced astrocytes. (n=3). **: p<0.01, ***: p<0.001. A line indicates comparison between the two bars connected by the line.



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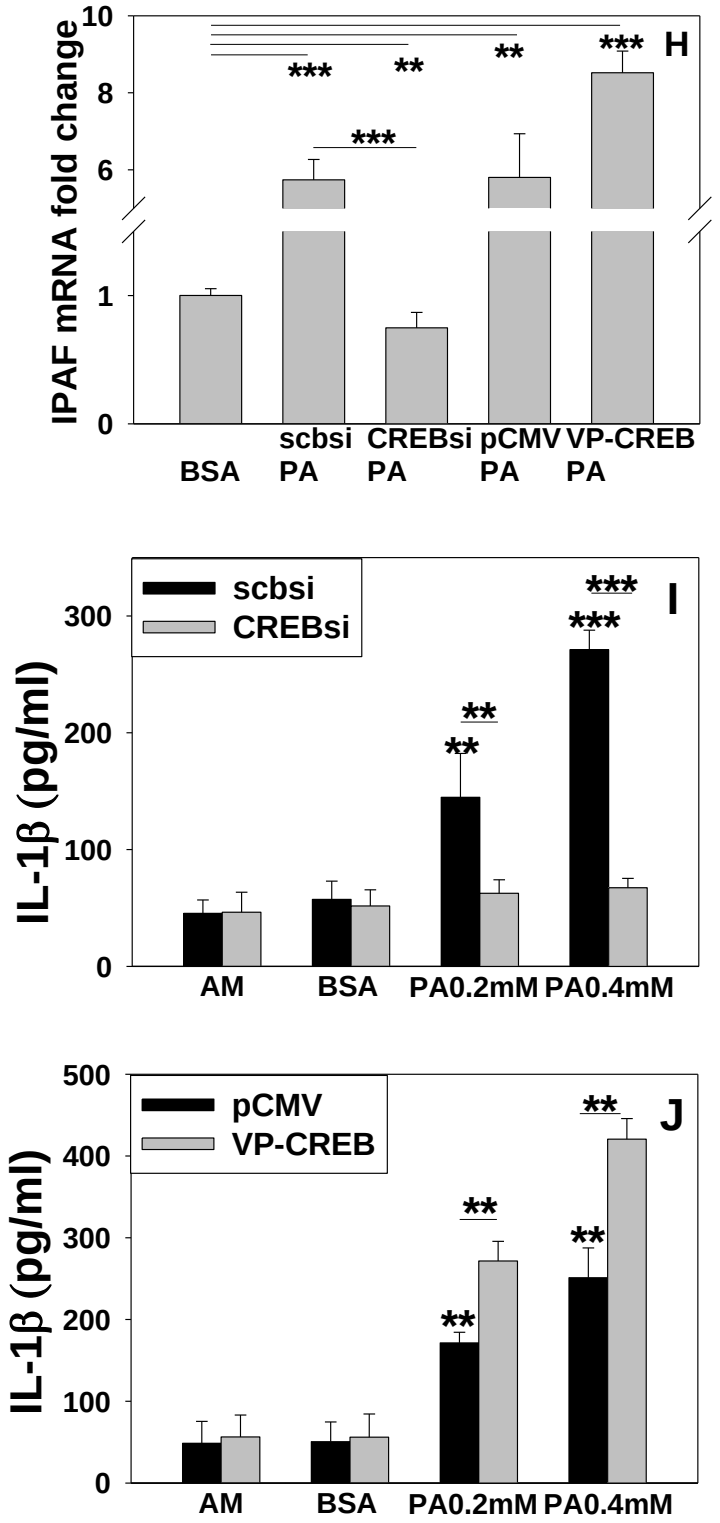


CHAPTER 5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 Concluding remarks

Alzheimer's disease is an irreversible degeneration of the brain causing disruptions in memory, cognition, personality, and other functions that eventually result in death from complete brain failure. Worldwide, 38 million people are suffering from AD currently, and this number of patients will double by 2030, and more than triple by 2050, barring the development of medical breakthroughs to prevent, slow or stop the disease [5, 184]. In the United States, AD is the sixth-leading cause of death, and 1 in 3 seniors dies due to AD or another dementia [184]. Without effective treatment, mostly due to the lack of knowledge on the etiology of AD, current approaches on the treatment as well as the research have focused on helping people maintain mental function, manage behavioral symptoms, and slow or delay the symptoms of the disease [5, 184]. Epidemiological finding suggested the relationship between diet and the risk of AD. It was reported that high fat diet increases the risk of AD, while diet rich in ω-3 and ω-6 polyunsaturated fats lower the risk of AD [136, 307-311]. These reasons provide, in part, the rationale for this work. Nevertheless, the role of elevated fatty acids in the pathogenesis of AD has been inconsistent [312, 313]. The conflicting results have been suggested to be related to several factors, such as age, the stage of the disease, other dietary components, cooking processes [314]. Environmental factors, i.e. fatty acids, have been strongly indicated in the pathogenesis of AD [17, 148-152, 154, 167]. Especially, palmitate, the most abundant fatty acid in the western diet, has been shown to trigger AD-like changes in primary neurons mediated by astrocytes [151, 152, 154, 184], but the mechanisms by which fatty acids induce amyloidogenesis in neurons are not well understood. Therefore the objective of this dissertation was to investigate the mechanisms by which environmental factor, namely palmitate, causes

amyloidogenesis and to provide insight into potential therapeutic targets. Based on our results, we confirm the relationship between fatty acid and pathogenesis of AD is mediated by proinflammatory molecules. Therefore, it is possible that the inconsistent results of dietary factors and AD observed in some epidemiological studies could be due to differences in inflammatory events or responses or intake of anti-inflammatory drugs by an individual. Diabetes and obesity have been suggested to be risk factors of AD [315]. In obese individuals, adipose tissue releases increased level of proinflammatory cytokines and other factors that are involved in the development of insulin resistance and type 2 diabetes mellitus [316]. It raises the possibility that the increased proinflammatory cytokines released by adipose tissue affect the development of AD.

Chapter 2 demonstrates that SPT in the astrocytes increased ceramide levels and was involved in the release of cytokines, TNFα and IL-1β, into the conditioned media of PA-treated astrocytes. The cytokines in the conditioned media mediated the activation of N-SMase and A-SMase in the neurons, to propagate the deleterious effects of palmitate. The activated N-SMase and A-SMase increased the ceramide levels in primary neurons, which resulted in the elevation of BACE1. This study suggests that tight regulation of ceramide production may be an important therapeutic approach for modulating BACE1 level [17]. In addition, it is likely that the upregulation in BACE1 level enhances the generation of Aβ level from neurons, which in turn could further increase intracellular ceramide levels in both the astrocytes and neurons, and reinforce the ceramide-Aβ-ceramide cascade [17].

In regards to the upregulation of both BACE1 and presenilin-1 in primary neurons, leading to amyloidogenesis, this research identified a signaling pathway that led to the transcriptional regulation of both BACE1 and γ-secretase. Specifically, conditioned media from

PA-treated astrocytes rapidly elevated the calcium level in neurons, which subsequently increased calpain activity, a calcium-dependent protease, resulting in the enhancement of p25/Cdk5 activity, and in turn the phosphorylation and activation of STAT3, a transcription factor. STAT3 transcriptionally regulated both BACE1 and presenilin-1 expression in neurons, suggesting that STAT3 could be a potential therapeutic target for AD (Chapter 3). This is a novel study that STAT3 regulates the expression of presenilin-1 expression in neurons cultured in CM-P.

In Chapter 2, an increase in the cytokine levels in the conditioned media of PA-treated astrocytes, in particular IL-1β, was found to be involved in the activation of a SMases-ceramide-BACE1 signaling pathway in primary neurons [17]. IL-1β was demonstrated to elevate calcium levels [266-268], and the elevated calcium is involved in the transcriptional regulation of both BACE1 and γ-secretase in neurons cultured in CM-P (Chapter 3). Chapter 2 showed that PA induces the release of IL-1β from astrocytes [17], and in Chapter 4 a possible molecular mechanism that initiates the generation of IL-1β in astrocytes cultured with PA is uncovered. This study investigated and identified that PA induced the activation of IPAF-ASC inflammasome in astrocytes causing the maturation of IL-1β, thereby implicating not only pathogen-related factors can activate IPAF-ASC inflammasome. The matured IL-1β can be secreted by four different mechanisms. They include exocytosis of IL-1β-containing secretory lysosomes, release of IL-1β from shed plasma membrane microvesicles, fusion of multivesicular bodies with the plasma membrane and subsequent release of IL-1β-containing exosomes, and export of IL-1β through the plasma membrane using specific membrane transporters [295]. Moreover, downregulating IPAF to decrease IL-1β secretion from the astrocytes reduced the generation of Aβ42 in the primary neurons treated with CM-P and IPAF was found to be

potentially regulated by CREB. In addition, the expression levels of IPAF and ASC were found significantly elevated in a subgroup of sporadic AD patients, suggesting an involvement of IPAF-ASC inflammasome in the inflammatory response associated with AD, and a possibility that IPAF-ASC could be a potential therapeutic target for AD (Chapter 4). This is the first time that IPAF inflammasome has been identified to be expressed in primary astrocytes, and activated by non-pathogen associated factors.

In summary, this chapter provides insights into the mechanism of how dietary fats could contribute to the development of AD, and reveals the mechanism by which PA induces amyloidogenesis in primary neurons mediated by astrocytes. The study demonstrated two novel signaling pathways that are activated in primary neurons cultured in CM-P, and identified the molecular mechanism by which IL-1β matures in astrocytes treated with PA. Through this study, several potential therapeutic targets for AD are highlighted. An overview of the mechanism of amyloidogensis in neurons cultured in CM-P is summarized in the schematic below (Figure 5.1).

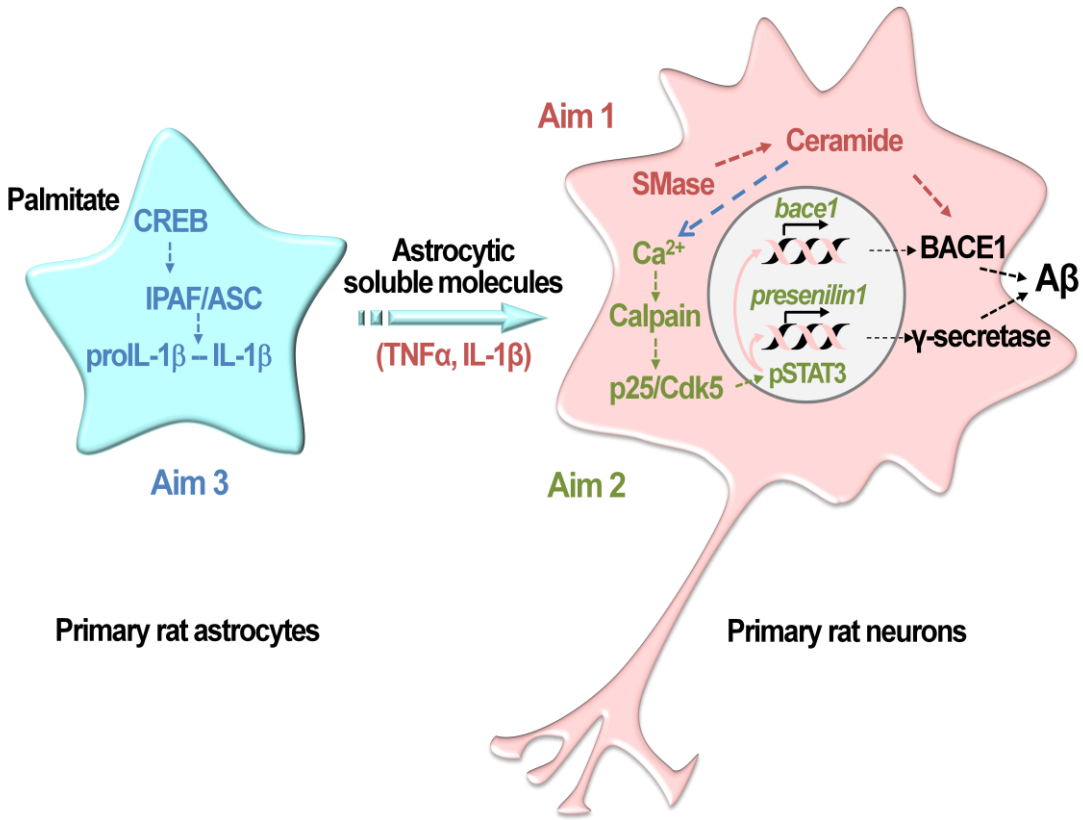


Figure 5.1 Schematic representation of the mechanisms of palmitate induced amyloidogenesis in AD mediated by astrocytes. Palmitate activates astrocytes to induce the maturation of IL-1β through the activation of IPAF-ASC inflammasome and release cytokines (i.e. IL-1β) into the conditioned media. This could be due to a possible regulation of IPAF by CREB, given that CREB can be activated by PA [85]. The released cytokines in the conditioned media, i.e. TNFα and IL-1β, activate SMases and in turn upregulates ceramide levels, leading to an increase in BACE1 level in the neurons. Concomitantly, the CM-P elevates calcium levels in the neurons, triggered by TNFα and IL-1β [268] in the CM-P, or by the increased ceramide levels [267] in the neurons. The elevated intracellular calcium level upregulates calpain activity, and in turn causes STAT3 phosphorylation through cdk5 activation [317]. Phosphorylated STAT3 increases the expression of both BACE1 and presenilin-1 to elevate Aβ production in the neurons [317].

5.2 Future direction

5.2.1 Potential therapeutic targets and their *in vivo* studies

In the present study, SMases in the primary neurons and SPT in the astrocytes led to ceramide generation which regulated Aβ biogenesis in our cell-based experiments. In an AD transgenic mice study, SPT has been confirmed to be positively correlated with Aβ and tau hyperphosphorylation levels and inhibiting SPT has been suggested as a potential therapeutic target [166, 292]. Apart from SPT, SMases also are involved in ceramide generation, and in the upregulation of BACE1 level in neurons (Chapter 2) [17]. Therefore, the risk of SMases associated with AD should be further confirmed in AD transgenic animal models using pharmacological inhibitors of ASMase and NSMase in AD, which remains open to investigation.

The signaling pathway, calcium-calpain-Cdk65/p25-pSTAT3, was identified in this study to regulate the mRNA of both BACE1 and presenilin-1 in primary neurons mediated by CM-P. Mounting reports suggest the role of calcium, calpain and p25 as well as STAT3 in the development of AD [34, 189, 193-195, 229, 263]. In light of these facts, the role of each component on this signaling cascade in the development of AD require further study in animal models, possibly through pharmacological inhibition of each target in AD transgenic mice to assess their potential as therapeutic targets.

5.2.2 *In vivo* study of IPAF inflammasome

This study identified that the IPAF-ASC inflammasome regulates maturation of IL-1β in primary rat astrocytes cultured with PA, and the release of IL-1β from astrocytes into the CM-P is involved in the elevation of BACE1 and Aβ generation in primary rat neurons. Moreover, the upregulation of IPAF and ASC was found in sporadic AD patients' brains, suggesting their potential role in the development of AD. Increased caspase-1 expression, activated by

inflammasome, was observed in human mild cognitively impaired patients, in AD brains as well as in AD transgenic mouse [83]. However, it remains unknown whether IPAF activation contributes to AD *in vivo*. AD transgenic mice carrying Ipaf (-/-) or Asc (-/-) can be used in *in vivo* experiments to monitor the memory loss and other characters associated with AD, such as Aβ and hyperphosphorylation of tau. These results would show an important role of IPAF/ASC in the pathogenesis of AD, and would further suggest that inhibition of IPAF inflammasome could be a novel therapeutic intervention for the disease.

5.2.3 Mechanism of inducing hyperphosphorylation of tau in primary neurons cultured in CM-P

Neurofibrillary tangles (NFTs), accumulated in the neuron cell bodies and dendrites in human AD brains, are considered another hallmark of AD [318, 319]. The hyperphosphorylation of tau protein accumulates as insoluble fibrils to form paired-helical filaments that coalesce into NFTs [320]. Two major known functions of the tau protein are to promote polymerization and to maintain the structure of microtubules by the microtubule-binding domain of tau [321]. Hyperphosphorylated tau, caused by an imbalanced action between kinases and phosphatases, neither binds to microtubules nor promotes microtubule assembly, and further inhibits the assembly of microtubules by sequestering normal tau protein and depolymerizing microtubules [322-324]. The toxicity of hyperphosphorylated tau in AD appears to be solely due to its abnormal hyperphosphorylation since dephosphorylation of tau converts it into a normal-like protein [322-325]. Tau is a substrate for several protein kinases, in particular cyclic AMP-dependent protein kinase (PKA), *glycogen synthase kinase* 3β (GSK3β), dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dryk1A) [326-329], and phosphatases, including phosphoseryl/phosphothreonyl protein phosphatase-2A (PP-2A) [330-333]. However, the mechanism of abnormal phosphorylation is unclear thus far. The hyperphosphorylation of tau

was also observed in primary neurons exposed to CM-P, although PA does not induce the hyperphoshporylation of tau in primary neurons directly [154]. However, the molecular mechanism of the hyperphosphorylation of tau induced by the CM-P has not yet been studied. Several possibilities exist regarding this. The main possibility is the potential regulation of hyperphosphorylation of tau by an imbalance between the kinases and phosphotases. The upregulation of tau expression may be also correlated with hyperphosphorylation, thus raising the question of the regulation of tau expression. Therefore a promoter analysis was performed and the results suggest that Nfatc2, Foxq1, Foxd3, Zfp423, and HNF1A could be putative transcription factors that regulate tau. Overexpression or silencing of the transcription factor to, respectively, enhance or reduce tau expression could be tested to determine which transcription factor is involved in regulating tau in primary neurons. ChIP and EMSA assays could be used to verify that the transcription factor directly regulates tau expression. Understanding the mechanism of tau hyperphosphorylation will provide further insight into the etiology of AD in regards to high fat diet, and could establish other therapeutic targets and biomarkers for AD.

APPENDICES

APPENDICES

APPENDIX A. SUPPLEMENTARY METHODS AND FIGURES FOR CHAPTER 2

Supplementary Materials and Methods

Quantitative real time polymerase chain reaction

mRNA was extracted using the RNA extraction kit (Qiagen, Valencia, CA, USA), then mRNA was reverse transcribed into cDNA using the cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The following primer sets (Operon, Huntsville, AL, USA) were used for PCR: ATase1 (5'-gagtgatcgtggctcaggtt-3', 5'-ctggagcacagttctgacca-3'), ATase2 (5'-ctgctcccatttttgtggtt-3', 5'-agagctgcaactgcttcctc-3'), actin (5'-ctcttccagccttccttcct-3', 5'-aatgcctgggtacatggtg-3'). Amplifications of the cDNA templates were detected by SYBR Green Supermix (Bio-Rad) using Real-Time PCR Detection System (Bio-Rad). The cycle threshold values were determined by the MyIQ software.

Western blot

Whole cell extracts lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer were assayed for protein concentrations by Bradford assay (Bio-Rad). 15-30 μg protein samples were separated by 10% Tris–HCl gel and transferred to nitrocellulose membrane. Membranes were then blocked with 5% milk or 5% BSA in 0.05% Tween 20-TBS (Tris buffered saline) (USB corporation, Fremont, CA, USA) for 1 hr and incubated with primary antibodies, PKR and p-PKR (Novus, Littleton, CO, USA) overnight at 4℃. Next day, anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific, Asheville, NC, USA) was added and the blots were incubated for 1 hr at room temperature. The blots were washed three times with 0.05%

Tween 20-TBS, and then visualized by SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

Supplementary Figures

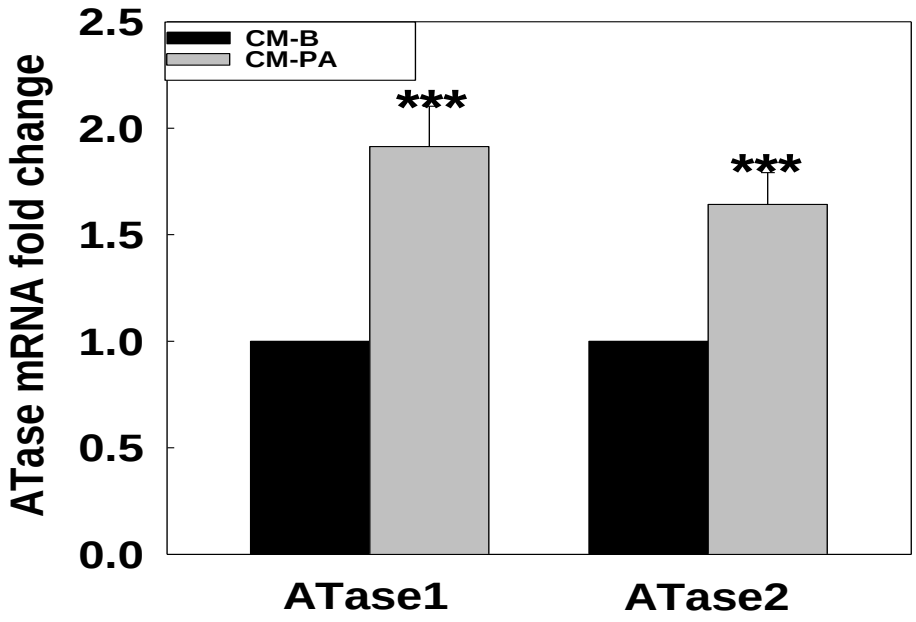


Figure A.1 mRNA levels of ATase1 and ATase2 in the neurons upon treatment with astrocyte-conditioned media. Primary rat neurons were incubated with CM-B or CM-PA for 12 hrs and mRNA levels were detected by real time PCR (n=3). Statistical analysis was performed using an unpaired, two tail student T-test. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

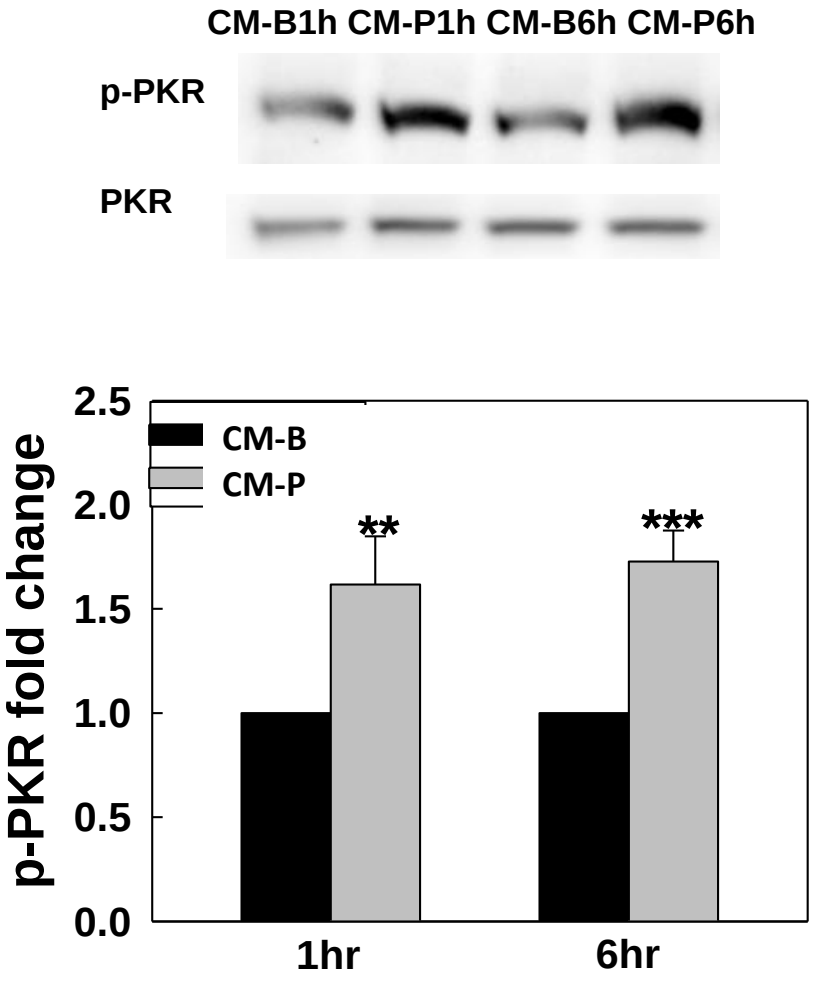


Figure A. 2 Effect of CM-P on phospho-PKR (p-PKR) level in the neurons. Primary rat neurons were incubated with CM-B or CM-PA for 1 or 6 hr. Left panel shows a representative western blot of p-PKR. Right panel shows the quantification of p-PKR. Statistical analysis was performed using an unpaired, two tail student T-test. *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

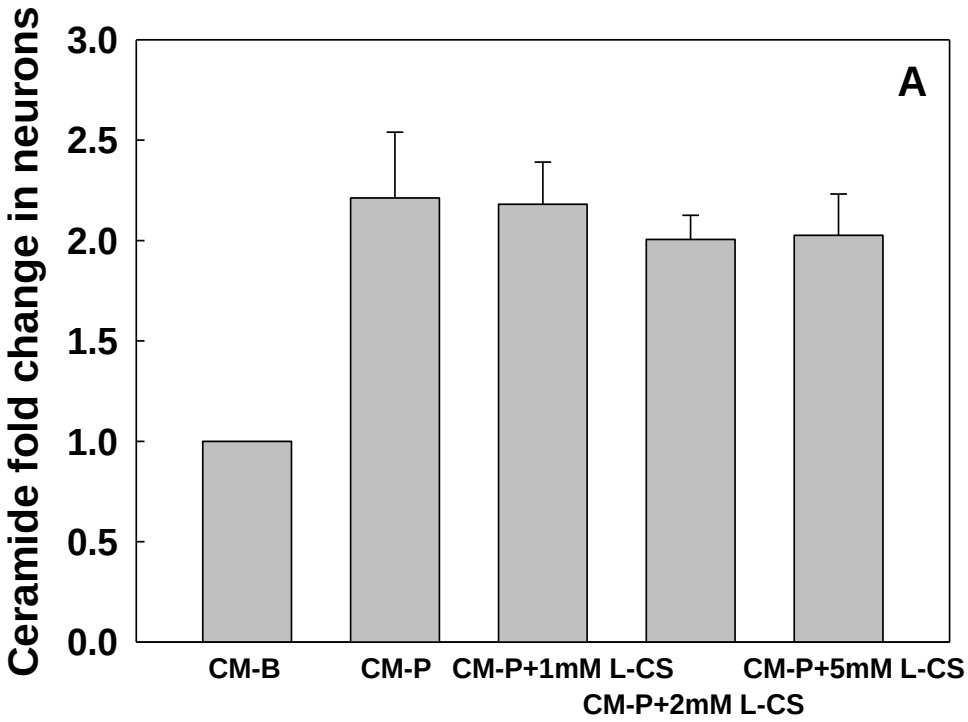
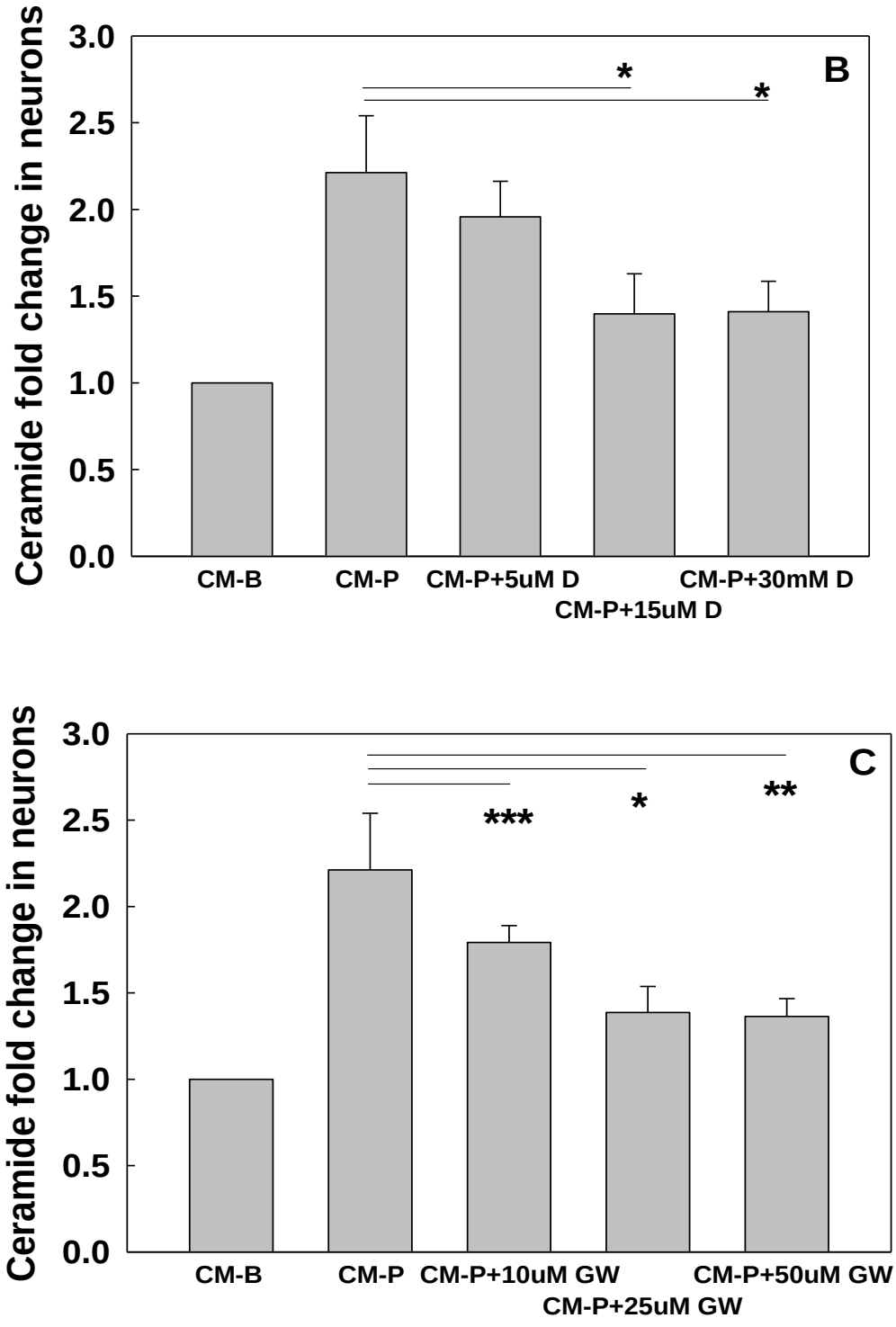


Figure A. 3 Changes in ceramide levels in primary rat neurons. (A) CM-B, CM-P, CM-P plus 1mM, 2mM, or 5mM L-CS were used to treat primary rat neurons for 12 hrs. (B) CM-B, CM-P, CM-P plus 5 μM, 15 μM, or 30 μM desipramine (A-SMase inhibitor) was used to treat primary rat neurons for 12 hrs. (C) CM-B, CM-P, CM-P plus 10 μM, 25 μM, or 50 μM GW4869 (N-SMase inhibitor) were used to treat primary rat neurons for 12 hrs. All ceramide levels were detected by LC/MS/MS and normalized to CM-B (ctrl) (n=3). Statistical analysis was performed using an unpaired, two tail student T-test. *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

Figure A. 3 (con'd)



APPENDIX B. SUPPLEMENTARY METHODS AND FIGURES FOR CHAPTER 3

Supplementary Figures

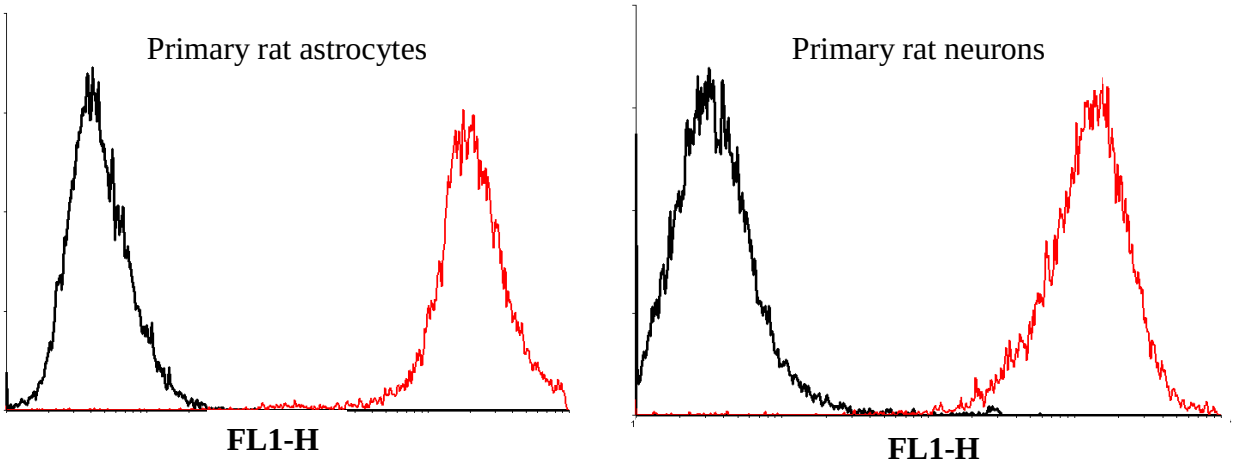


Figure B.1 Purity of the primary rat astrocytes and neurons. Cells were stained with astrocytic and neuronal markers, GFAP and βIII Tubulin, respectively. The control (black line) is the unstained cells. Cytofluorometric analysis shows that the purities of the astrocyte and neuronal cell isolations are above 90%.

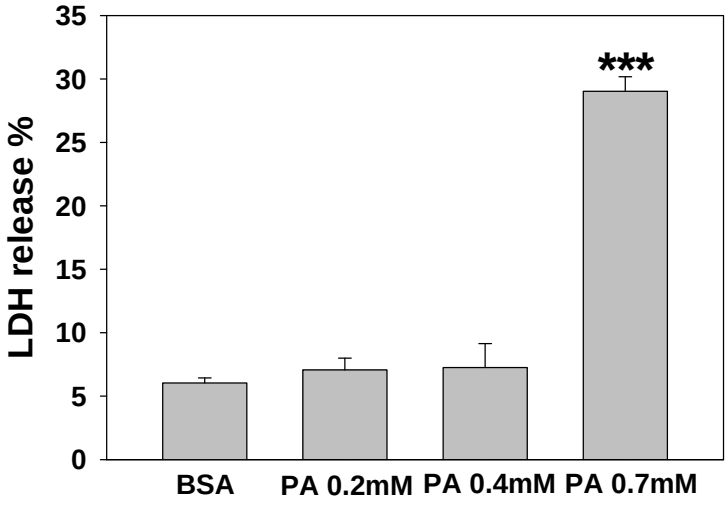


Figure B.2 LDH release from astrocytes. Primary astrocytes were treated with BSA (ctrl) and different concentrations of PA as indicated for 12 hr (n=3). ***: $p<0.001$.

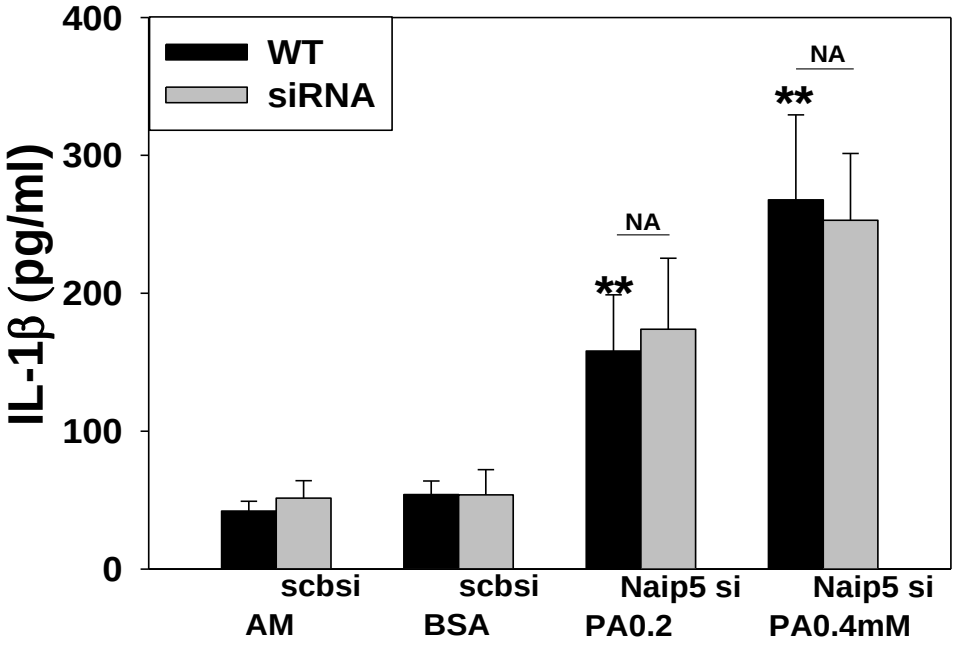


Figure B.3 IL-1β expression level. Wild-type astrocytes or astrocytes silenced with scramble siRNA (scbsi) or Naip5 siRNA (Naip5 si) were cultured for 12hr with astrocyte medium (AM), BSA, or PA. ELISA was performed and the expression levels of IL-1β were normalized to BSA treated wild-type or silenced astrocytes ($n \geq 3$). **: $p < 0.01$. A line indicates comparison between the two bars connected by the line.

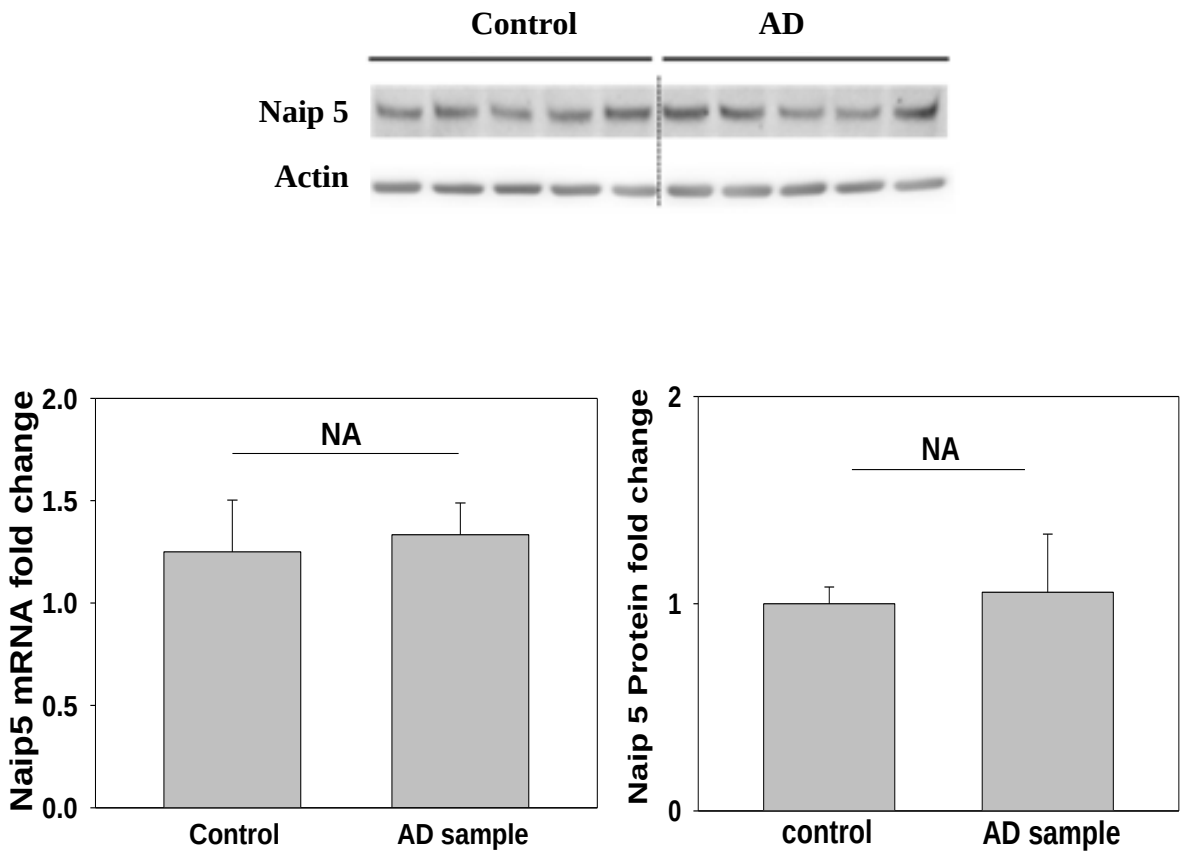


Figure B.4 Naip 5 expression levels in human samples. Human neocortical brain samples (AD: Alzheimer's disease patients, control: normal healthy aged matched subjects) were analyzed for Naip5 by western blot and actin was used as loading control. The mRNA level of Naip5 was analyzed by real-time PCR (n=5) na: no significant change between two bars connected by the line.

A JASPAR Matrix

```
CTTTACCTGGGAGAAATGTGAACAGACATCCAGCCACTCACTCCACAAGT      50
CTCTCTAGTTTGTTGAACCAGTGGGCCTGGTGGTCTTACTTGTCAGAGTC     100
CAGATAAGTGGTAACCCAAGGTACCCACATCTCTGCCAAGCCCTGTATCA     150
GCGTGGATGACAATTCCATATATATATATATATATATATATATATATATA      200
TGGAATTCATATATATATATGGAATTCATATATATATATATATATATATA     250
TATATATATGAATGGCTGGGAGAGAGACATAGAAAGAGAGTAGCTGGACT     300
CTCAGGTAGGACCTAATGATCCCTTCCCACCCCCCACCACTCCTCCTTCT     350
TTTTTTTTTTTTTTTTCTTTTTTTTCGGAGCTGGGGACCGAACCCAGGGC     400
CTTGCGCTCGCTAGGCAAGCGCTCTACCGCTGAGCTAAATCCCCAACCCC     450
GCCACTCCTCCTTCTAAAGGAGAATGTAGATAAGATGGTAATAGCTCTTA     500
TGCTTGGAGGATAGAGCCATTCATTACTTCAAAACCCTTCACTCTCCCTT     550
TTCCTCCTTCCTTCTCTCCCTCCTGCTCCCCATCCTCTTCCTCCTCCTCC     600
TCCCACTCTCCTTCCTCCTTTCTTCTGGTCTCATCATGTAGCCCTGGTTA     650
TCCTAGAACTCACAGAGATCTTGGCTGCCTCTGCCTCCTGAGTCTGGGAT     700
TAAAGACGGGCGCCACCACACCAGGCACTCACTTGAGGCTCAAATCAGTG     750
GACAAAATTACCCACCTTGCCTCATCACCAAAGCAAAACCATCTTGCTCA     800
CGGACTGAAGTTATGGCTGTAGAGTTTAACAGAGCACTTCTTGAGGGCCT     850
GAGCATAAAGCATCCGATATCCCCATGGCTTGAGATGAGCCAGGGGGAGG     900
GAAATATGCAGCTCTGATTCACCGGTGACAGAAATGTTTCTTTCATGTAT     950
AGTAAAATAAAGAAATGAACTAATAAACAAATATGTACTCCATCTCTTCA    1000
GTGAACTTTATAAAGGAAAACAGCCAAGCCCTTATTCAGAGG            1042

1 [152 .. 163] GTGGATGACAAT (score=2.39)
2 [628 .. 639] TCTCATCATGTA (score=0.14)
3 [792 .. 803] CTTGCTCACGGA (score=0.73)
4 [800 .. 811] CGGACTGAAGTT (score=1.77)
5 [836 .. 847] CTTCTTGAGGGC (score=0.55)
```

Figure B.5 Transcription factors binding sites on promoter. (A) CREB binding sites on the promoter of IPAF. (B) NFκB binding sites on the promoter of ASC. We extracted the promoter sequence of IPAF and ASC from the UCSC Genome Browser database (http://genome.ucsc.edu/), and searched for CREB and NFκB binding sites by comparing the promoter sequences with PWM (position weight matrix). Several putative CREB and NFκB binding sites were predicted on the promoter of IPAF and ASC, respectively, using the JASPAR (http://jaspar.genereg.net/) and the TRANSFAC (www.generegulation.com) databases.

Figure B.5 (cont'd)

A TRANSFAC Matrix

```
CTTTACCTGGGAGAAATGTGAACAGACATCCAGCCACTCACTCCACAAGT      50
CTCTCTAGTTTGTTGAACCAGTGGGCCTGGTGGTCTTACTTGTCAGAGTC     100
CAGATAAGTGGTAACCCAAGGTACCCACATCTCTGCCAAGCCCTGTATCA     150
GCGTGGATGACAATTCCATATATATATATATATATATATATATATATATA     200
TGGAATTCATATATATATATGGAATTCATATATATATATATATATATATA     250
TATATATATGAATGGCTGGGAGAGAGACATAGAAAGAGAGTAGCTGGACT     300
CTCAGGTAGGACCTAATGATCCCTTCCCACCCCCCACCACTCCTCCTTCT     350
TTTTTTTTTTTTTTTTCTTTTTTTTCGGAGCTGGGGACCGAACCCAGGGC     400
CTTGCGCTCGCTAGGCAAGCGCTCTACCGCTGAGCTAAATCCCCAACCCC     450
GCCACTCCTCCTTCTAAAGGAGAATGTAGATAAGATGGTAATAGCTCTTA     500
TGCTTGGAGGATAGAGCCATTCATTACTTCAAAACCCTTCACTCTCCCTT     550
TTCCTCCTTCCTTCTCTCCCTCCTGCTCCCCATCCTCTTCCTCCTCCTCC     600
TCCCACTCTCCTTCCTCCTTTCTTCTGGTCTCATCATGTAGCCCTGGTTA     650
TCCTAGAACTCACAGAGATCTTGGCTGCCTCTGCCTCCTGAGTCTGGGAT     700
TAAAGACGGGCGCCACCACACCAGGCACTCACTTGAGGCTCAAATCAGTG     750
GACAAAATTACCCACCTTGCCTCATCACCAAAGCAAAACCATCTTGCTCA     800
CGGACTGAAGTTATGGCTGTAGAGTTTAACAGAGCACTTCTTGAGGGCCT     850
GAGCATAAAGCATCCGATATCCCCATGGCTTGAGATGAGCCAGGGGGAGG     900
GAAATATGCAGCTCTGATTCACCGGTGACAGAAATGTTTCTTTCATGTAT     950
AGTAAAATAAAGAAATGAACTAATAAACAAATATGTACTCCATCTCTTCA    1000
GTGAACTTTATAAAGGAAAACAGCCAAGCCCTTATTCAGAGG            1042

1 [805 .. 812] TGAAGTTA (score=4.83)
2 [880 .. 887] TGAGATGA (score=0.11)
```

Figure B.5 (cont'd)

B JASPAR Matrix

```
AAACTGAGAGTCCTGTGTGGCTGGAGCTTGAAGATAAAGGTAAACTGGTA      50
GTATAGTATAAAACAAGACTGAATCGATCAAAACTGGGGTGTACCAGTAG     100
ACCCTTCCCAGCCATGTTTAAAGCAGGTTATGAATTCTTAGAGCAGGCTC     150
CAAGGTCATCCTAACCAGCCCGAGTAAGGAGTAGCTAGCTCTCCACTCAG     200
GTCTCTCTTCACTTTCGTGTTTTATCTAGTGGTGATTGTCATCACACTAC     250
AAAATATTTAACTACTGGGGAACTTGGTAGGATTTCAAGAAGCCTTAGCC     300
CTTCCAACCCAACCATCATGACCCCAGTGAGACAGAGTGACGAAAATGTT     350
TAATAAATGTAGCTGAAAGTAAGGTGAAGACATATAGTCACACTCTTTTC     400
ATCCAAGTCTGTGGAATTCAGGGCGCTTAGTGTTCTAGCTGTTAAAACTC     450
TAATATCCAACGGCATGTGAGAGGATCCTAAGATCTGGGGAAACGAGATT     500
CAGGGCATCTAGATTTCCAAGATTCTAAGCTTCTATGCTCCAAGAAACAA     550
CAGAAACAGCAACAGCTGGTTTACAAAAAAGCTCTATTTGAGCCTCTCAG     600
AAACTCAGGACCTGCCGGAGTAACAATTTAGCTCCACCCTCTGTCCCCAC     650
CCGACATCCTCTGCCGACCCAACTCCACTTTCGGCAGCCTAGAAAAAAAG     700
CCAAATTTGAAAGTCTTGGGGCGGAAACCAAAACTAGCTCGCTGGGGAAA     750
GAACAGGCGCTGTAAGTGGAAGAGGGTGGAGTCCTGGCACGCCCATTGGC     800
CTAAGCAGATGACTTCCTGGTCTTGAAGGGCTGGCAGCAGGCAGGCTGCA     850
GCAGGCGAGCTGCAGCAGGCGAGCTGCAGCAGGCGAGCTGCAGCAGGCGA     900
GCTGCAGCAGGCGAGCTGCAGCAGGCGAGCTGCAGCAGGCGAGCTGCAGC     950
AGGCTAGCAGCGGCAAGAGTAAAAGGTGACCGCGGCTGCCCACCTGAGCC    1000

1 [85 .. 94] GGGGTGTACC (score=5.02)
2 [744 .. 753] GGGAAAGAAC (score=2.32)
3 [775 .. 784] GTGGAGTCCT (score=0.55)
4 [981 .. 990] GCGGCTGCCC (score=2.39)
```

Figure B.5 (cont'd)

B TRANSFAC Matrix

```
AAACTGAGAGTCCTGTGTGGCTGGAGCTTGAAGATAAAGGTAAACTGGTA      50
GTATAGTATAAAACAAGACTGAATCGATCAAAACTGGGGTGTACCAGTAG     100
ACCCTTCCCAGCCATGTTTAAAGCAGGTTATGAATTCTTAGAGCAGGCTC     150
CAAGGTCATCCTAACCAGCCCGAGTAAGGAGTAGCTAGCTCTCCACTCAG     200
GTCTCTCTTCACTTTCGTGTTTTATCTAGTGGTGATTGTCATCACACTAC     250
AAAATATTTAACTACTGGGGAACTTGGTAGGATTTCAAGAAGCCTTAGCC     300
CTTCCAACCCAACCATCATGACCCCAGTGAGACAGAGTGACGAAAATGTT     350
TAATAAATGTAGCTGAAAGTAAGGTGAAGACATATAGTCACACTCTTTTC     400
ATCCAAGTCTGTGGAATTCAGGGCGCTTAGTGTTCTAGCTGTTAAAACTC     450
TAATATCCAACGGCATGTGAGAGGATCCTAAGATCTGGGGAAACGAGATT     500
CAGGGCATCTAGATTTCCAAGATTCTAAGCTTCTATGCTCCAAGAAACAA     550
CAGAAACAGCAACAGCTGGTTTACAAAAAAGCTCTATTTGAGCCTCTCAG     600
AAACTCAGGACCTGCCGGAGTAACAATTTAGCTCCACCCTCTGTCCCCAC     650
CCGACATCCTCTGCCGACCCAACTCCACTTTCGGCAGCCTAGAAAAAAAG     700
CCAAATTTGAAAGTCTTGGGGCGCGAAACCAAAACTAGCTCGCTGGGGAAA     750
GAACAGGCGCTGTAAGTGGAAGAGGGTGGAGTCCTGGCACGCCCATTGGC     800
CTAAGCAGATGACTTCCTGGTCTTGAAGGGCTGGCAGCAGGCAGGCTGCA     850
GCAGGCGAGCTGCAGCAGGCGAGCTGCAGCAGGCGAGCTGCAGCAGGCGA     900
GCTGCAGCAGGCGAGCTGCAGCAGGCGAGCTGCAGCAGGCGAGCTGCAGC     950
AGGCTAGCAGCGGCAAGAGTAAAAGGTGACCGCGGCTGCCCACCTGAGCC    1000

1 [3 .. 13] CTGAGAGTCCT (score=1.52)
2 [84 .. 94] TGGGGTGTACC (score=0.70)
3 [410 .. 420] GTGGAATTCAG (score=0.81)
4 [650 .. 660] CCGACATCCTC (score=0.50)
5 [719 .. 729] GGCGGAAACCA (score=0.43)
6 [720 .. 730] GCGGAAACCAA (score=0.57)
7 [743 .. 753] GGGGAAAGAAC (score=0.05)
8 [744 .. 754] GGGAAAGAACA (score=2.62)
9 [774 .. 784] GGTGGAGTCCT (score=4.06)
10 [784 .. 794] TGGCACGCCCA (score=1.18)
```

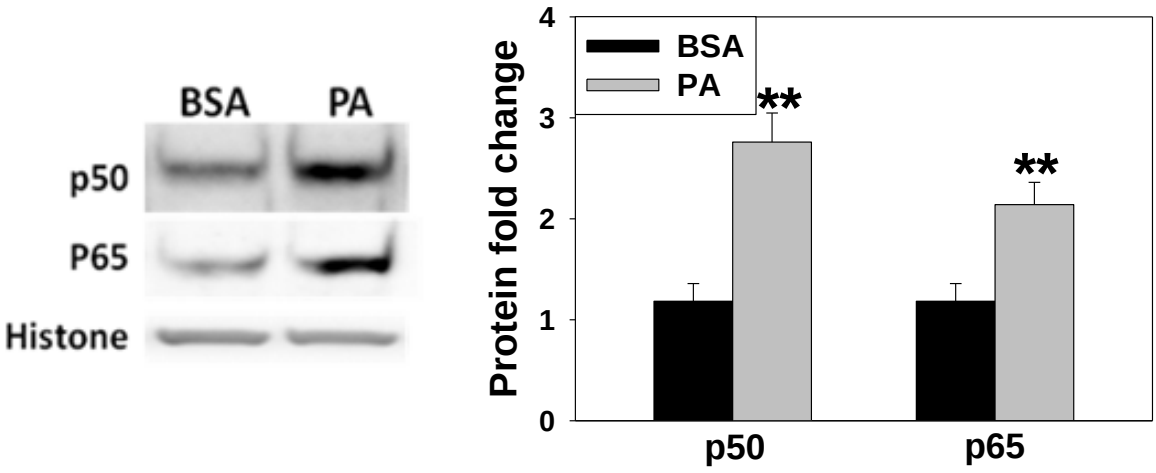


Figure B. 6 NFκB levels in nucleus of primary astrocyte. Primary astrocytes were treated with BSA (ctrl) and 0.4mM PA for 12 hr. Nuclear extraction was performed and p50 and p65 in nucleus were measured with western blot and histone was used as loading control (n=3). **: $p<0.01$.

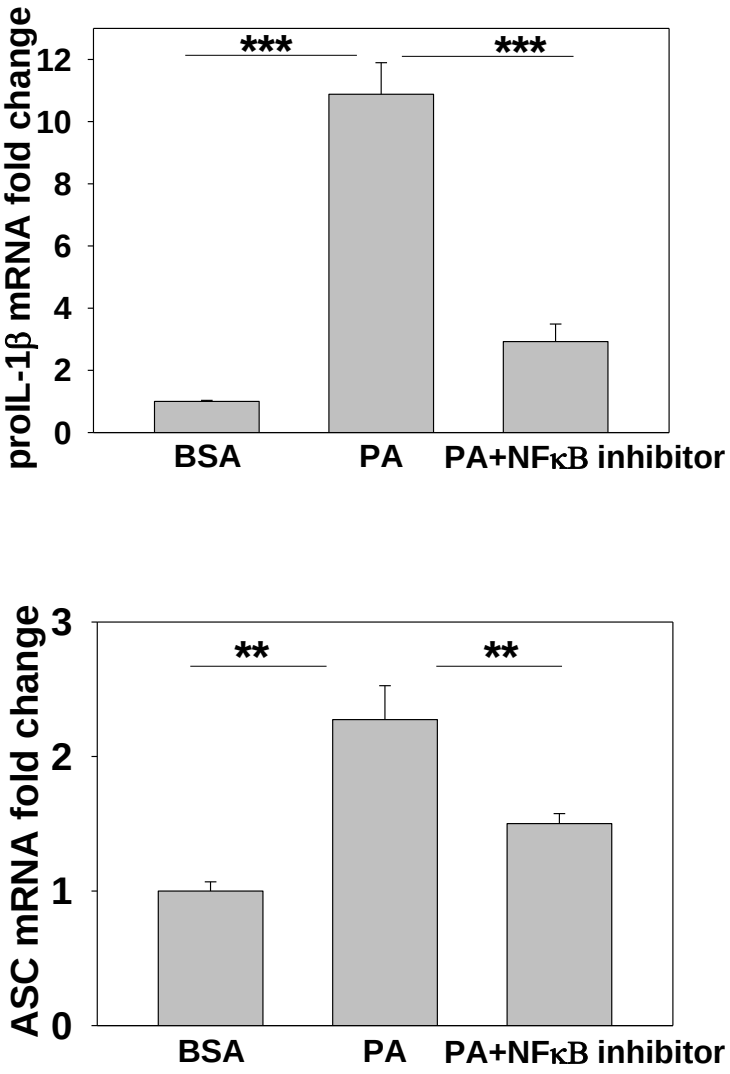


Figure B.7 Pro-IL-1β and ASC mRNA levels in primary astrocytes. Primary astrocytes were cultured in BSA (ctrl), 0.4mM PA, or 0.4mM PA plus NFκB inhibitor for 12hr. The inhibitor for NFκB (EMD Millipore, Billerica, MA, USA) at 150nM was used to pre- and co-treated the astrocytes along with PA. The mRNA of Pro-IL-1β and ASC were detected by real-time PCR (n=3). **: $p<0.01$, ***:$p<0.001$. A line indicates comparison between the two bars connected by the line.

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