Investigation of the Modulating Effect of Cholesterol Derivatives on the Processing of the β-Amyloid Precursor Protein (AβPP)

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ABSTRACT

Alzheimer's disease involves the formation of amyloid plaques in the brain, which lead to neuronal death and loss of brain function. The Aβ peptide is the major component of these plaques and is produced through proteolytic cleavage of the amyloid precursor protein (AβPP). Recent data suggest a link between cholesterol levels and the proteolytic processing of AβPP, from this we hypothesized that cholesterol derivatives, such as cholesterol-sulphate (CS) could modulate AβPP processing, potentially inhibiting Aβ formation. We demonstrate a shift in AβPP processing through the non-amyloidogenic pathway following treatment of cultured cells with μM quantities of CS; additionally, the investigation of CS-modulation of α-, β-, and γ-secretase enzyme activities provides insight into the mechanism responsible for the changes in AβPP processing. These findings implicate CS and other sterol derivatives, as possible novel regulators of Aβ production with applications in the treatment of Alzheimer's disease arising from abnormal Aβ secretion.

ACKNOWLEDGEMENTS

I would like to thank Dr. James J. Cheetham for his supervision, direction, and for starting me down the path of discovery which has led to the work published herein. Also the majority of this work was greatly aided by the wisdom and patience of Dr. Bill Willmore, who I recognize here as having been integral in teaching me many of the techniques used in completing this research, as always being available for consultation when working through a problem, and for allowing me the use of his laboratory and equipment.

I want to thank my family, friends, and the Carleton University Biology Department for their support during my time in academia.

Finally, and most of all, I want to acknowledge and thank Michelle Lee Liddiard for being by my side for nearly the entire duration of my graduate studies...and for making it the time of my life.

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ABBREVIATIONS

Aβ	β-Amyloid
AβPP	Amyloid-β precursor protein
$AβPP_α$	Secreted AβPP ectodomain derived specifically through α-cleavage
$AβPP_β$	Secreted AβPP ectodomain derived specifically through β-cleavage
ABCA1	ATP-binding cassette transporter-1, a lipid efflux regulator
ACAT	Acyl-CoA acyltransferase
AD	Alzheimer's disease
AICD	AβPP intracellular C-terminal domain
APH-1	Anterior pharynx-defective 1, component of γ-secretase protein complex
BCA	Bicinchoninic acid
C	5-Cholesten-3β-ol , Cholesterol
CHEMS	5-Cholesten-3β-ol-3-hemisuccinate, Cholesterol-Hemisuccinate
COS-7	African green monkey kidney cell
CS	5-Cholesten-3β-ol-sulfate, Cholesterol-Sulfate
CSF	Cerebrospinal fluid
CTF	C-Terminal Fragment
DABCYL	4-((4-(dimethylamino)phenyl)azo)-benzoic acid, fluorescence quencher
DMEM	Dulbecco's Modified Eagle's Medium
DNP	2,4-dinitrophenyl, fluorescence quencher
EDANS	5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid, fluorescence donor
EtOH	Ethyl alcohol
FRET	Fluorescence resonance energy transfer
HST2B	Hydroxysteroid sulfotransferase 2B
LRP	Low density lipoprotein receptor-related protein
MeOH	Methyl alcohol
NMA	2-(N-methylamino)benzoyl, fluorescence donor
NPC-1	Niemann-Pick Disease type C1 protein
PBS	Phosphate-buffered saline
PC-12AC	Rat pheochromocytoma adherent clone cell line
PCR	Polymerase chain reaction
PKC	Protein Kinase C
PS	Presenilin, a component of γ-secretase protein complex
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute cell culture media formulation 1640
sAβPP	Secrete amyloid-β precursor protein
SDS	Sodium dodecyl sulfate
SH-SY5Y	Human neuroblastoma cell line
SREBP	Sterol regulated element binding protein
TBS	Tris-buffered saline

I. INTRODUCTION

1.1 *Aging and Alzheimer's Disease*

Improvements in healthcare, nutrition, and sanitation standards along with an increased adoption of social behaviours favouring improved quality of life have been responsible for establishing a trend which has more than doubled the human life expectancy since the early 19th century (Oeppen and Vaupel, 2002). The increase in lifespan consequently gives rise to a much larger segment of the population made up of the elderly, prompting a significant portion of 20th and 21st century research to focus on the investigation of disorders arising in an aging populous. Of the health concerns associated with advanced age, a decline in cognitive function into senility is a grave concern from both a medical as well as an economic standpoint. Impairment of an individual's memory, attention, perception, language, and critical thinking skills greatly reduces the psychological and physical wellbeing of the suffering person, as well as the dependants and caretakers close to them. It is the high social and economic costs combined with the anxiety associated with the loss of mental faculties that makes the cognitive decline of dementia such a disturbing consequence for all age groups (Rice *et al*, 2003; Wimo *et al*, 2007).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder first described by German psychiatrist Alois Alzheimer in 1906, and is now understood to be the most common form of dementia in the elderly human population (Tanzi and Bertram, 2005). The disease progresses through stages characterized by the behavioural

symptoms observed or neuropathological changes in brain tissue (Braak and Braak, 1991; Cummings, 2004). Symptoms at the early stages include memory impairments, disinhibition, and minor aberrant delusions, which grow in severity until patients in the later stages suffer serious loss in memory and recall, communication deficits, difficulty in maintaining control over physical movements and bodily functions, and increasingly degraded mental abilities (Cummings *et al*, 1998; Cummings, 2004).

The molecular mechanisms underlying the severe cognitive deficits experienced by Alzheimer's patients are still not fully clear. The two major hypotheses for the cause of neuronal death leading to dementia in Alzheimer's stem from the major lesions found in AD patients' brains: the aberrant formation of extracellular β-amyloid "plaques" and intracellular neurofibrillary tangles of the tau protein (Small and Duff, 2008). Evidence for the "amyloid-hypothesis" suggests that the cellular secretion and accumulation of the Aβ protein creates inter-neuronal plaques leading to dysfunction of synaptic signaling, local inflammation, and neuronal death; while the "tau-hypothesis" emphasizes the importance of tau-specific hyperphosphorylation in inducing uncoupling and subsequent aggregation of the microtubule-stabilizing protein, leading to disintegration of the microtubule network and the deposition of tangled tau protein threads within the cell causing the observed gross pathology (Selkoe, 2001; Williams, 2006). The traditional amyloid-hypothesis has held that Aβ accumulation initially drives the deregulation of cellular processes leading to tau hyperphosphorylaton and eventual

development of AD; however recent research has implicated that both lesions may in fact be driving the progression of AD is a converging pathway (Small and Duff, 2008).

1.2 *β-Amyloid*

The aggregation and deposition of insoluble fibrillar β-amyloid (Aβ) plaques in the brain is implicated as a major hallmark of the neurodegenerative pathology in Alzheimer's disease. Aβ is a small 39-43 amino acid protein fragment derived through proteolytic-processing of a type-1 transmembrane glycoprotein recognized as the amyloid-β precursor protein (AβPP) (Selkoe, 1994; Nunan and Small, 2000). Initiation of the pathology involves the onset of an aberrant cycle of high Aβ secretion combined with a low rate of clearance, with the increasing levels of Aβ forming oligomeric and fibrillar aggregates or "plaques" in the extracellular space between neurons, triggering inflammation and eventually leading to neuronal dysfunction and damage (Tanzi and Bertram, 2005). Processing of the Aβ precursor may yield short or long isoforms with differing apparent toxicities depending on individual mutations in AβPP or a class of proteins known as presenilins, which function as part of the γ-secretase complex (Scheuner *et al*, 1996). While isoform-specific events are currently being investigated, the general hypothesis holds that a significant increase in Aβ secretion will initiate or cause the progression of AD-like pathology (Selkoe, 2001). The *in vivo* biological function of the Aβ precursor protein itself is poorly understood, with evidence suggesting possible roles as a growth factor receptor, apoptotic mediatior, and possible factor in

axonic and dendritic expansion (De Strooper and Annaert, 2000). Due to the differences in domains present in the long ($A\beta PP_{751}$/$A\beta PP_{770}$) and short ($A\beta PP_{695}$) isoforms, the relevant interaction partners for the long ectodomain of this molecule have been difficult to identify; specifically in neurons where $A\beta PP_{695}$ predominates and lacks the Kunitz-type protease inhibitor domain, which has been identified as necessary for longer isoforms to bind LRP (De Strooper and Annaert, 2000). Additional function is attributed to the AβPP intracellular C-terminal domain (AICD), which has been shown to interact with intercellular adapter proteins that potentially link AβPP metabolism to nuclear signaling, synapse function, cell adhesion, and phosphorylation dependant pathways (De Strooper and Annaert, 2000).

Understanding of the contributing factors in deregulation of normal AβPP processing leading to increased Aβ secretion requires the examination of the processing pathway itself, specifically the secretase enzymes associated directly with the production of Aβ.

1.3 *Secretase Enzymes and AβPP Processing*

AβPP undergoes processing in two stages, with an initial "deterministic" cleavage step by either α- or β-secretase enzymes followed by a second intramembranous cleavage via the γ-secretase enzyme complex to release the final products (Nunan and Small, 2000). Initial cleavage of AβPP isoforms by α-secretase (a

group of zinc-proteases related to the adamalysin family) yields soluble $A\beta PP_{\alpha}$ ($A\beta PP$ ectodomain derived specifically from α-cleavage) and a shorter C-terminal fragment (CTF) which is further processed by γ-secretase to yield an $A\beta PP$ intracellular C-terminal domain (AICD) and a small fragment designated 'p3' with the cleavage products having no pathogenic activity (Allinson *et al*, 2003). Processing in this way is the major route of $A\beta PP$ cleavage (Figure 1B). Conversely $A\beta PP$ processing through the β pathway generates an extracellular soluble fragment of $A\beta PP_{\beta}$ ($A\beta PP$ ectodomain derived specifically from β-cleavage) and a shorter 'C99' CTF which is processed into an AICD and isoforms of $A\beta$ post γ-secretase cleavage; in this way β-secretase is the rate-limiting protease for $A\beta$ generation (Vassar *et al*, 1999; Nunan and Small, 2000) (Figure 1C). The properties of a given isoform of $A\beta PP$ may predetermine protein cleavage through different γ-complexes, or even vary processing between α and β cleavage (Henriques *et al,* 2007). Processing of $A\beta PP$ in this way is thought to be conserved among eumetazoans, with cleavage of the $A\beta PP$ homolog yielding similar sized products (Ramabhadrant *et al*, 1993) and γ-complex-related homologs identified in such model organisms as *D. melanogaster* and *C. elegans* (Hong and Koo, 1997; Francis *et al*, 2002); however the pathway has been most studied in mammalian models where $A\beta PP$ expression is relatively ubiquitous across cell types (De Strooper and Annaert, 2000).

The components required for γ-secretase activity (presenilin, nicastrin, APH-1, and PS-enhancer-2) have been reported to localize within the cholesterol-rich detergent-resistant liquid-ordered domains of plasma membranes often known as lipid

raft domains, allowing for the reconstitution of a functional γ-protease complex. Additionally, a portion of total cellular β-secretase also localizes to lipid rafts, implicating the raft domains as minor sites of β-cleavage (Wahrle *et al*, 2002; Kalvodova *et al*, 2005; Hattori *et al*, 2006; Reid *et al*, 2007; Hur *et al*, 2008). Evidence for the importance of such membrane domains existing in distinct liquid-ordered states composed of saturated phospholipids, cholesterol, and sphingolipids has been reviewed by Simons and Toomre (2000). Raft domains may be conceptualized as higher-order lipid regions suspended in the liquid-disordered lipid matrix of plasma membranes (rafts may also occur in intracellular membranes of the biosynthetic pathway and endocytic pathway), with the ability to include and exclude proteins and allow a specific environment for biological reactions to take place (Simons and Toomre, 2000). Cholesterol and sphingolipids are integral structural components of raft domains. Cholesterol intercalates between saturated phospholipids and acts to further stabilize their acyl chains giving the domains a higher liquid-ordered state; while sphingolipids act to give a more stable structure, potentially coupling cytoplasmic and exoplasmic leaflets of the membrane domain to each other (Simons and Toomre, 2000). Changes in sterol distribution may alter lipid domain characteristics consequently altering AβPP localization and permitting raft-independent α-cleavage preferentially while possibly avoiding fragment excision from the membrane via γ-cleavage (Bodovitz and Klein, 1996; Kojro *et al*, 2001; Wahrle *et al*, 2002; Vetrivel *et al*, 2005) (Figure 3). The initiation of AβPP cleavage is dependent primarily on the association of the protein with active

secretase enzymes under conditions which will permit either α-, β-, or γ-cleavage; most of the total cellular AβPP protein is left whole, with only a small fraction of the total going through secretase processing (De Strooper and Annaert, 2000; Nunan and Small, 2000).

The set of secretase enzymes is then ultimately responsible for regulation of nearly all Aβ production and subsequent secretion from a cell, conferring a high-priority to the AβPP processing system as a potential target of therapeutic agents. The importance of the lipid membrane environment in the processing of AβPP (Ehehalt *et al*, 2003) can be looked at as a point for further investigation of the Aβ generating system.

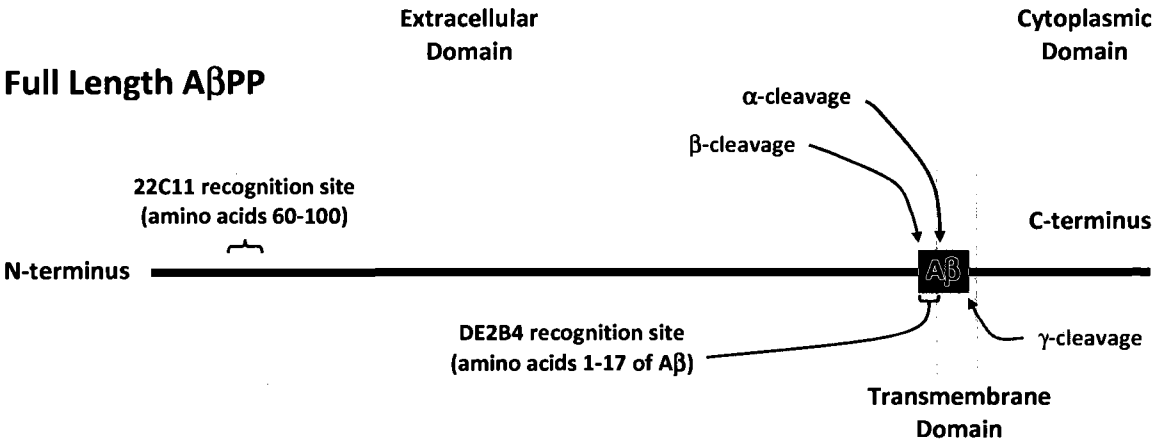
Figure 1

A) Schematic of full length AβPP showing the points of enzymatic cleavage by α-, β- and γ-secretases. The top diagram also shows the recognized epitopes for the two primary antibodies used in this study for detection of either net AβPP or the $AβPP_α$ isoform allowing for qualitative recognition of differential processing within treated cells (while DE2B4 is able to detect the Aβ peptide as well, detection of amyloid was not carried out).

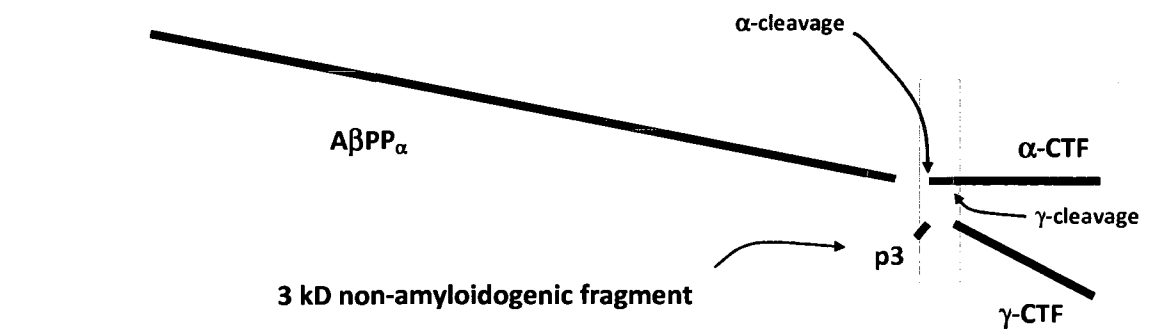
B) Representation of the two-step cleavage of AβPP leading to the non-amyloidogenic pathway with the release of $AβPP_α$, an α-derived C-terminal fragment (CTF), a small non-amyloidogenic peptide, p3, and the γ-CTF also known as the AICD.

C) Representation of the two-step cleavage of AβPP leading to the amyloidogenic pathway with the release of $AβPP_β$, a β-derived CTF, an isoform of the Aβ protein, and the γ-CTF also known as the AICD.

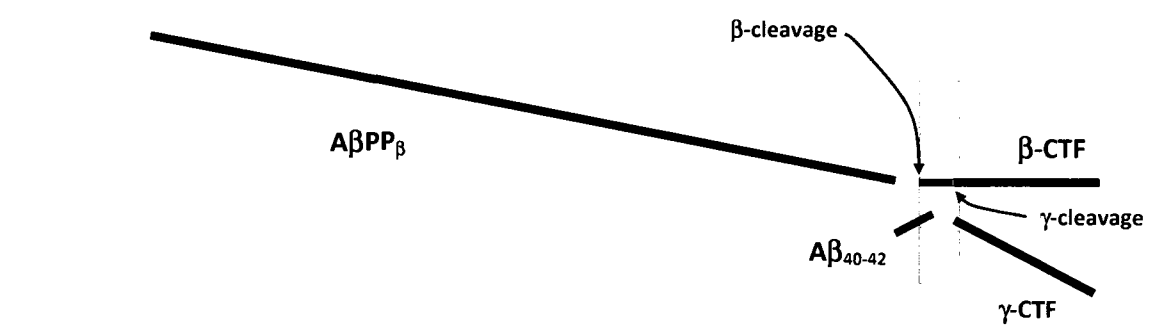
A


Extracellular Domain
Cytoplasmic Domain
Full Length AβPP
α-cleavage
β-cleavage
22C11 recognition site (amino acids 60-100)
C-terminus
N-terminus
Aβ
DE2B4 recognition site (amino acids 1-17 of Aβ)
γ-cleavage
Transmembrane Domain

B


Non-Amyloidogenic Cleavage
α-cleavage
AβPPα
α-CTF
γ-cleavage
p3
3 kD non-amyloidogenic fragment
γ-CTF

C


Amyloidogenic Cleavage
β-cleavage
AβPPβ
β-CTF
γ-cleavage
Aβ40-42
γ-CTF

The relationship between Aβ lesions in AD and cellular cholesterol homeostasis has been well established (Canevari and Clark, 2007). Accumulation of cholesterol has been associated with Aβ-pathology via modification of membrane characteristics vital to normal secretase enzyme function (such as lipid raft domains). While cholesterol promotes the formation of tightly packed lipid domains, its sulfoconjugate, 5-Cholesten-3β-ol-sulfate (CS) has a disruptive effect due to the acidity of its polar sulfate head group and much shallower insertion depth between saturated membrane phospholipids (Figure 2A and 2B) (Xu and London, 2000). Incorporation of cholesterol-sulfate into membranes could destabilize lipid domains associated with γ-complex function and may even be responsible for liberation and subsequent upregulation of α-secretase enzymes sequestered in rafts, potentially leading to a drop in Aβ produced (Cordy *et al*, 2006). The incorporation of another anionic cholesterol deriviative such as carboxyl-group bearing cholesterol-hemisuccinate (CHEMS) would reportedly stabilize membrane phospholipids and mimic C in some of its interaction with the lipid environment, with the sterol orienting in a similar conformation as the less polar hydroxyl-group bearing cholesterol moiety but inserting deeper into the membrane bilayer and increasing interfacial charge (Figure 2C) (Lai *et al*, 1985; Massey, 1998). While the direct action of sterol substitution into membrane domains may play a role in controlling the processing of AβPP, it is also possible that cholesterol derivatives may activate signaling pathways leading to different expression/activation levels of secretase enzymes, support proteins,

or regulatory enzymes thereby altering the preference for either the α– or β-deterministic cleavage pathways (Smith and Johnson, 1989; Kuroki *et al*, 2000; Canevari and Clark, 2007; Ishimaru *et al*, 2008). The reported ability of CS to act as an activator of select isoforms of the widely acting protein kinase C (PKC) (Kuroki *et al*, 2000) may result in just such an activation of α-secretase and thereby shunt AβPP processing through the non-amyloidogenic pathway (Jolly-Tornetta, and Wolf, 2000; Etchberrigaray *et al*, 2004); furthermore both CS and CHEMS are reported to inhibit animal DNA metabolic enzymes such as polymerases α, β, and γ, and topoisomerase II (Ishimaru *et al*, 2008). The consequences of affecting such pervasive enzymes may go well beyond modulating the function of secretases. Other signaling effects may be through indirect sterol or metabolite interaction with lipoproteins, which in turn interact with ABCA1, LRP, SREBP, ACAT, NPC-1, etc (Canevari and Clark, 2007). Whether such processes are internally regulated or occur only upon exogenous administration of disruptive sterols may also give clues to the initial causes of amyloidogenic pathologies.

In order to better understand how sterol homeostasis affects processing of AβPP with respect to AD onset, an experiment to determine the effects of polar-sterol incorporation in cell membranes on Aβ generation was undertaken in part by Dr. James J. Cheetham at the University of Aveiro with some dramatic results (Figure 4). It was observed that treatment of mammalian cell cultures with acidic-sterol moieties (CS and CHEMS) resulted in a cessation of Aβ secretion. CS was chosen as a test compound due to its relatively significant presence in mammalian tissue (Table 1), its reported function

as a regulatory molecule (Strott and Higashi, 2003), and the existence of previously published data showing treatment of cell cultures with the 24S-hydroxycholesterol, and 27-hydroxycholesterol sterol derivatives producing a modulating effect on secretase enzyme function (Famer *et al* 2007). The polar sterol occurs mainly in membranes where it has an equilibrating effect, it is primarily found in human plasma, urine, bile, seminal fluid, spermatozoa, platelets, red blood cells, skin, hair, nails, aorta, adrenal glands, liver, kidneys, and cerebral synaptosomal membranes (Schofield *et al*, 1998; Stortt and Higashi 2003). The Aveiro study was the first step in quantifying the effect CS has on the specific processing of AβPP, further investigation would allow for a better understanding of the link between CS-homeostasis and AD progression.

The potential for novel endogenous regulation of secretase function by cholesterol-sulfate and cholesterol-hemisuccinate may open up new avenues for both therapeutic and causative investigation of amyloid management. Current therapeutic strategies for AD focus on enhancing neurotransmitter availability and efficiency using acetyl-cholinesterase inhibitors and *N*-methyl-D-aspartate receptor antagonists to maintain cognitive function despite loss of neurons (Findeis, 2007). This approach is of a palliative nature in lieu of strategies with the potential to reverse AD progression. Therapeutics currently in clinical development include Aβ aggregation inhibitors, γ-secretase modulators and inhibitors, immunization strategies against Aβ, and Aβ-ROS-directed metal chelators (Findeis, 2007). The secretase enzymes are attractive drug targets for therapies seeking to directly reign in AD progression due to their direct

involvement in AβPP cleavage and Aβ generation. Traditional inhibitors of the γ-secretase complex face the challenge of having to preserve processing of the Notch receptor (an essential cell-signaling pathway for differentiation), which competes with AβPP for γ-secretase, in order to minimize complications resulting from cell cycle arrest or other unanticipated outcomes (Nunan and Small, 2000). To this end the discovery of selective modulators of γ-secretase activity has been required in order to allow for some functionality of the complex while minimizing AβPP processing (Findeis, 2007). The potential for CS to deliver a two-pronged therapeutic impact by downregulating γ-cleavage through membrane alteration and possibly promoting processing through the non-amyloidogenic α-cleavage pathway makes the sterol an excellent candidate for further study.

Figure 2

Schematic of hypothesized membrane-domain-dependant AβPP processing, illustrating a possible lipid raft contribution to the selection of a preferential mode of AβPP cleavage where α-secretase function is specific to non-raft domains and γ-secretase functions solely upon association of the complex proteins in lipid raft domains (adapted from Reid *et al*, 2007).

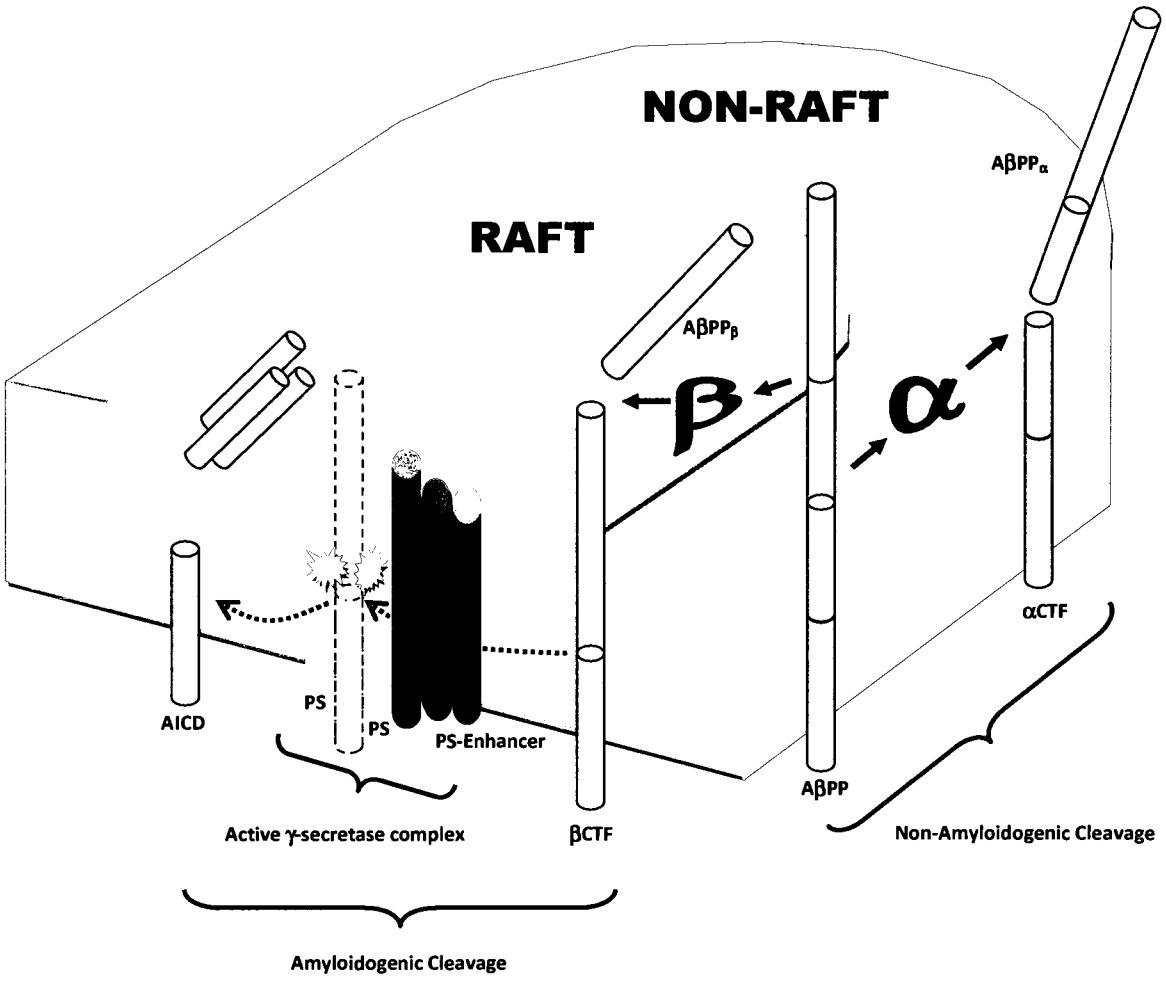

NON-RAFT
RAFT
AβPPα
AβPPβ
β
α
αCTF
AICD
PS
PS
PS-Enhancer
AβPP
Active γ-secretase complex
βCTF
Non-Amyloidogenic Cleavage
Amyloidogenic Cleavage

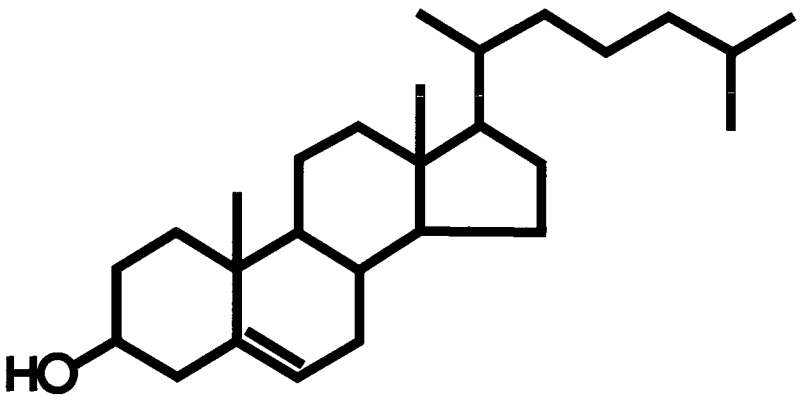
Figure 3

A) Chemical structure diagram for cholesterol (C). When incorporated into a lipid bilayer C is oriented such that the hydroxyl head group interacts with the polar groups of neighbouring phospholipids (Schofield *et al*, 1998).

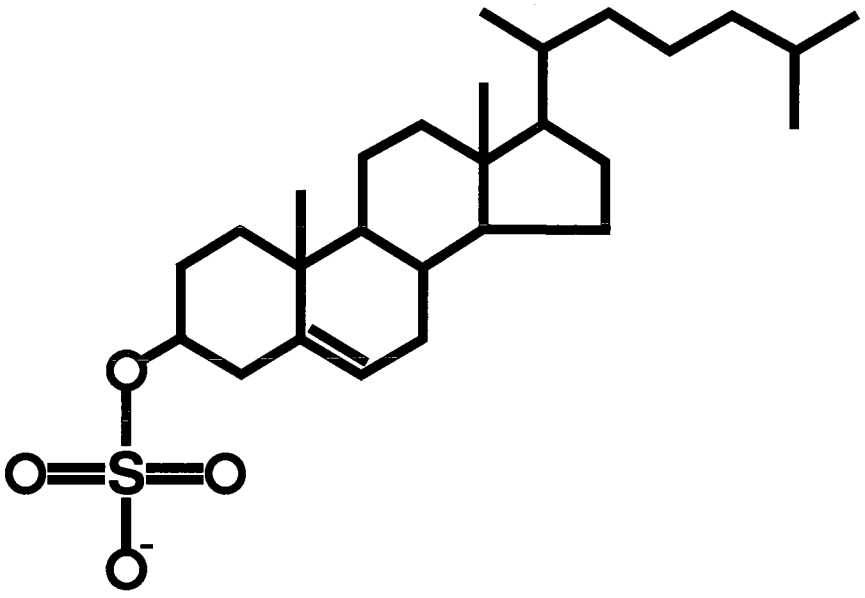
B) Structure diagram for cholesterol sulfate (CS). When incorporated into a saturated lipid bilayer the highly polar sulfate head group of CS facilitates a shallower insertion depth and a much greater degree of destabilization of neighbouring phospholipids (Schofield *et al*, 1998).

C) Structure diagram for cholesterol hemisuccinate (CHEMS). The sterol has a significantly greater aqueous solubility than C due to the replacement of the hydroxyl head group with a succinate moiety. Interactions within the phospholipid membrane environment are reported to be similar to those of cholesterol (Lai *et al*, 1985). While not a significant physiological derivative, it is commonly used to mimic cholesterol effects on membrane bilayers due to its greater solubility.

A



B



C

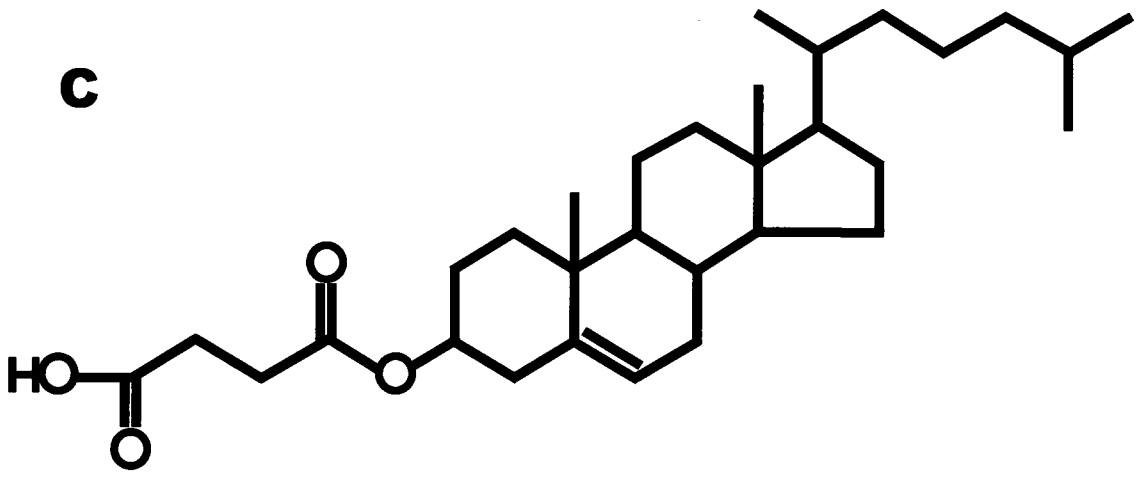
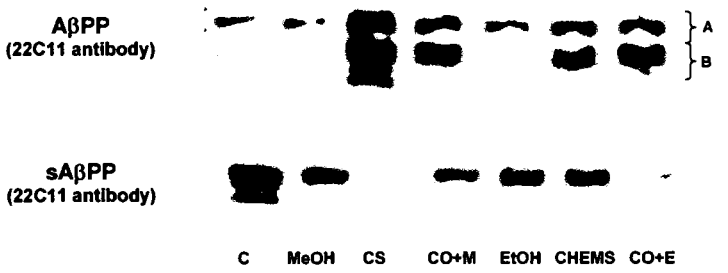


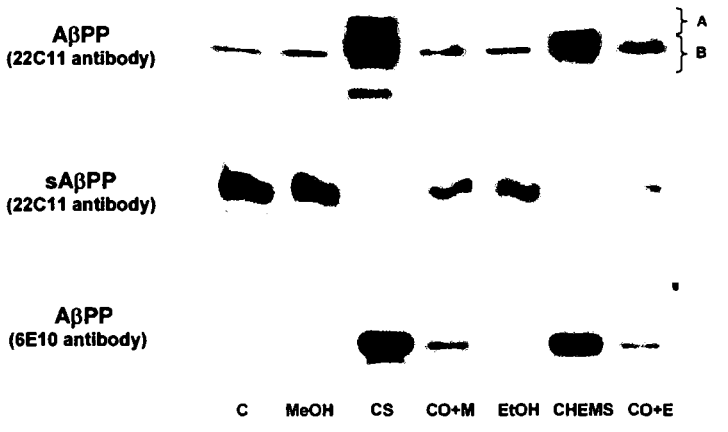
Figure 4

Initial immunoblot results completed at the University of Aveiro for AβPP production following treatment with sterol derivatives. The data presented in the figure served as the precedent for the experiments outlined in this thesis. Data shows the action of CS on PC-12AC and COS-7 cell lines eliminating the secretion of net AβPP compared to controls. In addition cells were treated with C, MeOH, an oxysterol in MeOH (CO+M), EtOH, CHEMS, and an oxysterol in EtOH (CO+E). These initial findings are unpublished and were carried out in part by Dr. James J. Cheetham of Carleton University and Dr. Edgar F. da Cruz e Silva of the University of Aveiro. Replication of these results was the first step in better understanding the phenomenology they presented.

PC12AC



COS-7



A. Mature $A\beta PP_{751/770}$

B. Mature $A\beta PP_{695}$
Immature $A\beta PP_{751/770}$
Immature $A\beta PP_{695}$

Table 1

Comparison of cholesterol to cholesterol-sulfate concentrations in human and rodent (adapted from Liu *et al*, 2003). Despite the comparatively low concentration of CS, it is the most important sulfated sterol in plasma (Strott and Higashi, 2003).

Sterol	Chemical Formula	Human Plasma	Human CSF	Human Brain	Rodent Brain
C	$C_{27}H_{46}O$	2 mg/mL	3 µg/mL	7-8 µg/mg	Mouse 20 µg/mg
CS	$C_{27}H_{46}O_4S$	50-300 ng/mL	No Data	No Data	Rat 1.2 ng/mg

1.5 *Overview of Thesis*

The purpose of this study was to determine the extent to which the anionic cholesterol derivatives CS and CHEMS could act as novel promoters of the non-amyloidogenic pathway and the molecular mechanisms behind such an effect. A combination of two likely hypotheses may be applied to explaining the Aveiro results (Figure 4): cellular uptake of these sterols may disrupt high-order lipid domains necessary for membrane-embedded secretase enzymes to associate and function due to their added capacity for electrostatic interaction with membrane phospholipids on top of the hydrogen-bonding exhibited by the more prevalent cholesterol molecule (Massey, 1998; Schofield *et al*, 1998); additionally treatment may induce activation of signal pathways within the cell leading to changes in internal protein activation state, possibly PKC activation leading to increased α-secretase activity (Jolly-Tornetta and Wolf, 2000; Kuroki *et al*, 2000).

Herein are described the conditions under which the interaction of CS with the cellular machinery responsible for AβPP production induces complete internalization of all AβPP molecules in the PC-12AC and COS-7 cell lines, and how this interaction may be responsible for preferentially shunting AβPP processing through the α-cleavage pathway, precluding formation of Aβ; in addition the effect of C and CS on the activities of α-, β-, and γ-secretase enzymes in cultured COS-7 cells were determined using a FRET-based quenching assay in order to resolve the mechanisms by which these sterols modulate AβPP processing. Cell lines used in experiments were chosen on the basis of

mammalian origin and previous use in the Aveiro study. The effects of cholesterol on the processing of Aβ are by now well established, but for the purposes of this study treatment of cells with C provided a means of contrasting the putative non-amyloidogenic effect of CS exposure. The experiments herein were attempted to firstly replicate and confirm the findings of the Aveiro group study, and secondly to find evidence allowing for a mechanistic explanation of the phenomenon presented by the immunoblot data.

II. MATERIALS AND METHODS

2.1 *Sterol Reagents*

Purified 5-Cholesten-3β-ol-sulfate (CS) and 5-Cholesten-3β-ol-3-hemisuccinate (CHEMS) were obtained from Steraloids (Newport, RI); CS stock solutions were prepared in methanol (MeOH) in concentrations ranging from 1-6 mg/mL and stored at -80°C, CHEMS stock was prepared in absolute ethanol (EtOH) ranging from 1-6 mg/mL and stored at -80°C. Purified 5-Cholesten-3β-ol (C) was obtained from Sigma-Aldrich (St. Louis, MO); stock was prepared between 1-2 mg/mL in absolute EtOH and stored at -80°C.

2.2 *Treatment of Cell Cultures with C, CS, and CHEMS*

Rat pheochromocytoma adherent-clone cells (PC-12AC), a clonal derivative of the PC12 line (Brewer *et al*, 2002) were cultured in 100mm cell culture dishes using RPMI-1640 supplemented with 5% newborn calf serum, 10% horse serum, 300 Units penicillin/mL, 300μg streptomycin/mL, and 0.25mg amphotericin-B/mL (Invitrogen, Carlsbad, CA) at 5% CO_2 and 37°C. Sub-culturing of cells was carried out by first incubating with 0.05% trypsin (Invitrogen, Carlsbad, CA) to detach adhered cells from the culture dish, followed by suspension in media, determination of cell number using a hemocytometer (Hausser Scientific, Horsham, PA), centrifugation, re-suspension, and finally plating to new culture dishes at an appropriate predetermined density. Upon

reaching ≈80% confluence, determined by examination of cultures under a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Göttingen, Germany), the culture media was replaced with serum and antibiotic-free RPMI-1640 in which the cultures were allowed to equilibrate for 1 hour at 5% CO_2 and 37°C. Following equilibration, the cells were treated with 100µL CS, CHEMS, or C from a 1mg/mL stock of each sterol and incubated at 5% CO_2 and 37°C for a period of 24 hours. Two controls, consisting of untreated cultures and cultures treated with 100µL MeOH, were used as well. The treatment volumes of C and CHEMS solubilized in EtOH, and CS solubilized in MeOH were not high enough to observe reported solvent-related effects on cell viability (Polo *et al*, 2003; Forman *et al*, 1999). African Green Monkey Kidney cells (COS-7) (Gluzman, 1981) were cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum, 300 Units penicillin/mL, 300µg streptomycin/mL, and 0.25mg amphotericin-B/mL (Invitrogen, Carlsbad, CA) at 5% CO_2 and 37°C. Treatment of COS-7 cells adhered to the procedure already described for PC-12AC cells, differing only in that the sterol concentrations used for the treatments were standardized to final concentrations of 10µM/mL_{media} (following an informal range-finding study between 0.5-20µM/mL_{media}) and that multiple treatment periods were observed (1hr, 2hr, 4hr, 6hr, 12hr, and 24hr). Cells were examined for viability by trypan blue dye exclusion assay adapted from Freshney, 1994; sterol treatments did not appearing to compromise the normal morphology or growth seen in untreated cultures under observation with a Zeiss Axiovert 200. Culture of human neuroblastoma cells (SH-SY5Y) was attempted as

well; however no relevant analyses to gauge cellular response to sterol treatment were completed due to technical difficulties encountered in the culture facility, resulting in contamination and subsequent loss of culture integrity.

2.3 *Sample Collection and Immunoblot analysis*

Sterol treated PC-12AC, COS-7, and control cells were harvested using a cell lifter prior to pelleting via bench top centrifuge for 5 minutes at 1,000 x g; trypsin was not used when harvesting cells in order to minimize damage to cell membranes and integral proteins of interest. Cultured media was collected and the cell pellets were washed with ice cold PBS and lysed using lysis buffer adapted from Abu-Farha *et al*, 2005 (PBS containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, and 0.01% protease inhibitor cocktail). Cultured media was passed through a 0.22μm filter (MilliPore, Billerica, MA) in order to exclude whole-cell debris from downstream sample analysis; ensuring only secreted proteins would be detected. Sample protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) which relies on the reduction of Cu^{2+} by interaction with peptide bonds followed by chelation of the Cu^{1+} by two moieties of BCA forming an intense purple-coloured complex with strong absorbance at 562nm (Smith *et al*, 1985). Western blot analysis was carried out with a protocol adapted from Gallagher *et al*, 2008. Standardized quantities of 30μg crude protein from cell lysates or constant volumes of 15μL cultured media were boiled

in 2X Laemmli sample buffer for 5 minutes and subjected to 10% Tris-HCL, 8% Bis-Tris, or 12% Bis-Tris SDS-PAGE at constant voltage until the dye front from the loading buffer approached the bottom edge of the gel. Two different protein standards (Bio-Rad Laboratories, Hercules, CA) were loaded into the gel alongside the samples to ensure transfer efficiency to nitrocellulose (Kaleidoscope prestained standards) and to determine relative protein sizes following immunodetection (Recombinant Precision Plus Protein unstained standard with a *Strep*-tag). Separated proteins were then transferred to a nitrocellulose membrane overnight in Towbin transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3) under constant current. In order to prevent non-specific binding, the membranes were blocked in 5% non-fat dry milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 3 hours on a rocker panel then probed overnight with primary antibody at 4°C. Monoclonal 22C11 antibody (Chemicon, Billerica, MA), recognizing the AβPP N-terminus (amino acids 66-81, Hilbich *et al*, 1993) of both immature and mature forms (Hoffmann *et al*, 2000), was used for the detection of net endogenous AβPP (1:3,500-6,000 dilution in 1% non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20)), and the monoclonal DE2B4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), which binds the N-terminus of Aβ (amino acids 1-17 of Aβ), was used for the detection of secreted $A\beta PP_{\alpha}$ (1:1,000-5,000 dilution in 1% non-fat dry milk in TBST). Following primary incubation, the membrane was washed four times for 15 minutes in TBST. Detection of proteins was carried out using a horseradish peroxidase-conjugated anti-mouse IgG+IgM (H+L) (KPL Gaithersburg, MD) secondary-

antibody (1:15,000 dilution) (detection of the Precision Plus protein standard involved the additional incubation with StrepTactin-HRP). Four final 15 minute washes in TBST were carried out and immediately followed by incubation in enhanced chemiluminescence solution (MilliPore, Billerica, MA) for 5 minutes in the dark, visualization was carried out by either exposure of the blot to x-ray film (Kodak, Rochester, NY), or using a Flurochem Q imaging system (Alpha Innotech Corporation, San Leandro, CA). Nitrocellulose membranes were stripped of antibody by immersion in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) at 50°C for 30 minutes with agitation, followed by two 10 minute washes in large volumes of TBST. Monoclonal mouse anti-β-tubulin E7 (1:3,000 dilution) (Developmental Studies Hybridoma Bank, Boulder, CO) was used to probe stripped and re-blocked membranes as a loading control after visualization of AβPP was completed, the detection of the β-tubulin followed the same procedure as that for AβPP.

2.4 *Determination of α-, β-, and γ-Secretase Activity in COS-7 Cells*

The FRET-based assay used for determination of secretase activity was dependent on a secretase-specific peptide substrate with an attached fluorescence donor molecule in close proximity to a fluorescence quenching molecule; upon cleavage of the substrate at residues between the donor and quencher, the distance between the

two fragments increases and the fluorescence signal would cease to be quenched, signaling cleavage of the substrate has occurred.

COS-7 cells treated and collected from culture dishes as previously described (with the omission of CHEMS treatment) were lysed with extraction buffer adapted from Farmery *et al*, 2003 (20 mM HEPES, pH 7.0, 150 mM KCl, 2 mM EGTA and 1% (w/v) Brij-35). Protein concentration from each lysate was determined using the BCA assay (Pierce, Rockford, IL). Volumes corresponding to 100 μg (for α- and γ-secretase activity assays) and 150 μg (for the β-secretase activity assay) crude protein from each sample lysate were added to an equal volume of 2X reaction buffer adapted from Farmery *et al*, 2003 (100 mM sodium acetate, pH 4.5, 2mM EDTA and 0.25% (w/v) Brij-35), individual assays for specific secretase activity were then carried out with fluorogenic α- ($RE_{(EDANS)}$VHHQKLVF$K_{(DABCYL)}$R), β- ($RE_{(EDANS)}$EVNLDAEF$K_{(DABCYL)}$R), and γ- ($_{(Nma)}$GGVVIATV$K_{(Dnp)}$-*D-Arg-D-Arg-D-Arg*. (D-arginine residues increase solubility of the probe)) secretase substrates (Calbiochem, San Diego, CA), added at 50nM, 1μL, and 15μM respective final quantities (β-secretase substrate was obtained from a Calbiochem kit and the exact stock concentration was not known, thus a constant volume recommend by the manufacturer was used). Additional reactions were set up with active β-secretase enzyme (Calbiochem, San Diego, CA) as a positive control, and a background protein-free control containing only extraction/reaction buffers and secretase substrate. Samples were gently mixed by tapping and incubated in the dark for 90 minutes at 37°C. Samples were placed in optical glass cuvettes and readings of

relative fluorescence were taken on a Hitachi F-2000 fluorescence spectrophotometer (α-secretase: excitation $\lambda \approx 340$nm, emission $\lambda \approx 490$nm, β-secretase: excitation $\lambda \approx 350$nm, emission $\lambda \approx 490$nm, and γ-secretase: excitation $\lambda \approx 355$nm, emission $\lambda \approx 440$nm). Fluorescence intensity was correlated with enzyme activity. Optimization of reaction buffer pH, incubation time, and substrate concentration was carried out in order to ensure fluorescence intensity readings occurred within the middle of the Hitachi F-2000 detection range.

2.5 *Statistical Analysis*

Densitometric analysis of immunoreactive bands in the immunoblot assay was carried out using AlphaView Q software (Alpha Innotech Corporation, San Leandro, CA). Data are expressed as a mean ± SEM (standard error of the mean) of combined experimental trials. Statistical significance was determined by analysis of variance (ANOVA) and application of Student's *t*-test using Microsoft Excel Analysis ToolPak software. Statistical significance was determined at $P < 0.05$.

III. RESULTS

3.1 *AβPP Processing and Secretion in PC-12AC and COS-7 Cells Exposed to Sterol Derivatives*

Immunoblot analysis comparing the concentration of AβPP in cell lysates and cultured media across treatment groups from PC-12AC and COS-7 cells revealed a complete cessation of AβPP secretion following CS treatment in both cell lines (Figure 5 and 6). CS exposure appears to significantly increase AβPP band intensity in cell lysates compared to untreated control cells or those treated with C, while abolishing signal in the cultured media; this was interpreted as a dramatic shift in the processing of AβPP leading to the retention of all immune-detectable AβPP isoforms within the cell. Confirmation of equal protein distribution across wells was obtained by inspection of β-tubulin bands on re-probed nitrocellulose membranes (Figure. 3.1.2). Relative densitometry comparing 22C11-probed CS treated sample AβPP-band intensities to those of the untreated control established a 2.26 fold increase in mature $A\beta PP_{751/770}$ isoform retention within cell lysates and a 2.88 fold increase in retention of immature $A\beta PP_{751/770}$ (as well as mature/immature $A\beta PP_{695}$) isoforms in PC-12AC cells. COS-7 lysates exhibited 4.00- and 1.28-fold increases in mature and immature AβPP content respectively compared to the untreated control. Cells treated with cholesterol exhibited similar quantitative expression of AβPP as the naive control in both lysates and cultured media, with exposure causing slight increases in respective pools of mature and immature AβPP of 1.8% and 5.74% in PC-12AC cells, and a slight decrease of 6.26% in

COS-7 mature AβPP compared to the control. Treatment of both cell lines with CHEMS resulted in a peculiar case where a moderate decrease of 16.27% in total AβPP content in PC-12AC cells was found, while total AβPP was found to increase by 1.23 fold in COS-7 cells. No secreted AβPP was detectable in the cultured media of either cell line post CS treatment, while the remaining three treatment groups exhibited comparable reactivity to probing with both 22C11 and DE2B4 antibodies. Probing lysates with DE2B4 antibody confirmed that CS exposure led to a significant increase in the presence of the $A\beta PP_{\alpha}$ isoform compared to the other treatment groups.

Effects of treatments (C, CS, CHEMS, MeOH) on cell viability were monitored throughout the culturing process by maintaining counts of living cells post trypan blue exclusion (Table 2), with no significant deviation from the average cell viability seen in the untreated cultures.

Figure 5

A) Western blot results showing the effect of CS on AβPP processing in COS-7 cells. Cells were treated with 100μM final concentration (10μM/ml $_{media}$) Cholesterol (C), Cholesterol-Sulfate (CS), Cholesterol-Hemisuccinate (CHEMS), or left untreated. CS induced a significant increase in AβPP content of cells, while appearing to completely stop secretion of AβPP into the media.

B) Visualization of reprobed blot using anti-β-tubulin as a loading control to ensure any differences in detected AβPP are in fact a result of cellular response to sterol treatment and do not arise from a disparity in the masses of crude protein loaded onto the gel for analysis.

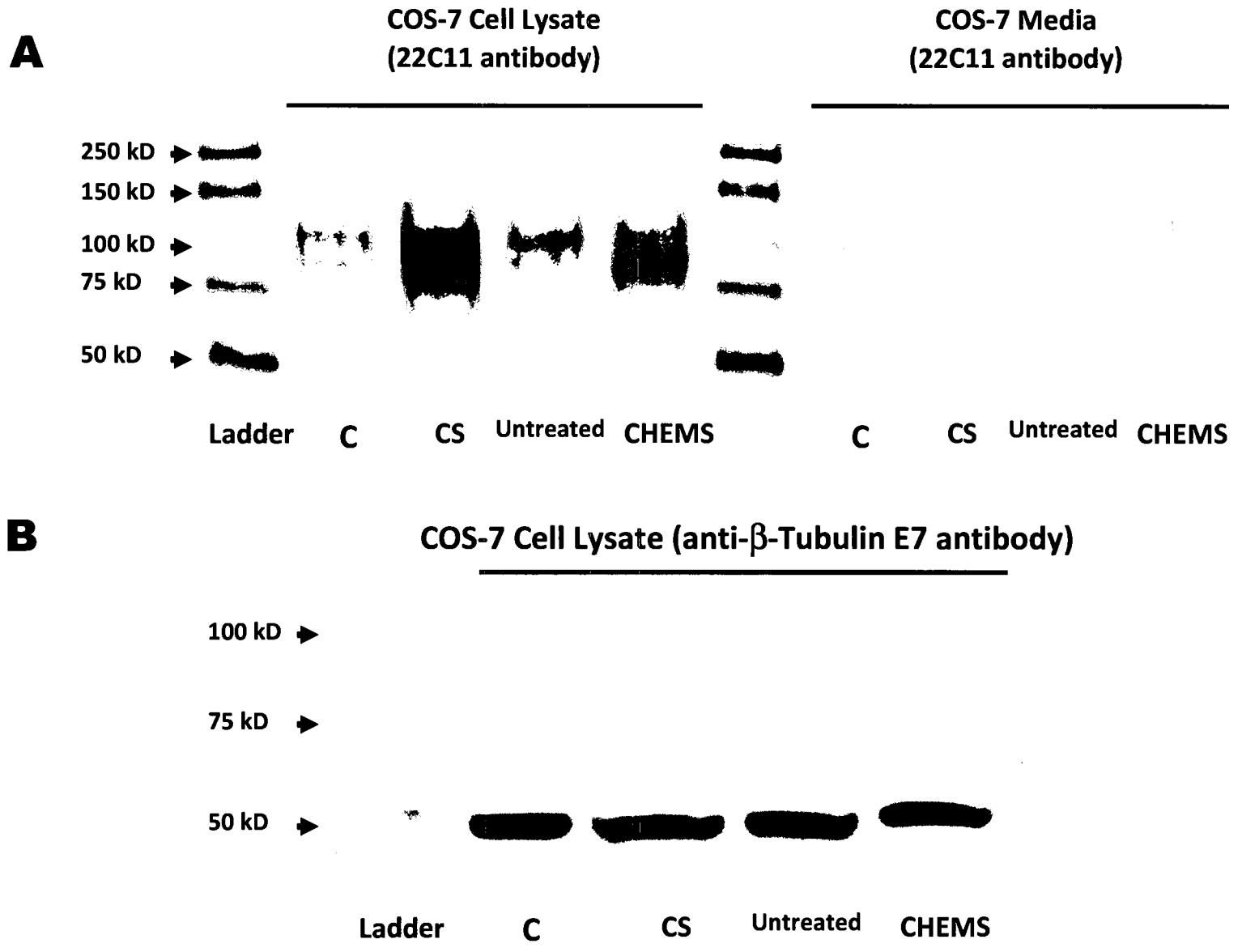

A
COS-7 Cell Lysate
(22C11 antibody)
COS-7 Media
(22C11 antibody)
250 kD
150 kD
100 kD
75 kD
50 kD
Ladder
C
CS
Untreated
CHEMS
C
CS
Untreated
CHEMS
B
COS-7 Cell Lysate (anti-β-Tubulin E7 antibody)
100 kD
75 kD
50 kD
Ladder
C
CS
Untreated
CHEMS

Figure 6

Immunoblot results showing the effect of CS on AβPP processing in PC-12AC cells.

A) PC-12AC lysates probed with 22C11 antibody to detect net-AβPP, large increase in cellular AβPP (both mature and immature isoforms) was seen in cells treated with 100μL of 1mg/mL CS.

B) PC-12AC lysates probed with DE2B4 antibody to detect the $A\beta PP_{\alpha}$ isoform, a significant increase in CS-treated cells suggests increased processing via the non-amyloidogenic pathway.

C) PC-12AC cultured media probed with DE2B4 antibody to detect the secreted $A\beta PP_{\alpha}$ isoform, a complete cessation of secretion was observed for CS-treated cells.

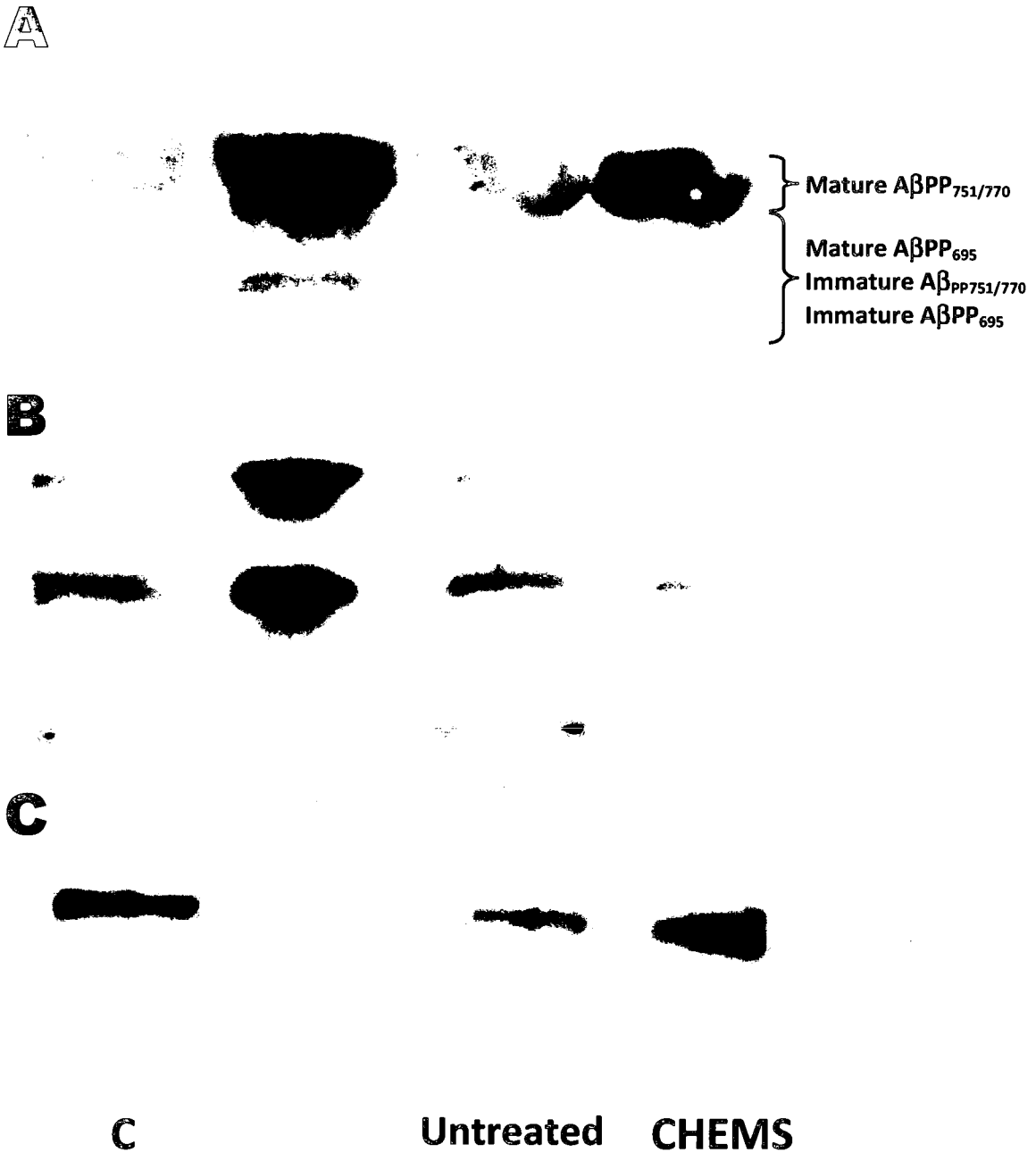

A
Mature AβPP751/770
Mature AβPP695
Immature Aβ PP751/770
Immature AβPP695
B
C
C
Untreated
CHEMS

Table 2

Cell counts and viability ratios of harvested cells determined through trypan blue dye exclusion using a hemocytometer under an inverted microscope.

A) PC-12AC cell culture cell counts of untreated cells compared to counts post treatment with C, CS, CHEMS, and MeOH. Viability was determined by counting cells exhibiting blue staining as non-viable (dead) and clear cells as viable (living), no significant differences in viability were evident. (n=72)

B) COS-7 cell culture cell counts of untreated cells compared to counts post treatment with C, CS, CHEMS, and MeOH. Viability was determined by counting cells exhibiting blue staining as non-viable (dead) and clear cells as viable (living), no significant differences in viability were evident. (n=80)

A

PC-12AC	Treatment Group				
	Untreated	C	CS	CHEMS	MeOH
Cell Number (Cells/mL)	4.28×10^5	4.33×10^5	4.34×10^5	4.24×10^5	4.41×10^5
Viability Ratio (Dead/Living)	0.064	0.067	0.062	0.064	0.066

B

COS-7	Treatment Group				
	Untreated	C	CS	CHEMS	MeOH
Cell Number (Cells/mL)	2.89×10^5	2.85×10^5	2.96×10^5	2.92×10^5	2.91×10^5
Viability Ratio (Dead/Living)	0.058	0.056	0.059	0.055	0.056

3.2 *α-, β-, and γ-Secretase Activity in COS-7 Cells*

Individual enzyme activities were assayed for each of α-, β-, and γ-secretases in COS-7 cells using the fluorogenic quenching substrates described in the methods. The fluorescence intensity of cleaved substrate in CS treated cell lysates was compared to the flouroescence in lysates of cells treated with MeOH as the control. Cells were harvested at 1, 2, 4, 6, 12, and 24h intervals to determine the onset of the AβPP response following sterol treatment (α-secretase activity was not assayed for 1, and 12h time points due to a loss of cultures immediately prior to treatment, additional measures were not taken to reassess the activity at these timepoints following acquisition of the data presented herein). AβPP processing via both β- and γ-secretase enzymes was affected within the first hour of CS exposure, with the activity of β-secretase dropping to 68.8% of the MeOH control within the first hour of exposure and remaining inhibited to a lesser degree for the duration of the 24h treatment window, and γ-secretase activity in relative decline to a minimum of 26.2% of control at 6h post treatment (Figure 8 and 9 respectively). No relevant difference in α-secretase activity could be observed prior to 4h post CS treatment at which point the maximum observed increase in activity of 2.36 fold over the MeOH treated control was recorded (Figure 7), upregulation in α-secretase activity persisted to a lesser extent through the 24h treatment period. Cells treated with C and assayed for α-secretase activity showed a marginal but significant 6.24% upregulation at 4h post treatment and 9.15% downregulation of the enzyme 24h post exposure compared to the MeOH treated

control. Similarly, cholesterol treatment appeared to have a small, but statistically significant, effect on β- and γ-secretase function as well; with β-secretase exhibiting downregulation after 1h (15.25%), 4h (17.8%), 12h (10.4%), and 24h (24.66%), and slight upregulation at 2h (3.09%), while γ-secretase was downregulated at 2h (1.99%), 4h (5.36%), and 12h (9.46%) and upregulated at 6h (5.37%). The additional comparison of secretase-activity profiles between those samples treated with C and those with CS showed that the significance of the cholesterol-sulfate induced upregulation of α-secretase and downregulation of β- and γ-secretases was maintained relative to the effects of cholesterol treatment. The composite graph of secretase activity post CS exposure in Figure 10 allows for efficient identification of the multi-secretase modulating effect leading to the dramatic shift in AβPP processing identified by immunoblot analysis.

Figure 7

Relative α-secretase activity in COS-7 cells treated with cholesterol (C) and cholesterol-sulfate (CS) for intervals of 2, 4, 6, and 24 hours. Determined using a fluorogenic substrate with paired quenching fluorophores ($RE_{(EDANS)}VHHQKLVFK_{(DABCYL)}R$).

Error bars represent standard error of the mean. $*p < 0.01$, $**p < 0.005$, $***p < 0.001$, $****p < 0.0005$.

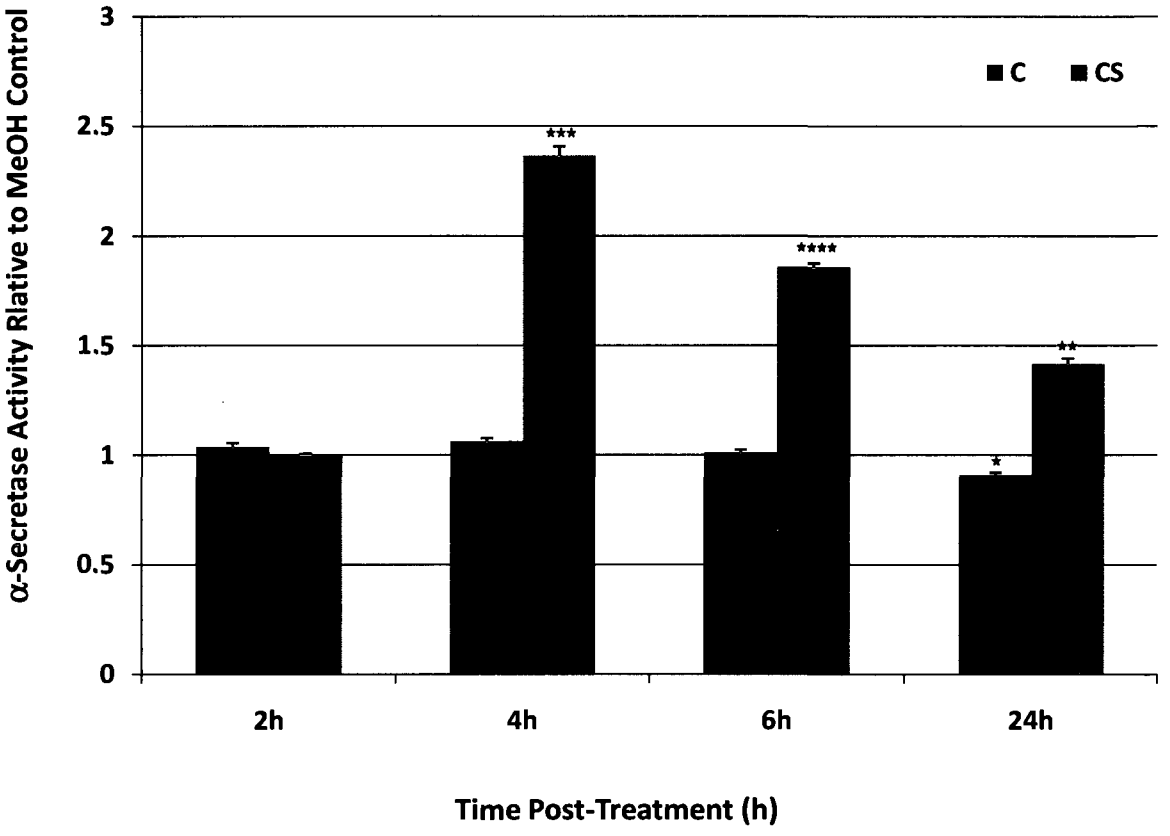

α-Secretase Activity Rlative to MeOH Control
3
2.5
2
1.5
1
0.5
0
C
CS
***
****
*
**
2h
4h
6h
24h
Time Post-Treatment (h)

Figure 8

Relative β-secretase activity in COS-7 cells treated with cholesterol (C) and cholesterol-sulfate (CS) for intervals of 1, 2, 4, 6, 12, and 24 hours. Determined using a fluorogenic substrate with paired quenching fluorophores ($RE_{(EDANS)}EVNLDAEFK_{(DABCYL)}R$).

Error bars represent standard error of the mean. *$p < 0.01$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0005$, *****$p < 0.0001$.

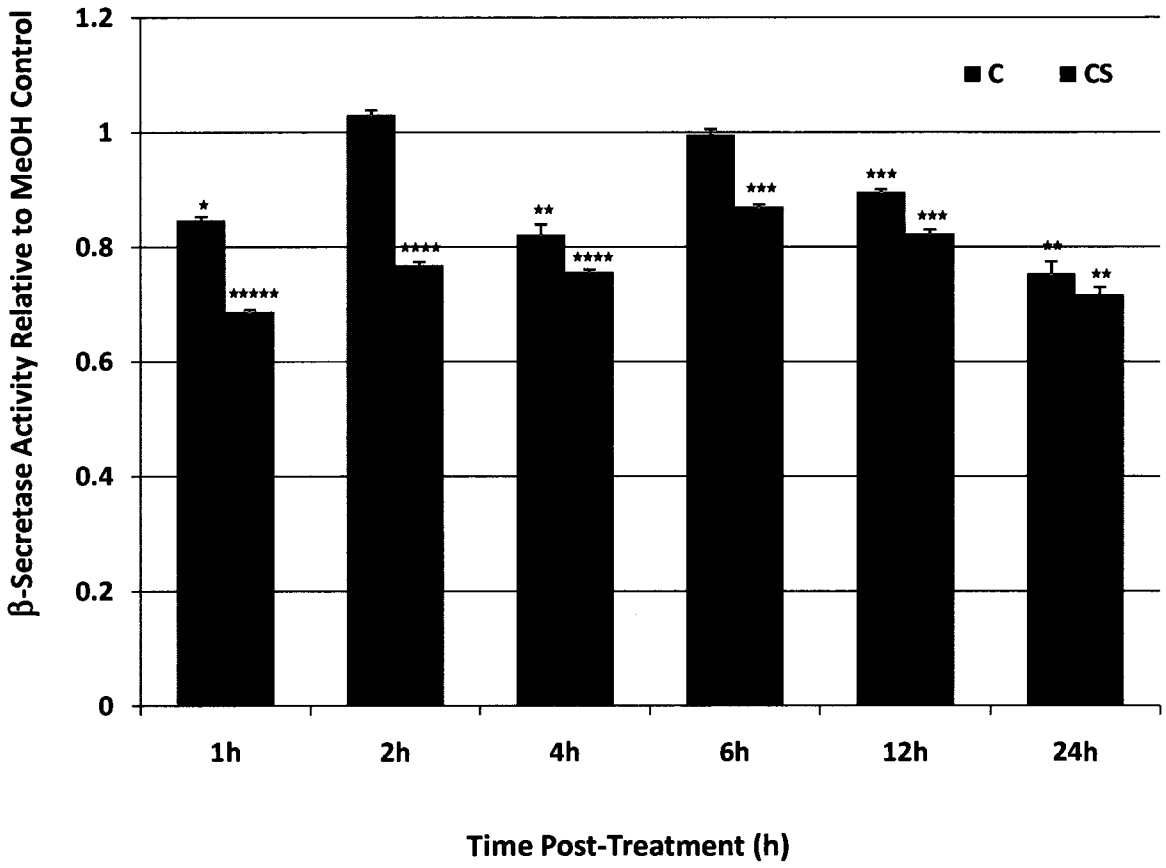

β-Secretase Activity Relative to MeOH Control
1.2
1
0.8
0.6
0.4
0.2
0
C
CS
*
*****
****
**
****
***
***
***
**
**
1h
2h
4h
6h
12h
24h
Time Post-Treatment (h)

Figure 9

Relative γ-secretase activity in COS-7 cells treated with cholesterol (C) and cholesterol-sulfate (CS) for intervals of 1, 2, 4, 6, 12, and 24 Hours. Determined using a fluorogenic substrate with paired quenching fluorophores ($_{(Nma)}$GGVVIATVK$_{(Dnp)}$-*D-Arg-D-Arg-D-Arg*). Error bars represent standard error of the mean. *$p < 0.01$, **$p < 0.005$, ****$p < 0.0005$, *****$p < 0.0001$.

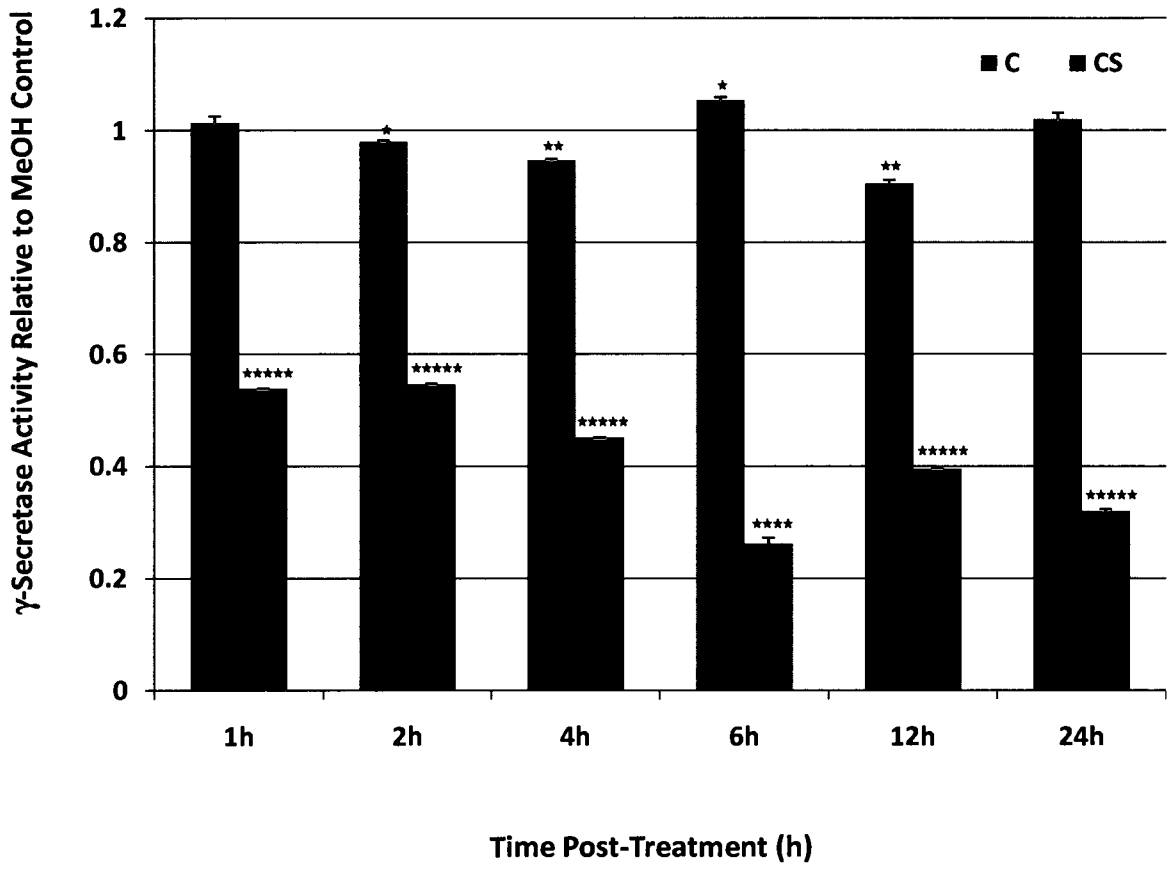
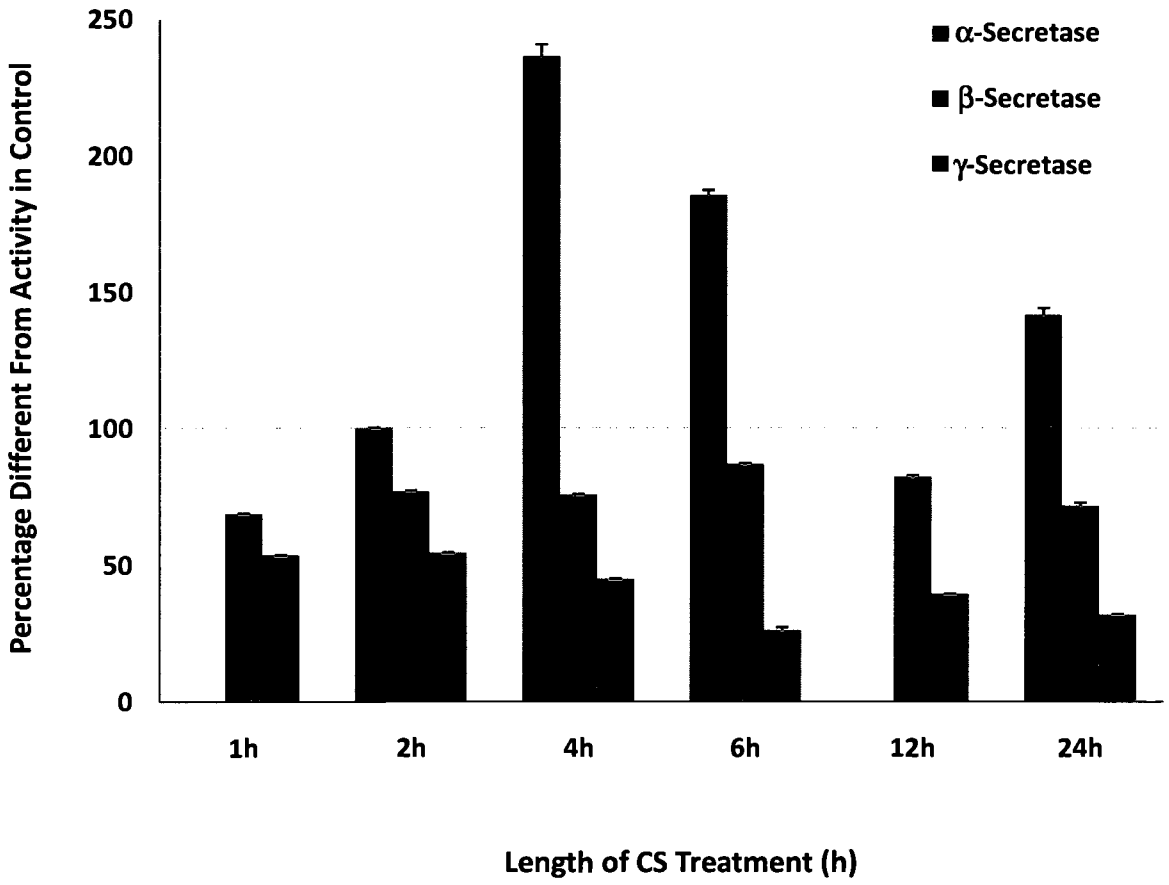

γ-Secretase Activity Relative to MeOH Control
1.2
1
0.8
0.6
0.4
0.2
0
C
CS
*
**
**
*
**
1h
2h
4h
6h
12h
24h
Time Post-Treatment (h)

Figure 10

Summary of α-, β-, and γ-secretase activity in COS-7 cells following CS treatment over a 24 hour period. The line at 100% represents the presumed normal activity from control cells treated with MeOH. Error bars represent standard error of the mean.


250
200
150
100
50
0
Percentage Different From Activity in Control
α-Secretase
β-Secretase
γ-Secretase
1h
2h
4h
6h
12h
24h
Length of CS Treatment (h)

IV. DISCUSSION

4.1 *Sterol Modulation of Secretase Activity*

The investigation of the moderating effect of sterol homeostasis specific to Alzheimer's disease pathology has been gaining momentum since the association of AD risk with the inheritance of the apolipoprotein E4 allele variant (Corder *et al*, 1993). Current research has broadened to cover numerous pathways of sterol action (Canevari and Clark, 2007), with increasing focus on potentially therapeutic properties of cholesterol metabolites (Famer *et al*, 2007; Brown *et al*, 2004). Here we present evidence for modulation of AβPP processing by cholesterol-sulfate, an abundant sterol sulfoconjugate in the body responsible for several regulatory roles and present in mammalian brain tissue (Strott and Higashi, 2003; Liu *et al*, 2003). Treatment of PC-12AC and COS-7 cell cultures with μM concentrations of CS completely abolished secretion of AβPP in addition to significantly increasing the quantity of detectable $A\beta PP_{\alpha}$ produced. Immunoblot results show a great deal of accumulated AβPP in CS treated cells compared to controls, further supporting the hypothesis of CS-induced full length AβPP retention due to cessation of secretion. This phenomenon is predicted to stem from the CS-induced destabilization of membrane lipid domains necessary for embedded secretase proteins to associate and function (likely γ-complexes and to a lesser extent β) (Hur *et al*, 2008; Hattori *et al*, 2006; Hunt and Turner, 2009) along with an upregulation in α-secretase activity due to either increased membrane fluidity (Bodovitz and Klein, 1996; Kojro *et al*, 2001), or CS-mediated activation of PKC signaling

leading to a subsequent increase in the activity of inducible α-secretase enzyme (Kuroki *et al*, 2000; Jolly-Tornetta, and Wolf, 2000; Skovronsky *et al*, 2000). To determine how the activity of the three secretase enzymes was being affected, COS-7 cells were divided into treatment groups of C, CS, MeOH, and an untreated group (the effect of CHEMS was not assayed as immunoblot data from this study did not show consistent changes in AβPP production/secretion as significant as those observed for CS-exposed cells) and subjected to testing of α-, β-, and γ-secretase activity. The comparatively delayed onset of CS-mediated increase in α-secretase activity suggests a different mechanism of regulation from its effects on β-, and γ-secretases, giving potential support for further study of the PKC-mediation hypothesis. The short interval required to see downregulation in the β- and γ-secretase enzymes gives evidence in support of the hypothesis which holds that these enzymes are sensitive to changes in membrane lipid composition and that timely incorporation of CS into the bilayer modulates their ability to process AβPP. The strong downregulation of the γ-secretase complex limits secretion of processed AβPP into the extracellular medium as observed in the immunoblot data; additionally CS-induced downregulation of β-secretase and strong upregulation of α-secretase presumably decreases the production of Aβ some hours following treatment as the ratio of α/β secretase activity more than doubles after 4 hours of CS exposure. The overall effect of CS incorporation then is to drive AβPP processing preferentially through the non-amyloidogenic pathway, potentially abolishing Aβ pathology. The mechanism for the overall shift in processing was not fully uncovered in the present

study, however the evidence presented clearly shows CS works to modulate secretase enzyme function in a manner from which a more refined process of control may be inferred. Other effects on cells treated with sterols were not directly monitored; however the continued observation of cell morphology and viability during treatments did not yield any perceptible differences from untreated controls. While treatment with CS at the dosages in the experiments previously described may likely induce vast changes in cells, it does not appear to have an associated toxicity.

While CS-induction of benign AβPP processing was confirmed from the Aveiro University group's results, the significant effect of CHEMS on AβPP processing, which can be seen in Figure 4, was not observed despite reported similarity to CS in effects on other cellular processes (Ishimaru *et al*, 2008). Due to the similarities between CS and CHEMS, with respect to their anionic head groups, a similar disruptive effect on γ-secretase (and thus secretion of Aβ) following cellular exposure to CHEMS was expected, even if shunting of AβPP processing through the α-pathway did not occur. The most likely explanation for the lack of a CHEMS-induced effect on AβPP processing in this experiment is that unlike CS, CHEMS actually works to stabilize phospholipids in bilayers, increasing the order of the membrane (Massey, 1998). As previously explained, it is the membrane domain destabilizing properties of CS which are thought to induce the downregulation of ordered-lipid domain-dependent enzymes such as β- and γ-secretase. The explanation for the discrepancy in the Aveiro results post-CHEMS

treatment would require further study, however if the sterol is indeed capable of inducing an effect it is likely through a different mechanism than that of CS.

4.2 *Follow-up Investigation*

The correlation of changes in AβPP processing with a reduction in Aβ output can only be made tenuously without direct analysis of Aβ production levels following treatment. Such experiments were attempted; however as production of Aβ by either PC-12AC or COS-7 cell lines was minimal, the immunoblot detection of the peptide using the DE2B4 antibody was made extremely difficult, and unfortunately could not be completed. Future attempts at gauging Aβ response to CS directly must include some form of enrichment of the relatively rare peptide in order to yield meaningful results when interrogating low molecular weight proteins with DE2B4 or another suitable antibody such as 6E10. Additionally the potential upregulation of genes coding for the secretase enzymes or AβPP directly would need to be examined as a possible cause of the CS-induced shift in processing. While based on the short time of onset for changes in secretase activity shown in this study the potential for CS to modulate gene expression is unlikely to be the primary factor in the cessastion of AβPP secretion or $A\beta PP_{\alpha}$ increase, changes in gene expression could be examined by performing reverse-transcriptase PCR analysis to determine if CS induces an increase in mRNA transcripts of secretase enzymes, AβPP or support proteins; 2D gel electrophoresis may also be

performed on cell lysates post treatment to observe changes in protein expression patterns.

It is possible that polar sterol derivatives similar to CS may have comparable effects on AβPP processing; with the status of CHEMS as a modulator of secretase function still unconfirmed it would be prudent to reexamine the possibility using higher calibrated doses in milder solvents to maximize potential for incorporation of the sterol into cells while maximizing post-treatment viability. The investigation of secretase activity in CHEMS treated cells, without initial immunoblot confirmation of secretase modulation, as an indirect means of determining a regulatory relationship between CHEMS exposure and secretase enzyme function may also be undertaken.

A final experiment which had been undertaken without yielding significant results was the attempted visualization using immunofluorescence of any differences in AβPP localization in cells treated with CS compared to controls. Observation of where the internalized AβPP accumulates post treatment would provide a better understanding of what cellular compartments the regulatory step is occurring.

Further investigation of the mechanisms underlying the upregulation of α-secretase and whether the outcomes described in these results may be induced by endogenous deregulation of CS production would allow for a better understanding of the role CS has as a novel promoter of the non-amyloidogenic pathway; such understanding may usher in potential for the use of the naturally occurring sterol derivative in regulation of the AD pathology. A system for tracking membrane CS

content and efficiency of uptake post-treatment would establish the real thresholds necessary for induction of the non-amyloidogenic pathway by CS. Experimentation with the modulation of endogenous machinery for CS production via the HST2B enzyme (Javitt *et al*, 2001) would determine if CS-regulation of AβPP can occur without exogenous addition of the sterol.

Finally the elucidation of the exact mechanism(s) responsible for α-secretase upregulation by CS would be worthwhile. The involvement of PKC signaling induced by CS could be ruled-out or confirmed by setting up an experiment similar to that of Jolly-Tornetta and Wolf (2000) where CS would be used as the potential PKC activator in place of (or compared to) PMA, and the use of PKC inhibitors would allow for the discrimination of PKC-dependent from PKC-independent increases in α-secretase activity. Once it is determined if PKC is involved, a more defined mechanistic representation of how cholesterol-sulfate influences the fate of AβPP can be adopted and expanded upon. The ability of CS to activate PKC would imply that the sterol could have a far greater effect on the cell, going well beyond secretase enzyme modulation. In order to quantify the extent of this effect 2D electrophoresis combined with an antibody recognizing phosphorylated tyrosine and serine/threonine residues could be employed to compare global protein phosphorylation states between CS-treated and untreated cells. Investigation of the effects CS has outside of the secretase-pathway would allow for inferences to be made about possible adverse side-effects that increased levels of the sterol might have on cells or tissues.

4.3 *Conclusions*

The experimental findings presented here support the assertion that CS is a novel enhancer of α-secretase activity, either via modulation of membrane dynamics or through the interactions with a more ubiquitous signal molecule such as PKC, as well as its ability to significantly decrease Aβ secretion from exposed cells via β- and γ-secretase inhibition. With the secretase activity data clearly showing the magnitude to which CS is an inhibitor of the amyloidogenic pathway, as well as the separation of secretase up- or down-regulation, some clarification of the phenomenon occurring in the Aveiro Western blot as well as the immunoblot data collected from this work can be made. It is the combination of α-upregulation and β-, γ-downregulation which makes CS a potentially potent compound for the blockage of Aβ secretion and subsequent aggregation leading to an AD pathology. The relatively minor effects seen in cells treated with cholesterol or CHEMS are an excellent contrast to the effects seen with CS treatment at similar doses. The modulation of secretase function leading to measureable changes in AβPP production and secretion reaffirms the importance of membrane composition to the appropriately benign processing of the Aβ precursor.

V. References

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