Effect of Cholesterol Accumulation on the Metabolism of Amyloid Precursor Protein (APP) in Cultured N2a Cells

by

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder believed to be triggered by the accumulation of β-amyloid (Aβ) peptides derived from the proteolytic processing of amyloid precursor protein (APP). Accumulated evidence has shown that alterations in the levels and/or subcellular distribution of cholesterol can influence Aβ metabolism and development of AD pathology, but the underlying mechanisms remain unclear. A number of recent studies have shown that AD exhibits some striking parallels with Niemann-Pick Type C (NPC) disease – an autosomal recessive disorder caused primarily by loss-of-function mutations in the *NPC1* gene. NPC disease, which is neuropathologically characterized by the intracellular accumulation of cholesterol, exhibits tau-positive neurofibrillary tangles and increased levels of Aβ peptides that are also the hallmarks of AD brains. To determine how alterations of intracellular cholesterol levels/distribution can influence APP processing, we evaluated the effects of U18666A, a class II amphiphile which triggers intracellular redistribution of cholesterol, on cultured $N2a_{wt}$, APP_{wt} and APP_{sw} cells in the presence and absence of different concentrations of fetal bovine serum (FBS). Our results revealed that U18666A treatment, but not increasing concentrations of FBS, triggered accumulation of cholesterol in cultured $N2a_{wt}$, APP_{wt} and APP_{sw} cells. U18666A treatment, but not FBS concentrations, differentially increased levels of APP and its cleaved products α-CTF and β-CTF in $N2a_{wt}$, APP_{wt} and APP_{sw} cells. The levels of APP secreatses and their activities remained unaltered following U18666A treatment in all three cell lines, but the cellular levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ were markedly increased in APP_{wt} or APP_{sw} cells. Collectively, these results suggest that cholesterol accumulation can significantly influence APP levels and its metabolism, especially in cells stably overexpressing either wild-type or mutant human APP.

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ABBREVIATIONS

Aβ	Amyloid-β peptide
AD	Alzheimer's disease
NFT	Neurofibrillary tangle
ADAM	A Disintegrin and Metalloprotease
sAPPα	Soluble amyloid precursor protein alpha
AICD	Amyloid precursor protein intracellular domain
APH1	Anterior pharynx-defective phenotype 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BSA	Bovine serum albumen
BACE	β-site APP cleaving enzyme
C83	83 a.a. residues C-terminal fragment
C99	99 a.a. residues C-terminal fragment
CTF	C-terminal fragment
DMEM	Dulbecco's modified Eagles medium
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NTF	N-terminal fragment
PBS	Phosphate-buffered saline
PEN2	Presenilin enhancer 2
PFA	Paraformaldehyde
PHF	Paired helical filaments
PS1/PS2	Presenilin 1/Presenilin 2
PVDF	Polyvinylidenefluoride
SAD	Sporadic Alzheimer's disease
sAPPβ	Soluble amyloid precursor protein beta
SDS	Sodium dodecyl sulfate

1. Introduction

1.1 Alzheimer's disease pathology

Alzheimer's disease (AD) is a progressive, multifactorial, and heterogeneous neurodegenerative disease with no prevention or treatment at present. Found in one out of eight people aged 65 years and older, it is the most common type of dementia affecting elderly populations and the sixth leading cause of death in the United States of America (Alzheimer's Association, 2012). AD patients are initially affected with mild memory deficits resembling Mild Cognitive Impairment (MCI). As the disease develops, it progressively deteriorates a broad spectrum of cognitive functions including memory, attention, language, and visuospatial functions. In the mid-stage, psychosis and personality changes may also afflict the patients, and the ability to carry out the basic daily activities, such as getting dressed and going to the washroom, becomes impaired. Eventually the patients stop responding to their environment and their motor functions decline (Maulik et al., 2013).

The hallmarks of AD pathology are intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques. NFTs are formed mainly by hyperphosphorylated tau protein, which polymerize into filaments and subsequently become paired helical filaments (PHFs). Under normal physiological conditions, tau, a microtubule-associated protein, binds and stabilizes microtubules *via* a reversible phosphorylation and dephosphorylation process. A phosphorylated tau that has not been dephosphorylated can polymerize and form PHF (Iqbal et al., 1998; Brion et al., 2001). Because the PHF-tau are unable to bind and stabilize microtubules, cellular transport becomes impaired and cells die as a consequence. It has been reported that NFTs are found in large numbers in the brains of AD patients, particularly in the cortex, hippocampus, amygdala, and some subcortical regions (Iqbal et al., 1998; Johnson & Jenkins, 1999; Brion et al., 2001). The number of cortical NFTs has been found to be positively correlated with the severity of dementia (Arriagada et al., 1992).

Extracellular plaques consist of reactive astrocytes, activated microglia, dystrophic neurites, and insoluble beta amyloid (Aβ) peptide deposits (Lewis et al., 2001). The highest densities of these plaques have been found in the most severely damaged brain regions by AD, such as the

entorhinal cortex, neocortex, and hippocampus (Dickson, 1997; Selkoe, 2001), suggesting a direct link between these plaques and AD pathology. Some have reported a correlation between elevated Aβ peptide levels in the brain and cognitive decline (Naslund et al., 2000), and others have shown that the levels of Aβ peptides and amyloid plaques increase before the appearance of other pathology in AD brains (Tanzi, 1996). Fibrillar Aβ peptides can be neurotoxic themselves, (Pike et al., 1993; Selkoe, 2001) or the Aβ aggregates can facilitate the formation of NFTs and, in turn, neuronal death (Mawuenyega et al., 2010). Together, these data support the hypothesis that the accumulation of Aβ peptides is the initiating factor of AD pathology. Anatomically, substantial synaptic and neuronal loss occurs in regions such as neocortex, hippocampus, entorhinal cortex, amygdala, and some subcortical areas including serotonergic neurons in the dorsal raphe and noradrenergic neurons in the locus coeruleus (Dekosky et al., 1996; Lasner & Lee, 1998). Interestingly, cholinergic neurons in the basal forebrain are also severely affected, whereas cholinergic neurons in other areas such as the brainstem or the striatum are not affected until the later stages (Muir, 1997; Lasner & Lee, 1998; Francis et al., 1999).

1.2 Familial AD

While the majority of AD cases appear sporadically after the age of 65 (Hartmann et al., 2007), a small percentage of cases (6-8%) are inherited as an autosomal dominant trait in an early-onset form (Maulik et al., 2013). To date, mutations on three genes [i.e. the Amyloid Precursor Protein (*APP*) gene on chromosome 21, the Presenilin 1 (*PSEN1*) gene on chromosome 14, and the *PSEN2* gene on chromosome 1] have been found to cause early-onset form of AD, accounting for about 50-60% of all known familial AD cases.

All of the APP mutations implicated in familial AD so far have been found within or near the Aβ sequence region. “Swedish” (KM670/671NL) and “Indiana” (V717F) mutations are some examples of these mutations (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Chen et al., 2000). These mutations accelerate the pathology by either increasing the Aβ production or the aggregation of Aβ into β-sheet fibrils. Mutations on *PSEN1/2* have been shown to alter the γ-secretase’s preferred cleavage site, favouring the more toxic $A\beta_{1-42}$ isoform which has a high propensity to aggregate (Borchelt et al., 1997; Holcomb et al., 1998). Many lines of transgenic mice harbouring APP or APP/PS1 mutations exhibit many of the pathological

hallmarks of AD, such as extracellular Aβ plaques, phosphorylated tau, reactive gliosis, and synaptic loss in the regions that are known to be affected by AD in humans (Games et al., 1995; Hsiao et al., 1996; Borchelt et al., 1997; Sturchler-Pierrat et al., 1997; Holcomb et al., 1998; Chen et al., 2000). These studies of familial AD further support the importance of APP metabolism/Aβ production in AD pathology.

1.3 Sporadic AD

Although there is no firm evidence of gene involvement in sporadic cases, some genetic factors do seem to influence the risk of developing sporadic AD. The best known factor is the Apolipoprotein E (ApoE) gene which codes for a protein involved in cholesterol transport. Located on chromosome 19, ApoE gene has three distinct alleles: ε2, ε3, and ε4. Out of these, ε4 alleles dramatically increase the risk for AD while decreasing the age of onset in a gene-dose-dependent manner. Having one or two copies of ApoE ε4 allele indicates 45% and 50-90% chance, respectively, of developing AD by the age of 85 (Reiss & Voloshyna, 2012). In addition, ApoE ε4 allele lowers the age of onset by 7-9 years per allele. The basis of such dramatic effect seems to be in each allele's efficiency in aiding the formation of Aβ fibrils. ApoE has been shown to accelerate Aβ fibrilosis, with ApoE ε4 being the most efficient and ApoE ε2 the least efficient. In a study where the Aβ and ApoE interaction was blocked using a synthetic peptide, the Aβ fibril formation was reduced *in vitro* and the levels of Aβ plaques were lowered *in vivo* (Sadowski et al., 2004). In addition, mice expressing ApoE ε4 and APP have been reported to exhibit heightened levels of Aβ deposits compared with mice expressing no or other ApoE isoforms (Holtzman et al., 2000; Carter et al., 2001). ApoE ε2 is the least efficient at accelerating Aβ fibrilosis, and this allele has even been shown to have a protective effect against AD (Corder et al., 1994).

Research has also demonstrated that ApoE plays an important role in promoting the clearance of Aβ from the brain. Here, the ε4 allele is the least effective, resulting in a higher accumulation of Aβ peptides and increased vulnerability to AD pathogenesis (Dematttos et al., 2004; Bell et al., 2007). A number of genomic studies have also identified several genes involved in cholesterol metabolism that may influence AD pathology. Some of these genes are involved in the transport of cholesterol (ApoJ) (Johnson & Jenkins, 1999; Lambert et al., 2009), the efflux of cholesterol

from the brain (ABCA1) (Katzov et al., 2004; Shibata et al., 2006), the catabolism of cholesterol (cholesterol 24-hydroxylase) (Kolsch et al., 2002), or the esterification of cholesterol (ACAT1) (Bertram et al., 2007). The association of AD with these genes strengthens their connection to cholesterol metabolism, supporting the need to investigate the potential role of cholesterol in the pathogenesis of AD. Other environmental and biological factors such as age, diabetes, traumatic brain injuries, female gender, and stress are also known to increase the risk of developing AD (Kivipelto et al., 2001; Schipper, 2011).

1.4 APP metabolism and Aβ synthesis

Aβ peptides are generated from amyloidogenic processing of APP, a type I integral membrane protein thought to be associated with the regulation of various cellular processes including cell survival/death, synaptogenesis, synaptic plasticity, neuronal excitability, calcium and metal homeostasis, and cell adhesion (Reinhard et al., 2005; O'Brien & Wong, 2011). However, despite being attributed to a range of key cellular processes, deletion of the APP gene in mice does not cause any significant change in their phenotype or their life expectancy (Zheng et al., 1995). Rather than being responsible for certain functions of its own, APP seems to share partially overlapping functions with other members of the APP gene family such as amyloid-precursor-like protein 1 and 2 (ALPL1 and APLP2). In a study, knocking out APP/APLP2 as well as knocking out APLP1/APLP2 caused early postnatal death in mice, whereas knocking out APP/APLP1 did not (Anliker & Muller, 2006). Triple knockout mice lacking APP/APLP1/APLP2 were also found to die shortly after birth, supporting the idea that the members of the APP gene family share the responsibility of certain critical functions in cells (Herms et al., 2004).

The link between APP and AD is built mainly on the role of APP as a precursor for Aβ peptide. Three enzymes are involved in the cleavage of APP: α-secretase called a disintegrin and metalloprotease (ADAM 10 or ADAM 17), β-secretase called β-site APP cleaving enzyme 1 (BACE1), and γ-secretase. The initial cut is carried out by either α- or β-secretase, and the identity of the initial enzyme determines the end product. The enzyme α-secretase cleaves APP within the Aβ domain, thus precluding the production of Aβ. This process, known as the non-amyloidogenic pathway, synthesizes a C-terminal fragment α-CTF which, upon further cleavage

by γ-secretase, leads to the generation of p3 peptide in place of Aβ peptide. Cleavage by β-secretase leaves the Aβ sequence intact in a C-terminal fragment called β-CTF, which is subsequently cleaved by γ-secretase within the transmembrane domain. This process, called the amyloidogenic pathway, leads to the formation of intact Aβ peptide and the amyloid precursor protein intracellular domain (AICD). The cleavage site for γ-secretase varies, and results in Aβ peptides of different lengths (Chavez-Gutierrez et al., 2012). The most common isoforms are $A\beta_{1-40}$ and $A\beta_{1-42}$. $A\beta_{1-40}$, which makes up about 90% of the Aβ peptides, is the soluble type that only slowly converts into an insoluble β-sheet form. On the other hand, $A\beta_{1-42}$ is highly fibrillogenic and more toxic to cells (Selkoe, 2001). The endocytic pathway has been shown to be the main site for Aβ generation, but some Aβ peptides are also synthesized in the membrane, endoplasmic reticulum and golgi apparatus (Koo & Squazzo, 1994; Cook et al., 1997; Greenfield et al., 1999).

The functional γ-secretase contains four factors: nicastrin, presenilin 1/2 (PS1/PS2), anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2). Studies have shown that the PS1/2 is the catalytic subunit that acts as the active site of γ-secretase (Wolfe et al., 1999), and the other subunits are cofactors required for recognizing substrate and assembling the γ-secretase complex. PS1 is stabilized by nicastrin and APH1 and then lastly by PEN2 to form the γ-secretase complex (Iwatsubo, 2004). Given the catalytic role of PS1/2 in the γ-secretase complex and the directness of its action on Aβ synthesis, it makes sense that mutations on PSEN1/2 genes act as culprits for some of the inheritable types of AD.

1.5 Cholesterol

Cholesterol is an essential substance for the body, necessary for the formation of cellular membranes and the synthesis of hormones such as vitamin D, estrogen, and testosterone. In the nervous system, it plays a critical role in neural development and maintaining synaptic plasticity, regulating the formation of synapses, neurite outgrowth, synaptic vesicle transport, as well as neurotransmitter release (Pfrieger, 2003). As an integral part of the plasma membrane, it controls ionic homeostasis, endocytosis, and intracellular signalling mechanisms. The importance of cholesterol in the nervous system is evident in the amount of cholesterol contained in the brain. Twenty-five percent of the total body cholesterol resides in the brain, an organ which only

occupies 2% of the total body mass (Maron et al., 2000; Dietschy & Turley, 2004). About 70-80% of this cholesterol is located in the specialized membranes of myelin, while others are located in the neurons and glia (Martins et al., 2009). Because it serves as a major regulator in such a wide range of cellular functions discussed above, cholesterol itself receives a close monitoring and tight regulation. A failure in cholesterol regulation can lead to a series of critical impairments at the cellular level.

1.6 Cholesterol metabolism in the Brain

In peripheral tissues, cholesterol can either be taken up from the dietary lipids or synthesized in the endoplasmic reticulum. The brain, on the other hand, needs to make its cholesterol *de novo* because of the Blood Brain Barrier (BBB) blocking the entry of peripheral cholesterol into the central nervous system (CNS). Neurons can synthesize most of their own cholesterol during development. However, they gradually lose this ability as they mature and have to rely on the astrocytes which can synthesize 2-3 times more cholesterol than neurons (Maron et al., 2000; Dietschy & Turley, 2004).

The first step of cholesterol synthesis is the conversion of the precursor, acetyl CoA, into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. HMG-CoA is then converted into mevalonate by HMG-CoA reductase, which is the rate-limiting enzyme. In three steps, mevalonate is phosphorylated into isopentyl pyrophosphate and the isomer dimethylallyl pyrophosphate, which are converted into squalene in another three steps. Squalene then becomes catalyzed into lanosterol, which finally turns into cholesterol (Maulik, 2013). This process of cholesterol synthesis is mainly regulated by the levels of cholesterol as well as sterol regulatory element binding proteins (SREBP2) (Maulik, 2013). High cholesterol levels exert a negative feedback on the conversion of HMG-CoA into mevalonate by activating ubiquitination of the enzyme HMG-CoA reductase and causing its proteasomal degradation. High cholesterol levels also exert a feedback inhibition on the SREBPs, the ER-bound transcription factors which

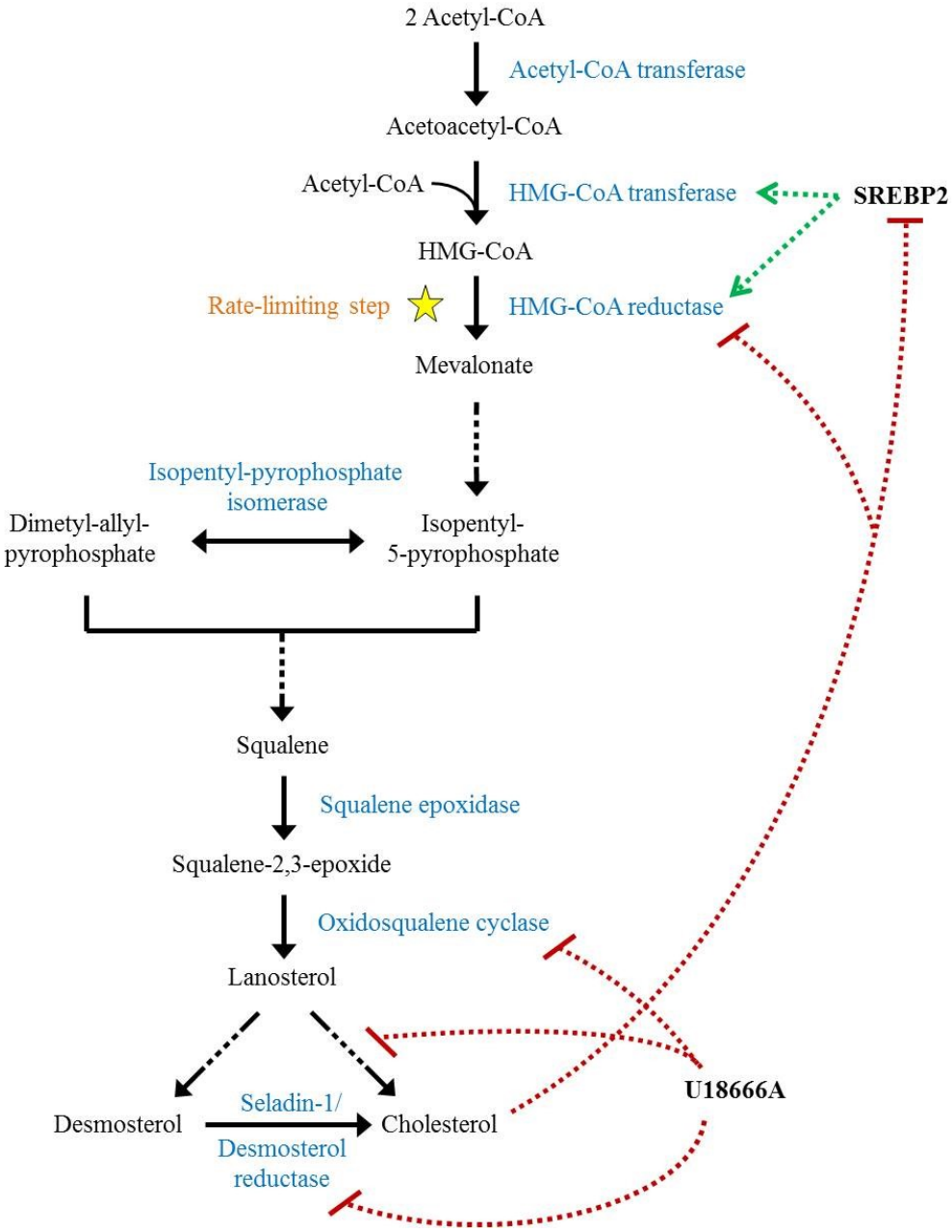


Fig. 1. Main steps involved in the synthesis of cholesterol and the positive/negative feedback exerted by SREBP or U18666A. Low levels of cholesterol triggers SREBP2 to increase the expression of HMG-CoA transferase and HMG-CoA reductase. High levels of cholesterol inactivate the SREBP2 and directly inhibit the action of HMG-CoA reductase as well. U18666A attenuates cholesterol synthesis by disrupting the enzymes oxidosqualene cyclase, sterol Δ8-Δ7 isomerase (involved in the transformation of lanosterol into cholesterol), and desmosterol reductase.

can increase the expression of HMG-CoA synthase and HMG-CoA reductase. Cholesterol regulates SREBP by restricting it to the ER, but when cholesterol is depleted, SREBP cleavage-activating protein (SCAP) interacts with the full-length SREBP in the ER and transports it to the Golgi (Goldstein et al., 2006). Here, two proteases – Site-1 protease (S1P) and Site-2 protease (S2P) – cleave SREBP and release the N-terminal domain, which acts as the transcription factor enhancing cholesterol synthesis.

Once made, cholesterol from astrocytes is transported to neurons *via* an ApoE-dependent mechanism. First, cholesterol and ApoE form complexes and are actively exported from astrocytes *via* members of the ATP-binding cassette (ABC) membrane transport protein family (Karten et al., 2006). Subsequently, ApoE binds to members of the low-density lipoprotein receptor (LDLR) family – e.g. LDLR, low-density lipoprotein receptor related protein (LRP), and very low-density lipoprotein receptor (VLDL) (Beffert et al., 1998) – and the ApoE-cholesterol complexes become internalized into neurons. Once internalized, cholesterol is transported to late endosomal/ lysosomal compartments where it is released as free cholesterol. This free cholesterol then exits the endosomal/lysosomal compartments, via a mechanism involving Neimann Pick type C1 (NPC1) and NPC2 proteins and then transported to other cellular compartments including ER and plasma membrane. In the ER, excess cholesterol gets esterified by acyl-coenzyme-A cholesterol acetyltransferase (ACAT) and stored in lipid droplets in the cytoplasm (Puglielli, 2001; Poirier, 2003). ER also senses the levels of cholesterol and accordingly regulates the expression of genes involved in cholesterol synthesis such as HMGCR and LDLR.

1.7 Eliminating cholesterol

Even though peripheral cholesterol cannot cross the BBB to enter the brain, it is possible for cholesterol to cross from the brain to the periphery. This occurs when excess cholesterol needs to be eliminated from the brain. Because neurons and glia do not have the ability to degrade cholesterol on their own, they dump it into the peripheral circulatory system where it can get collected in the liver and released as bile. Neurons regulate the majority (i.e. approximately two thirds) of cholesterol elimination (Martin et al., 2010). In order for cholesterol to move across BBB, it needs to either be converted into a molecule that can cross the barrier, or else be

transported actively. In most cases the brain converts cholesterol into 24-hydroxycholesterol using cholesterol 24-hydroxylase (Bjorkhem et al., 1998). As a lipophilic metabolite, the 24-hydroxycholesterol can cross the BBB freely. In other cases, cholesterol gets eliminated by a reverse cholesterol transport pathway, where brain cholesterol gets translocated by membrane transport proteins such as ABCA1 (Kim et al., 2007).

1.8 Cholesterol and Neurodegenerative Diseases

Since cholesterol is an essential substance for normal brain function, any compromise in its usually tight regulatory mechanisms can lead to serious problems. One such problem which has been linked to abnormal brain cholesterol metabolism is the impaired functioning/death of neurons observed in neurodegenerative diseases such as AD, Huntington's disease, and Parkinson's disease. Disturbance in cholesterol regulation would also have a dire effect on oligodendrocytes which harbour the majority (70-80%) of cholesterol within the CNS for the synthesis and maintenance of the myelin sheath. Consisting of 80% lipids and 20% protein, the myelin sheath insulates and protects nerves as well as allowing fast transmission of nerve signals. Aberrant cholesterol regulation has the potential to compromise these functions, which can contribute to demyelinating neurodegenerative diseases such as Multiple Sclerosis (Adibhatla & Hatcher, 2007)

Another neurodegenerative disease which has been connected with disturbances in cholesterol regulation is NPC disease. Found in 1 in 120,000 – 150,000, NPC disease is a rare autosomal recessive hereditary disease caused by mutations in the *NPC1* (~95% of the cases) or *NPC2* (~5%) genes. These genes encode NPC1 and NPC2 proteins, which are hydrophobic-polytopic transmembrane proteins necessary for exporting cholesterol from the endosomal-lysosomal system. Thus, the loss-of-function mutations on *NPC1* or *NPC2* gene impair the cholesterol trafficking mechanism and disturb the cell's ability to maintain homeostasis (Walkley & Suzukim, 2004). NPC disease has shown an accumulation of unesterified cholesterol and other lipids such as sphingomyelin, sphingosine, and gangliosides within the late endosomal-lysosomal system in the CNS. A significant dystrophy has been found in the axons of affected neurons and NFTs have been found in the hippocampus, cingulate gyrus, and entorhinal regions of NPC-diseased brains.

Interestingly, NPC patients who carry two copies of ApoE ε4 have been shown to exhibit extracellular Aβ deposits in addition to NFT formation (Saito et al., 2002). The NFTs observed in NPC and AD pathologies are found to be structurally and immunologically indistinguishable. These findings go hand in hand with recent studies which have outlined the striking similarities between NPC and AD, including NFTs, neuroinflammation, altered cholesterol levels, and endosomal-lysosomal abnormalities. Additionally, increased levels of Aβ-related peptides have been reported in the vulnerable brain regions of NPC patients (Jin et al., 2004), suggesting the involvement of Aβ peptides in NPC pathology. Furthermore, altered levels of NPC1 mRNA/protein have been observed in the vulnerable regions of AD brains (Kagedal, 2010). The similarity between NPC and AD has attracted the attention of many researchers investigating the effect of intracellular cholesterol accumulation in AD pathology. NPC mutations have been incorporated into cellular or animal models for AD, which recapitulate many features associated with AD brain, including Aβ pathology. These investigations discovered that intracellular cholesterol accumulation can exacerbate behavioural and pathological features associated with AD (Maulik et al., 2012).

1.9 Cholesterol and AD

Multiple epidemiological studies have provided evidence to support the link between cholesterol and AD. Lower prevalence of AD was found in patients who take statins (a popular cholesterol-lowering drug used to treat cardiovascular disease) in comparison to the total population (Rudel et al., 2001; Zamrini et al., 2004; Haag et al., 2009; Reiss & Voloshyna, 2012). Some have demonstrated that high midlife serum cholesterol levels act as a risk factor for cognitive impairment and AD development, while a study of post-mortem brains found higher levels of plasma LDL in AD patients than in control subjects (Kuo et al., 1998). Brain tissues from AD patients and animal models of AD revealed changes in many different lipids in the affected regions such as prefrontal cortex and entorhinal cortex (Chan et al., 2012), whereas another study revealed higher levels of cholesterol in neurons containing NFTs (Matsuzaki et al., 2011). These observations are further supported by biochemical experiments. For example, putting animals on a high cholesterol diet increases the levels of APP and Aβ peptides, and enhances the formation of Aβ-containing plaques in the affected brain regions such as hippocampus, whereas treating

animals with cholesterol-lowering drugs such as atorvastatin decreases the amount of Aβ deposit/levels in the brain (Refolo et al., 2001; Petanceska et al., 2002; Grhibi et al., 2006).

Another line of evidence that has tied cholesterol to AD is genomic studies involving genes regulating cholesterol metabolism. As mentioned earlier, the ApoE gene is by far the most important risk marker for AD. Out of the three alleles (i.e. ε2, ε3, and ε4), ε4 allele increases the risks of AD in a dose-dependent manner; possessing one or two copies of ApoE ε4 allele represents 45% or 50-90% chance of developing AD by the age of 85 (Petanceska et al., 2001). Along with the ApoE gene, which codes for the protein involved in cholesterol transport, many other genes related to cholesterol metabolism and regulation such as ApoJ, ABCA1, and ACAT1 have been associated with AD. Despite the positive correlation between cholesterol levels and AD pathogenesis, some studies have found Aβ levels/deposits are unaffected or even decreased in animals on high cholesterol diet (George et al., 2004) or after statin treatment (Höglund et al., 2004). However it is worth noting that many of these studies only examined the total cholesterol level. There has been evidence showing that the influential factor in AD is the increase of cholesteryl esters (Puglielli, 2001).With the contradictory data in the field, we believe that a continued effort to understand better the role of cholesterol in the development of AD pathology is critical.

1.10 Cholesterol and APP metabolism

If cholesterol indeed plays a role in the development of AD, how does it influence the pathology? To address this question, many investigators have studied the effect of aberrant cholesterol regulation on APP metabolism. Some found that increased cholesterol level/accumulation enhances Aβ levels by increasing the amount of APP being metabolized *via* the amyloidogenic pathway. Additionally, some recent reports in the literature have suggested a role for lipid raft regions in APP metabolism and as an important connection between cholesterol and AD pathology.

Lipid rafts are membrane microdomains enriched with cholesterol and sphingolipids. These microdomains play an integral role in regulating membrane trafficking, cell migration and a number of cell signalling cascades (Korade & Kenworthy, 2008). The makeup of lipid rafts gives

them a detergent-insoluble character, which distinguishes them from non-raft regions. Many studies have demonstrated that the influence of cholesterol disturbance on APP metabolism stems not only from the change in total cholesterol levels, but also from the distribution of cholesterol within neurons (Runz et al., 2002; Davis, 2008; Kosicek et al., 2010). It is believed that α-secretase is mainly localized in the low-cholesterol regions of the cellular membrane, whereas β- and γ-secretases reside in cholesterol-rich lipid-raft domains. Their substrate, APP, is thought to exist in two distinct pools. The bigger pool lies within various intracellular organelles, while a smaller pool is linked to the detergent-resistant rafts. It has been suggested that, in a normal physiological condition, the majority of APP is metabolized through a non-amyloidogenic pathway because α-secretase has a better access to the larger pool of substrates. However, when cellular cholesterol increases and/or the amount of cholesterol in lipid raft region increases, the β- and γ-secretases gain better access to their substrates and the amyloidogenic processing is promoted (Ehehalt et al., 2003). There is also evidence that the same effect is achieved by lipid rafts clustering together in response to an increase in cholesterol levels (Marquer et al., 2011). In support of this hypothesis, antibodies cross-linking APP and BACE1 co-patched with lipid raft markers showed a significant increased production of Aβ peptides in a cholesterol-dependent manner. Apart from the localization of β-secretase activity to the lipid rafts, inhibition of γ-secretase results in an accumulation of APP-CTFs in the raft domains. (Ehehalt et al, 2003)

1.11 U18666A and its effect on cholesterol

The connection between cholesterol and AD has been studied primarily by i) creating an environment where there is increase/decrease cholesterol compared to normal, ii) performing a genetic mutation which endogenously interferes with cholesterol metabolism, leading to decreased/increased cellular cholesterol levels, or iii) administering a drug that alters cholesterol metabolism and lowers/heightens cellular cholesterol levels. One such drug which has been used, to some extent, in studying the relationship between cholesterol and APP metabolism is U18666A - a class II amphiphile known to mimic NPC disease-related phenomena by triggering redistribution of cholesterol to the endosomal-lysosomal system.

The compound U18666A was initially studied as a potential hypocholesterolemic drug due to its ability to inhibit cholesterol biosynthesis (Cenedella, 2009). At least three enzymes involved in sterol metabolism are disrupted by the drug: i) oxidosqualene cyclase, which catalyzes the transition of squalene-2,3-epoxide into lanonsterol; ii) sterol Δ8-Δ7 isomerase (emopamil binding protein (EBP) in vertebrates), which is involved in the transformation of lanosterol into cholesterol (catalyzes the shift of the C_{8-9} double bond of zymosterol to C_{7-8}) (Moebius et al., 1998); and iii) desmosterol reductase, which catalyzes the conversion of desmosterol into cholesterol (Bae & Paik, 1997). In 1983, Sexton et al. found that U18666A inhibits oxidosqualene cyclase, which causes diversion of its substrate, squalene oxide (SO), into squalene dioxide (SDO) (Sexton et al., 1983). A year later, the same group reported that adding a low concentration (<1μg/ml) of U18666A into the culture media decreases the activity of HMG-CoA reductase, whereas a higher concentration of this drug has an opposite effect (Panini et al., 1984). They concluded that low levels of U18666A only partially inhibit oxidosqualene cyclase, thus simultaneously allowing for the formation of SDO as well its conversion into polar sterols, which impede HMG-CoA reductase. In contrast, high levels of U18666A can fully inhibit the cyclase so that SDO cannot be converted into polar sterols. The same study which observed the inhibition of desmosterol reductase by U18666A also found that, at higher concentrations, the drug has an inhibitory effect at an additional site preceding the formation of squalene (Bae & Paik, 1997).

Another important effect of U18666A is the disruption of cholesterol trafficking. It broadly interferes with the pathways involved in intracellular trafficking of cholesterol, especially the movement out of lysosomes (i.e. lysosome to plasma membrane, lysosome to ER, and plasma membrane to ER). This effect was discovered when researchers observed the abolition of negative feedback on HMG-CoA reductase normally caused by low density lipoprotein (LDL). In normal physiological conditions, internalized LDL gets degraded by lysosomes and the resulting esterified cholesterol is subsequently de-esterified into free cholesterol. The free cholesterol is then released by lysosomes and suppresses the activity of HMG-CoA reductase in the ER. In cells treated with U18666A, this release from lysosomes does not happen and therefore free cholesterol accumulates in lysosomes. The exact mechanism of inhibition by U18666A still remains unclear. However it is notable that the degree of inhibition by U18666A

is different for each part of the trafficking pathway. The concentration of U18666A which reduces lysosome-ER trafficking by 90% has been shown to reduce the lysosome-PM trafficking by only 10% (Nixon & Cataldo, 2006). Overall, the direct effect of U18666A on cholesterol synthesis itself is complex. Nevertheless, it provides a useful model for intracellular cholesterol accumulation through redistribution from the ER and the plasma membrane into endosomal-lysosomal system.

1.12 U18666A and APP metabolism

A few studies have taken advantage of the effect of U18666A to examine the link between cholesterol and APP metabolism. These attempts mostly found a reduction in the secretion of Aβ peptides in both primary and cultured cell lines (see Table 1), suggesting the involvement of cholesterol trafficking system in APP metabolism. However, these studies produced contradictory results in other aspects of this system, with some reporting an increase of $A\beta_{1-42}$ production paired with increased intracellular β-CTF levels, and others reporting reduced Aβ synthesis paired with decreased intracellular β-CTF levels. Such inconsistencies in current literature may be due to the use of different experimental paradigms such as the type of cells and the growth/treatment condition. For example, composition of the culture media determines what nutrients are available for the growth/survival of cells which may subsequently influence the way cells respond to experimental variables. Fetal bovine serum (FBS) is a widely used media supplement which contains various nutrients including growth factors, cholesterol, and other lipids. Varying the concentration of FBS in the media would change the exogenous levels of cholesterol which may be an important factor to consider in studies investigating the effect of intracellular cholesterol accumulation on APP metabolism.

Considering the aforementioned evidence, we want to verify whether cholesterol accumulation triggered by U18666A can differentially influence APP metabolism with varying FBS concentrations in the media. To address this issue we used three lines of cells: a well-characterized mouse neuroblastoma ($N2a_{wt}$) cells, N2a cells overexpressing wild-type human APP (APP_{wt}), and N2a cells overexpressing human APP carrying Swedish mutation (APP_{sw}). Swedish mutation represents double mutations of APP gene near the site for BACE1 cleavage which causes early onset familial AD by enhancing Aβ production. Use of APP_{wt} and APP_{sw}

cells would allow us to compare the effect of cholesterol on cell overexpressing normal *vs* mutant human APP that underlies the cause of predisposition to familial AD. $N2a_{wt}$ cells express native mouse APP serve as control for the transfected cells. In APP_{wt} and APP_{sw} cells, the expression of human APP is approximately 15-20 times higher than the baseline mouse APP, thus allowing for most of the visible changes to be attributed to the human APP.

1.13 Hypothesis

Many studies, albeit contradictory, have provided compelling evidence that cholesterol contributes to AD pathology by influencing Aβ synthesis. Intriguing similarities between AD and NPC diseases lend credence to cholesterol's significance in regulating amyloidogenesis. We believe that part of the variability of cholesterol's role on APP metabolism may be due to the cell types used and/or the experimental paradigms applied. Thus, we hypothesize that **endosomal-lysosomal accumulation of cholesterol which may partly depend on the conditioned media can influence APP metabolism by regulating amyloidogenic processing of APP**. To address this hypothesis, we evaluated the effect of U18666A on:

a) the levels/expression of APP and Aβ peptides
b) the levels/expression and activity of α- and β-secretases
c) lipid rafts and its association to APP metabolism

Table 1. Effects of U18666A on APP processing under *in vitro* conditions

Cell type used	Experimental condition	Effects on APP metabolism	References
i) Cultured primary mouse cortical neurons expressing APP695	i) Serum-free Neurobasal medium plus B27 supplement and maintained at 37°C in 5% CO_2 24hr treatment with 3μg/ml U18666A	i) ↑intracellular levels of β-CTF/C99 i) ↑intracellular levels & aggregation of $Aβ_{1-42}$	Jin et al., 2004
ii) MC65	ii) Serum-free Opti-MEM medium without tetracycline to induce β-CTF/C99 expression for 24 hours, followed by 24 hr treatment with 3μg/ml U18666A	ii) unchanged intracellular $Aβ_{1-40}$ ii) unchanged γ-secretase activity ii) unchanged intracellular C99 levels	
i) N2a-APP_{wt}	i) DMEM/OptiMem I (50:50) supplemented with 2 mM glutamine and 1% Penstrep	i): ↑APP	Warren Davis Jr., 2008
ii) N2a-APP_{sw}	ii) DMEM/OptiMem I (50:50) supplemented with 2 mM glutamine and 1% Penstrep	ii): ↑APP; ↑secretion of sAPPα ↑intracellular levels of α-CTF/C83 ↓secretion of $Aβ_{1-40}$ ↓intracellular levels of β-CTF/C99	

i) SH-SY5Y cells expressing APP695	i) Maintained in DMEM/5% fetal calf serum (FCS); FCS boosted to 10% for 24hr treatment with 50 μg/ml LDL and 3μg/ml U18666A	i) ↓intracellular levels of β-CTF ↑ γ-secretase activity ↓secretion of $A\beta_{1-40}$ & $A\beta_{1-42}$ ↓intracellular levels of Aβ ↓secretion of sAPP unchanged intracellular APP	Runz et al., 2002
i) CHO cells expressing APP751	i) Maintained in DMEM containing 10% FCS, 24hr treatment with 3μg/ml U18666A	i) ↑intracellular levels of $A\beta_{1-42}$	Yamazaki et al., 2001

2. Materials and Methods

2.1 Materials

U18666A was purchased from Enzo Life Sciences, Inc. (Ann Arbor, MI, USA). Opti-MEM® I Reduced-Serum Medium, Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin (10,000 U/mL), Fetal Bovine Serum (FBS), Geneticin® Selective Antibiotic (G418 Sulfate, 50 mg/mL), Protease inhibitor, Phosphatase inhibitor, 4-12% NuPAGE Bis-Tris gels, Prolong Gold Antifade reagent, Amplex Red Cholesterol Assay Kit, Enzyme-Linked Immunosorbent Assay (ELISA) kits for the detection of human $A\beta_{1-40}$, human $A\beta_{1-42}$, mouse $A\beta_{1-40}$, and mouse $A\beta_{1-42}$ were purchased from Life Technologies Corp. (Burlington, ON, Canada). Bicinchoninic Acid Protein Assay (BCA) kit, Enhanced Chemiluminescence (ECL) kit, and Restore Western Blot Stripping Buffer were purchased from Thermo Fisher Scientific (Montreal, QC, Canada). Information for all primary antibodies used in the study are listed in Table 2.1. All horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Paso Robles, CA). All other chemicals used in our study were from Sigma-Aldrich or Thermo Fisher Scientific.

2.2 Cell culture

All cells (i.e. $N2a_{wt}$, APP_{wt} and APP_{sw})

were maintained in DMEM/OptiMem I (50:50) supplemented with 2 mM glutamine and 1% Penstrep. These cell lines, obtained as gift from Dr. G. Thinakaran (University of Chicago, IL, USA) were first plated in media containing 5% FBS for 48 hours. Subsequently, the cells were treated with or without 3µg/ml U18666A in media containing 0%, 5%, or 10% FBS to trigger intracellular accumulation of cholesterol. After 24 hour treatment, cells were washed and collected in phosphate-buffered saline (PBS, pH 7.4) and then centrifuged at 400*g* for 5 minutes. The supernatant was discarded and the pellets were processed according to the protocol for each experiment.

2.3 Filipin

To determine intracellular accumulation of cholesterol, control and U18666A-treated cells from various experimental conditions were washed in PBS (pH 7.4) and fixed with 4%

paraformaldehyde before 1 hour incubation with filipin (dilution 1:1000). After washing with PBS, slides were mounted with ProLong Gold Antifade Reagent, and then viewed and photographed with a Zeiss Axioskop-2 microscope (Carl Zeiss, Germany).

2.4 BCA Assay

Protein content of each sample was determined using a BCA kit following the manufacturer's instructions. The absorbance values were converted into concentration of protein using a standard curve. All samples were processed in triplicate.

2.5 Western blot

After 24 hour incubation with or without 3µg/mL U18666A in media containing 0%, 5%, or 10% FBS, cells (i.e. $N2a_{wt}$, APP_{wt}, and APP_{sw}) were washed and collected as described above. Cultured cells were sonicated in ice-cold Radioimmunoprecipitation Assay (RIPA) buffer containing 1X protease inhibitor, and then centrifuged for 10 minutes at 10,000*g*. Only the supernatants were collected and their protein concentrations were determined using BCA assay. All protein samples were normalized and processed for Western blot as described earlier (Maulik et al., 2012). In brief, 10-15µg of protein was separated on 7-17% gradient polyacrylamide gels or 4-12% NuPAGE Bis-Tris gels. After separation, the proteins were transferred onto Polivinylidene Fluoride membranes overnight, and then blocked with 10% skimmed milk for 1 hour. The membranes were then washed 3 times, 10 minutes each in Tris-Buffered Saline containing 0.2% Triton-X (TBST) and incubated overnight at 4°C with the primary antibodies at dilutions listed in Table 2.1. After Incubation, membranes were washed in TBST and exposed for 1 hour with the appropriate HRP-conjugated secondary antibody (1:2000) in 5% milk and immunoreactive proteins were detected using an ECL detection kit. Blots were stripped with stripping buffer and re-probed with anti-β-actin or anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antisera to validate the loading control. Subsequently blots were quantified using a MCID image analyzer (Imaging Research, Inc.) as described earlier (Kodam et al., 2010). All experiments were repeated at least three to four times.

2.6 ELISA for human and mouse $A\beta_{1-40}$ and $A\beta_{1-42}$

All materials used for this assay were from ELISA kits specified to detect specific Aβ peptide. After 24 hour incubation with or without 3μg/mL U18666A in media containing 0%, 5%, or 10% FBS, cultured cells (i.e. $N2a_{wt}$, APP_{wt}, and APP_{sw}) were collected and processed with respective standard as described earlier (Maulik et al., 2015; Wang et al., 2015).

2.6.1. Detection of human $A\beta_{1-40}/A\beta_{1-42}$: The plates were incubated with detection antibody for 3 hours at room temperature, washed and then exposed to HRP anti-rabbit antibody for 30 minutes at room temperature. Subsequently, plates were incubated with stabilized chromogen for an additional 30 minutes. The reaction was terminated using stop solution, absorbance was measured at 450nm. The absorbance values were then converted into concentration of Aβ using the standard curve. Each experiment was replicated 2 to 3 times in duplicate.

2.6.2. Detection of mouse $A\beta_{1-40}/A\beta_{1-42}$: The ELISA plates were incubated for 2 hours at room temperature. One hour incubation with detection antibody was followed by 30 minutes incubation with HRP anti-rabbit antibody, and 30 minutes with stabilized chromogen. Subsequently, the stop solution was added and the absorbance was read at 450nm. The absorbance values were converted into $A\beta_{1-40}/A\beta_{1-42}$ concentrations using the standard curve. All experiments were repeated 2-3 times in triplicates.

2.7 Cholesterol assay

After 24 hour incubation in media containing 0%, 5%, or 10% FBS with or without 3μg/mL U18666A, control and treated cells (i.e. $N2a_{wt}$, APP_{wt}, and APP_{sw}) were washed and collected as described above. Subsequently, cells were sonicated in PBS containing 1X protease inhibitor and centrifuged at 10,000*g* for 10 minutes. Only the supernatants were collected and their protein concentrations were determined using BCA assay. The amount of total and free cholesterol was determined using Amplex Red cholesterol assay kit. In brief, Amplex Red reagent, HRP, and cholesterol oxidase were added to control and experimental samples and standards. After incubation, fluorescence was measured using excitation at 550nm and emission at 590nm. These

fluorescence readings were converted into cholesterol concentration using a standard curve. All experiments were done in duplicate.

2.8 Cell fractionation

After 24 hour incubation with 5% FBS with or without 3µg/mL U18666A, control and APP_{sw} cells were washed with PBS three times in order to eliminate all media from interfering with the assay. Cells were then collected in TNE buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, pH7.4) containing 2X protease inhibitor and lysed in 1% Triton X using 22G and 25G needles. After normalizing the amount of protein with BCA, the lysate was centrifuged for 5 minutes at 10,000*g*. Only the supernatant was used to build a gradient. The cellular components were separated by 24 hour centrifugation at 100,000*g* in 5%, 30%, and 40% sucrose gradient created by OptiPrep (Sigma Aldrich, St. Louis, MO, USA). The resulting gradient was divided into 13 fractions of 380µl each.

2.9 Dot blot

Nitrocellulose membranes were activated in 1X TBS buffer and samples were loaded for gravity filtration. After 45 minutes, samples were vacuum filtered and each well was washed 3 times with 1X TBS, followed by 3 times with 1X TBST. The membrane was allowed to dry completely before 1 hour blocking with 10% (w/v) milk. Subsequently the membrane was incubated for 30 minutes with cholera toxin (1:1000). After the incubation, the membrane was washed with 1X TBST three times, 10 minutes each. Cholera toxin bound to mono-sialo ganglioside 1 (GM-1), a raft marker, was detected using ECL kit. For the detection of other proteins on the dot blots, membranes were treated using the same procedure as described in Western blot.

2.10 Data analysis

Data are expressed as means ± SEM. Statistical significance of differences was determined by ANOVA followed by Bonferroni's post-hoc analysis for multiple comparisons or unpaired two-tailed Student's t-test for single comparison with a significance set at $p<0.05$. All analyses were performed using GraphPad Prism Software.

Table 2. Details of the primary antibodies/Filipin used in this study

Antibody	Type	Dilution	WB Dilution	Source
APP (clone Y188)	Monoclonal	n/a	1:5000	Abcam Inc.
BACE1	Polyclonal	n/a	1:2000	Abcam Inc.
ADAM10	Polyclonal	n/a	1:2000	EMD Millipore Co.
Nicastrin	Polyclonal	n/a	1:800	Santa Cruz
PS1	Polyclonal	n/a	1:2000	Dr. G. Thinakaran
Pen2	Polyclonal	n/a	1:2000	Dr. G. Thinakaran
APH1	Polyclonal	n/a	1:800	Dr. G. Thinakaran
β-Actin	Monoclonal	n/a	1:5000	Sigma-Aldrich, Inc
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Monoclonal	n/a	1:3000	Sigma-Aldrich, Inc
Filipin	n/a	1:2000	n/a	Sigma-Aldrich, Inc

WB; Western blot

1. Results

3.1 Effects of FBS and U18666A on cholesterol redistribution in cultured N2a cells

Filipin, which specifically labels unesterified cholesterol, makes it possible to visualize distribution of cholesterol within cells. Under normal conditions, no accumulation of cholesterol was evident in either $N2a_{wt}$, APP_{wt} or APP_{sw} cells. Faint filipin labeling appeared throughout the cytoplasm of control cells, whereas their cell membranes showed a somewhat increased intensity of staining. Culture media containing 10%, 5% or 0% FBS did not alter filipin labelling in any of the three cell lines used in the studied (Figs. 2A-C, 3A-C, 4A-C). The intensity of labelling, however, markedly increase in all three cell lines following 24 hour exposure to 3µM U18666A (Figs. 2D-F, 3D-F, 4D-F). The staining, unlike the control cells, appeared to be aggregated mostly in the endososomal-lysosomal compartments as reported in earlier studies (Amritraj et al., 2009). Interestingly, no marked alteration in the intensity of staining was evident among three cell lines following exposure to UA18666A. Additionally, U18666A treatment did not influence either the distribution or intensity of filipin staining when $N2a_{wt}$, APP_{wt} and APP_{sw} cells were cultured in media containing 10%, 5% or 0% FBS (Figs. 2D-F, 3D-F, 4D-F).

3.2 Influence of FBS and U18666A on cellular cholesterol levels in $N2a_{wt}$, APP_{wt} and APP_{sw} cells: To determine whether cellular levels of cholesterol are altered in $N2a_{wt}$, APP_{wt} or APP_{sw} cells cultured in different FBS concentrations, or following 24 hour treatment with 3µM U18666A we measured total and free cholesterol levels using a commercially available cholesterol assay kit (Fig.5A-C). Interestingly, we observed an increasing trend in the levels of total and free cholesterol with higher FBS conditions in all three cell lines but none of them reached a statistically significant difference. U18666A treatment also did not alter either total or free cholesterol levels in any of the cell lines grown in media containing 10% or 5% FBS. Surprisingly, U18666A treatment significantly decreased the levels of cholesterol when $N2a_{wt}$, APP_{wt} and APP_{sw} cells were grown in cultured media containing 0% FBS (Fig.5A-C).

3.3 Differential effects of FBS and U18666A treatment on APP metabolism in $N2a_{wt}$, APP_{wt} and APP_{sw} cells: To investigate how cholesterol redistribution can influence APP metabolism in $N2a_{wt}$, APP_{wt} and APP_{sw} cells, we first measured steady state APP holoprotein levels using

Western blotting in cells grown with 10%, 5%, and 0% FBS-containing media. In parallel, we also evaluated the influence of U18666A treatment on APP metabolism in $N2a_{wt}$, APP_{wt}, and APP_{sw} cells grown with 10%, 5%, and 0% FBS-containing media (Fig. 6A-C). In $N2a_{wt}$ cells, we did not observe any significant alteration in APP levels when cells were cultured with 10%, 5% or 0% FBS media. Furthermore, U18666A treatment resulted in no marked changes in APP levels either at 10%, 5% or 0% FBS compared to respective controls (Fig 6A). In keeping with $N2a_{wt}$ cells, normal APP_{wt} cells showed no marked alteration in APP levels with increasing concentration of FBS. However, U18666A treatment significantly enhanced APP levels at 0% and 5% FBS but not when cells were cultured with 10% FBS (Fig. 6B). As for APP_{sw} cells, no marked alteration in APP levels, as observed with APP_{wt} cells, was evident with increasing concentrations of FBS. Moreover, U18666A treatment significantly enhanced the levels of APP when cells were grown with either 10%, 5% or 0% FBS. It is also of interest to note that the magnitude of change was more at 0% followed by 5% and10% FBS conditions (Fig. 6C).

In keeping with altered APP expression, we subsequently analysed levels of APP cleavage products α-CTF and β-CTF, generated by α- and β-secretases, in control and U18666A-treated $N2a_{wt}$, APP_{wt}, and APP_{sw} cells grown with 10%, 5%, and 0% FBS-containing media (Fig. 7A-C, 8A-C). In normal $N2a_{wt}$ cells, the levels of α-CTF did not alter significantly with increasing concentrations of FBS in the cultured media. However, U18666A treatment significantly enhanced the levels of α-CTF at 10% FBS, but not when cells were grown with either 0% or 5% FBS conditions (Fig. 7A). Normal APP_{wt} cells, as observed for $N2a_{wt}$ cells, did not depict any altered levels of α-CTF either at 0%, 5% or 10% FBS conditions. U18666A treatment, on the other hand, significantly enhanced α-CTF levels in all cultured conditions compared to untreated controls (Fig. 7B). The steady state levels of α-CTF did not alter significantly with increasing concentrations of FBS in APP_{sw} cells. As observed with APP_{wt} cells, U18666A treatment significantly enhanced α-CTF levels in all conditions, and the relative change exhibited a parallel increase with increasing concentrations of FBS (Fig. 7C).

The levels of β-CTF exhibited more or less similar changes as α-CTF in cultured $N2a_{wt}$, APP_{wt} and APP_{sw} cells grown with 10%, 5% and 0% FBS (Fig. 8A-C). The $N2a_{wt}$ cells did not exhibit any alterations in β-CTF levels either with increasing concentrations of FBS or following

treatment with U18666A (Fig. 8A). On the contrary, both APP_{wt} (Fig. 8B) and APP_{sw} (Fig. 8C) cells showed significantly increased levels of β-CTF following 24 hour treatment with 3μM U18666A in all cultured conditions used in the study. Additionally, the relative increase was found to be higher with increasing concentrations of FBS in both APP_{wt} and APP_{sw} cells (Fig. 8B, C).

3.4 Influence of FBS and U18666A on APP secretases in $N2a_{wt}$, APP_{wt} and APP_{sw} cells: Since levels of α-CTF and β-CTF are differentially altered in $N2a_{wt}$, APP_{wt} and APP_{sw} cells independently as well as in conjunction with U18666A treatment, we measured the steady levels of α-secretase ADAM10 (Fig. 9A-C), β-secretase BACE1 (Fig. 10A-C), and components of the γ-secretase complex (Figs. 11A-C, 12-A-C) using Western blotting. The steady state levels of ADAM10 did not exhibit any significant variation in any of the cell lines either with increasing concentrations of FBS or following treatment with 3μM U18666A for 24 hours (Fig. 9A-C). Similar results were obtained with BACE1 in cultured $N2a_{wt}$, APP_{wt} and APP_{sw} cells (Fig. 10A-C). The levels of four components of the γ-secretase complex which are involved in the processing of α-CTF and β-CTF were subsequently evaluated in cell lysates obtained from various experimental conditions. However, we did not observe any significant alterations in the steady state levels of the catalytic subunit PS1 (Fig. 11A-C) or other complex components, i.e, nicastrin (Fig.12A-C), APH1 (data not shown) and Pen2 (data not shown) either with increasing concentrations of FBS or following treatment with 3μM U18666A for 24 hours in any of the three cell lines.

3.5 Influence of FBS and U18666A on α-secretase and β-secretase activity: A number of earlier studies have indicated that activity of enzymes involved in APP processing can be altered in the absence of any changes in enzyme levels (Thinakaran et al., 1997; Thinakaran and Koo, 2008). Thus, we evaluated activities of α-secretase ADAM10 and β-secretase BACE1 in $N2a_{wt}$, APP_{wt} and APP_{sw} cells grown in 5% FBS with or without U18666A treatment (Fig. 13A, B). Interestingly, no significant alteration was observed in ADAM10 activity in any of the cell lines following treatment with 3μM U18666A (Fig. 13A). Similarly, we did not observe any marked alteration in β-secretase activity in $N2a_{wt}$, APP_{wt} and APP_{sw} cells cultured with 5% FBS following treatments with 3μM U18666A for 24 hours (Fig. 13B). These data indicate that

higher levels of α-CTF and β-CTF observed in our study may possibly be due to decreased clearance of these proteins or decreased activity of γ-secretase enzyme.

3.6 Influence of FBS and U18666A on $A\beta_{1-40}$ and $A\beta_{1-42}$ in $N2a_{wt}$, APP_{wt} and APP_{sw} cells: To determine the influence of cholesterol on intracellular Aβ levels in $N2a_{wt}$, APP_{wt}, and APP_{sw} cells, we first measured $A\beta_{1-40}$ and $A\beta_{1-42}$ levels using ELISA in cells grown in 10%, 5%, and 0% FBS-containing media. Additionally, we also evaluated the effects of U18666A treatment on $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the aforementioned three cell lines grown in 10%, 5%, and 0% FBS-containing media (Figs. 14A-C, Fig. 15A-C). In $N2a_{wt}$ cells, we did not observe any significant alteration in $A\beta_{1-40}$ or $A\beta_{1-42}$ levels with increasing concentration of FBS in the cultured media. Furthermore, U18666A treatment did not induce significant alterations in $A\beta_{1-40}$ or $A\beta_{1-42}$ levels either in 10%, 5% and 0% FBS compared to respective controls (Figs. 14A, 15A). Unlike $N2a_{wt}$ cells, normal APP_{wt} cells showed a gradual increase in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels with increasing concentrations of FBS. Additionally, U18666A treatment drastically enhanced the levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$ in 0%, 5% and 10% FBS (Figs. 14B, 15B). The normal APP_{sw} cells, as observed with the APP_{wt} cell line, showed a gradual increase in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels with increasing concentrations of FBS. Furthermore, U18666A treatment markedly enhanced the levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$ when cells were grown in 10%, 5% or 0% FBS. Interestingly, the magnitude of increase in U18666A-treated cells was found to be enhanced with higher concentrations of FBS (Figs. 14C, 15C). Additionally, we observed that U18666A treatment increased $A\beta_{1-40}$ (Fig. 14B, C) and $A\beta_{1-42}$ (Figs. 15B, C) levels more profoundly in APP_{sw} cells than those observed with APP_{wt} cells.

3.7 Influence of FBS and U18666A on lipid raft distribution in $N2a_{wt}$, APP_{wt} and APP_{sw} cells: Assimilated evidence suggests that the α-secretase ADAM10 resides mostly in the low-cholesterol containing non-raft domains, whereas a subset of BACE1 and γ-secretase complex are associated with cholesterol-rich lipid-raft microdomains of the plasma membrane and intracellular organelles. APP, on the other hand, exists in both the raft and non-raft microdomains of the cellular membranes. These observations raised the possibility that amyloidogenic *vs* non-amyloidogenic processing of APP occurs in different microdomains of the membrane, but an alteration in the distribution of APP or its processing enzymes may influence

APP processing (Kojro et al., 2001; Wahrle et al., 2002; Ehehalt et al., 2003; Vetrivel et al., 2004; Cheng et al., 2007). To determine whether U18666A treatment can influence distribution and/or processing of APP in the raft *vs* non-raft domain, we analyzed control and U18666A-treated APP_{sw} cells cultured with 5% FBS by lipid-raft fractionation. Cells were lysed with 1% Triton X-100 on ice followed by discontinuous Optiprep gradient centrifugation to separate the detergent-resistant microdomains from the detergent-soluble non-raft domains. The presence of major constituents of lipid-rafts, namely prion protein in fractions 3-5, and non-raft marker transferrin receptor in fractions 8-12 validated our fractionation protocol. Consistent with published studies, a fraction of APP and its metabolite CTFs were found in Triton X-100-resistant raft fractions in control and U18666A treated APP_{sw} cells. Overall, the relative amounts of APP and APP-CTFs found in lipid-raft *vs* non-raft fractions were found to be higher after U18666A treatment, but statistically insignificant.

Fig. 2. In control $N2a_{wt}$ cells, faint filipin labeling appeared throughout cell cytoplasm, with somewhat increased intensity of staining on the cell membrane (A-C). Culture media containing 10%, 5% or 0% FBS did not markedly alter filipin labelling in $N2a_{wt}$ cells used in the study. However, the intensity of labelling increased following 24 hour exposure to 3μM U18666A and the staining appeared to be aggregated in the endosomal-lysosomal compartments as expected. Interestingly, U18666A treatment did not influence either the distribution or intensity of filipin staining in $N2a_{wt}$ cells cultured in media containing 10%, 5% or 0% FBS.

Fig. 2

$N2a_{wt}$

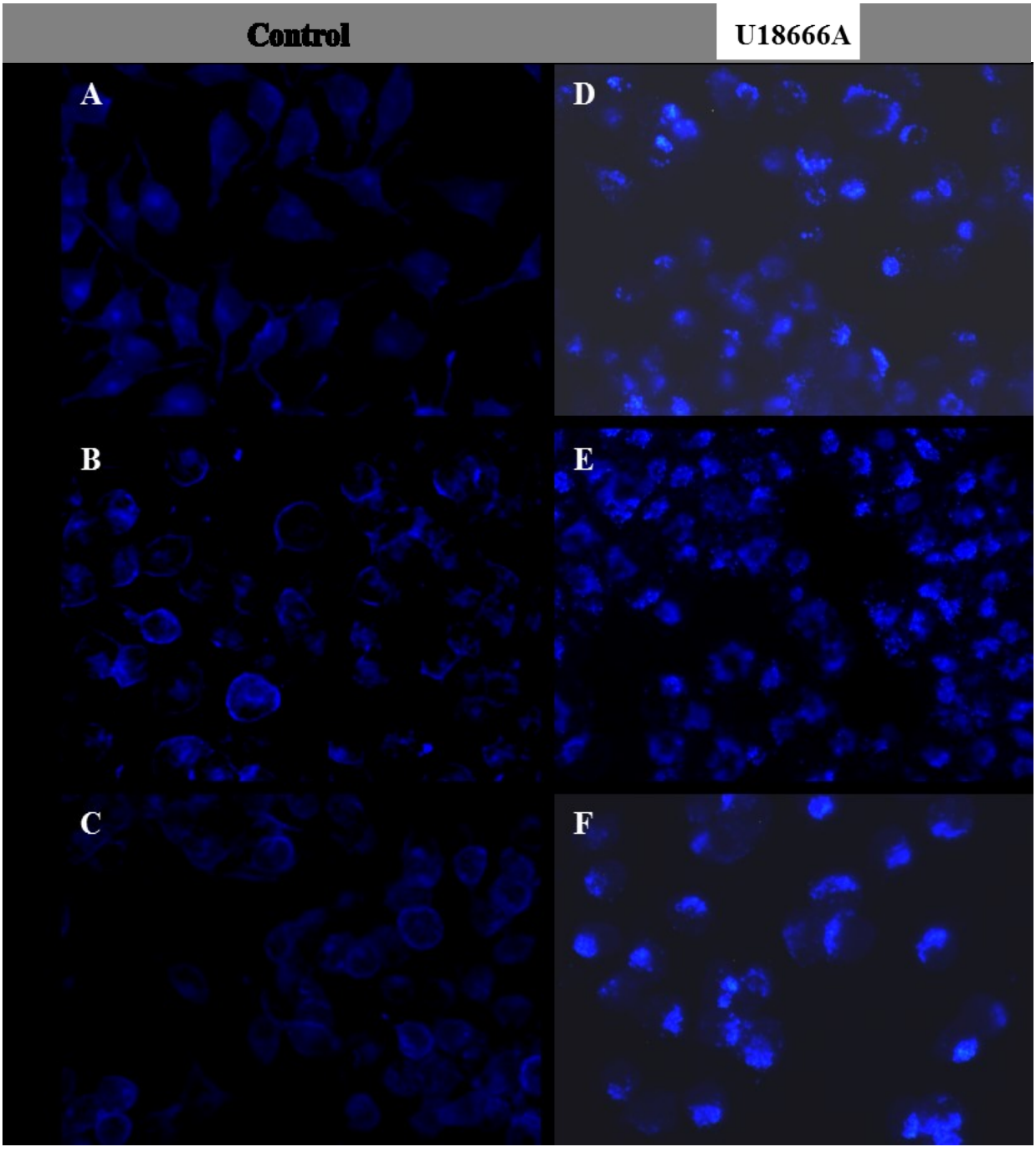


Fig. 3. In control APP_{wt} cells, faint filipin labeling appeared throughout cell cytoplasm, with somewhat increased intensity of staining on the cell membrane (A-C). Culture media containing 10%, 5% or 0% FBS did not alter filipin labelling in APP_{wt} cells used in the study. However, the intensity of labelling increased following 24 hour exposure to 3μM U18666A and the staining appeared to be aggregated in the endosomal-lysosomal compartments as expected. Interestingly, U18666A treatment did not influence either the distribution or intensity of filipin staining in APP_{wt} cells cultured in media containing 10%, 5% or 0% FBS.

Fig. 3

APP_{wt}

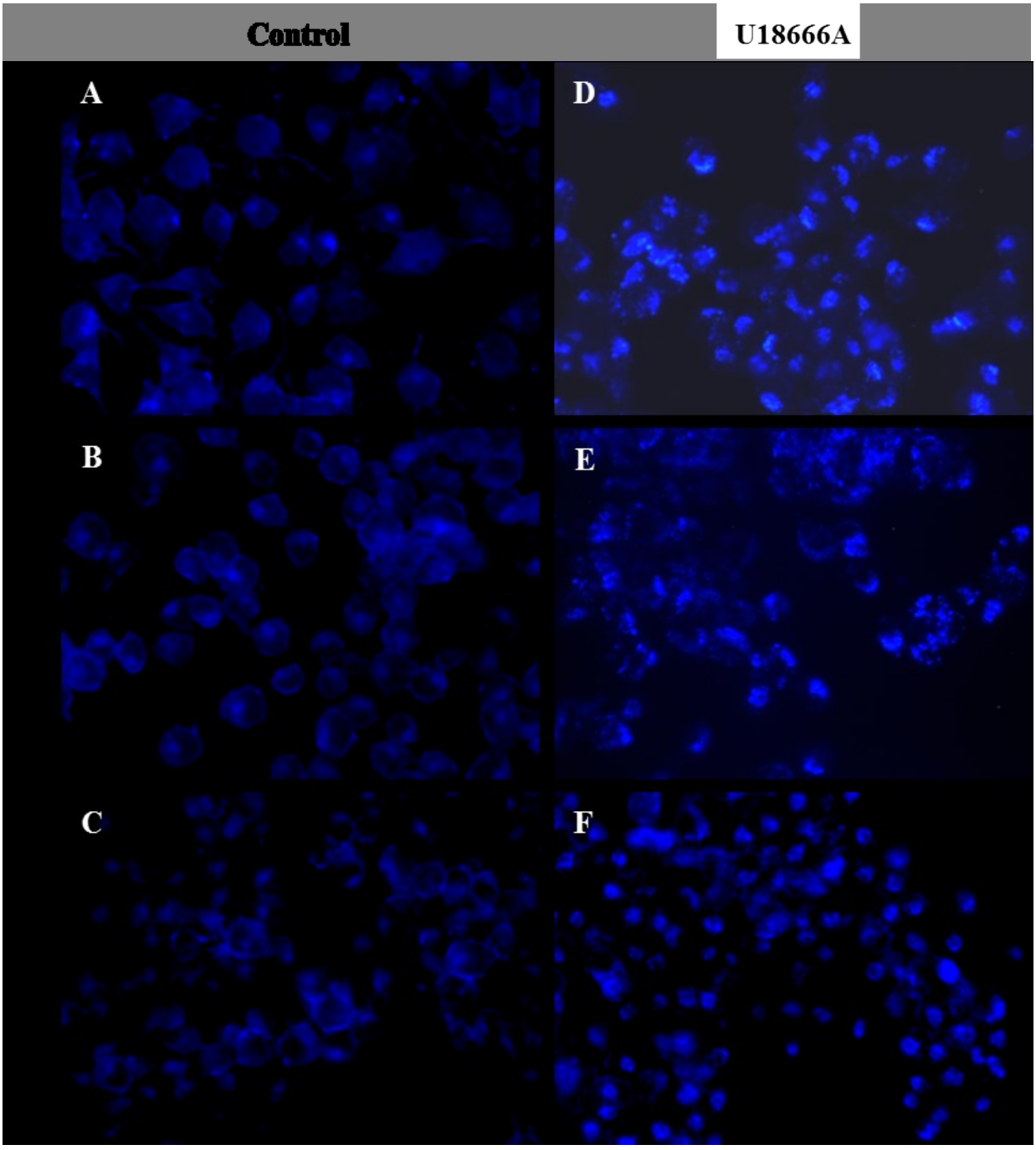


Fig. 4. In control APP_{sw} cells, faint filipin labeling appeared throughout cell cytoplasm, with somewhat increased intensity of staining on the cell membrane (A-C). Culture media containing 10%, 5% or 0% FBS did not alter filipin labelling in APP_{sw} cell line used in the study. However, the intensity of labelling increased following 24 hour exposure to 3μM U18666A and the staining appeared to be aggregated in the endosomal-lysosomal compartments as expected. Interestingly, U18666A treatment did not influence either the distribution or intensity of filipin staining in APP_{sw} cells cultured in media containing 10%, 5% or 0% FBS.

Fig. 4

APP_{sw}

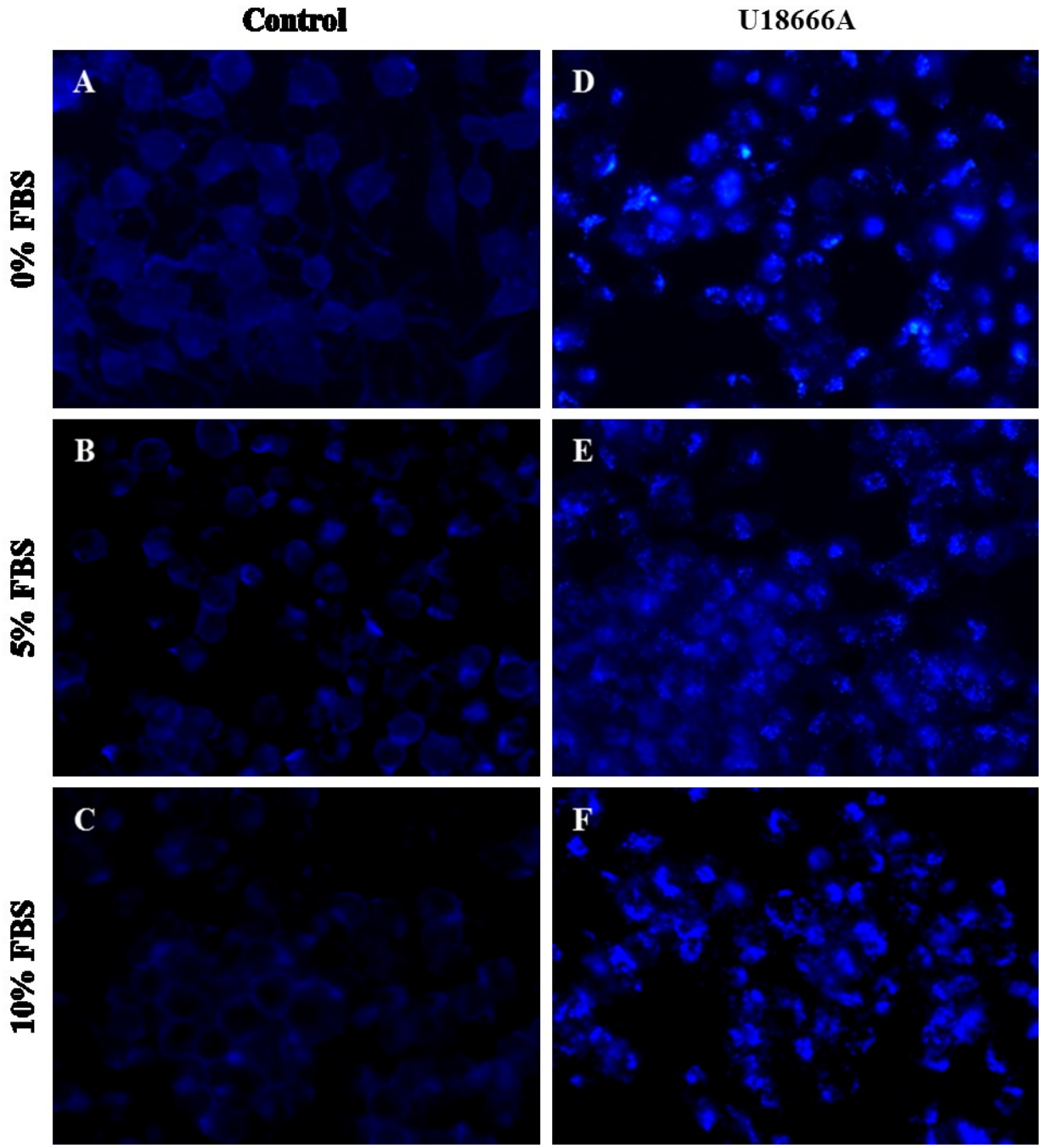


Fig. 5. U18666A treatment significantly decreases the levels of total and free cholesterol at 0% but not at 5% or 10% FBS conditions in $N2a_{wt}$ (**A**), APP_{wt} (**B**), and APP_{sw} (**C**) cells compared to respective untreated control cells. Bars represent means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Fig. 5

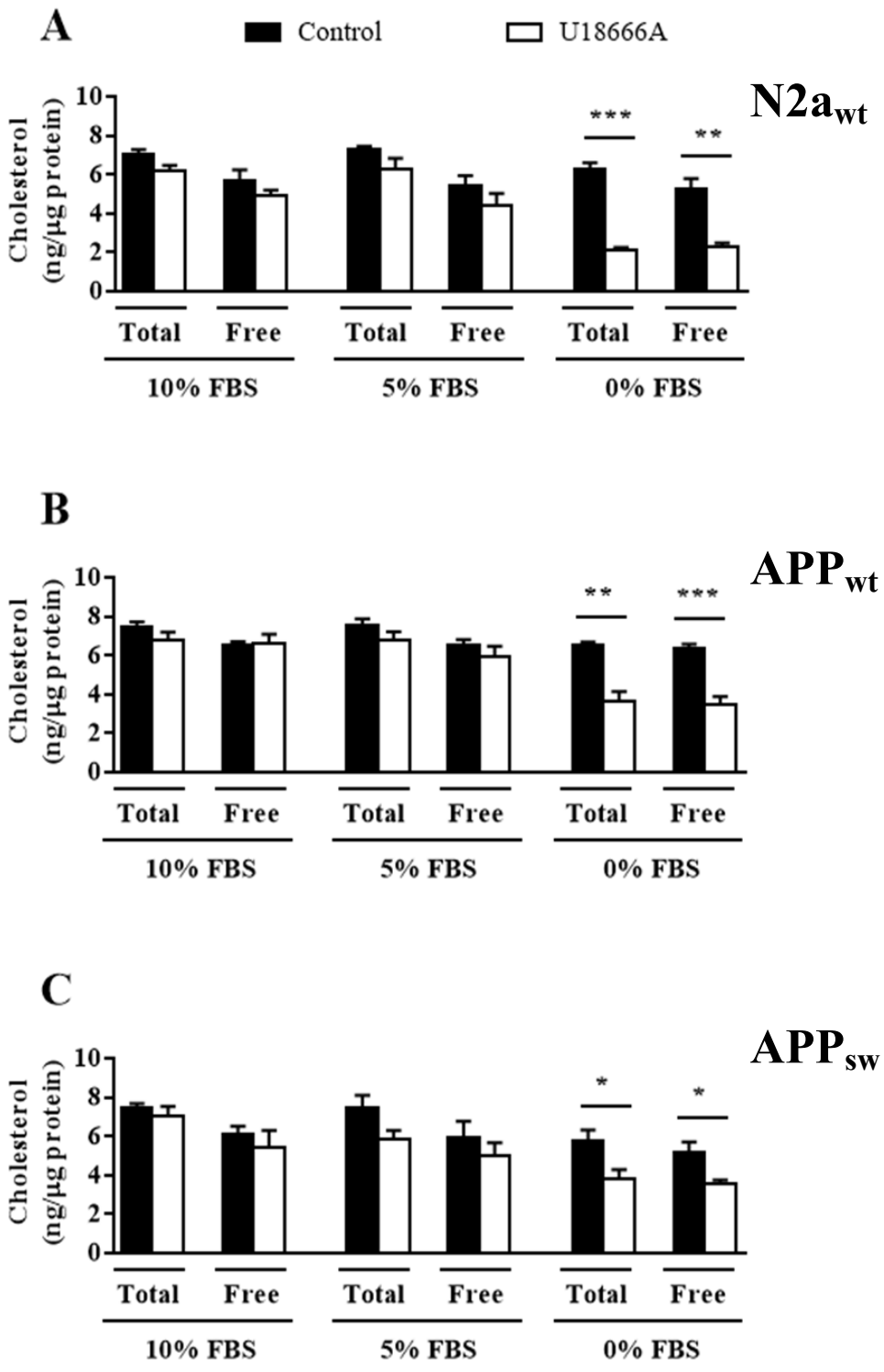

A
Control
U18666A
N2a$_{wt}$
Cholesterol (ng/µg protein)
10
8
6
4
2
0
***
**
Total
Free
Total
Free
Total
Free
10% FBS
5% FBS
0% FBS
B
APP$_{wt}$
Cholesterol (ng/µg protein)
**
***
Total
Free
Total
Free
Total
Free
10% FBS
5% FBS
0% FBS
C
APP$_{sw}$
Cholesterol (ng/µg protein)
*
*
Total
Free
Total
Free
Total
Free
10% FBS
5% FBS
0% FBS

Fig. 6. Levels of APP holoprotein are not changed following U18666A treatment in cultured $N2a_{wt}$ cells at any FBS conditions (**A)**, but increased only at 5% and 10% FBS in APP_{wt} cells (**B)** and at 0%, 5% and 10% FBS in APP_{sw} cells (**C)**. Bars represent means ± SEM. ***P < 0.01,* ****P < 0.001*

Fig. 6

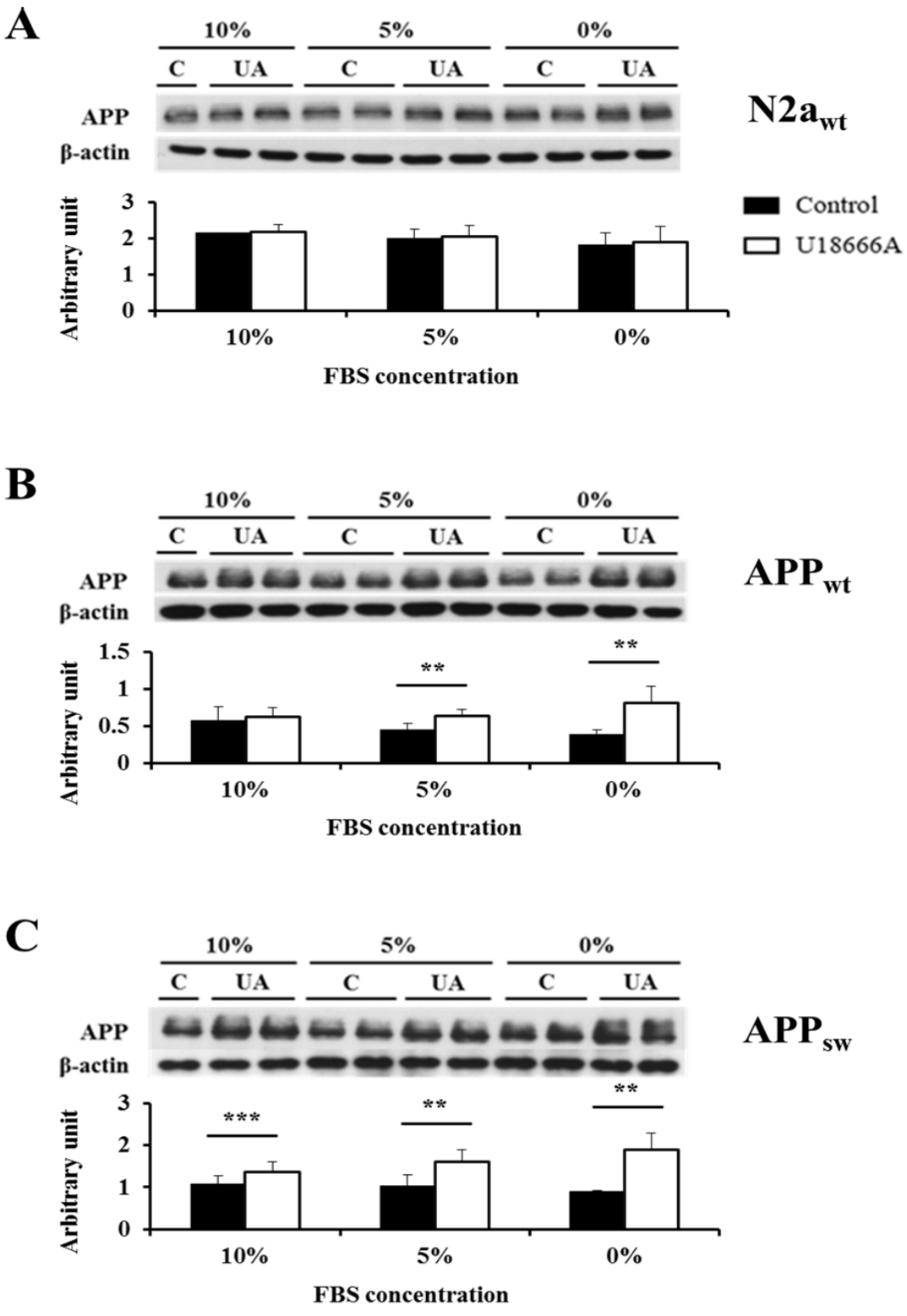

A
10%
5%
0%
C
UA
C
UA
C
UA
APP
β-actin
N2a$_{wt}$
Control
U18666A
Arbitrary unit
3
2
1
0
10%
5%
0%
FBS concentration
B
10%
5%
0%
C
UA
C
UA
C
UA
APP
β-actin
APP$_{wt}$
Arbitrary unit
1.5
1
0.5
0
**
**
10%
5%
0%
FBS concentration
C
10%
5%
0%
C
UA
C
UA
C
UA
APP
β-actin
APP$_{sw}$
Arbitrary unit
3
2
1
0
***
**
**
10%
5%
0%
FBS concentration

Fig. 7. Levels of α-CTFs are increased in all three media conditions in both APP_{wt} (**B**) and APP_{sw} cells (**C**), but remain unchanged in $N2a_{wt}$ cells (**A)**. Bars represent means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Fig. 7

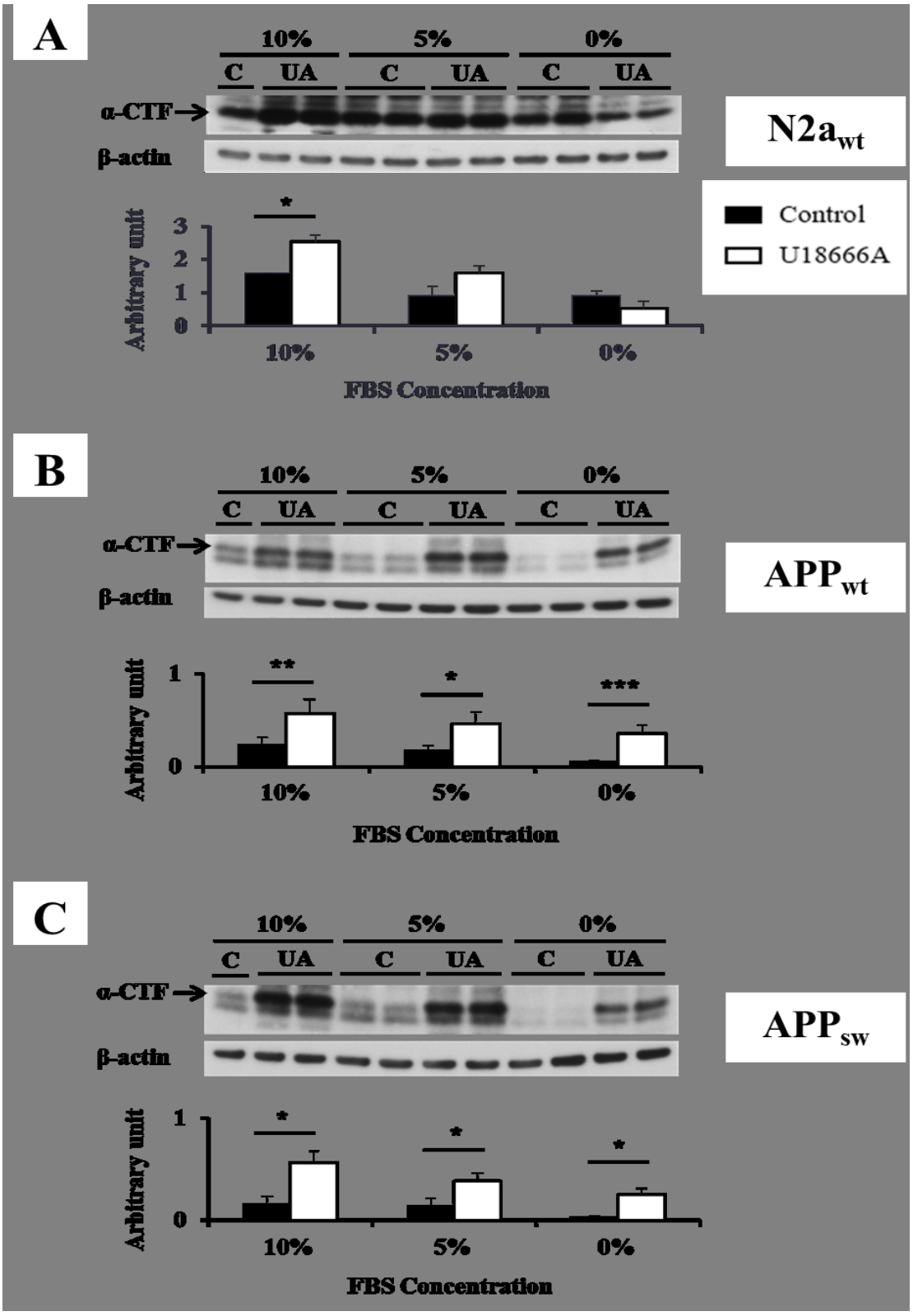

A
10%
5%
0%
C
UA
C
UA
C
UA
α-CTF
β-actin
N2a$_{wt}$
Control
U18666A
Arbitrary unit
*
3
2
1
0
10%
5%
0%
FBS Concentration
B
10%
5%
0%
C
UA
C
UA
C
UA
α-CTF
β-actin
APP$_{wt}$
Arbitrary unit
**
*
***
1
0
10%
5%
0%
FBS Concentration
C
10%
5%
0%
C
UA
C
UA
C
UA
α-CTF
β-actin
APP$_{sw}$
Arbitrary unit
*
*
*
1
0
10%
5%
0%
FBS Concentration

Fig. 8. Levels of β-CTFs are increased in all three media conditions in both APP_{wt} (**B**) and APP_{sw} cells (**C**), but remain unchanged in $N2a_{wt}$ cells (**A)**. Bars represent means ± SEM. **P <0.05, ***P < 0.001*

Fig. 8

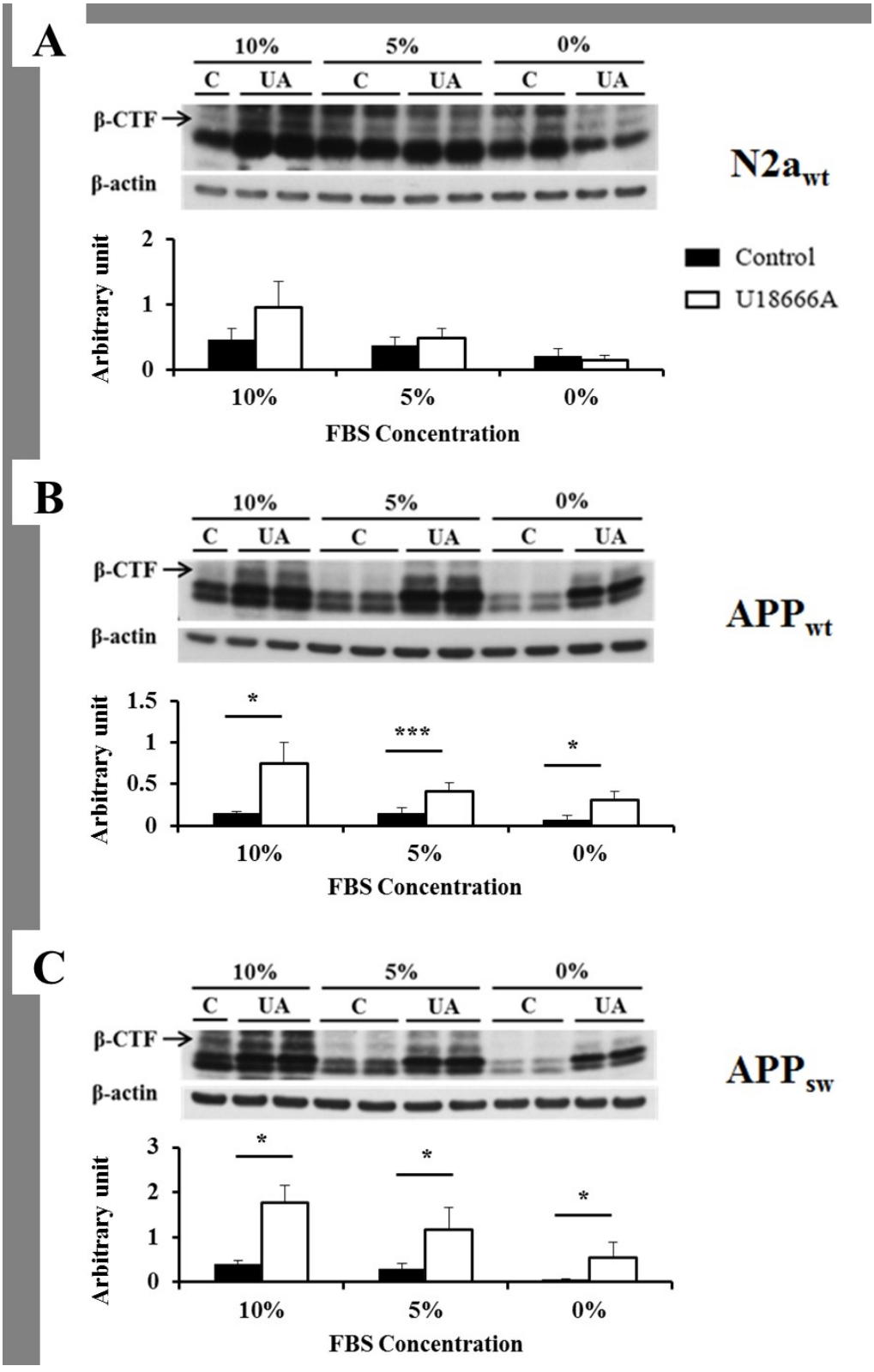

A
10%
5%
0%
C
UA
β-CTF
β-actin
N2a$_{wt}$
Arbitrary unit
Control
U18666A
FBS Concentration
B
APP$_{wt}$
C
APP$_{sw}$

Fig. 9. Levels of ADAM10 were not significantly altered in $N2a_{wt}$ (**A**), APP_{wt} (**B**), or APP_{sw} (**C**) cells either with different media conditions (i.e.. 0%, 5% and 10% FBS) or following treatment with 3μM U18666A for 24 hours compared to their respective control cells. Bars represent means ± SEM.

Fig. 9

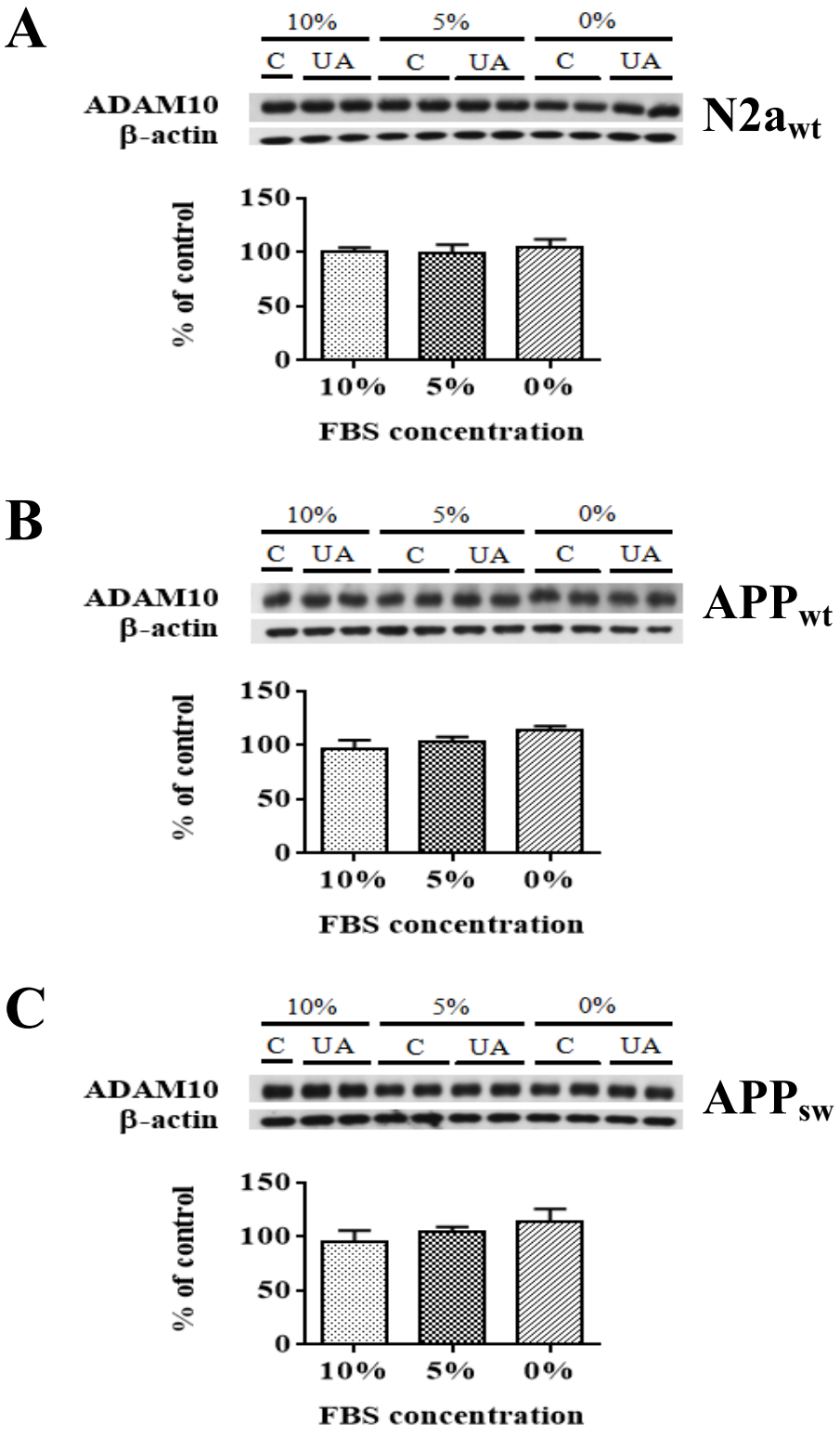

A
10%
5%
0%
C
UA
C
UA
C
UA
ADAM10
β-actin
N2a$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
B
10%
5%
0%
C
UA
C
UA
C
UA
ADAM10
β-actin
APP$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
C
10%
5%
0%
C
UA
C
UA
C
UA
ADAM10
β-actin
APP$_{sw}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration

Fig. 10. Levels of β-secretase BACE1 were not significantly altered in $N2a_{wt}$ (**A**), APP_{wt} (**B**), or APP_{sw} (**C**) cells either with different media conditions (i.e.. 0%, 5% and 10% FBS) or following treatment with 3μM U18666A for 24 hours compared to their respective control cells. Bars represent means ± SEM.

Fig. 10

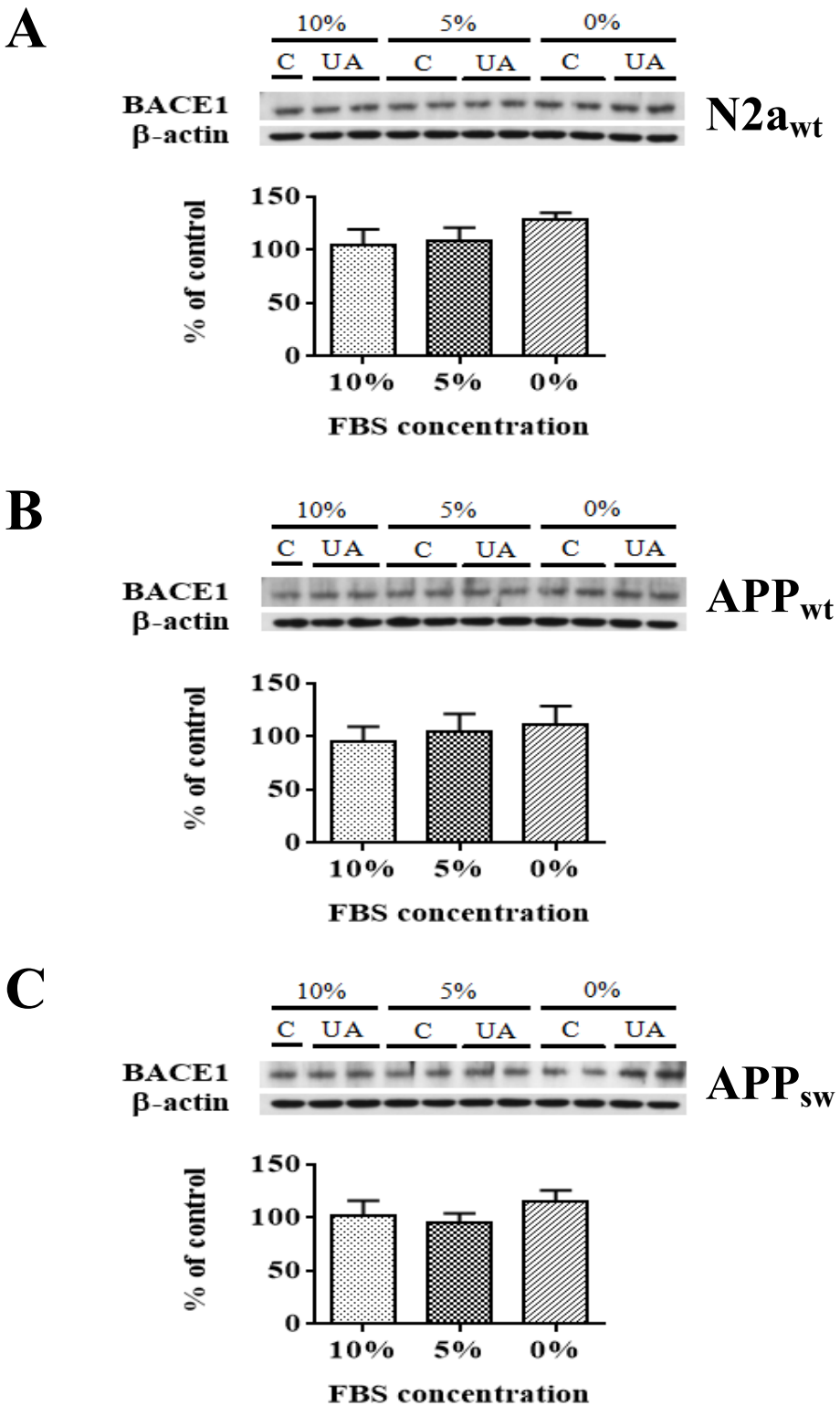

A
10%
5%
0%
C
UA
C
UA
C
UA
BACE1
β-actin
N2a$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
B
10%
5%
0%
C
UA
C
UA
C
UA
BACE1
β-actin
APP$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
C
10%
5%
0%
C
UA
C
UA
C
UA
BACE1
β-actin
APP$_{sw}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration

Fig. 11. Levels of PS1 were not significantly altered in $N2a_{wt}$ (**A**), APP_{wt} (**B**), or APP_{sw} (**C**) cells either with different media conditions (i.e.. 0%, 5% and 10% FBS) or following treatment with 3μM U18666A for 24 hours compared to their respective control cells. Bars represent means ± SEM.

Fig. 11

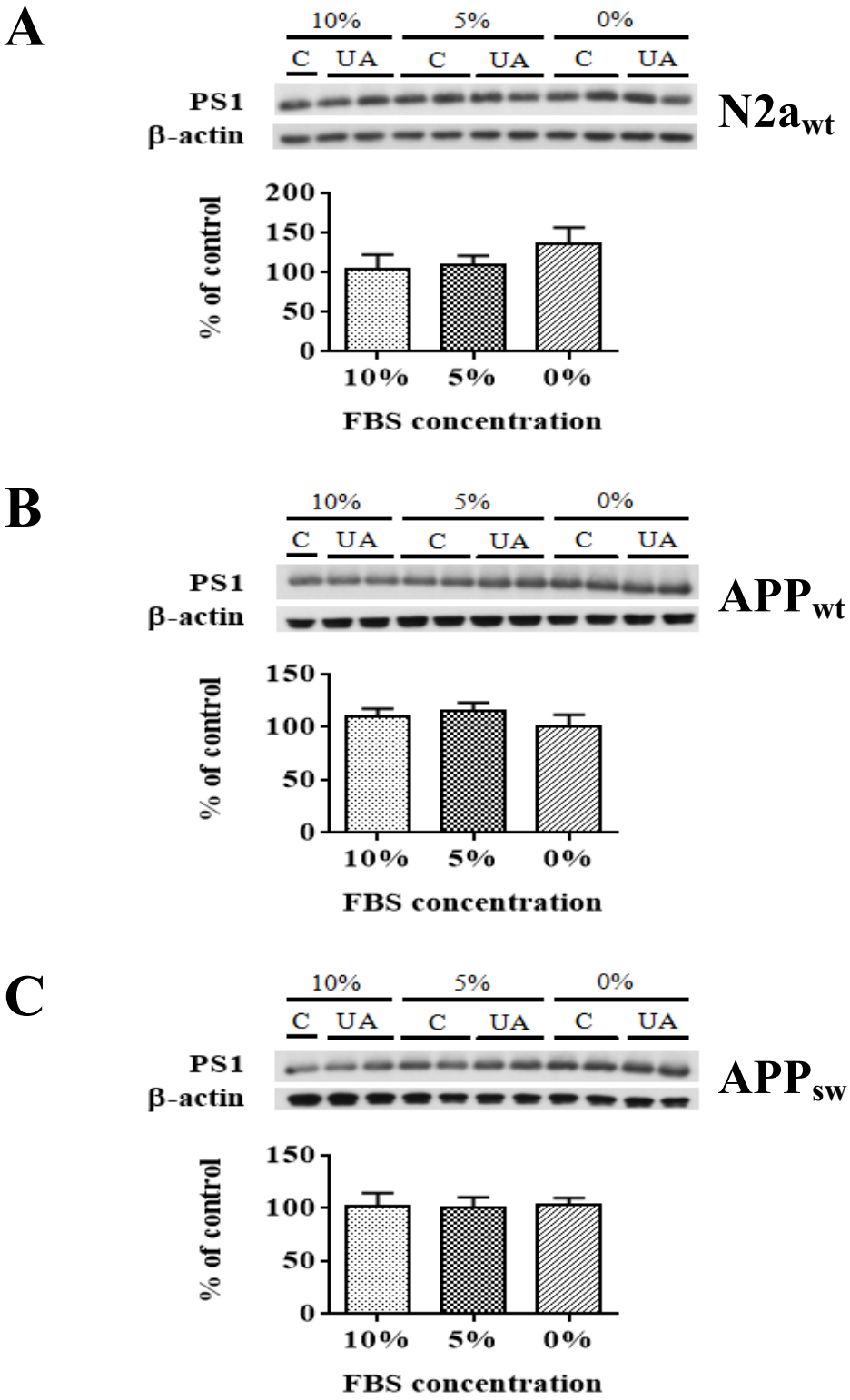

A
10%
5%
0%
C
UA
C
UA
C
UA
PS1
β-actin
N2a$_{wt}$
% of control
200
150
100
50
0
10%
5%
0%
FBS concentration
B
10%
5%
0%
C
UA
C
UA
C
UA
PS1
β-actin
APP$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
C
10%
5%
0%
C
UA
C
UA
C
UA
PS1
β-actin
APP$_{sw}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration

Fig. 12. Levels of nicastrin were not significantly altered in $N2a_{wt}$ (**A**), APP_{wt} (**B**), or APP_{sw} (**C**) cells either with different media conditions (i.e.. 0%, 5% and 10% FBS) or following treatment with 3μM U18666A for 24 hours compared to their respective control cells. Bars represent means ± SEM.

Fig. 12

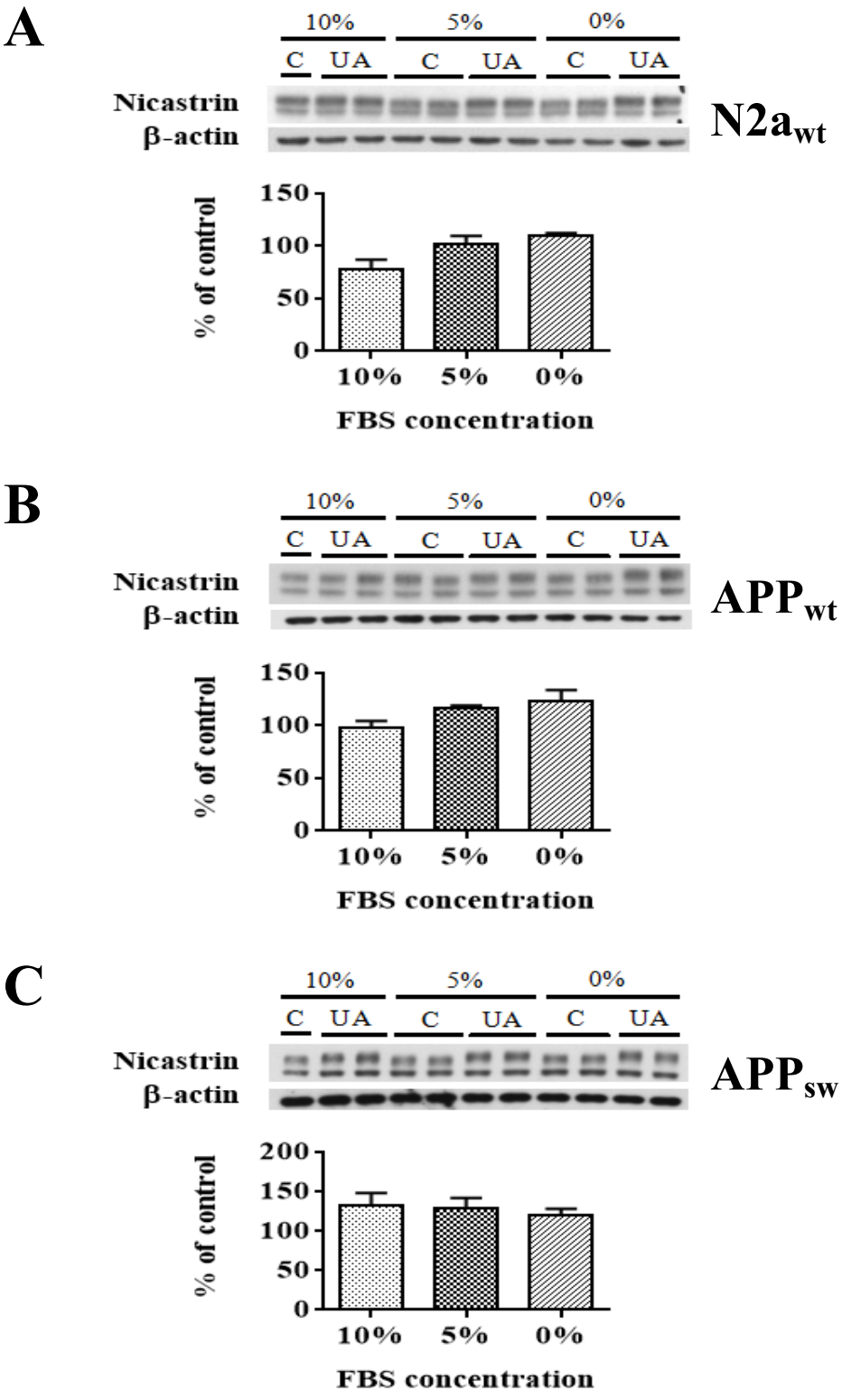

A
10%
5%
0%
C
UA
C
UA
C
UA
Nicastrin
β-actin
N2a$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
B
10%
5%
0%
C
UA
C
UA
C
UA
Nicastrin
β-actin
APP$_{wt}$
% of control
150
100
50
0
10%
5%
0%
FBS concentration
C
10%
5%
0%
C
UA
C
UA
C
UA
Nicastrin
β-actin
APP$_{sw}$
% of control
200
150
100
50
0
10%
5%
0%
FBS concentration

Fig. 13. No significant alteration was observed in α-secretase ADAM10 (**A**) or β-secretase BACE1 (**B**) activity in either $N2a_{wt}$, APP_{wt}, or APP_{sw} cells following treatment with 3μM U18666A for 24 hour in 5% FBS conditioned media. Bars represent means ± SEM.

Fig. 13

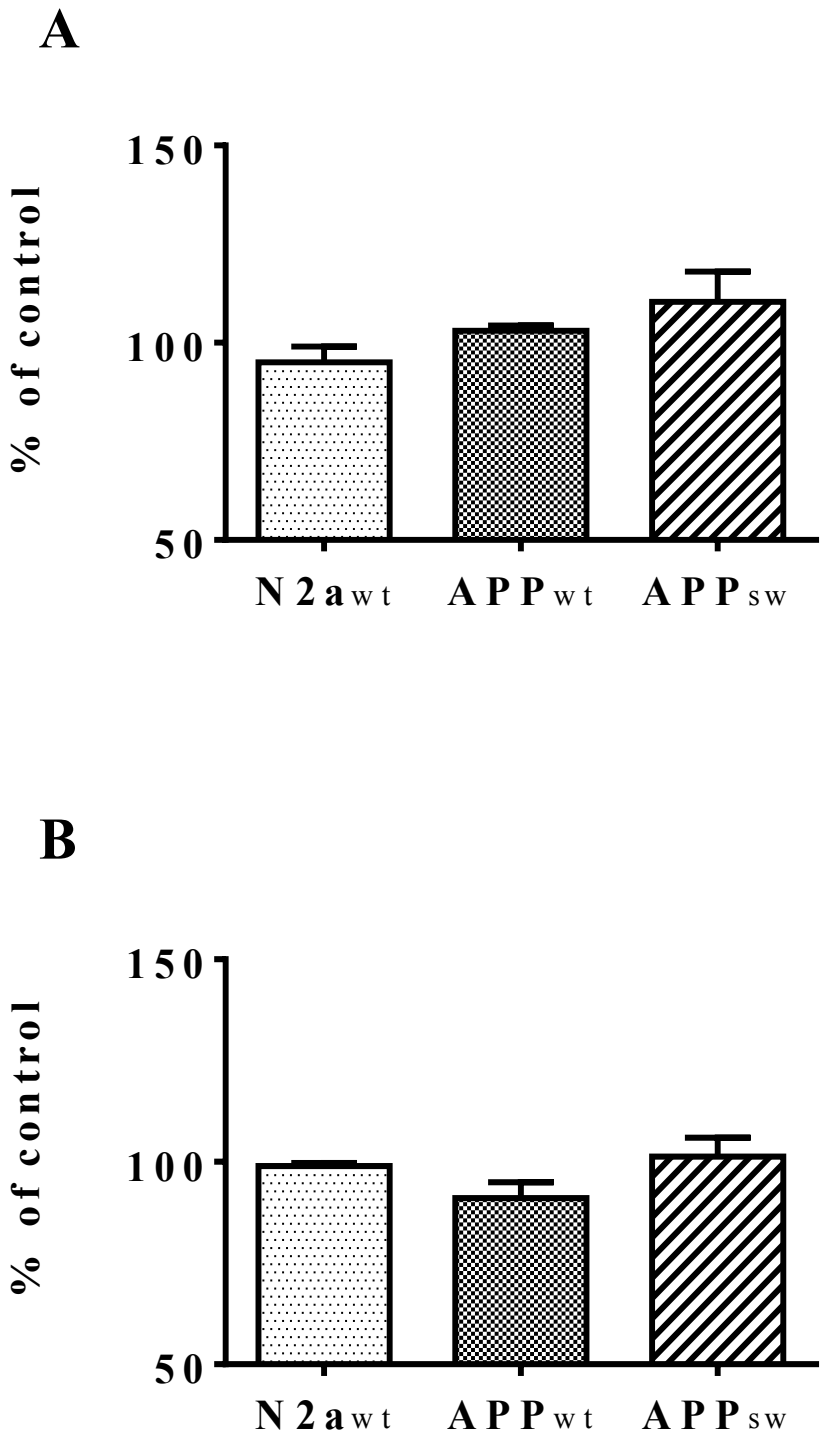

A
150
100
50
% of control
N2awt
APPwt
APPsw
B
150
100
50
% of control
N2awt
APPwt
APPsw

Fig. 14. Levels of $A\beta_{1\text{-}40}$ were not changed following U18666A treatment in cultured $N2a_{wt}$ cells at any FBS conditions (**A)**, but markedly increased at all FBS conditions in APP_{wt} (**B)** as well as APP_{sw} cells (**C)**. Bars represent means ± SEM. **P <0.05, **P < 0.01.*

Fig. 14

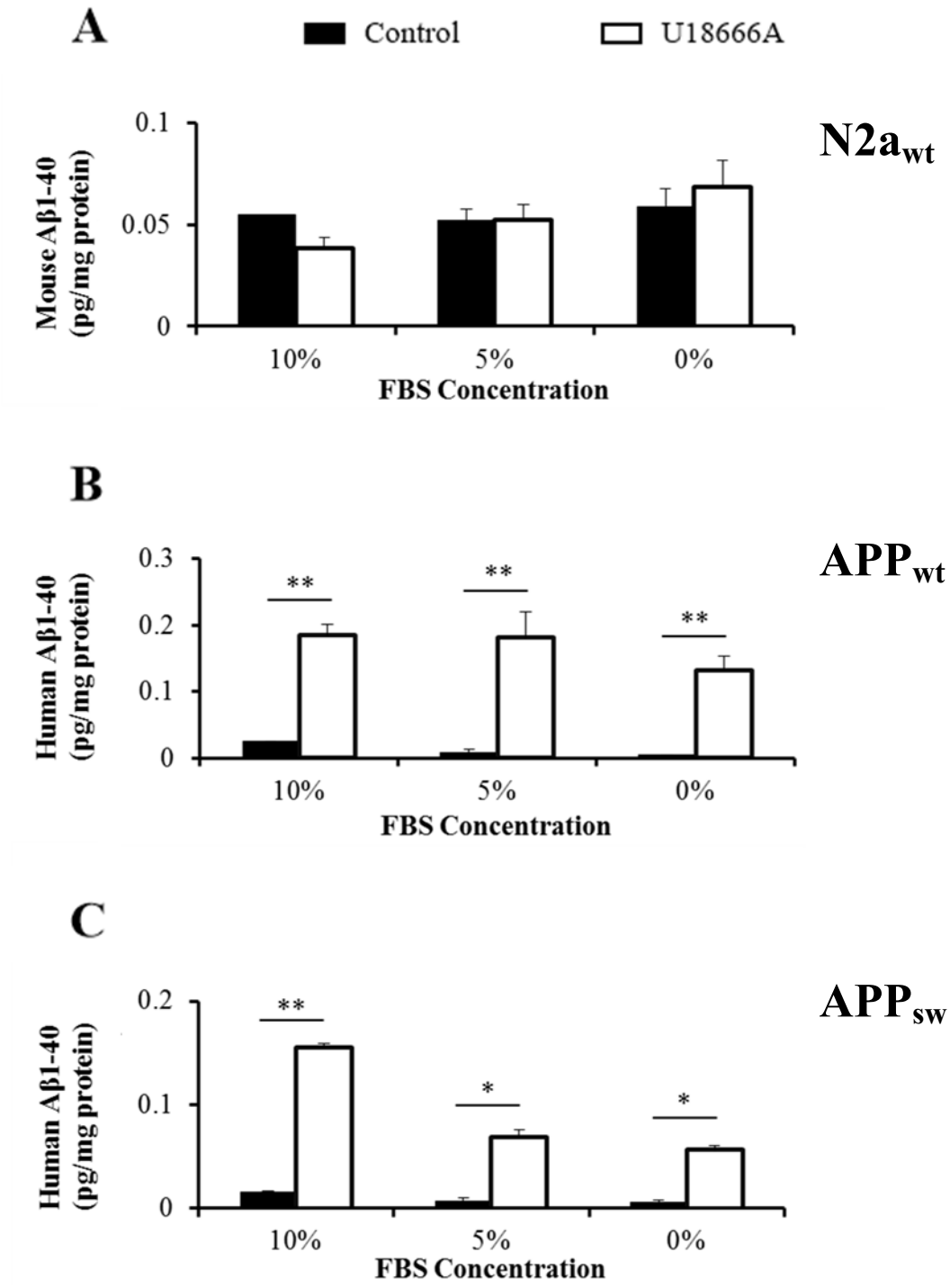

A
Control
U18666A
Mouse Aβ1-40 (pg/mg protein)
0.1
0.05
0
10%
5%
0%
FBS Concentration
N2a$_{wt}$
B
Human Aβ1-40 (pg/mg protein)
0.3
0.2
0.1
0
**
**
**
10%
5%
0%
FBS Concentration
APP$_{wt}$
C
Human Aβ1-40 (pg/mg protein)
0.2
0.1
0
**
*
*
10%
5%
0%
FBS Concentration
APP$_{sw}$

Fig. 15. Levels of $A\beta_{1\text{-}42}$ were not changed following U18666A treatment in cultured $N2a_{wt}$ cells at any FBS conditions (**A)**, but markedly increased at all FBS conditions in APP_{wt} (**B)** as well as APP_{sw} cells (**C)**. Bars represent means ± SEM. ***P < 0.01, ***P < 0.001*

Fig. 15

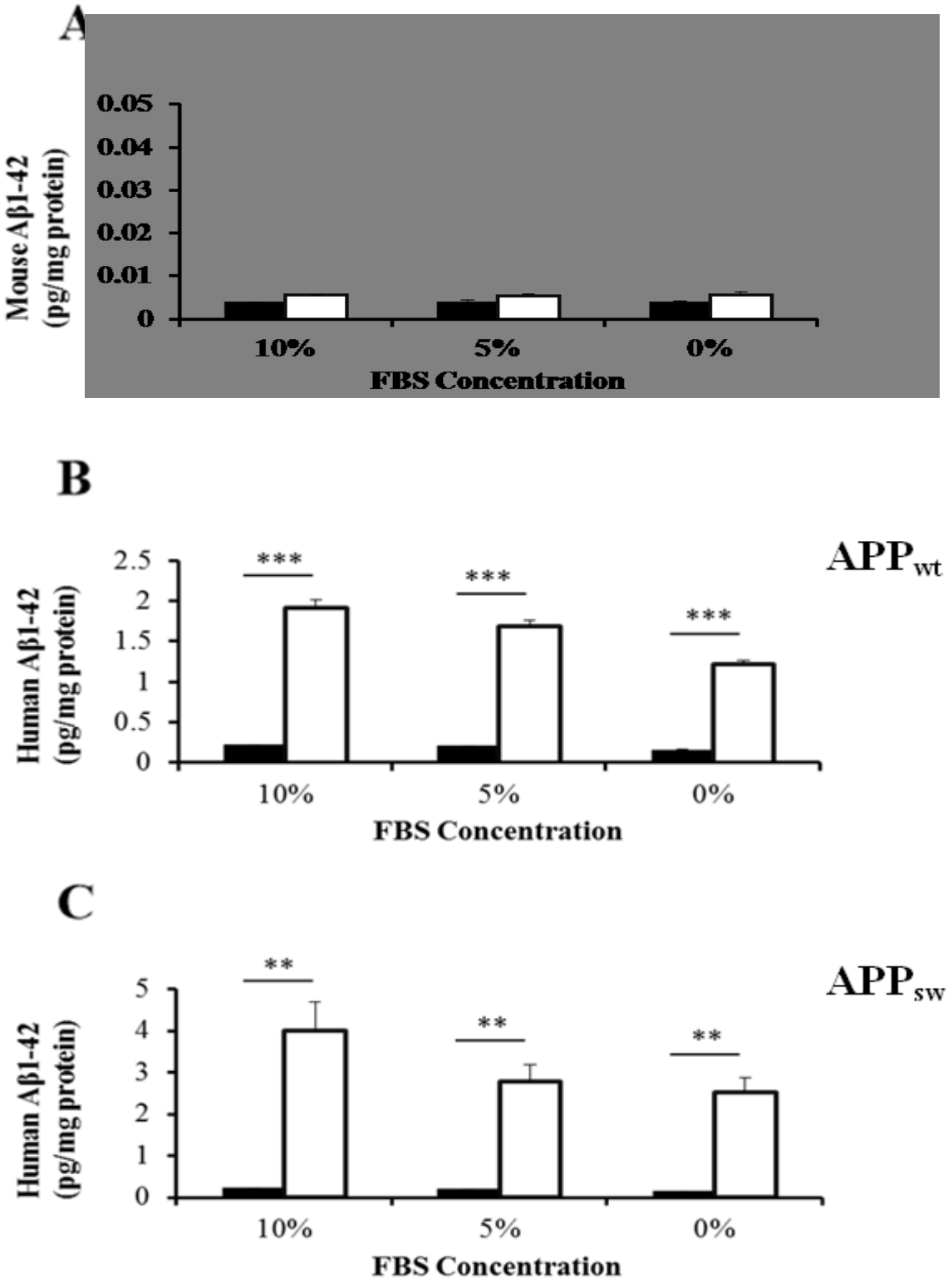
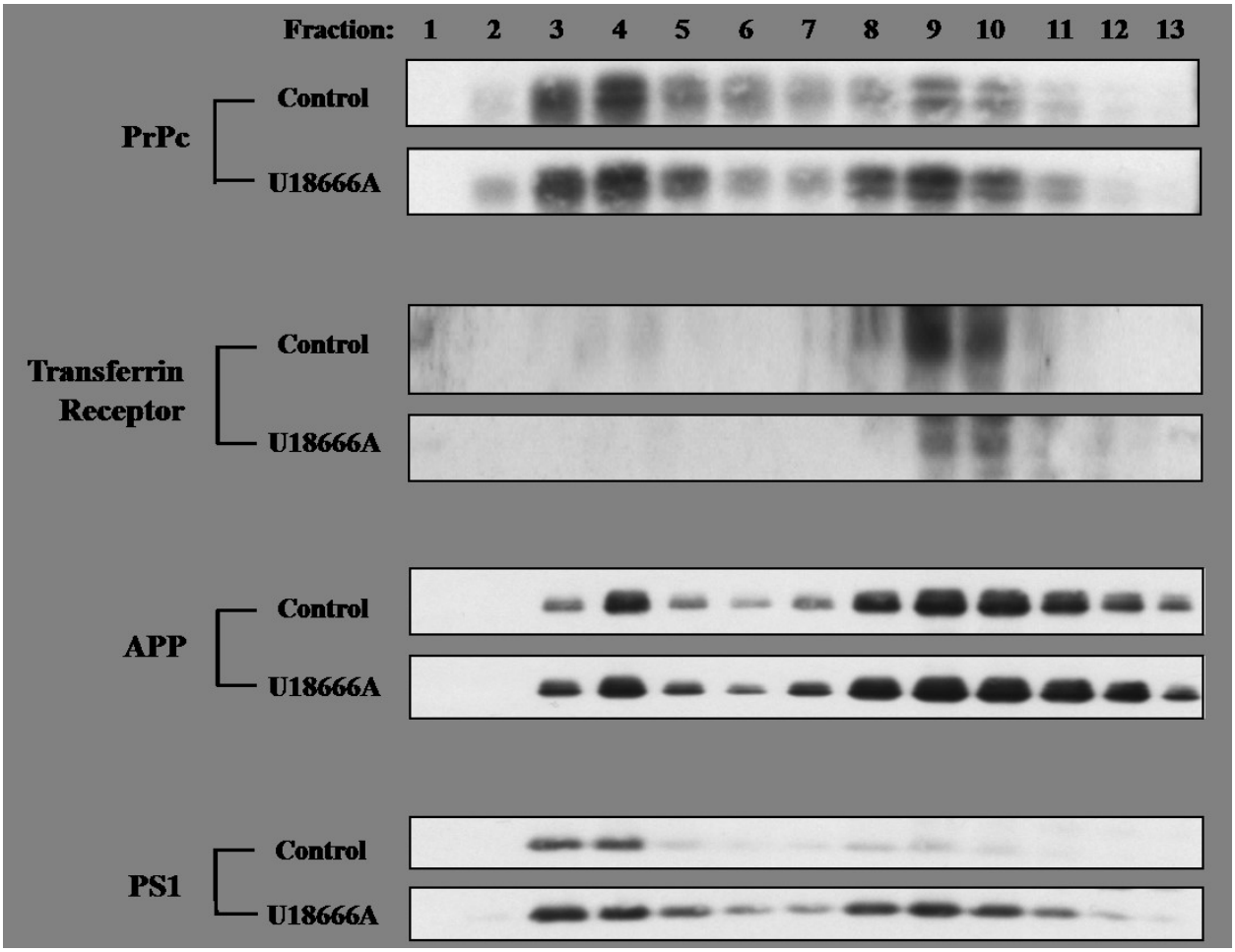

A
Mouse Aβ1-42 (pg/mg protein)
0.05
0.04
0.03
0.02
0.01
0
10%
5%
0%
FBS Concentration
B
APPwt
Human Aβ1-42 (pg/mg protein)
2.5
2
1.5
1
0.5
0
***
***
***
10%
5%
0%
FBS Concentration
C
APPsw
Human Aβ1-42 (pg/mg protein)
5
4
3
2
1
0
**
**
**
10%
5%
0%
FBS Concentration

Fig. 16. Raft-markers PrPc show the separation of raft fractions (fractions 2-7) and non-raft fractions (fractions 9-13). Some signal is detected in the non-raft region as well, especially after U18666A treatment. Non-raft marker Transferrin receptor shows non-raft regions. U18666A also increases the overall expression of APP and PS1 across all cell fractions, with the most change evident in the non-raft region.

Fig. 16


Fraction: 1 2 3 4 5 6 7 8 9 10 11 12 13
PrPc
Control
U18666A
Transferrin Receptor
Control
U18666A
APP
Control
U18666A
PS1
Control
U18666A

4. Discussion

The present study clearly shows that intracellular accumulation of cholesterol within the EL system can differentially alter the level of APP and its processing in cultured $N2a_{wt}$, APP_{wt} or APP_{sw} cells. Additionally, cultured conditions with variable FBS concentrations can, to some extent, influence APP metabolism in the three different cell types used. Our results revealed that i) U18666A treatment, but not increasing concentrations of FBS, triggered intracellular accumulation of cholesterol in cultured $N2a_{wt}$, APP_{wt} and APP_{sw} cells; ii) FBS concentrations did not significantly alter total or free cholesterol levels in any cell line, although U18666A treatment decreased the levels of both total and free cholesterol at 0% FBS in all three cell lines; iii) FBS concentrations did not alter APP levels in any of the three cell lines, while U18666A treatment differentially increased holoprotein levels only in APP_{wt} and APP_{sw} cells; iv) levels of α-CTF and β-CTF were not altered with increasing concentration of FBS but were differentially enhanced following treatment with U18666A in $N2a_{wt}$, APP_{wt} and APP_{sw} cells; v) steady state levels of APP secretases were neither altered with increasing concentrations of FBS nor following treatment of U18666A in any of the three cell lines; vi) activity of α-secretase and β-secreatse was not altered in any of the cell lines following treatment with U18666A; vii) cellular levels of $A\beta_{1-40}$ or $A\beta_{1-42}$ were not altered with increasing concentrations of FBS or U18666A treatment in cultured $N2a_{wt}$ cells, but were markedly increased in APP_{wt} or APP_{sw} cells, especially after treatment with U18666A; and viii) U18666A treatment somewhat altered the distribution profile of PS1, but not APP, from the raft to non-raft fractions in cultured APP_{sw} cells. Taken together, these results suggest that cholesterol accumulation specifically within the EL system rather than enhanced cellular level, can significantly influence APP levels and its metabolism, especially in cells stably overexpressing either wild-type or mutant human APP.

Evidence accumulated from three distinct lines of experiments suggest a potential role for cholesterol in AD pathogenesis. First, genetic studies revealed a dose-dependent association between the incidence of late-onset AD and the inheritance of the *APOE* ε4 genotype (Corder et al., 1993). Additionally, genome-wide association studies have linked single nucleotide polymorphisms in several genes related to cholesterol metabolism such as *APOJ* (*CLU*), *LRP*, *CYP46A1*, *ABCA1* and *ACAT1* to AD, although the strength of some of these associations remain

to be established (Bertman et al., 2007; Hollingworth et al., 2011; Lambert and Amouyel, 2011). Second, several epidemiological studies have indicated that a high level of total plasma cholesterol in mid-life is a risk factor for developing AD. Indeed, previous retrospective studies reported that the prevalence of AD was lower in patients taking statins compared with those receiving other medications used in cardiovascular disease (Wolozin et al., 2000), or even compared to the total population (Rockwood et al., 2002). Some prospective studies, however, have brought the efficacy of statins in slowing the progression of AD into question (Zandi et al., 2005; Sparks et al., 2008; Hang et al., 2009; Reiss and Voloshyna, 2012). Third, studies from a variety of *in vitro* and *in vivo* models have reported that cholesterol can influence APP processing, leading to Aβ generation which can subsequently influence AD pathology. Since a subset of cellular APP, as well as β- and γ-secretases, are localized in cholesterol-rich lipid-raft domains (Tun et al., 2002; Wahrle et al., 2002; Vetrivel et al., 2005), a number of *in vitro* studies have shown that the crucial factor influencing Aβ production and the risk of AD may depend not only on total cellular levels of cholesterol but also on the subcellular distribution of the lipid (Yamazaki et al., 2001; Puglielli et al., 2001; Runz et al., 2002; Davis, 2008; Kosicek et al., 2010; Malnar et al., 2010). Since the EL system is a major site of APP metabolism and exhibits marked changes in "at risk" neurons prior to Aβ deposition in AD brains (Nixon 2005; Haass et al., 2012), we believe it is critical to determine how alterations in EL cholesterol levels can influence production and clearance of Aβ peptides.

In the present study, we used the hydrophobic amine U18666A to define the effects of cholesterol accumulation within EL system on APP metabolism. U18666A is one of the best class-2 amphiphilic compounds known to trigger accumulation of cholesterol within EL system through dysfunction of lipid storage and inhibition of cholesterol movement from plasma membrane to ER and from the lysosome to the plasma membrane. Given the evidence that a subset of AD can be caused by mutation of the APP gene (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Chen et al., 2000), we have studied the effects of U18666A not only in normal and wild-type human APP expressing N2a cells, but also in N2a cells that stably overexpress Swedish mutation - which has been used for the development of transgenic mouse models of AD. Although the the molecular mechanism through which the drug exerts its effects remains to be established (Lange et al., 1994; Lang et al., 1999; Koh and Cheung 2006), it is

believed that U18666A may act on the activity or synthesis of a protein or lipid which facilitates cholesterol movement and possibly alters cellular distribution of the NPC1 protein, thus triggering EL accumulation of cholesterol to pathologically high levels (Neufeld et al., 1999; Lange et al., 2000; Lang et al., 2002; Liscum and Sturley 2004). Apart from cholesterol, U18666A has been shown to potently alter the trafficking and cellular distribution of a variety of intracellular membrane proteins. For example, U18666A has been shown to prevent cycling of CD63 lysosome-associated membrane protein-3 from late-endosomes to specialized secretory granules known as Weibel-Palade bodies in human endothelial cells (Kobayashi et al., 2000). Recent studies have also reported that U18666A treatment can alter the trafficking pathway of cation-independent mannose 6-phosphate receptor and can increase intracellular calcium concentrations (Ikeda et al., 2005). The 3μg/ml U18666A used in the present study has previously been shown by us to trigger accumulation of cholesterol without any significant effect on cell viability over a 24 hour period (Yamazaki et al., 2001; Runz 2002; Jin et al., 2004; Amritraj et al., 2013). It is of interest to note that, apart from evaluating the effects of U18666A on APP metabolism, we have also defined how altered levels of FBS with varying concentrations of cholesterol can influence APP metabolism in the absence and presence of U18666A. This experimental paradigm is designed to address two critical issues: first, whether increasing extracellular levels of cholesterol along with its intracellular accumulation triggered by U18666A can have an additive effect on APP metabolism; second, whether the FBS concentrations used in culturing cells may underlie the cause of discrepancy reported for APP metabolism following U18666A treatment.

Our results clearly showed that treatment with U18666A, as reported in earlier studies (Jin et al., 2004; Davis, 2008; Amritraj et al., 2013), triggered the accumulation of cholesterol in all three cell lines. The levels of total and free cholesterol in U18666A-treated cells, however, did not alter at 5% and 10% FBS but decreased at 0% FBS in all three cell lines. Although earlier studies reported that cholesterol sequestration into the EL system in NPC1 knockout cells did not alter total levels of cellular cholesterol (Karten et al., 2002; Martin et al., 2010), it is rather intriguing to observe a reduction in cholesterol levels at 0% FBS following U18666A treatment. Since U18666A can inhibit synthesis of cholesterol, apart from blocking its intracellular trafficking

(Lang et al., 1999; Koh and Cheung 2006), it is likely that the decreased levels of cholesterol represent an inhibition of cholesterol synthesis which is compensated for with higher concentrations of FBS.

The levels of APP holoprotein are found to be significantly increased in both APP_{wt} and APP_{sw} cells in most conditions, but not in $N2a_{wt}$ cells following treatment with U18666A. Earlier studies have in fact shown that U18666A treatment can lead to increased levels of APP holoprotein in APP_{wt} as well as APP_{sw} cells (Davis, 2008). Although no study thus far has been carried out on $N2a_{wt}$ cells, there is evidence that treatment of primary cultured neurons with U18666A did not alter APP holoprotein levels (Jin et al., 2004). Consistent with APP, we observed increased levels of α-CTF and β-CTF in both APP_{wt} and APP_{sw} cells. In $N2a_{wt}$ cells, the levels of α-CTF, but not β-CTF, were found to be increased only with 10% FBS. A number of studies have reported that U18666A treatment can lead to either an increase in α-CTF/β-CTF (Jin et al., 2004; Boland et al., 2010) or an increase in α-CTF and decrease in β-CTF (Davis 2008; Runz 2002) levels. This could be attributed to dose and duration of U18666A treatment or the specific cell lines used in various studies. Notwithstanding these results, we did not observe any alteration in the steady state levels of either ADAM-10, BACE1 or the four components of the γ-secretase complex (i.e. PS1, nicatrin, APH1 and Pen2) following treatment with U18666A in any of the three cell lines used in the study. Although it is reported that U18666A treatment can lead to transcriptional up-regulation of PS1 (Crestini et al., 2006) without any alteration in protein levels, there is evidence of intracellular redistribution of PS1 in Rab7-positive late-endosomes implicated in cholesterol sorting (Runz et al., 2002). At present, it is unclear whether U18666A treatment in our experimental paradigm can lead to redistribution of PS1 in late-endosomes in the absence of any change in its steady state levels. Accompanying ADAM-10 and BACE1 levels, we observed no alteration in either α-secretase or β-secretase activity in U18666A-treated $N2a_{wt}$, APP_{wt} and APP_{sw} cells, thus raising the possibility that intracellular accumulation of APP and its CTFs may be the consequence of decreased turnover. In fact, cholesterol accumulation within the EL system has been shown to impair clearance of APP and its CTFs *via* autophagic-endosomal-lysosomal pathways (Boland et al., 2010; Kodam et al., 2010; Maulik et al., 2015).

With regard to Aβ peptides, we observed that cellular levels of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ are increased in APP_{wt} and APP_{sw} cells at all FBS conditions. However, no significant alteration was evident either in $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ levels in U18666A-treated $N2_{wt}$ cells. Several previous studies have examined Aβ metabolism after U18666A treatment, but the results are difficult to compare due to differences in cell models and concentrations of drugs used in the studies. For example, U18666A treatment has been shown to reduce the cellular and secretory levels of $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ in neurons as well as APP695-transfected SH-SY5Y neuroblastoma cells (Runz et al., 2002). In contrast, Yamazaki et al., found no effect of U18666A treatment on secretion of $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$, but increased levels of intracellular Aβ peptides in late endosomes of CHO cells (Yamazaki et al., 2001). Additionally, U18666A treatment has also been shown to trigger accumulation of $A\beta_{X\text{-}42}$ in APP695 transfected primary mouse cortical neurons (Jin et al., 2004) as well as in mouse cortical neurons expressing endogenous APP (Koh et al., 2006). At present, however, it remains unclear whether a change in the activity of the γ-secretase complex and/or turnover of Aβ-related peptides may underlie the altered levels/accumulation of Aβ peptides observed in various U18666A-treated cells. Since U18666A is able to redistribute certain components of APP processing enzymes (e.g. PS1) from raft to non-raft fractions as well as within the intracellular organelles (Runz et al., 2002), it will be of interest to determine their significance, if any, in regulating the levels of Aβ-related peptides following intracellular accumulation of cholesterol within the EL system.

A number of earlier studies have demonstrated that U18666A inhibits both trafficking of exogenous cholesterol derived from lipoprotein uptake and cholesterol synthesis – the two sources of intracellular cholesterol which play an important role on APP processing. Members of the low-density lipoprotein (LDL) family mediate uptake of exogenous cholesterol contained in lipoprotein particles. The LDL receptor-related protein (LRP) binds cell surface APP and mediates its internalization (Kounnas et al., 1995). There is also evidence that LRP binds the cholesterol-containing lipoprotein ApoE and regulates its endocytosis as a source of cholesterol in cultured cells (Herz 2001). The significance of LRP is highlighted by results which showed that blocking its function affects APP metabolism. Additionally, treatment with the LRP antagonist RAP increased cell surface APP and reduced Aβ levels; this effect could be reversed

by expression of LRP in LRP-deficient cells (Ulery et al., 2000). Cholesterol biosynthesis has also been shown to directly influence APP processing. A key enzyme in cholesterol biosynthesis is seladin-1/DHCR24, which is down-regulated in AD brain areas exhibiting high levels of amyloid deposition (Greeve et al., 2000; Iivonen et al., 2002). Loss of saladin-1 expression has been shown to cause displacement of BACE-1 from detergent-resistant lipid-rafts to APP-containing membrane fractions. This displacement of BACE-1 was associated with elevated β-cleavage of APP and Aβ generation (Davis, 2008). Mice deficient in the cholesterol synthesizing enzyme seladin-1 showed lowered brain cholesterol levels and increased production of Aβ peptides through amyloidogenic pathway, whereas opposite effects were found in seladin-1 overexpressing mice (Crameri et al., 2006). While hippocampal samples from normal human brains showed only a small pool of APP is colocalized with BACE1 in the detergent-soluble membrane fraction, samples from AD brains with a moderate reduction in membrane cholesterol levels exhibited much higher levels of APP and BACE1 colocalization. These results suggest that a mild reduction in membrane cholesterol levels may enhance the colocalization of APP and BACE1 in the detergent-soluble membrane fraction which can lead to increased production of Aβ peptides (Ledesma et al., 2003; Abad-Rodriguez et al., 2004).

Chronic exposure to U18666A has been shown to trigger toxicity, possibly *via* apoptosis in a variety of experimental conditions (Koh and Cheung, 2006; Amritraj et al., 2013). Interestingly, co-treatment with pravastatin, a HMG-CoA reductase inhibitor, or cyclodextrin, a water-soluble compound which can remove cholesterol from the plasma membrane, exhibited significant attenuation of U18666A toxicity, thus indicating that cholesterol dysfunction may underlie U18666A-mediated toxicity (Cheung et al., 2004; Koh et al., 2006). The effect of U18666A is associated with activation of caspase 3 in a time-dependent manner and co-treatment with the pan caspase inhibitor Z-VAD-FMK provides significant neuroprotection (Cheung et al., 2004). There is evidence of involvement of calpain and other caspases, suggesting a potential crosstalk between the caspase and calpain pathways during apoptosis mediated by U18666A (Koh et al., 2006). As procaspase-12 is predominantly found in the ER, activation of caspase-12 at an earlier time point before the activation of calpain and other caspases suggested that ER stress might play a role in initiating U18666A-mediated apoptosis. Caspase-12 can also activate other effector

caspases in the downstream of the caspase activation cascade (Koh and Cheung, 2006). Apart from caspases, a number of recent studies have indicated that U18666A treatment can lead to increased intracellular accumulation of both $A\beta_{1-40}$ and $A\beta_{1-42}$ possibly due to increased production and/or decreased secretion of the peptides (Koh et al., 2006). Since Aβ-related peptides are toxic to neurons and a variety of other cells, a potential role for the peptides in triggering U18666A-induced degeneration of neurons/cells cannot be eliminated. Interestingly, some studies have shown that treatment of cultured cortical neurons with U18666A can induce tau phosphorylation (Koh et al., 2006). These mechanistic features - i.e. intracellular accumulation of Aβ peptides, together with increased phosphorylation of tau protein - provide a basis to suggest that cholesterol dysfunction observed in AD and NPC pathology may have a role not only in regulating APP metabolism leading to increased levels of Aβ-related peptides, but also in the degeneration of neurons observed in selected regions of the brain (Maulik et al., 2012; Maulik et al., 2013).

Although the current study clearly indicates that U18666A-induced cholesterol dysfunction can trigger altered APP metabolism leading to increased accumulation of Aβ-related peptides, there are several caveats that need to be addressed in our studies. First of all, we need to measure secretory levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ following treatment with U18666A. Secondly, we have to complement our results with evaluation of both soluble APPα and APPβ along with the activity of γ-secretase enzyme complex which will reinforce the potential role of U18666A on APP metabolism. Finally, we have to examine whether attenuation of cholesterol dysfunction can lead to a reversal of APP levels and metabolism and possibly grant the cells a resistance against toxicity.

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