Implication of exosomes derived from cholesterol-accumulated astrocytes in Alzheimer’s disease pathology

Qi Wu¹, Leonardo Cortez¹, Razieh Kamali-Jamil², Valerie Sim¹, Holger Wille² and Satyabrata Kar¹,²

Departments of ¹Medicine (Neurology) and ²Biochemistry, Center for Prions and Protein Folding Diseases, University of Alberta, Edmonton, Alberta, Canada

E-mails: Q. Wu - qw6@ualberta.ca; L. Cortez - lcortez@ualberta.ca; R. Kamali-Jamil - kamalija@ualberta.ca; V. Sim - valerie.sim@ualberta.ca; H. Wille - wille@ualberta.ca; S. Kar - skar@ualberta.ca

Address correspondence to:
Satyabrata Kar, Ph.D.
Centre for Prions and Protein Folding Diseases
Departments of Medicine (Neurology) and Psychiatry
University of Alberta
Edmonton, Alberta, Canada T6G 2M8
Tel. no: (780) 492 9357; Fax no: (780) 492 9352
E-mail: skar@ualberta.ca

Abbreviations: Aβ, amyloid β; AD, Alzheimer’s disease; ADAM10; A Disintegrin and metalloproteinase domain-containing protein 10; ALIX, Alg-2 interacting protein; APP, amyloid precursor protein; AM-a, Astrocyte Medium-animal; BACE, β-site APP cleaving enzyme; BCA, bicinechinonic acid; CTF, C-terminal fragment; DLS, dynamic light scattering; ECL, enhanced chemiluminescence; EL, endosomal-lysosomal; ELISA, Enzyme-linked immunosorbent assay; EM, electron microscopy; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; HRP, Horseradish peroxidase; KPI, Kunitz family of serine protease inhibitor; LAMP1, Lysosomal-associated membrane protein 1; LC3, microtubule-associated protein 1 light chain 3; MBCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PFA, paraformaldehyde; PI3K, phosphatidylinositol-3-kinase; PS1/2, presenilins 1/2; RIPA, Radioimmunoprecipitation assay; TBS, Tris-buffered saline.

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Summary Statement

We describe that cholesterol accumulation within astrocytes can influence secretion of exosomes containing beta-amyloid-related peptides. Uptake of these exosomes can induce neurotoxicity suggesting their potential implication in Alzheimer’s disease pathogenesis.

ABSTRACT

Amyloid β (Aβ) peptides generated from the amyloid precursor protein (APP) play a critical role in the development of Alzheimer’s disease (AD) pathology. Aβ-containing neuronal exosomes, which represent a novel form of intercellular communication, have been shown to influence function/vulnerability of neurons in AD. Unlike neurons, the significance of exosomes derived from astrocytes remains unclear. In this study, we evaluated the significance of exosomes derived from U18666A-induced cholesterol-accumulated astrocytes in the development of AD pathology. Our results show that cholesterol accumulation decreases exosome secretion, whereas lowering cholesterol level increases exosome secretion from cultured astrocytes. Interestingly, exosomes secreted from U18666A-treated astrocytes contain higher levels of APP, APP-CTFs, soluble APP, APP secretases and Aβ1-40 than exosomes secreted from control astrocytes. Furthermore, we show that exosomes derived from U18666A-treated astrocytes can lead to neurodegeneration, which is attenuated by decreasing Aβ production or by neutralizing exosomal Aβ peptide with an Aβ antibody. These results, taken together, suggest that exosomes derived from cholesterol-accumulated astrocytes can play an important role in trafficking APP/Aβ peptides and influencing neuronal viability in the affected regions of the AD brain.

Key words: Alzheimer’s disease, β-amyloid, Astrocytes, Exosomes, U18666A, Cholesterol

INTRODUCTION

Alzheimer’s disease (AD), the most common type of dementia affecting the elderly population, is characterized neuropathologically by the presence of intracellular neurofibrillary tangles, extracellular neuritic plaques, gliosis and the loss of neurons in
selected brain regions (Chen and Mobley, 2019; Dawkins and Small, 2014; Lane et al., 2018). While neurofibrillary tangles contain hyperphosphorylated microtubule-associated tau protein, neuritic plaques are composed of a central deposit of β-amyloid (Aβ) peptides surrounded by dystrophic neurites, activated microglia and reactive astrocytes. The Aβ peptides are generated from amyloid precursor protein (APP) which is known to be processed proteolytically by either the non-amyloidogenic α-secretase or the amyloidogenic β-secretase pathways. The α-secretase pathway is mediated by a family of disintegrin and metalloproteinase domain-containing proteins (mainly ADAM10) that cleave APP within the Aβ domain, generating soluble APPα (sAPPα) and a C-terminal fragment (α-CTF) which is further processed by γ-secretase to produce Aβ_{17-40}/Aβ_{17-42} fragments. The β-secretase pathway, on the other hand, is mediated by β-site APP cleaving enzyme (BACE1) which cleaves APP to produce sAPPβ and an Aβ-containing β-CTF that can be processed by γ-secretase to generate full-length Aβ_{1-40}/Aβ_{1-42} peptides (Andrew et al., 2016). Unlike α-/β-secretases, γ-secretase is a tetrameric complex that is comprised of the aspartyl protease presenilin 1 or 2 (PS1/PS2) and three cofactors: nicastrin, presenilin enhancer 2 (Pen2) and anterior pharynx defective 1 (APH1) (Andrew et al., 2016; Grimm et al., 2015). Evidence suggests that an overproduction and/or a lack of clearance may lead to increased Aβ levels which, in turn, contribute to loss of neurons and development of AD. Although neurons are the major source of Aβ (Calhoun et al., 1999; Zhao et al., 1996), the activated astrocytes associated with plaques are also known to express APP resulting in Aβ production (Jo et al., 2014; Nagele et al., 2003). Since astrocytes under normal conditions do not generate Aβ (Avila-Munoz and Arias, 2014; Pihlaja et al., 2011; Thal, 2012), it is important to define the role of astrocytic Aβ in AD pathogenesis.

Astrocytes, the most abundant glial cells in the central nervous system, play vital roles in maintaining brain homeostasis, including regulation of the blood-brain barrier, trophic support, synaptic activity and synapse remodeling (Nag, 2011; Sidoryk-Wegrzynowicz et al., 2011). Upon activation, which may result from injury or development of diseases such as AD, astrocytes lose some of their normal functions and contribute to the loss of neurons (Allaman et al., 2011; Seifert et al., 2006; Steele and Robinson, 2012). A role for activated
astrocytes in AD is supported by evidence that i) they increase neuronal vulnerability to toxicity by impairing glutamate recycling (Steele and Robinson, 2012) and/or generating reactive oxygen and nitrogen species (Farfara et al., 2008; Luth et al., 2002), ii) they express pro-inflammatory molecules such as tumor necrosis factor-α and interleukin-1β that can increase Aβ production (Blasko et al., 2000; Li et al., 2011; Medeiros and LaFerla, 2013), iii) they are unable to regulate efficient Aβ clearance (Mulder et al., 2012; Wyss-Coray et al., 2003) and exacerbate Aβ-mediated toxicity (Domenici et al., 2002; Garwood et al., 2011). We and others have previously reported that exposure to increasing concentration of cholesterol or sequestration of cholesterol within the endosomal-lysosomal (EL) system by treatment with U18666A, a class II amphiphile inhibiting intracellular cholesterol transport, can increase APP levels/processing leading to enhanced Aβ production (Yang et al., 2017). Nevertheless, very little is known about the role of astrocytic Aβ in the development of AD pathology.

A number of recent studies have shown that exosomes, which are single membrane small vesicles (30-200nm diameter) belonging to a large family of membrane extracellular vesicles (EVs), represent a novel form of intercellular communication (Pegtel and Gould, 2019). They originate from endosomes and are secreted by most cells including neurons and glial cells (Mathieu et al., 2019; Thery et al., 2002). The exosomes, which contain a variety of proteins, lipids, glycoconjugates, mRNA, miRNA and genomic DNA, have been suggested to act as vehicles for the transfer of biomolecules/pathogens in various diseases including AD (Bellingham et al., 2012; Coleman and Hill, 2015; De Toro et al., 2015; Kalani et al., 2014; Pegtel and Gould, 2019; Simpson et al., 2008; Vingtdeux et al., 2012; Yanez-Mo et al., 2015). The initial link with AD was established not only by the identification of Aβ in exosomes but also the presence of exosomal proteins Alg-2 interacting protein (ALIX) and flotillin in Aβ-containing plaques in AD brains (Kokubo et al., 2005; Rajendran et al., 2006). Subsequent studies revealed that APP and its cleaved products are secreted with exosomes derived from neuroblastoma cell lines and primary cortical neurons (Fernandes et al., 2018; Laulagnier et al., 2018; Rajendran et al., 2006; Vingtdeux et al., 2007; Xie et al., 2019). Exosomes containing APP and its CTFs can also
be taken up by other cells where CTFs can be processed further by γ-secretase (Laulagnier et al., 2018). Inhibition of exosome secretion (Dinkins et al., 2014) or infusion of exosomes derived from cortical neurons can influence Aβ levels/deposition in mutant APP-transgenic mice (Yuyama et al., 2015), suggesting an important role for exosomes in AD pathology and its propagation. Unlike neurons, very little is known about exosomes secreted by astrocytes and their implication in AD pathology. An earlier study reported that exposure of astrocytes to Aβ triggers release of proapoptotic exosomes which can increase cell death (Wang et al., 2012). In the present study, we show the presence of APP, APP-cleaved products (α-CTF and β-CTF), Aβ and BACE1 in exosomes derived from cultured astrocytes. Cholesterol accumulation following U18666A treatment can decrease the secretion but enhance the levels of APP and Aβ-related peptides in exosomes. Additionally, we show that exosomes derived from U18666A-treated astrocytes can be taken up by primary cortical neurons in a phosphatidylinositol-3-kinase (PI3K)-dependent manner and trigger cell death, suggesting an important role for astrocyte-derived exosomes in AD-related pathology.

RESULTS

Effects of U18666A on astrocyte-derived exosomes: U18666A is one of the most well characterized class-2 amphiphilic compounds to attenuate cholesterol movement from the plasma membrane to endoplasmic reticulum and from the late-endosomes/lysosomes to the plasma membrane leading to accumulation of cholesterol within the EL system (Koh and Cheung, 2006; Martin et al., 2010). As reported earlier (Yang et al., 2017), U18666A triggered sequestration of cholesterol in rat cultured astrocytes, which are characterized using astrocyte specific marker glial fibrillar acidic protein (GFAP) (Fig. 1A and B). In untreated cells, staining of unesterified cholesterol with filipin showed only faint labelling without any accumulation, whereas 24hrs exposure to 5μg/ml U18666A markedly increase filipin staining suggesting intracellular sequestration of cholesterol (Fig. 1C and D). Since cholesterol sequestration enhances APP processing leading to increased secretion of Aβ peptides (Chung et al., 2018; Yang et al., 2017), we wanted to establish if exosomes
derived from UA-treated astrocytes may have a role in the development of AD pathology. As a first step, we revealed that exosomes isolated from control astrocytes using PEG-based precipitation method (Rider et al., 2016) display established exosomal markers flotillin-1, ALIX, TSG101, CD63 and CD81 (Perez-Gonzalez et al., 2012; Raposo and Stoorvogel, 2013), but not the negative marker calnexin (Zhang et al., 2019) (Fig. 1E, F).

The relative size of exosomes as measured by dynamic light scattering (DLS) is in the range of ~6-120nm diameter, which is reinforced by quantification of our electron micrographs of exosomes (Fig. 1G-I). To establish if intracellular cholesterol accumulation can influence the secretion of exosomes, astrocytes were treated with 5µg/ml U18666A for 24hrs and then exosomes isolated from control and treated astrocytes were processed for dot-blot, DLS and EM analysis, which showed a relative decrease in the levels of markers and number but not the size of exosomes secreted from astrocytes (Figs. 1E-I, 2E). To validate these data, astrocytes were labelled with Dil fluorescent dye and then treated with 5µg/ml U18666A for 24hrs. Labelling of the cholesterol with Dil did not affect astrocyte viability (Fig. 1J) but decreased the secretion of exosomes from U18666A-treated astrocytes, suggesting that cholesterol accrual can decrease the amount exosomes secreted from astrocytes (Fig. 1K).

To highlight the significance of cholesterol on the secretion of exosomes, cultured astrocytes were exposed to with or without Dil and then treated for 24hrs either with various modulators of cellular cholesterol levels such as cholesterol, methyl-β-cyclodextrin (MBCD) and wortmannin (Chung et al., 2018; Costa Verdera et al., 2017; Maulik et al., 2012; Tobert, 2003). As expected, total cholesterol levels in astrocytes detected using gas-liquid chromatography (Fig. 2A; Supplementary Fig. 1A) and Amplex red cholesterol assay kit (data not shown) were increased with cholesterol and 10% fetal bovine serum (FBS) treatment but decreased following exposure to MBCD, wortmannin and lovastatin. Cholesterol treatment as observed with U18666A showed increased sequestration of intracellular cholesterol in cultured astrocytes (Fig. 2B, C). Interestingly, treatment with aforementioned cholesterol regulating drugs/agents did not affect viability of cultured astrocytes (Fig. 2D; Supplementary Fig. 1B). Our dot-blot and Western blot analyses
further revealed that cholesterol and 10% FBS treatment, as observed with UA18666A-treated astrocytes, decreased the levels of exosomal markers flotillin-1, ALIX and TSG101, whereas the levels of these markers were increased following exposure to MBCD, wortmannin or lovastatin (Fig. 2E-G; Supplementary Fig. 1C). This was accompanied by a decreased secretion of exosomes from cholesterol- and 10% FBS-treated astrocytes, as evident from DLS analysis and/or quantification of fluorescence labelled exosomes. The secretion of exosomes, on the other hand, increased following MBCD, wortmannin and lovastatin treatments (Fig. 2H-J; Supplementary Fig. 1D). These results, taken together, suggest that intracellular cholesterol accumulation inversely regulates the secretion of exosomes from astrocytes.

Effects of U18666A on exosomal APP and APP-cleaved products: Previous studies have shown that exosomes derived from cultured neurons contain APP, APP-CTFs and Aβ peptides (Fernandes et al., 2018; Laulagnier et al., 2018; Rajendran et al., 2006; Vingtdeux et al., 2007; Xie et al., 2019). However, very little is known about the occurrence of APP and its cleaved products in exosomes derived from astrocytes or its regulation by U18666A. Our Western blot and dot-blot analysis revealed the presence of APP holoprotein in cell lysates as well as exosomes derived from control astrocytes and its upregulation following U18666A treatment (Fig. 3A, B). This was evident not only with APP antibody (clone Y188) that labels APP and APP-CTFs, but also with the antibody that identifies the Kunitz family of serine protease inhibitor (KPI)-domain containing APP, known to be expressed mostly in astrocytes (Fig. 3A, B). We also observed increased levels of APP-CTFα and APP-CTFβ in U18666A-treated astrocytes and secreted exosomes compared to control astrocytes (Fig. 3C-F). The steady-state levels of sAPPα, but not sAPPβ, were slightly increased both in cell lysates and exosomes derived from U18666A-treated astrocytes (Fig. 3G, H). Since cultured astrocytes secrete primarily Aβ_{1-40} (Yang et al., 2017), we measured the levels of rat Aβ_{1-40} using ELISA in cell lysates and exosomes following U18666A treatment. Interestingly, the levels of Aβ_{1-40} were markedly increased in U18666A-treated astrocytes and in secreted exosomes compared to control cultures (Fig. 3I).
Effects of U18666A on exosomal APP secretases: Earlier studies have shown that exosomes derived from cultured neurons contain APP-secretases such as ADAM10, BACE1 and components of γ-secretase complex (i.e., nicastrin, presenilin, PEN2 and APH1). Since α-CTF/β-CTF and sAPPα are evident in exosomes of cultured astrocytes, we evaluated the levels of ADAM10, BACE1 and two components of the γ-secretase-complex (nicastrin and PS1) both in cell lysates and exosomes derived from astrocytes using Western blot as well as dot-blot analysis. While all secretases or their components were evident in cell lysates, we were able to detect ADAM10, PS1 and nicastrin, but not BACE1, in secreted exosomes using Western blot and dot-blot analysis (Fig. 4A, B). However, using a sensitive BACE1-specific ELISA we could detect BACE1 in secreted exosomes of cultured astrocytes (Fig. 4G). Interestingly, treatment of astrocytes with U18666A, as reported earlier (Yang et al., 2017), increased the cellular levels of ADAM10, but not BACE1, PS1 or nicastrin compared to untreated control astrocytes (Fig. 4C-F). In contrast to cell lysates, U18666A treatment did not affect the exosomal levels of PS1 or nicastrin but decreased BACE1 levels (as detected by ELISA), compared to control exosomes (Fig. 4E-G).

Effects of U18666A on autophagy-lysosomal markers in exosomes: We have previously reported that U18666A treatment can enhance the levels of lysosomal (LAMP1) and autophagy (LC3-II) markers along with lysosomal enzyme cathepsin D in cultured astrocytes and/or neuronal cells (Amritraj et al., 2013; Yang et al., 2017). Since exosomes originate from the EL system, which plays a critical role in APP metabolism (Malm et al., 2016), we wanted to determine if LAMP1, LC3-II and cathepsin D are evident in exosomes and altered following U18666A treatment. Our results, in keeping with the earlier studies (Amritraj et al., 2013; Yang et al., 2017), revealed an up-regulation of cellular LAMP1, LC3-II and cathepsin D levels in U18666A-treated astrocytes (Fig. 5A-F). While cathepsin D level was increased in exosomes in Western blot and dot-blot analysis (Fig. 5G, H), a decreased level of exosomal LAMP1 and LC-3 was detected by dot-blot analyses (Fig. 5D, F).
Exosomes from U18666A-treated astrocytes affect neuronal viability: One of the critical functions of exosomes is to transport signaling and other molecules from donor to recipient cells where they can influence their functions (Mathieu et al., 2019; Thery et al., 2002). To determine if exosomes secreted from U18666A-treated astrocytes can affect neuronal function, we first characterized the purification of exosomes using dot-blot and DLS analyses (Fig. 6A, B) and then treated cortical neurons with DiI-labeled exosomes isolated from control or U18666A-treated astrocytes. As expected, DiI fluorescent signal was apparent only in treated cultured neurons (Fig. 6C-G) indicating uptake of exosomes by neurons. Since the PI3 kinase pathway plays a critical role in the neuronal uptake of extracellular vesicles including exosomes (Costa Verdera et al., 2017), we pre-treated cultured neurons with the established PI3 kinase inhibitor wortmannin and then incubated with DiI-labeled exosomes. Interestingly, wortmannin suppressed the uptake of exosomes, suggesting that the PI3 kinase pathway may be involved in regulating the uptake of exosomes into the neurons (Fig. 6F, G). Subsequently, our results revealed that viability of cultured neurons was decreased following exposure to exosomes derived from U18666A-treated astrocytes compared to control astrocytes (Fig. 6H). Interestingly, inhibiting neuronal uptake of exosomes by wortmannin attenuated the toxicity induced by U18666A-treated exosomes (Fig. 6I).

Since the level of Aβ was markedly increased in U18666A-treated exosomes, we wanted to determine the role of Aβ, if any, in neuronal vulnerability observed following exposure to U18666A-treated exosomes. Thus, we first showed that treatment with the γ-secretase inhibitor L-685,485 for 24hrs markedly decreased cellular Aβ1-40 levels both in control and U18666A-treated cultured astrocytes (Fig. 6J) without altering the characteristic or secretion of exosomes (Supplementary Fig. 2A, B). Subsequently, we exposed the cultured neurons to exosomes derived from control and U18666A-treated astrocytes treated with or without L-685,485 for 24hrs and then viability of neurons was assessed using MTT assay. As expected, U18666A-treated exosomes reduced neuronal viability compared to exosomes derived from control or L-685,485-treated astrocytes (Fig. 6K). Interestingly, exosomes derived from U18666A+L-685,485 co-treated astrocytes significantly increased
neuronal viability compared to U18666A-treated exosomes, suggesting a potential role for exosomal Aβ peptides in reducing neuronal viability (Fig. 6K). To validate this hypothesis, we subsequently evaluated neuronal viability following neutralization of exosomal Aβ with an Aβ antibody (i.e., A11 antibody). Since exosomal Aβ is apparent on the surface of the exosomal membrane (Rajendran et al., 2006; Yuyama et al., 2014), incubating exosomes derived from U18666A-treated astrocytes with A11 antibody overnight neutralized exosomal Aβ, as evident from our dot-blot analysis (Fig. 6L, M). Interestingly, treatment of cortical neurons with exosomes following neutralization of Aβ peptide did not influence the cellular uptake of exosomes (Supplementary Fig. 2C-F) but significantly increased neuronal viability compared to neurons treated with non-neutralized exosomes (Fig. 6N).

**DISCUSSION**

The present study reveals that U18666A-induced cholesterol sequestration within the EL system can decrease the secretion of exosomes derived from cultured astrocytes but increase the levels of APP and its cleaved products in exosomes. Additionally, exosomes derived from U18666A-treated astrocytes are found to induce toxicity following cellular uptake into primary cortical neurons. This is supported by results which showed that i) U18666A-triggered cholesterol sequestration in cultured astrocytes can decrease the secretion of exosomes, ii) levels of APP and its cleaved products APP-CTFs and soluble fragments are elevated both in cells as well as in secreted exosomes following U18666A treatment, iii) cellular as well as exosomal Aβ₁₋₄₀ levels are increased in U18666A-treated astrocytes, iv) exosomes derived from U18666A-treated astrocytes can be taken up by primary cortical neurons in a PI-3-kinase-dependent pathway and v) neuronal uptake of exosomes derived from U18666A-treated astrocytes can lead to neurotoxicity which is attenuated by treatment of the astrocytes with a γ-secretase inhibitor or neutralization of exosomal Aβ peptide with an Aβ antibody. Collectively, these results indicate that exosomes derived from cholesterol-accumulated cultured astrocytes can play an important role not only in trafficking APP and its cleaved products but also in influencing neuronal viability and spreading of AD pathology.
Evidence suggests that exosomes, generated in multi-vesicular bodies (MVBs), are secreted to the extracellular environment through the fusion of MBVs with plasma membrane (Colombo et al., 2014; Raposo and Stoorvogel, 2013). Cholesterol is not only a content of exosomes but also related closely to the process of secretion of exosomes (Pfrieger and Vitale, 2018; Tamboli et al., 2010; Xu et al., 2021). U18666A is one of the most well characterized class-2 amphiphilic compounds, which acts not only to reduce cholesterol movement from the plasma membrane to ER and from the late-endosomes/lysosomes to the plasma membrane, leading to accumulation of cholesterol within the EL system, but also to inhibit cholesterol biosynthesis by regulating enzymes involved in cholesterol biosynthesis. Additionally, U18666A affects membrane protein transport than can influence the composition of membrane and its ability to vesiculate (Cenedella, 2009; Kuzu et al., 2017). Some earlier studies reported that U18666A binds and inhibits Niemann Pick Type C1 protein, which plays a crucial role in the efflux of cholesterol out of the lysosomes, leading to its accumulation within the EL system (Liscum and Sturley, 2004; Lu et al., 2015). In the current study, we revealed that U18666A treatment can attenuate the secretion of exosomes from astrocytes but does not affect the size of exosomes. Additionally, we showed that upregulation of cellular cholesterol level following exposure to extracellular cholesterol or 10% FBS can decrease exosome secretion, while depletion of cellular cholesterol with MBCD, wortmannin or lovastatin treatments can increase exosome secretion, suggesting an inverse role for cholesterol in regulating exosomal secretion from astrocytes. Since the transport of MVBs along the microtubule towards the plasma membrane depends partly on the cellular cholesterol content, it is possible that inhibition of cholesterol trafficking and/or synthesis by U18666A may underlie the decrease secretion of exosomes observed in treated astrocytes (Pfrieger and Vitale, 2018). Although this could be a secondary consequence of the effects of U18666A on membrane protein/structure, the evidence that an upregulation and depletion in the cellular cholesterol levels can inversely regulate exosome secretion/markers, it is likely that cellular cholesterol levels rather than other effects of U18666A (Cenedella, 2009; Kuzu et al., 2017) may partly be associated with the observed decreased secretion/markers of exosomes from cultured astrocytes. This is also consistent with a previous study which
showed that U18666A can decrease the secretion of exosomes by blocking the formation of MVBs (Elgner et al., 2016). However, many studies reported differential role of cholesterol in regulating exosome secretion raising the possibility that variation in the levels/sites of cholesterol accumulation, cell types and/or experimental conditions may underlie the cause of discrepancy (Chung et al., 2018; Koh and Cheung, 2006; Martin et al., 2010).

The influence of cholesterol on APP metabolism has long been studied, both in cultured conditions and animal models of AD (Allinquant et al., 2014; Maulik et al., 2013), in view of the evidence that intracellular trafficking, localization and processing of APP are regulated by the levels of cholesterol within the cells. Consistent with earlier results, we showed that the cellular levels of APP holoproteins, including KPI-APP known to be expressed mostly in astrocytes, are increased following U18666A treatment. Accompanying APP, we observed an elevated level of α-/β-CTFs, ADAM10 and Aβ1-40, but not BACE1 or γ-secretase complex (PS1 and nicastrin) in U18666A-treated astrocytes. Additionally, the levels of LAMP1, LC3-II and cathepsin D, as reported in earlier studies (Amritraj et al., 2013; Yang et al., 2017), are found to increase in astrocytes following U18666A treatment. The observed changes are due to cholesterol sequestration rather than cellular degeneration as viability of astrocytes was not found to be affected following treatment with 5µg/ml U18666A over 24hrs. Although previous studies have shown that U18666A-induced cholesterol sequestration can lead an increased levels of APP and/or its cleaved products in cultured astrocytes/neurons/ cell lines (Boland et al., 2010; Chung et al., 2018; Davis, 2008; Jin et al., 2004; Koh and Cheung, 2006; Runz et al., 2002; Yamazaki et al., 2001; Yang et al., 2017), there is some discrepancy in the results which are likely due to cell types used in the studies along with the concentration and duration of the U18666A treatment.

Previous studies using cellular and/or animal models of AD have shown that exosomes secreted from neurons and astrocytes can serve as carriers for APP, APP-CTFs and/or Aβ peptides which can either exacerbate or attenuate AD pathology (Fernandes et al., 2018;
Laulagnier et al., 2018; Lauritzen et al., 2019; Rajendran et al., 2006; Sharples et al., 2008; Sullivan et al., 2011; Vingtdeux et al., 2007; Xie et al., 2019). However, very little is known how different treatment strategies can regulate the levels of APP or its cleaved products in exosomes secreted from neurons or astrocytes. Our results show that U18666A-induced cholesterol sequestration in astrocytes can trigger secretion of exosomes containing higher levels of APP and APP-CTFs compared to control astrocytes. Additionally, the levels of Aβ1-40 are increased in exosomes derived from U18666A-treated astrocytes. It is of interest to note that while the levels of BACE1 decrease, the levels of γ-secretase components nicastrin and PS1 remain unaltered in exosomes secreted from cholesterol-accumulated astrocytes. Since secretion of exosomes is decreased following U18666A treatment, it is likely that enhanced exosomal levels of APP and its cleaved products may have an intracellular origin due to increased cellular levels/processing of APP and their incorporation into intraluminal vesicles prior to release as exosomes. However, detection of APP and its secretases in exosomes raise the possibility that extracellular vesicles, as reported in earlier studies (Perez-Gonzalez et al., 2020), may also serve as a potential site for de novo APP metabolism. Consistent with our results, Aβ1-42 treatment which is known to increase cholesterol accumulation within cells (Mohamed et al., 2012) has been shown to decrease exosome secretion from astrocytes in a JNK-dependent pathway (Abdullah et al., 2016). However, an increase secretion of proapoptotic exosomes has also been reported from cultured astrocytes following Aβ treatment (Wang et al., 2012). Although levels of APP or its cleaved products have not been analyzed in the exosomes secreted following Aβ treatment, several studies have reported the presence of APP, APP-CTFs and/or Aβ peptides in astrocytic exosomes isolated from brain or serum of mutant APP transgenic mice (Elsherbini et al., 2020a; Lauritzen et al., 2019; Perez-Gonzalez et al., 2012; Perez-Gonzalez et al., 2020). A recent study further showed that astrocyte-derived exosomes isolated from serum contain markedly higher levels of BACE1, γ-secretase, sAPPα, sAPPβ and Aβ1-42 than neuronal-derived exosomes both in control and AD patients, highlighting the significance of astrocytic exosomes in regulating AD pathology (Goetzl et al., 2016).
Accompanying high levels of APP/Aβ-related peptides, we showed that cellular uptake of exosomes secreted from U18666A-treated astrocytes can render cortical neurons vulnerable to toxicity. This effect is ameliorated by inhibiting cellular uptake of exosomes as well as by attenuating Aβ production in U18666A-treated astrocytes with a γ-secretase inhibitor L-685,485 that did not affect exosomal characteristic/secretion, suggesting a role for exosomal Aβ-related peptides in the loss of neurons. This is further supported by the evidence that neutralization of exosomal Aβ with an Aβ antibody, which did not influence neuronal uptake, was found to attenuate toxicity induced by exosomes. Although the underlying mechanisms by which Aβ triggers neuronal loss remain unclear, a recent study revealed that Aβ-containing exosomes derived from astrocytes of 5xFAD mice and AD patients can promote neurodegeneration both under in vitro and in vivo conditions by inducing mitochondrial damage and caspase activation. The concentration of Aβ associated with exosomes inducing damage, however, was found to be several folds lower than those required for Aβ alone, indicating the contribution of other toxic factors in the degeneration of neurons (Elsherbini et al., 2020a; Elsherbini et al., 2020b). This is supported by an earlier study which showed that exosomes released from cultured astrocytes in response to Aβ treatment contain proapoptotic ceramide and prostate apoptosis response 4 that can trigger cell loss (Wang et al., 2012). Thus, it is of interest to determine if proapoptotic molecules other than Aβ such as ceramide may have a role in the loss of neurons triggered by exosomes derived from U18666A-treated astrocytes.

Unlike neurons, astrocytes generate very little Aβ under physiological conditions due to a low expression of APP and BACE1 (Thal, 2012; Zhao et al., 2011). Activated astrocytes that result from insults and pathological conditions such as AD, display higher levels of APP and/or its processing enzymes, which may enhance the generation of Aβ peptides (Hartlage-Rubsamen et al., 2003; Kodam et al., 2010; Kodam et al., 2019; Miake et al., 1999; Nadler et al., 2008; Nagele et al., 2003; Thal et al., 2000). Increased levels and/or sequestration of cholesterol within astrocytes have also been shown to enhance the production of APP and its cleaved products (Yang et al., 2017). Since cholesterol levels are increased in AD brains (Panchal et al., 2010; Xiong et al., 2008) and shown to be a risk
factor for AD (Maulik et al., 2013; Wolozin, 2004), it is possible that enhanced level/ altered subcellular distribution of cholesterol within astrocytes can contribute to the development/ propagation of AD pathology within the brain by triggering dysfunction/ degeneration of neurons following transport of APP/Aβ-related peptides into recipient neurons. This is supported by three lines of evidence i) Aβ toxicity is known to be enhanced in the presence of astrocytes (Domenici et al., 2002; Thal, 2012) and following exposure to conditioned media from Aβ-treated astrocytes but not from control astrocytes (Garwood et al., 2011; Paradisi et al., 2004), ii) Aβ-containing exosomes derived from astrocytes of 5xFAD mice and AD patients can trigger neuronal loss (Elsherbini et al., 2020a; Elsherbini et al., 2020b) and iii) Aβ peptides have been shown to accumulate within astrocytes prior to plaque formation and their accrual correlates with local tissue damage in AD brains (Nagele et al., 2003; Simpson et al., 2010; Thal et al., 2000). However, contradictory data suggesting that exosomes may act as potent scavengers for Aβ in the brain (Yuyama et al., 2014) and contribute to the clearance of toxic proteins by microglia uptake (Fitzner et al., 2011; Yuyama et al., 2012) or enzymatic degradation (Katsuda et al., 2013) favors a protective role for astrocyte-derived exosomes in the development of AD pathology. Considering the multifaceted roles for different subtypes astrocytes in various experimental conditions (Li et al., 2019; Liddelow and Barres, 2017), it is possible that astrocytes at early stages of AD may serve a protective role by relieving neurons from accumulated toxic materials. However, as the disease advances with compromised endosomal/ autophagic-lysosomal system, astrocytic exosomes may act not only to propagate disease pathology but also to trigger dysfunction/ degeneration of neurons in the affected brain regions (Kim et al., 2020; Upadhya et al., 2020; Venturini et al., 2019). Thus, characterizing the spatiotemporal function of exosomes derived from astrocytes from experiments relevant to disease pathology may provide a better understanding about the role of astrocytes in AD pathogenesis. In summary, the results presented in this study reveal that exosomes derived from cholesterol-accumulated astrocytes can play an important role in trafficking APP/Aβ peptides and also in influencing neuronal viability in AD brains.
MATERIALS AND METHODS

Reagents: Rat primary astrocytes as well as Astrocyte Medium-animal (AM-a) were purchased from ScienCell (Carlsbad, CA, USA), whereas polyethylene glycol (PEG), 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), wortmannin, cholesterol, MBCD, lovastatin, filipin and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) were from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kit for the detection of mouse Aβ1-40 was obtained from Life Technologies (Burlington, ON, Canada) and the ELISA kit for BACE1 was from Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan). U18666A was purchased from Enzo Life Sciences (Ann Arbor, MI, USA), whereas Radioimmunoprecipitation assay (RIPA) lysis buffer, bicinchoninic acid (BCA) protein assay kit, DiI cell-labeling solution, Amplex Red cholesterol assay kit and enhanced chemiluminescence (ECL) kit were from Thermo Fisher Scientific (Montreal, QC, Canada). Sources of all primary antibodies are listed in Table 1. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bio-rad. All other chemicals were from Sigma-Aldrich or Thermo Fisher Scientific.

Astrocytes cell culture: Rat primary astrocytes were cultured in AM-a media containing 2% FBS, 1% penicillin/streptomycin and 1% astrocyte growth supplement-animal (AGS-a) as described earlier (Ourdev et al., 2019; Yang et al., 2017). Cells were grown in T150 flask or 96-well plates at 37°C with 5% CO2 and the medium was changed every 2 or 3 days. At 90% confluence, cultured astrocytes were treated with 5µg/ml U18666A in AM-a media containing exosomes free FBS for 24hrs. In some experiments, cells were treated with 5µg/ml U18666A in the presence or absence of 100µM γ-secretase inhibitor L-685,458, 0.5µg/ml cholesterol, 5µM lovastatin, 10% FBS, 5µM MBCD or 5µM wortmannin in AM-a media for 24hrs. After treatment cells were washed, homogenized in RIPA lysis buffer containing protease inhibitor cocktail and stored at -80°C until further processing. In some experiments, exosomes isolated from control or U18666A-treated cultured astrocytes were incubated with or without Aβ antibody overnight at 4°C as reported earlier (Tang et al., 2020) and then processed for Dot-blot analysis or treatment of primary cultured neurons.
Filipin staining: Filipin labels unesterified cholesterol (Bornig and Geyer, 1974). To evaluate the intracellular cholesterol accumulation, control, U18666A and cholesterol-treated cultured astrocytes were washed in phosphate-buffered saline (PBS, pH 7.4), fixed with 4% paraformaldehyde (PFA) and then incubated in the dark with 125μg/ml filipin as described earlier (Yang et al., 2017). Stained coverslips were mounted with ProLong Gold Antifade Reagent and then visualized using a Zeiss Axioskop-2 microscope (Carl Zeiss, Germany).

Exosome isolation: Exosomes from the control and treated cultured astrocytes were isolated as described earlier (Rider et al., 2016). In brief, supernatant of the cultured astrocytes was centrifuged sequentially at 500g for 5min, 2,000g for 10min at 4°C followed by 10,000g for 30min. Once centrifuged, the media was added to an equal volume of 16% PEG at 4°C to achieve a desired final PEG concentration (8%). Samples were then mixed thoroughly and incubated at 4°C overnight. The next day, samples were centrifuged at 5,000g for 2hrs at 4°C, the pellet was suspended in 1mL of particle-free PBS and then ultracentrifuged at 120,000g for 90min to wash the particles of contaminating proteins and PEG. The pellet was resuspended in 60μl of particle free PBS by shaking for 30min at room temperature and the exosomes were isolated as described before (Rider et al., 2016).

Negative stain electron microscopy: Aliquots of 5μl exosomes derived from control and U18666A-treated cultured astrocytes were adsorbed onto freshly glow-discharged 400 mesh carbon-coated copper grids for 1min and then washed sequentially with 50μl 0.1M and 0.01M ammonium acetate solutions. The grids were then stained using a freshly filtered 2% uranyl acetate and air dried after removing the excess stain with filter paper. The stained samples were examined with a Tecnai G20 transmission electron microscope (FEI Company, The Netherlands) operating at an acceleration voltage of 200kV. Electron micrographs were recorded with an Eagle 4k x 4k CCD camera (FEI Company, The Netherlands).
Asymmetric flow field-flow fractionation and DLS: Asymmetric flow field-flow fractionation and DLS was used to fractionate and measure size distribution of exosomes isolated from control and treated cultured astrocytes. Eighty microlitres of sample were injected into an AF2000 Postnova system using PBS (pH 7.4) as running buffer. The channel was 26.5cm in length and 350µm in height, constructed with a trapezoidal spacer of maximal width 21mm at the inlet and lined with a 10-kDa cutoff polyethersulfone membrane at the accumulation wall. Samples were focused for 5min and then eluted at a channel flow of 0.5ml/min with cross-flow decreasing from 1.5 to 0.35ml/min in the first 15min, from 3.5 to 0ml/min in the next 30min, and running with no cross-flow for the last 10min. A slot pump was run at 0.3ml/min to concentrate the samples before they passed through the detectors. Fractions of 0.2ml were collected and then static and dynamic light scattering measurements were carried out on an in-line DAWN HELEOS II detector (Wyatt Technology) as described earlier (Eskandari-Sedighi et al., 2020; Zhang and Lyden, 2019).

Cholesterol assay: The amount of cholesterol level in the astrocytes obtained from the control and various experimental paradigms was determined using Amplex red cholesterol assay kit as described before (Maulik et al., 2012). Fluorescence was measured using a SpectraMax M5 spectrophotometer (Molecular Devices, LLC.) at excitation/emission wavelengths of 560/590 nm. All samples were assayed in quadruplicate and results were from three independent experiments. Apart from the Amplex red cholesterol assay kit, the mass of cholesterol from control and experimental samples was determined using gas-liquid chromatography. In brief, cultured samples were homogenized for 2hrs at 30°C with phospholipase C and then lipids were extracted after adding internal standard Tridecanoin and then processed to determine cholesterol mass using gas-liquid chromatography as described earlier (Amritraj et al., 2009). All samples were assayed in triplicate and results were from two independent experiments.
**Dil labeling:** Astrocytes suspended at a density of $1\times10^6$/ml in AM-a serum-free media were added with 5μl/ml Dil cell-labeling solution, incubated for 20min at 37°C and then centrifuged at 500g for 5min at room temperature as described recently (Viveiros et al., 2021). After removing the supernatant, cells were washed with AM-a media, centrifuged twice at 500g for 5min each and then seeded in T150 flask with normal Am-a media. The Dil fluorescence levels were measured using a SpectraMax M5 spectrophotometer at excitation/emission wavelengths of 549/565 nm.

**Dot-blotting and Western blotting:** The protein concentration of cell lysates collected from control and treated cultured astrocytes was determined using BCA kit. Dot-blotting was performed using the protocol of Bio-Dot Apparatus (Bio-rad). In brief, the nitrocellulose membrane after washing three times with 100μl Tris-buffered saline (TBS) was spotted with 5μl sample (containing equal amount of protein) in each well, washed with TBS, blocked with 5% milk and then incubated overnight at 4°C with various primary antibodies at dilutions listed in Table-1. After incubation, the membranes were washed, incubated with HRP-conjugated secondary antibodies (1:5000) for 1hr and immunoreactive proteins were detected with ECL kit. Western blotting of cultured cells and exosomes isolated from the cells was performed as described earlier (Wu et al., 2017; Yang et al., 2017). In brief, denatured samples were resolved on 10% or 12% gradient sodium polyacrylamide gels, transferred to polyvinylidene difluoride membranes, blocked with 5% milk and incubated overnight at 4°C with various primary antibodies at dilutions listed in Table-1. The membranes were then incubated for 1hr with HRP-conjugated secondary antibodies (1:5000) and immunoreactive proteins were detected with ECL kit. All blots were re-probed with anti-β-actin antibody and quantified using ImageJ (Ourdev et al., 2019).

**Mouse cortical neuronal cultures:** Timed pregnant BALB/c mice purchased from Charles River (St. Constant, Quebec, Canada) were maintained according to Institutional and Canadian Council on Animal Care guidelines. Primary cortical cultures were prepared from 18-day-old embryos of the pregnant mice as described previously (Kodam et al., 2019). In brief, frontal cortex from pup brains was dissected in Hanks’ balanced salt...
solution and then digested with 0.25% trypsin-EDTA. The cell suspension was filtered through a cell strainer and plated on 8-wells chamber slides or 96-well plates. The cultures were grown at 37°C in 5% CO₂ humidified atmosphere in Neurobasal medium supplemented with B27/N2, 50μM-glutamine, 15mM HEPES, 10units/ml penicillin, 10mg/ml streptomycin and 1% FBS. The medium was replaced 1 day later without FBS and all experiments were performed on day 6 or 7 after plating (Kodam et al., 2019). In brief, cortical neurons grown on 8-well chamber slides or 96-well plates were pre-treated with or without 5μM wortmannin for 24hrs and then incubated with exosomes derived from Dil-labeled control or U18666A-treated astrocytes for 4hrs (for neuronal uptake) or 24hrs (for neuronal viability). In parallel, neuronal uptake of exosomes treated with or without Aβ antibody was also evaluated. While cells from 8-well plates were fixed in 4% PFA and processed for DAPI staining to determine exosome uptakes, neurons of 96-well plates were processed for MTT cell assay. Stained sections were examined and photographed using a Zeiss confocal microscope equipped with a 63X Plan-apochromatic oil-immersion lens (LSM 700, Carl Zeiss, Inc.).

**Cell viability assay:** Viability of astrocytes, with or without DiI-labelling, following various experimental paradigms was analyzed using MTT assay as described earlier (Wu et al., 2015; Yang et al., 2017). Additionally, viability of cortical neurons was evaluated following exposure to exosomes isolated from control and UA-treated astrocytes either i) in the presence or absence of wortmannin or ii) after overnight incubation with or without Aβ antibody. In another set of experiments, viability of cortical neurons was assessed following exposure of exosomes isolated from astrocytes treated with or without L-685,458 in the presence or absence of U18666A. In brief, control and treated cultures seeded on 96-well plates were replaced with new media containing 0.5mg/ml MTT and then incubated for 4h at 37°C with 5% CO₂/95% air. After the reaction, formazan was dissolved in dimethyl sulfoxide and absorbance was measured at 570nm with a Spectramax M5 spectrophotometer. The experiments were repeated three times in triplicate.
**Immunostaining:** For characterization, cultured astrocytes grown on coverslips were fixed with 4% PFA and then incubated overnight at 4°C with anti-APP in combination with anti-GFAP antibodies at dilutions listed in Table 1. The coverslips were then exposed to Alexa Fluor 488/594-conjugated secondary antibodies (1:1000), washed and mounted with prolong gold anti-fade medium. Immunostained cells were visualized using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany) as described earlier (Maulik et al., 2015).

**ELISA for Aβ_{1-40} and BACE1:** The levels of Aβ_{1-40} and BACE1 in cell lysates as well as exosomes derived from astrocytes were measured by using respective rat Aβ_{1-40} and BACE1 ELISA kits as reported previously (Yang et al., 2017). The OD450 value was converted to pg/ml according to a standard curve. Data represent the total Aβ_{1-40} and BACE1 levels in cell lysates and exosomes derived from cultured astrocytes. All samples were assayed in duplicate and the data were from three independent experiments (Ourdev et al., 2015; Yang et al., 2017).

**Statistical analysis:** All data obtained from three to four experiments were expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s post-hoc analysis for multiple comparisons or Student’s t-test for single comparison with a significance set at $p < 0.05$. All analysis was performed using GraphPad Prism.

**Declaration**

**Competing interests:** The authors declare that they have no conflict of financial or competing interests.

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Authors contributions: QW did the major experiments, analysed the data related cultured astrocytes and provided first draft of the manuscript. LC and RKJ also performed experiments and analysed the data related to DLS and EM. SK designed the study, helped in analysing the data and corrected the manuscript with help from QW, LC, RKJ, VS and HW.

REFERENCES


Figure 1. U18666A treatment reduces exosome secretion from cultures astrocytes.

A and B: Representative confocal images showing immunoreactive GFAP and APP in control cultures astrocytes. C and D: Photomicrographs depicting the accumulation of cholesterol, as revealed by filipin staining, in cultured astrocytes treated with or without 5 µg/ml U18666A for 24hrs. E and F: Dot-blots and relative quantification showing the characterization and levels of markers of exosomes isolated from control and 5µg/mL U18666A-treated (24hrs) cultured astrocytes. Note the presence of Flotillin-1, ALIX, TSG101 and to some extent CD63 and CD81 both in cells and exosomes, whereas calnexin, as expected, is evident only in the cell-lysates but not in the exosomes. The levels of Flotillin-1, ALIX, TSG101, CD63 and CD81 are found to be markedly decreased in exosomes isolated U18666A-treated astrocytes compared to control astrocytes. G; Dynamic light scattering showing the relative size of secreted exosomes did not alter between control and U18666A-treated cultured astrocytes. H and I: Electron micrographs of exosomes isolated from control and U18666A-treated cultured astrocytes (scale bars = 200nm) (H) and their relative size and numbers measured from 20 random EM images. Note the decrease in the number of exosomes secreted from U18666A-treated astrocytes compared to control astrocytes. J; Histogram showing that neither U18666A treatment nor
labelling with fluorescent dye Dil affect viability of cultured astrocytes as revealed by MTT assay. Histogram depicting that U18666A treatment did not affect the cellular uptake of Dil fluorescent dye but decrease the secretion of Dil-labelled exosomes from U18666A-treated astrocytes compared to control astrocytes as detected by spectrometer. All results are presented as means ± SEM and obtained from three separate experiments. **p < 0.01. Con, control; UA, U18666A.
Figure 2. Cholesterol level inversely regulates exosome secretion from astrocytes.  
A: Histogram showing the cellular cholesterol levels following treatment 5μg/mL U18666A, 0.5μg/mL cholesterol, 5μM MBCD or 5μM wortmannin for 24hrs compared to control astrocytes as measured by gas chromatography. Note that cholesterol level was not altered in U18666A-treated cultured astrocytes but increased following exposure to extracellular cholesterol and decreased after treatment with MBCD and wortmannin. B and C: Photomicrographs depicting the accumulation of cholesterol, as revealed by filipin staining, in cultured astrocytes treated with or without 0.5μg/mL cholesterol for 24hrs. D: Histogram showing the viability of cultured astrocytes was not altered following exposure to 5μg/mL U18666A, 0.5μg/mL cholesterol, 5μM MBCD or 5μM wortmannin for 24hrs as revealed by MTT assay. E: Dot-blots showing the labelling of cell lysates and exosomes with Flotillin-1, ALIX and TSG101 following treatment 5μg/mL U18666A, 0.5μg/mL cholesterol, 5μM MBCD or 5μM wortmannin for 24hrs compared to control astrocytes. F and G: Western blots (F) and relative quantification (G) showing alterations of Flotillin-1, ALIX and TSG101 in exosomal markers in cells and secreted exosomes following treatments with 5μg/mL U18666A, 0.5μg/mL cholesterol, 5μM MBCD or 5μM...
wortmannin for 24hrs compared to control astrocytes. H: Dynamic light scattering showing the relative size and number of secreted exosomes following treatment U18666A, cholesterol, MBCD or wortmannin compared to control astrocytes. Note that number of exosomes secreted decreased from U18666A- and cholesterol-treated astrocytes but increased from MBCD- and wortmannin-treated astrocytes compared to control astrocytes. I: Histogram depicting that cellular uptake of Dil fluorescent dye, as measured using spectrometer, was not altered after treatment of astrocytes with U18666A, cholesterol, MBCD or wortmannin. J: Histogram depicting that secretion of Dil-labelled exosomes decreased from U18666A- and cholesterol-treated astrocytes but increased from MBCD- and wortmannin-treated astrocytes compared to control astrocytes. All results are presented as means ± SEM and obtained from three separate experiments. *p < 0.05, **p < 0.01 and ***p < 0.001. Con, control; UA, U18666A.
Figure 3. Cellular and exosomal levels of APP and its cleaved products in U18866A-treated astrocytes.

A: Western blots showing the levels of APP, KPI-APP, α-CTF and β-CTF in cell lysates and exosomes derived from control and U18666A-treated cultured astrocytes. Unlike cell lysates, no sAPPα or sAPPβ was detected by Western blot in the exosomes of control and U18666A-treated astrocytes. B: Dot-blots depicting the presence of APP, KPI-APP, sAPPα and sAPPβ in cell lysates and exosomes derived from control and U18666A-treated cultured astrocytes. C-H: Histograms showing quantification of Western blots depicting APP, KPI-APP, α-CTF and β-CTF in cell lysates and exosomes secreted from control and U18666A-treated astrocytes. Note the increased cellular and exosomal levels of APP, α-CTF, β-CTF and sAPPβ, but not sAPPα, following U18666A treatment compared control astrocytes. I: Histogram depicting increased cellular and exosomal levels of Aβ1-40, as detected by ELISA, following U18666A treatment compared control astrocytes. All results are presented as means ± SEM and obtained from three separate experiments. *p < 0.05 and **p < 0.01. Con, control; UA, U18666A.
Figure 4. Cellular and exosomal levels of APP secretases in U18866A-treated astrocytes. 

A and B; Western blots (A) and Dot-bLOTS (B) showing the presence/levels of ADAM10, BACE1 and γ-secretase components nicastrin and PS1 in cell lysates and exosomes derived from control and U18866A-treated cultured astrocytes. 

C-F; Histograms showing the quantification of Western blots depicting ADAM10, BACE1, nicastrin and PS1 in cell lysates and exosomes secreted from control and U18866A-treated astrocytes. Note the increased cellular and exosomal levels of ADAM10 but not BACE1, nicastrin or PS1 following U18866A treatment compared control astrocytes. 

G; Histogram depicting decreased exosomal, but not cellular, levels of BACE1, as measured by ELISA, following U18866A treatment compared control astrocytes. All results are presented as means ± SEM and obtained from three separate experiments. *p < 0.05 and **p < 0.01. Con, control; UA, U18866A.
Figure 5. Cellular and exosomal levels of LAMP1, LC3 and cathepsin D in U18866A-treated astrocytes.

A; Western blots showing the levels of LAMP1, LC3 and cathepsin D in cell lysates and exosomes derived from control and U18666A-treated cultured astrocytes. Unlike cell lysates, very low levels of LAMP1 but not LC3 was detected by Western blot in the exosomes of control or U18666A-treated astrocytes. The cellular and exosomal levels of cathepsin D, on the other hand, are found to be increased following U18666A treatment. B; Dot-blots depicting the presence of LAMP1, LC3 and cathepsin D in cell lysates and exosomes derived from control and U18666A-treated cultured astrocytes. C, E and G; Histograms showing quantification of Western blot depicting cellular levels of LC3-I (C), LC3-II (E) and cellular and exosomal levels of cathepsin D (G) from control and U18666A-treated astrocytes. Note the increased levels of LC3-II and cathepsin D following U18666A treatment compared to control astrocytes. D, F and H; Dot-blots depicting cellular and exosomal levels of LAMP1 (D), LC3 (F) and cathepsin D (H) from control and U18666A-treated astrocytes. Note the levels of LAMP1 and LC3 are increased in cells but decreased in exosomes, whereas cathepsin D levels are increased both in cells and exosomes following U18666A treatment compared control astrocytes. All results are presented as means ± SEM and obtained from three separate experiments. *p < 0.05. Con, control; UA, U18666A.
Figure 6. U18666A-treated exosomes affects viability of primary cortical neurons.

A; Dot-blots showing the labelling of cell lysates and secreted exosomes derived from Dil-labelled control and U18666A-treated cultured astrocytes with Flotillin-1, ALIX and TSG101. B; Dynamic light scattering showing the relative size of secreted exosomes derived from Dil-labelled control and U18666A-treated cultured astrocytes. C-G; Photomicrographs of primary cortical neurons without exposure to exosomes (C) and following cellular uptake of exosomes from Dil-labelled control (D) and U18666A-treated (E) astrocytes in the absence (D, E) and presence (F, G) of wortmannin. Note that uptake of labelled exosomes by primary cortical neurons decreased in the presence of wortmannin. H; Histograms showing quantification of relative fluorescence intensity representing decreased uptake of Dil-labelled exosomes by primary cortical neurons in the presence of Wortmannin. I; Histograms showing decreased viability of cortical neurons following uptake of exosomes derived from UA18666A-treated astrocytes compared to control astrocytes as revealed by MTT assay. Inhibiting cellular uptake of exosomes from UA18666A-treated astrocytes by wortmannin protects viability of neurons. J; Histograms showing the cellular levels of Aβ1-40, as detected by ELISA, in control and UA18666A-treated astrocytes in the absence and presence of γ-secretase inhibitor L-685,458. Note that treatment of astrocytes with L-685,458 significantly decreased the cellular levels of Aβ1-40. K; Histograms showing viability of cortical neurons after uptake of exosomes derived from UA18666A-treated astrocytes treated with and without γ-secretase inhibitor L-685,458. Note that reversal of neuronal viability following uptake
of exosomes derived from UA18666A-treated astrocytes exposed to L-685,458. L; Dot-blot showing the neutralization of Aβ peptide following overnight incubation of exosomes secreted from control and U18666A-treated cultured astrocytes with Aβ antibody. M; Purification of corresponding exosomes was depicted with dot-blot analysis using exosomal markers Flotillin-1 and ALIX. N; Histograms showing viability of cortical neurons after uptake of exosomes derived from UA18666A-treated astrocytes treated with and without Aβ antibody. Note increase viability of neurons following uptake of exosomes derived from UA18666A-treated astrocytes exposed to Aβ antibody. All results are presented as means ± SEM and obtained from three separate experiments. *p < 0.05, **p < 0.01 and ***p < 0.001. Con, control; UA, U18666A.

Table 1. Details of the primary antibodies used in this study

<table>
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WB: Western blot; DB: dot-blot.
Fig. S1. Effects Lovastatin and 10% FBS on exosome secretion from astrocytes.
A: Histogram showing the cellular cholesterol levels following treatment 5μg/mL U18666A, 5μM lovastatin and 10% FBS for 24hrs compared to control astrocytes as measured by gas chromatography. Note that cholesterol level was not altered in U18666A-treated cultured astrocytes but increased following exposure to 10% FBS and
decreased after treatment with lovastatin. **B**; Histogram showing the viability of cultured astrocytes was not altered following exposure to 5μg/mL U18666A, 5μM lovastatin and 10% FBS for 24hrs as revealed by MTT assay. **C**; Dot-blot showing the labelling of cell lysates and exosomes with Flotillin-1, ALIX and TSG101 following treatment 5μg/mL U18666A, 5μM lovastatin and 10% FBS for 24hrs compared to control astrocytes. **D**; Dynamic light scattering showing the relative size and number of secreted exosomes following treatment U18666A, lovastatin and 10% FBS compared to control astrocytes. Note that number of exosomes secreted decreased from U18666A- and 10% FBS-treated astrocytes but increased from lovastatin-treated astrocytes compared to control astrocytes. All results are presented as means ± SEM and obtained from three separate experiments. *p < 0.05, **p < 0.01 and ***p < 0.001. Con, control; UA, U18666A.
Fig. S2. Characterization of exosomes secreted following treatment with γ-secretase inhibitor and the neuronal uptake of exosomes after exposure to A11 antibody.  
A; Dot-blot showing the labelling of cell lysates and secreted exosomes derived from γ-secretase inhibitor-treated cultured astrocytes in the presence or absence of U18666A with Flotillin-1, ALIX and TSG101.  
B; Dynamic light scattering showing the relative size
of secreted exosomes derived from γ-secretase inhibitor-treated astrocytes in the presence or absence of U18666A. C-E; Photomicrographs of primary cortical neurons without exposure to exosomes (C) and following cellular uptake of exosomes from Dil-labelled U18666A (D) and U18666A + A11-treated (E) cultured astrocytes. F; Histograms showing quantification of relative fluorescence intensity representing no alteration in the uptake of Dil-labelled exosomes by primary cortical neurons in the presence of A11 antibody.